Circulating blood immunophenotyping and metabolite profiling in pulmonary vascular diseases

Submitted for the degree of MD
March 2018

Dr Kasia Isabel Zalewska

Cardiff University

Acknowledgements

I am sincerely grateful to the following individuals and organisations who have supported this work;

- My supervisors Dr Joanna Pepke Zaba and Dr Mark Toshner at Papworth Hospital and Professor Chris Pepper at Cardiff University for their guidance, nurturing, patience and constructive criticism.
- All the staff of the Pulmonary Vascular Diseases Unit and other departments at Papworth Hospital who have facilitated this work, in particular Dr John Cannon, Dr Dolores Taboada and Dr Emilia Swietlik.
- The Morrell Laboratory, University of Cambridge and Biomedical Research Centre Immunophenotyping Service, Addenbrookes Hospital for their assistance with immunophenotyping studies. In particular I would like to thank Emily Groves and Natalia Savinykh Yarkoni.
- Patients who have participated in this research.
- Papworth Hospital NHS Foundation Trust, Actelion, the NIHR-TRC and The Dinosaur Trust for their financial support.
- My husband David for his patience, understanding and good humour, which has been invaluable throughout the challenges of this work.
- My mother Hanna for her unwavering support and for always inspiring me and encouraging me to aim high and achieve my goals.

Summary

Pulmonary hypertension is an abnormal physiological state associated with a variety of medical conditions. However, the ability to accurately phenotype disease subtypes within this heterogeneous syndrome is limited.

In this thesis, I utilised advanced phenotyping techniques, guided by pathophysiological processes known to be dysregulated in pulmonary vascular diseases; immunity and metabolism. I used flow cytometry based immunophenotyping to study circulating leukocyte subpopulations and metabolomic analysis to study metabolite profiles in circulating blood. I hypothesised that there would be differences between disease and health, and differences between disease subgroups.

In the immunophenotyping studies, I identified an immune cell signature in Idiopathic Pulmonary Arterial Hypertension (IPAH) and Heritable Pulmonary Arterial Hypertension (HPAH) characterised by increased frequencies of T follicular helper (Tfh) cells, plasmablasts and PD1-expressing CD8+ T cells. This signature was not found in Chronic Thromboembolic Pulmonary Hypertension (CTEPH). These findings support the hypothesis that dysfunctional immune activation may be implicated in IPAH pathobiology, and that IPAH and HPAH may have shared immunopathological mechanisms.

In the metabolomic studies, I identified wide ranging metabolic changes in pulmonary vascular disease, including evidence of disrupted energy metabolism, increased cellular proliferation and reduction in antioxidant metabolites. Additionally, by comparing paired samples from different anatomical sites, it was possible to differentiate metabolic perturbations which are localised to specific anatomical sub-compartments.

Key to the clinical applications of this research, I have demonstrated immunological and metabolic alterations which are a shared feature amongst different pulmonary vascular disease subgroups, but also some changes which are specific to disease subsets. Future advances in disease phenotyping may facilitate effective new targeted therapy for pulmonary vascular diseases.

Presentations to learned societies arising from this work

Circulating metabolites in chronic thromboembolic pulmonary hypertension and chronic thromboembolic pulmonary vascular occlusion. KI Zalewska, EM Swietlik, D Taboada, JE Cannon, J Sanchez-Hernandez, C Hadinnapola, NW Morrell, MR Toshner, J Pepke Zaba. Oral presentation at British Thoracic Society Winter Conference-December 2016 and joint meeting of the Welsh Thoracic Society and Cardiff Chest Federation- April 2017.

Peripheral blood leukocyte phenotype in IPAH and CTEPH. KI Zalewska, E Groves, H Baxendale, J Pepke Zaba, NW Morrell, MR Toshner. Poster presentation. European Respiratory Society International Congress- September 2016.

Circulating Immune Cell Phenotype in Idiopathic Pulmonary Arterial Hypertension. KI Zalewska, E Groves, H Baxendale, J Pepke Zaba, NW Morrell, MR Toshner. Oral presentation at Cambridge Immunology Network- Annual Immunology PhD and Postdoc Day- May 2016.

Evidence of Dysregulated Humoral Immunity in Idiopathic Pulmonary Arterial Hypertension. E Groves, KI Zalewska, A Crosby, RM Salmon, Z Wei, PD Upton, C Hadinnapola, EF McKinney, M Southwood, H Baxendale, J Pepke-Zaba, NW Morrell, MR Toshner. Poster at American Thoracic Society International Conference- May 2016.

'Idiopathic' Pulmonary Arterial Hypertension- An autoimmune disease? KI Zalewska, E Groves, H Baxendale, J Pepke Zaba, NW Morrell, MR Toshner. Oral presentation at joint meeting of the Welsh Thoracic Society and Cardiff Chest Federation- April 2016.

Circulating Immune Cell Phenotype in Idiopathic Pulmonary Arterial Hypertension. KI Zalewska, E Groves, H Baxendale, J Pepke Zaba, NW Morrell, MR Toshner. Poster presentation at 15th International Pulmonary Hypertension Forum- March 2016.

Idiopathic Pulmonary Arterial Hypertension demonstrates a peripheral blood signature of dysregulated immunity. KI Zalewska, E Groves, H Baxendale, J Pepke Zaba, NW Morrell, MR Toshner. Oral presentation at British Thoracic Society Winter Meeting- December 2015.

Thesis Contents

List of Tables

List of Figures

Abbreviations

12-HETE	12-Hydroxyeicosatetraenoic acid
18F-FDG	18F-fluorodeoxyglucose
3H-BPAP	3H-benzoyl-Phe-Ala-Pro
5-HT	5-hydroxytryptamine (serotonin)
5-HTT	5-hydroxytryptamine transporter
6MWD	six minute walk distance
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
ALK 1	Activin receptor-like kinase type 1
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
BCAA	Branched chain amino acid
BMI	body mass index
BMPR2	bone morphogenetic protein receptor type 2
BNP	brain natriuretic peptide
BP	systemic blood pressure
BSA	bovine serum albumin
CAMPHOR	Cambridge Pulmonary Hypertension Outcome Review
CCB	calcium channel blocker
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster designation antigen
cGMP	cyclic guanosine monophosphate
CHD	congenital heart disease
CI	cardiac index
CO	cardiac output
COPD	chronic obstructive pulmonary disease
CRP	C reactive protein
CT	computerised tomography
CTD	connective tissue disease
CTD-PAH	connective tissue disease associated pulmonary arterial hypertension
CTED	chronic thromboembolic disease
CTEPH	chronic thromboembolic pulmonary hypertension
CTPA	Computerised tomography pulmonary angiography
CXCR	C-X-C motif chemokine receptor

Da	Dalton
DHEA-S	dehydroepiandrosterone-sulphate
DNA	deoxyribonucleic acid
dPAP	diastolic pulmonary artery pressure
ECG	electrocardiogram
ECHO	echocardiogram
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ERA	endothelin receptor antagonist
ERS	European Respiratory Society
ESC	European Society of Cardiology
ESI	electrospray ionisation
ET-A	endothelin receptor A
ET-B	endothelin receptor B
FA	formic acid
FACS	fluorescence-activated cell sorting
FcR	Fc receptor
FDR	false discovery rate
FEV1	forced expiratory volume in 1 second
FFA	free fatty acid
FMO	fluorescence minus one
FoxP3	forkhead box protein 3
FSC	forward scattered light
FSC(A)	forward scattered light area
FSC(H)	forward scattered light height
FSC(W)	Forward scattered light width
µ	micro (10^{-6})
FVC	forced vital capacity
g	gravitational force
GI	gastrointestinal
GPC	glycerophosphocholine
GPE	glycerophosphoethanolamine
H&E	haemotoxylin and eosin
Hg	mercury
hi	high expression
HILIC UPLC	hydrophilic interaction liquid chromatography
HILIC UPLC-MS/MS	HILIC UPLC with tandem mass spectrometry
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HLA-DR	human leukocyte antigen D related
HPAH	heritable pulmonary arterial hypertension
HR	heart rate

HRCT	high resolution computed tomography
IFN	interferon
Ig	immunoglobulin
IHD	ischaemic heart disease
IL	interleukin
ILD	interstitial lung disease
IPAH	idiopathic pulmonary arterial hypertension
IV	intravenous
K2 EDTA	potassium ethylenediaminetetraacetic acid
KCO	gas transfer coefficient
kg	kilograms
L	litres
LCFA	long chain fatty acid
LIP	lymphopenia induced proliferation
LV	left ventricle
m	metres
M	mega (10^{6})
m	milli (10^{-3})
M	molar
m/z	mass to charge ratio
m^2	metres squared
MACS	magnetic activated cell sorting
MCFA	medium chain fatty acid
min	minute
MIP 1 alpha	macrophage inflammatory protein 3
mL	millilitres
mM	millimoles
mm	millimetres
mmHg	millimetres of mercury
mPAP	mean pulmonary artery pressure
MRI	magnetic resonance imaging
MS	mass spectrometry
MS/MS	Tandem mass spectrometry
n	nano (10^{-9})
n	number
NIHR	National Institute for Health Research
NK	natural killer
NKT	natural killer T cell
NO	nitric oxide
NT-proBNP	N-terminal pro brain natriuretic peptide
O2	oxygen

PA	pulmonary artery
PAH	pulmonary arterial hypertension
PAH-CTD	pulmonary arterial hypertension associated with connective tissue disease
PAH-SSc	pulmonary arterial hypertension associated with systemic sclerosis
PAP	pulmonary artery pressure
PASMC	pulmonary artery smooth muscle cell
PBMCs	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCWP	pulmonary capillary 'wedge' pressure
PD-1	programmed death 1
PDE5	phosphodiesterase type 5
PDE5i	phosphodiesterase type 5 inhibitor
PDGF	platelet derived growth factors
PE	pulmonary embolus
PEA	pulmonary endarterectomy
PEG	polyethylene glycol
PET	positron emission tomography
PFO	patent foramen ovale
PFPA	perfluoropentanoic acid
PFT	pulmonary function test
pg	picogram
PH	pulmonary hypertension
pKa	acid dissociation constant (logarithmic scale)
PO_2	partial pressure of oxygen
ppm	parts per million
PUFA	polyunsaturated fatty acid
PV	pulmonary vascular
PVD	pulmonary vascular disease
PVR	pulmonary vascular resistance
Q	flow
QC	quality control
QOL	quality of life
r	correlation coefficient
RA	rheumatoid arthritis
RA	radial artery
RANTES	regulated on activation, normal T cell expressed and secreted
RAP	right atrial pressure
RHC	right heart catheterisation
RI	retention time index
RNA	ribonucleic acid
RP	reversed phase
RP UHPLC-MS/MS	RP ultra high performance liquid chromatography with tandem mass spectrometry

RR	respiratory rate
RSD	median relative standard deviation
RV	right ventricle
RVEDP	right ventricular end diastolic pressure
RVSP	right ventricular systolic pressure
s	second
SaO2	arterial oxygen saturation
SBP	systolic blood pressure
SD	standard deviation
SERT	serotonin transporter
sGC	soluble guanylyl cyclase
SLE	systemic lupus erythematosis
sPAP	systolic pulmonary artery pressure
SpO2	arterial oxygen saturation via pulse oximeter
SSc	systemic sclerosis
SSC-A	scattered light area
SSc-PAH	systemic sclerosis associated pulmonary arterial hypertension
ssDNA	single stranded DNA
SvO2	mixed venous oxygen saturations
TCA	tricarboxylic acid
TD	thermodilution
Tfh	T follicular helper cells
TGF	transforming growth factor
Th	T helper cells
TLC	total lung capacity
TLCO	diffusion capacity of the lung for carbon monoxide
TNF	tumour necrosis factor
TPG	transpulmonary gradient
Tregs	regulatory T cells
tRNA	transfer ribonucleic acid
TTCW	time to clinical worsening
UHPLC	ultra-high performance liquid chromatography
V/Q	ventilation/perfusion scan
VEGF	vascular endothelial growth factor
w/w	concentration by weight
WHO	World Health Organisation
WU	Wood unit

1 Chapter 1 - Introduction

1.1 Historical overview

The first accurate description of the pulmonary circulation was by the Arab Physician Ibn al-Nafis (1210–1288) in the 13th century AD. Ibn al-Nafis was the first to challenge the teachings of Galen, which had been accepted since the 2nd century AD. Galen had erroneously stated that blood passed from the right to the left ventricle through invisible pores in the interventricular septum and that only a small amount of blood passed into the pulmonary artery, solely to nourish the lungs.

Contrary to the belief that blood passed through the interventricular septum, Ibn al-Nafis deduced that the route of transit of blood from the right to left side of the heart was in fact via the lung, through the pulmonary circulation (West, 2008).

However, the insights of Ibn al-Nafis did not reach the Western world, and it was not until 300 years later that the European scholar Michael Servetus (1511-1553) reached a similar conclusion. Ibn al-Nafis had also recognised the existence of small communications between the pulmonary artery and vein, but it was not until 400 years later that the pulmonary capillaries were further described by Marcello Malpighi (1628-1698) (Azizi et al., 2008).

In the 17th century, development of experimental models by William Harvey (1578-1657) demonstrated the mechanistic principles of both the systemic and pulmonary circulation. However, invasive study of pulmonary haemodynamics in humans, finally providing direct insight into pulmonary circulation physiology, was not conducted until the 1940s (Cournand and Ranges, 1941).

The technique of right heart catheterisation, pioneered through self-experimentation by Forssman (Meyer, 1990), was used in human studies to measure multiple parameters including pressure, flow and gas content in the pulmonary circulation (Cournand et al., 1944, Cournand et al., 1945), thus providing information essential to our current understanding of both normal physiology and disease pathophysiology.

Over the last 50 years it has been increasingly recognised that the pulmonary circulation is not merely a passive conduit for blood flow. It is now known that it has a multitude of dynamic roles in homeostatic, metabolic and immunological processes (Comroe, 1966, Said, 1982, Mellins, 1982, Orfanos et al., 2004, Millar et al., 2016), and there is developing understanding of disease processes

that involve or affect the pulmonary circulation and therapeutic interventions which target this system.

1.2 Pulmonary vascular development

Pulmonary vascular development occurs through the processes of vasculogenesis- *de novo* formation of vessels, and angiogenesis- sprouting of new vessels from existing ones.

Embryologically, the main pulmonary arteries develop from the 6th aortic arch by angiogenesis. Capillaries later form by vasculogenesis, with differentiation of progenitor endothelial cells in the primitive mesenchyme and fusion of channels of endothelial cells with the existing vessels (deMello and Reid, 2000, Hislop and Pierce, 2000).

Growth factors regulating blood vessel formation include vascular endothelial growth factors (VEGFs) and angiopoietins (Hislop, 2005, Crivellato, 2011, Hato et al., 2009, Asahara et al., 1998, Suri et al., 1996). In addition to playing a critical role in lung development, in adult life they are involved in the response to lung injury (Voelkel et al., 2006, Lahm et al., 2007, Mura et al., 2006, Lomas-Neira et al., 2014, Wada et al., 2013, Schlosser et al., 2017, Uehara et al., 2016).

The foetal pulmonary circulation receives less than 10% of cardiac output, with the majority of circulating blood volume bypassing the lungs via the foramen ovale to the left atrium or ductus arteriosus to descending aorta. It is characterized by a high vascular resistance. However, with the closure of the ductus arteriosus at birth, there is a dramatic increase in pulmonary blood flow and rapid fall in pulmonary vascular resistance, resulting in the classic haemodynamic features of the normal pulmonary circulation- a high flow, low resistance system (Hislop, 2005).

1.3 Normal anatomical structure of the pulmonary circulation

The pulmonary circulation consists of a branching network of vessels, which run in parallel with the airways. This ‘pulmonary arterial tree’ branches from large proximal pulmonary arteries into repeatedly smaller divisions, transitioning to arterioles and finally the alveolar capillaries, prior to return of blood to the left heart via the pulmonary veins (Townsley, 2012, JMB and NW, 2001, Hughes and Morrell, 2001).

The reduction in vessel diameter as the pulmonary arterial tree divides is accompanied by morphological changes which reflect the properties of these vessels (Brenner, 1935).

The large proximal vessels (external diameter > 1mm) have thin walls relative to the size of the lumen and have a predominance of elastic fibres in the media. These proximal elastic vessels primarily serve a conducting function and act to protect against pressure fluctuations via their 'windkessel' effect (Lammers et al., 2012).

The small arterioles and capillaries are the primary determinants of the vascular resistance. (Bhattacharya and Staub, 1980, Bhattacharya et al., 1982). As the arteries become smaller, the elastic laminae are replaced by smooth muscle. Small arteries (0.1-1mm external diameter) have a predominance of smooth muscle constituting the vessel media. However, in the most distal pre-capillary segments of the pulmonary vascular bed (vessels of 20-30 micrometres internal diameter), the muscular layer is lost and there is subsequent transition into pulmonary capillaries, whose wall consists of only a thin layer of endothelial cells, which share their basement membrane with that of the type I pneumocytes lining the alveolar space (Townsley, 2012).

1.4 Normal pulmonary circulation haemodynamics

The normal pulmonary circulation is a high flow, low resistance system. Pulmonary circulation blood flow is approximately 3.5L/min/m^2 body area at rest (West, 2011). Flow into the pulmonary circuit, like the systemic circuit, is pulsatile, but pressures are much lower (Table 1.1). In a healthy individual at rest, mean pulmonary arterial pressure is around 1/6 of the systemic arterial pressure at rest (West, 2011).

Table 1.1- Normal systemic and pulmonary circulation pressures.

	Systemic	**Pulmonary**
Arterial pressure	120/80 mmHg; 95 mmHg (mean)	25/10 mmHg; 15 mmHg (mean)
Capillary pressure	30 mmHg (mean)	10 mmHg (mean)
Venous pressure	2-5 mmHg	2-5 mmHg

Due to gravity, intravascular pressures are lowest at the lung apex and highest at the base, resulting in regional distribution in pulmonary blood flow (Powell et al., 2016).

In health, low resistance to flow allows optimal conditions for gas exchange, prevents movement of fluid from the vessels into the interstitial space and enables the right ventricle to operate at minimal energy cost. Pulmonary vascular resistance falls below resting values upon exercise, facilitated by

distension of existing vessels, allowing increase in calibre, along with pulmonary vascular recruitment, whereby previously closed vessels open up (Frank L. Powell, Powell et al., 2016).

Pulmonary vascular resistance (PVR) can be considered according to Poiseuille's Law, which states that resistance to flow is inversely related to r^4 (where r refers to vessel radius). Therefore, small changes in vessel radius result in large changes in resistance and the pulmonary circulation is very sensitive to small changes in vessel calibre. However, it must be noted that Poiseuille's Law provides only an approximation of true PVR as it is intended to be applied to Newtonian fluid in laminar flow through a straight tube of constant cross section, whereas pulmonary blood flow is pulsatile, the pulmonary vascular tree has a complex branching structure and the circulation is both distensible and compressible.

In comparison with systemic arteries and arterioles, there is much less smooth muscle in the walls of the vessels of the pulmonary arterial tree (West, 2011). Additionally, there is a relative lack of supporting tissue surrounding the vessels. As a consequence of this, the pulmonary vessels are more distensible than systemic arteries. This distensibility (along with vascular recruitment) allows accommodation of relatively large increases in blood volume, such as occurs with exercise (Naeije and Chesler, 2012). However, the distensibility also means that pressure-flow relationships in the pulmonary circulation are sensitive to mechanical influences such as changes in alveolar and intrapleural pressures, which can have significant effects on PVR (Powell et al., 2016). In addition, intravascular pressures, blood viscosity, lung volume, gravity and RV output can all have significant effect on PVR without alterations in pulmonary vascular cross-sectional area (Powell et al., 2016).

Aside from the passive mechanisms affecting PVR, the pulmonary vasculature shows dynamic responses to various stimuli. For example, the vessels undergo constriction in response to alveolar hypoxia and release vasoactive substances such as nitric oxide, endothelins and prostacyclin in response to mechanical forces such as shear stress and cyclic stretch of the vessel wall (Powell et al., 2016). Although sympathetic, parasympathetic and sensory neural fibres are present in the proximal pulmonary arteries, this neural network plays a very limited role influencing overall pulmonary vascular resistance, as innervation does not extend beyond the small intrapulmonary vessels, with the more distal arterioles being devoid of innervation (Kummer, 2011).

In clinical practice, pulmonary vascular resistance (PVR) is calculated using measurements taken during right heart catheterisation (described further in chapter 2). Calculation is based on the principle that PVR equals inflow pressure minus outflow pressure, divided by mean pulmonary blood flow.

Using directly measured haemodynamic parameters;

PVR = (mPAP- PCWP)/CO

mPAP - mean pulmonary artery pressure

PCWP - pulmonary capillary wedge pressure (surrogate for left atrial pressure)

CO - Cardiac output (equal to pulmonary blood flow in absence of significant shunting)

1.5 Metabolic functions of the pulmonary circulation

1.5.1 Historical overview

For centuries, the lung was thought to have little metabolic activity and the pulmonary circulation was considered to be a passive conduit for blood transit to the lungs for gas exchange. However, the much wider roles of the pulmonary circulation are now increasingly recognised.

Perhaps the first recorded observation of the metabolic function of the pulmonary circulation was in the 1920s, by Starling and Verney (Starling and Verney, 1925). In their experiments, they were unable to perfuse an isolated kidney without a heart-lung circuit, reporting that a serum vasoconstrictor substance (later found to be serotonin) was detoxicated on passing through the lung.

However, it was not until 40 years later that studies were conducted specifically to assess pulmonary circulation metabolic function. This included confirmation of pulmonary circulation clearance of serotonin (Gaddum et al., 1953), providing a pathophysiological explanation for the predominance of right sided heart lesions found in carcinoid syndrome and thus, recognition of the role of the lungs in detoxification and regulatory processes. This was followed by studies demonstrating that prostaglandins E1, E2 and F2alpha are stable in systemic blood but 'rapidly inactivated by lung' (Ferreira and Vane, 1967), and that conversion of angiotensin I to angiotensin II occurs more rapidly in the lung vasculature than in other tissues (Ng and Vane, 1968).

Astonishingly, to this day, the metabolism of the pulmonary circulation has not been fully investigated and it is likely that the true extent of metabolic processes occurring in the pulmonary circulation is not yet appreciated. However, advances in currently available technologies may provide a means for us to address the gaps in our knowledge, and allow us to more fully appreciate the true extent and complexity of pulmonary circulation metabolic function.

1.5.2 Fate of substances in the pulmonary circulation

Substances present in pulmonary blood flow may undergo uptake or biotransformation, may be released as a result of endothelial cell activity, or may be unaffected during transit (Kayyali and Fanburg, 2009, Hughes and Morrell, 2011).

The pulmonary vascular bed has a vast surface area facilitating interaction between the endothelium and circulating factors in the bloodstream. By receiving almost the entire circulating blood volume, it has the potential to modify the composition of blood entering the systemic circulation, and through synthesis and release of substances, can influence biological activity at distal sites (Jernigan et al., 2009).

However, it is also vulnerable to particles which may become trapped in the small calibre vessels, such as emboli, and noxious substances such as toxins which may be transmitted to the lungs in circulating blood (Jorens et al., 2009).

The pulmonary vascular endothelial cell is recognised to be an important mediator of the pulmonary circulation metabolic function, and expresses proteins that facilitate different functions such as hydrolysis (e.g. ACE, lipoprotein lipase) and active transport (e.g. the serotonin transporter (SERT)) (Kayyali and Fanburg, 2009, Hughes and Morrell, 2011, Stan, 2009). It is also responsible for the transmission of communications from the endothelial surface to vascular smooth muscle cells and fibroblasts (Grinnell and Harrington, 2009).

The metabolic function of the pulmonary vascular endothelium shows structural specificity (Table 1.2). For example; although >95% of circulating serotonin (5-HT) is removed in a single transpulmonary passage, melatonin (which is very closely related in structure to 5-HT) is not significantly cleared by the lungs. Likewise, 30% of noradrenaline is removed in a single pass through the lungs while adrenaline is unaffected. Similarly, although prostaglandins E_1 E_2 and $F_{2\alpha}$ undergo 90% first pass metabolism, prostaglandins A2 and I2 pass through with very little clearance (Said, 1982, Ryan et al., 1971, Hughes et al., 2001).

In addition to these selective processes, the endothelium is involved in non-specific binding of other substances. For example, many drugs show non-specific uptake onto the endothelial cell membrane, which allows the lungs to act as a temporary site of retention and may provide a buffering function. Specific binding or uptake of drugs by pulmonary endothelial cells is limited to a small number of substances (Geddes et al., 1979, Boer, 2003, Roerig DL, 1989, Suhara et al., 1998).

Table 1.2-Fate of substances in blood upon pulmonary circulation transit.

Cleared	**Unaffected**
Arachidonic acid (> 90%)	Prostaglandin A2, I2
Prostaglandins E 1+2, F2α (>90%)	Leukotrienes
Adenosine + derivatives (> 95%)	Adrenaline
Serotonin (98%)	Dopamine
Angiotensin I (80%)	Histamine
Bradykinin (80%)	Angiotensin II
Endothelin 1 (50%+)	ANP
Noradrenaline (30-40%)	Majority of hormones

1.5.3 Metabolic function of the pulmonary circulation in disease states

In the same way that dysfunction of the liver or kidney can alter blood metabolite profile, it can be expected that the disease processes of the lung vasculature will alter the metabolite profile of blood passing through the pulmonary circulation**.**

Changes in metabolites present may be a primary reflection of processes occurring as an intrinsic part of the disease pathobiology, such as endothelial damage. Conversely, the effects of disease upon the local environment may impact upon metabolic activity.

For example, a reduction in functional surface area in diseases such as emphysema, acute lung injury and chronic thromboembolic pulmonary hypertension affect receptor availability and binding and biotransformation of substances (Boer, 2003, Maniatis et al., 2008, Orfanos et al., 2008, Orfanos et al., 2000). Also hypoxaemia is known to has a significant effect on some metabolic pathways e.g. ACE activity is reduced if PO_2 is low (Milledge, 1984), and acidosis results in changes in the ratio of ionised to non-ionised forms of drugs and other substances, thereby altering their properties and uptake (Boer, 2003).

Therefore, alterations in lung metabolic function may occur both as a direct result of the disease itself, but the disease process may also result in structural and environmental changes which may further affect metabolic processes.

1.6 Pulmonary Hypertension

Pulmonary hypertension (PH) is an abnormal physiological state where there is elevated pressure in the pulmonary circulation. It is defined as a mean pulmonary artery pressure ≥ 25mmHg at rest, when measured in the supine position by cardiac catheterisation (Hoeper et al., 2013b). Normal mean pulmonary artery pressure (mPAP) at rest is 14 ± 3 (Kovacs et al., 2009). This haemodynamic state is associated with a variety of medical conditions and can occur due to primary pressure elevation in the pulmonary arterial system, or secondary to elevations in the pulmonary venous system (as may occur with left heart disease).

Pulmonary Hypertension is classified into five main clinical groups (Galiè et al., 2015):

1- Pulmonary Arterial Hypertension (PAH) including Idiopathic Pulmonary Arterial Hypertension (IPAH) and Heritable Pulmonary Arterial Hypertension (HPAH)

2- Pulmonary Hypertension due to left heart disease,

3- Pulmonary Hypertension due to lung diseases and/or hypoxia

4- Chronic Thromboembolic Pulmonary Hypertension (CTEPH), and

5- Pulmonary Hypertension with unclear and/or multi-factorial mechanisms.

Regardless of the aetiology of pulmonary hypertension, the physiological effects of sustained elevation in pulmonary circulation pressure are shared by the groups. The ultimate consequences of this are the development of right heart dysfunction and failure, leading to premature death. The expanded classification of disease subtypes is shown in Table 1.3 (Galiè et al., 2015).

The research presented in this thesis is primarily focussed on Group 1 (Pulmonary Arterial Hypertension) and Group 4 (Chronic Thromboembolic Pulmonary Hypertension), which are described further in sections 1.8-1.13 and 1.15-1.16.

Table 1.3- Clinical classification of Pulmonary Hypertension.

1. Pulmonary arterial hypertension (PAH)
1.1 Idiopathic 1.2 Heritable 1.2.1 BMPR2 mutation 1.2.2 Other mutations 1.3 Drugs and toxins induced 1.4 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'. Pulmonary veno-occlusive disease and/or pulmonary capillary haemangiomatosis 1". Persistent pulmonary hypertension of the newborn
2. Pulmonary hypertension due to left heart disease
2.1 Left ventricular systolic dysfunction 2.2 Left ventricular diastolic dysfunction 2.3 Valvular disease obstruction and congenital cardiomyopathies 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies 2.5 Other
3. Pulmonary hypertension due to lung diseases and/or hypoxia
3.1 Chronic obstructive pulmonary disease 3.2 Interstitial lung disease 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern 3.4 Sleep-disordered breathing 3.5 Alveolar hypoventilation disorders 3.6 Chronic exposure to high altitude 3.7 Developmental lung diseases
4. Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions
4.1 Chronic thromboembolic pulmonary hypertension 4.2 Other pulmonary artery obstructions
5. Pulmonary hypertension with unclear and/or multifactorial mechanisms
5.1 Haematological disorders 5.2 Systemic disorders 5.3 Metabolic disorders 5.4 Others

1.7 Relationship between the right ventricle and pulmonary circulation

The function of the right ventricle (RV) is intricately linked with the function of the pulmonary circulation (Pinsky, 2016, Champion et al., 2009). Compared to the left ventricle, which needs to generate sufficient pressure to perfuse the whole systemic circulation, the right ventricle usually has to generate a much lower pressure to perfuse the pulmonary vasculature.

However, in pulmonary arterial hypertension, where there is a sustained increase in pulmonary vascular resistance, the increase in RV afterload necessitates a compensatory increase in RV systolic pressure. Chronic pressure overload of the RV results in initial hypertrophy, but with increasing afterload the RV is overwhelmed, leading to decompensation (Champion et al., 2009, Vonk Noordegraaf and Galiè, 2011, Naeije and Manes, 2014).

Decompensation is characterised by dilatation, reduced ejection fraction and a drop in cardiac output (Figure 1.1). Development of RV failure is strongly associated with poor prognosis in PAH and this is the primary mechanism of death in the disease (Forfia et al., 2006, Ghio et al., 2011, Raymond et al., 2002, Vonk Noordegraaf and Galiè, 2011).

The reduction in cardiac output is further exacerbated by the mechanical effects of the RV on the left heart, where compression of the left heart by the dilated, pressure and volume overloaded right heart impairs left ventricular filling and results in further drop in cardiac output (Puwanant et al., 2010, Chua et al., 2013, Naeije and Manes, 2014).

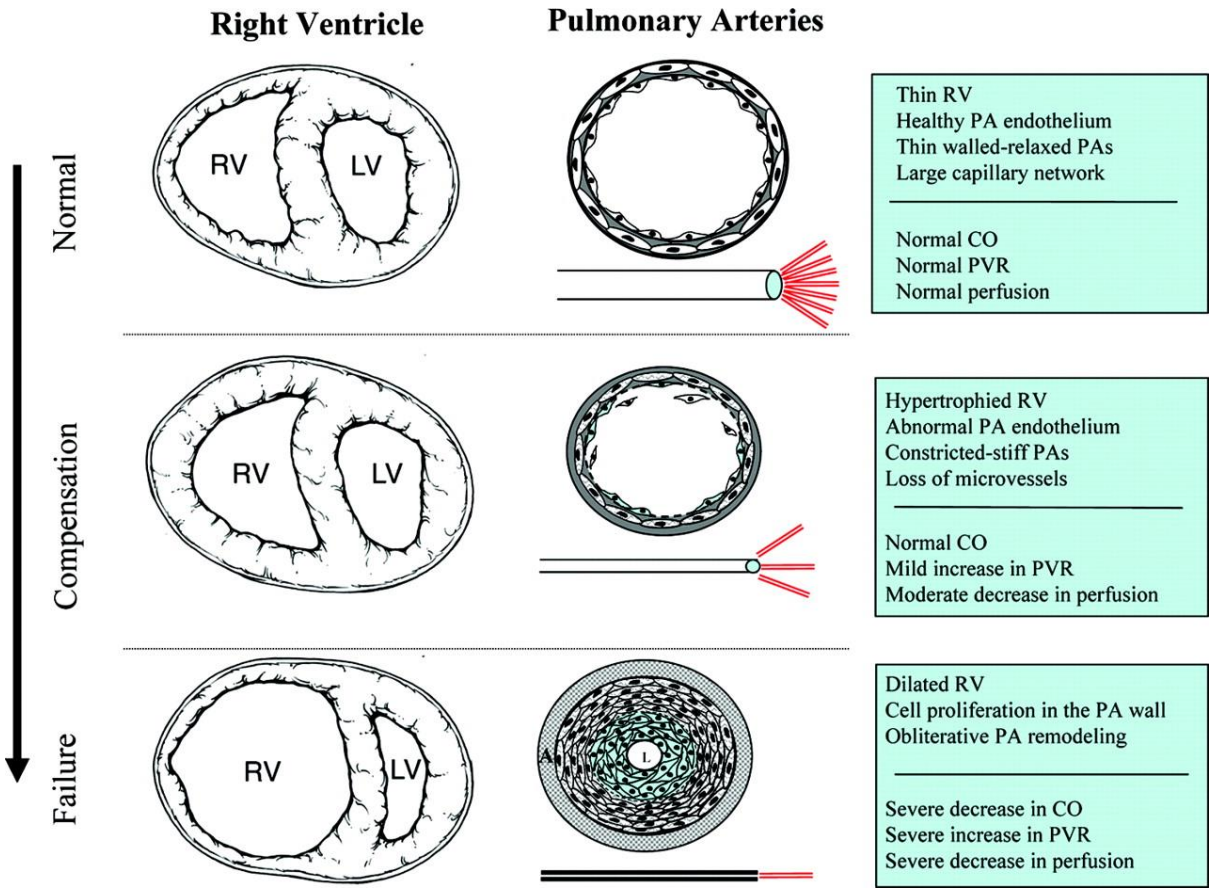


Figure 1.1-Progression of pulmonary vascular disease and subsequent effect on RV function. Schematic showing the theoretical progression of pulmonary vascular disease: as pulmonary vascular resistance progressively resistance increases, RV remodelling and subsequent RV failure occur (adapted from Champion et al., 2009).

1.8 Group 1 - Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (clinical classification group 1) is a rare condition with an annual incidence of 1.1 cases per million population in the UK, a prevalence of 6.6 cases per million (Ling et al., 2012). Median age at diagnosis is 50 years (Ling et al., 2012). It is defined by the presence of pre-capillary pulmonary hypertension (mPAP ≥ 25mmHg and PCWP ≤ 15 mmHg) with PVR >3 Wood units, in the absence of other causes of PH such as lung disease and chronic thromboembolic disease (Hoeper et al., 2013b). Within group 1 (PAH) there are a number of subtypes, defined by the presence or absence of associated features or a genetic basis for the disease (see Table 1.3).

1.9 PAH diagnosis and treatment

Although non-invasive investigations may suggest the presence of pulmonary arterial hypertension, unlike systemic blood pressure, there is no non-invasive way of accurately determining pulmonary circulation haemodynamics. Confirmation of the diagnosis can only be made by specialist

assessment which includes invasive measurement of haemodynamics by right heart catheterisation (Galie 2015). There is no non-invasive test or biomarker which can reliably diagnose PAH.

Existing treatment of the disease centres on the use of drugs which primarily act as vasodilators, with some drugs also demonstrating weak anti-proliferative effect. Currently available treatments include prostacyclin pathway agonists, endothelin receptor antagonists and phosphodiesterase 5 inhibitors, calcium channel blockers and a guanylate cyclase stimulant (Lang and Gaine 2015) (Figure 1.2). These treatments primarily focus on reducing pulmonary vascular resistance, thereby reducing right heart strain, delaying or ameliorating right heart failure and improving cardiac output. However, they do not arrest or reverse the underlying vascular remodelling process.

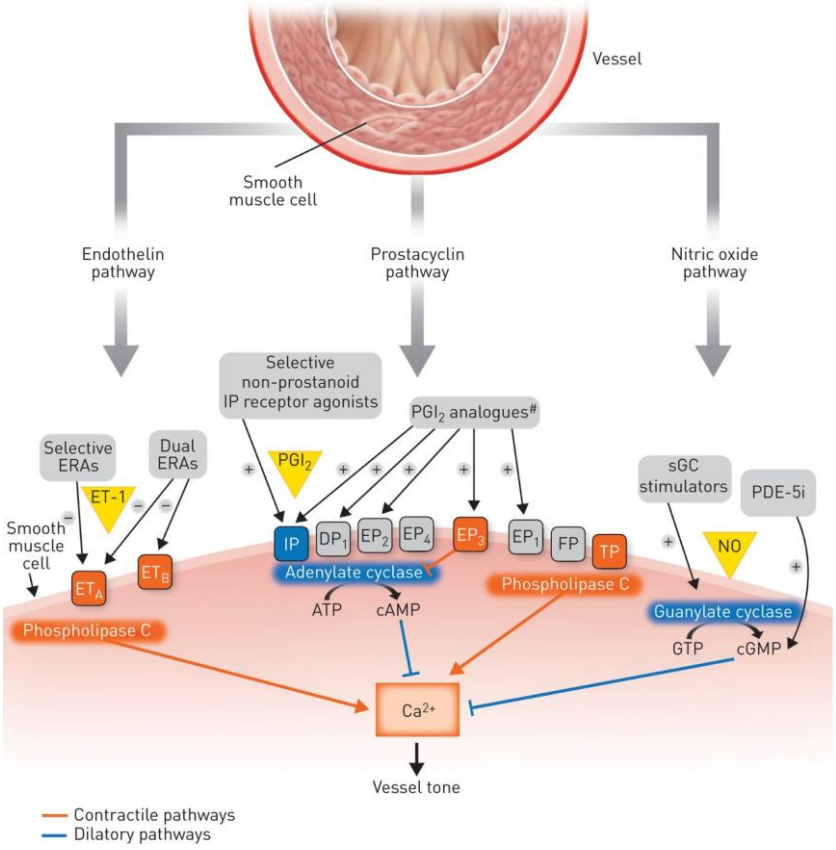


Figure 1.2- Involvement of the endothelin, nitric oxide and prostacyclin pathways in the pathogenesis of pulmonary arterial hypertension. In the endothelin pathway, the effects of endothelin (ET)-1 are mediated via the ETA and ETB receptors. Receptor binding leads to activation of phospholipase-C and mobilisation of calcium, resulting in vasoconstriction. Selective and dual endothelin receptor antagonists (ERAs) inhibit this pathway. In the pulmonary artery the prostanoid receptors IP, EP3 and TP regulate vessel tone. The prostacyclin pathway involves prostacyclin binding to the IP receptor, which belongs to a family of prostanoid target receptors. Prostanoid binding to the IP receptor induces adenylate cyclase activity, cAMP production and ultimately reduction of Ca2+ concentrations, and leads to vasodilation. TP binding activates phospholipase C, mediating mobilisation of calcium and vasoconstriction. EP3 receptor binding leads to a decrease in cAMP, which blocks vasodilation. Prostacyclin analogues activate this pathway (EP3 pathway). The nitric oxide (NO) pathway involves the production of cGMP, which leads to inhibition of calcium entry, resulting in vasodilation. Phosphodiesterase type 5 inhibitors (PDE-5i) and soluble guanylate cyclase (sGC) stimulators activate this pathway (figure reproduced from Lang and Gaine, 2015).

1.10 Heritable PAH

Heritable pulmonary arterial hypertension (HPAH) refers to pulmonary arterial hypertension occurring due to mutations in predisposing genes or in a familial context. In 2000, mutations in BMPR2 (bone morphogenetic protein receptor type 2) were identified as the first known genetic variant predisposing to PAH (Lane et al., 2000, Deng et al., 2000). Mutations in this gene remain the most common genetic abnormality implicated in cases of HPAH. Pathogenic mutations vary in nature (e.g. deletion, missense, duplication or nonsense mutation) but all result in a loss of function. The ultimate consequence of this loss of function is an adverse response of the pulmonary circuit to injury, with increased endothelial cell susceptibility to apoptosis and loss of inhibitory effects of BMPR2 upon vascular proliferation (Teichert-Kuliszewska et al., 2006).

BMPR2 mutations implicated in PAH are transmitted as an autosomal dominant trait with incomplete penetrance and are found in around 80% of cases where there is familial PAH. However, only 20-30% of those with a mutation develop the disease and therefore it is thought that a 'second hit' is required (Fessel et al., 2011, Austin and Loyd, 2014). BMPR2 mutations are also found in seemingly sporadic cases of PAH, where it has either not manifested in relatives due to the low penetrance, or occurs as a *de novo* mutation.

Although BMPR2 mutations associated with HPAH are germline in nature and therefore distributed throughout bodily cells, the pathology caused by BMPR2 mutations appears to be localised to the pulmonary vasculature. The reasons for this lung-specific susceptibility remain unclear. PAH patients with a BMPR2 mutation are less likely to respond to vasodilator treatment (Rosenzweig et al., 2008), develop clinical manifestations of the disease at a younger age, have more severe haemodynamics and a worse prognosis (Sztrymf et al., 2008).

To date, a number of other genes implicated in PAH have also been identified including ALK 1 (Harrison et al., 2003), endoglin (Pousada et al., 2016), BMPR1B (Chida et al., 2012), NOTCH3 (Chida et al., 2014), CAV1 (Austin et al., 2012), CBLN2 (Germain et al., 2013) and KCNK3 (Ma et al., 2013). This is an area of continuing interest and it is likely that more cases than are currently recognised involve genetic mutations or genetic modifiers.

1.11 Idiopathic PAH: Epidemiology, clinical presentation and natural history

Idiopathic pulmonary arterial hypertension (IPAH) is a diagnosis of exclusion and refers to cases of pulmonary arterial hypertension in which no underlying cause can be identified. IPAH shows a

female predominance with the disease being 2-4 times more common in women as in men (Badesch et al., 2010, Frost et al., 2011). There are no apparent ethnic differences in incidence.

Presentation of IPAH is often insidious, with non-specific symptoms including breathlessness, exercise intolerance, fatigue, palpitations, chest pain, dizziness, syncope and oedema. The rare nature of the disease, along with lack of early distinguishing features, means that the condition may not be recognised without a high index of suspicion. As a consequence, diagnosis and initiation of treatment is often delayed (Ling et al., 2012), and the disease is most frequently diagnosed when it is already at an advanced stage (Humbert et al., 2010). Although survival rates have improved over the last 20 years, there are no curative treatments for IPAH and long-term prognosis remains poor (D'Alonzo et al., 1991, Strange et al., 2017, Peacock et al., 2007, Humbert et al., 2010).

1.12 Idiopathic PAH- Pathobiology

IPAH is a vasculopathy characterised by remodelling of the small pulmonary arteries ≤500 µm in diameter (Figure 1.3). Remodelling affects all the vessel layers- endothelium, neointima, media and adventitia and involves endothelial cell, fibroblast and vascular smooth muscle cell activation and proliferation (Tuder et al., 1994, Pietra, 1994, Dorfmüller, 2013). Ultimately this process results in reduction of the luminal diameter and altered function of the vascular endothelium.

Characteristic histological lesions which are found in the small muscular pulmonary arteries in IPAH include:

1. Medial hypertrophy/hyperplasia
   Increased muscularity of the vessel develops by enlargement and proliferation of smooth muscle cells within the tunica media. In health, the cross-sectional diameter of a single layer of the media is approximately 5% of the thickness of the external vessel diameter, but in PAH this is increased to >10% of the vessel diameter.

2. Thickening and fibrosis of the intima
   Normally in health, the intima consists of a single layer of endothelial cells overlying the internal elastic lamina. In the disease, thickening and fibrosis occurs due to migration and proliferation of fibroblasts and myofibroblasts, with subsequent collagen deposition. This thickening may be concentric (with layers resembling an onion skin) or eccentric.

3. Complex vascular lesions

    a) Plexiform lesions - the classical vascular lesion in IPAH, consisting of disorganised proliferation of endothelial cells to form a capillary like plexus of channels. They are often located at arterial branching points.
    b) Dilatation lesions - vein-like dilated vessels, most frequently found distal to plexiform lesions. In contrast to the muscular arteries which precede them, they have very thin walls. Occasionally, extremely dilated branches may cluster together and are referred to as angiomatoid lesions.
    c) Classical arteritis - this is rare and is manifest by fibrinoid necrosis of the vessel walls.

The factors which precipitate and propagate this abnormal vascular remodelling remain unclear. However, there is mounting evidence to suggest a role for inflammation, immune disturbances and metabolic alterations in the disease. This is discussed further in Section 1.18 and 1.19.

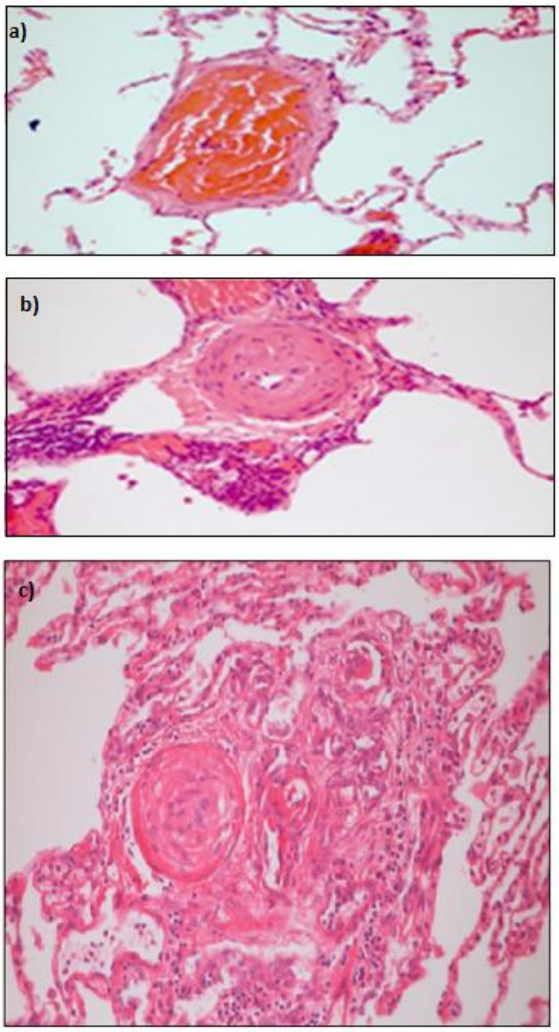


Figure 1.3- Histopathology of IPAH. Haematoxylin and eosin staining of; a) normal small pulmonary artery b) remodelled vessel in IPAH c) lung section containing remodelled pulmonary artery and plexiform lesion. Images courtesy of Mark Southwood, Papworth Hospital.

1.13 Clinical phenotypes in IPAH

The population of patients classified as having IPAH is clinically heterogenous. Different disease phenotypes within the IPAH population are evident and individuals with the disease differ significantly in haemodynamic profile, responses to treatment and clinical outcomes (Ling et al., 2012, Hoeper and Gibbs, 2014, Dweik et al., 2014, Halliday and Hemnes, 2017).

The mean age of diagnosis varies significantly among different cohorts (Idrees et al., 2015, Badesch et al., 2010, Frost et al., 2011, Humbert et al., 2006). However, registries suggest that the mean age at diagnosis is increasing and there is a growing proportion of elderly male patients being diagnosed with IPAH (McGoon et al., 2013, Frost et al., 2011, Ling et al., 2012). Therefore, the classical description of IPAH as a disease typically affecting young women is changing.

Younger patients have been noted to have more severe hemodynamic impairment but better survival, compared with older patients who have more comorbidities, including systemic hypertension, hyperlipidaemia, obesity and type 2 diabetes mellitus (Ling et al., 2012). Age-related changes in the pulmonary and cardiovascular systems are likely to play some part in the differences observed. These changes include progressive decline in lung function, systemic vascular stiffening and decreased left ventricular compliance leading to LV diastolic dysfunction. LV diastolic dysfunction with preserved systolic function (HEFPEF) can be difficult to clinically discern, and in older individuals it can be challenging to discriminating true pulmonary vasculopathy from the consequences of ageing and comorbidities (Lam et al., 2009).

Over recent years there has been a shift towards identifying different patient phenotypes in various diseases. Examples of this include asthma and COPD (Miravitlles et al., 2013, Wenzel, 2012) where disease phenotyping is being increasingly used to tailor treatment. In this evolving era of ‘personalised medicine’, improved characterisation of different IPAH phenotypes may enhance our ability to understand subgroups within this heterogeneous disease classification and may lead to more targeted and personalised approaches to treatment. This is discussed further in section 1.14.

1.14 Future directions in PAH

1.14.1 Disease specific biomarkers

Despite expansion of treatment options for PAH over the last 30 years, prognosis remains poor. Due to the insidious and non-specific nature of presenting symptoms, there is often significant delay between symptom onset and diagnosis (Strange et al., 2013). Early detection and treatment have been shown to improve outcomes (Humbert et al., 2010), but remains challenging.

Unfortunately, there is no biomarker available for clinical use which is specific for PAH, nor any biomarker which can reliably identify early or pre-clinical disease.

Diagnosis relies on assessment of haemodynamics by right heart catheterisation, which is not only invasive, but is costly and requires expertise. A number of non-invasive investigations are used in subsequent longitudinal follow up (such as 6MWD, echocardiography and blood BNP or NT-proBNP level), however, there are significant limitations associated with the use of these methods (Galiè et al., 2015), necessitating repeated cardiac catheterisation in many cases.

For example, although BNP correlates with haemodynamics, exercise capacity and survival in PAH (Leuchte et al., 2004, Nagaya et al., 2000), it is not disease specific, being elevated in a wide range of conditions causing cardiac failure and is affected by other factors such as renal function (Balion et al., 2008). In essence, BNP is only a surrogate marker for PAH, reflecting the RV dysfunction which occurs in established disease, rather than the primary vascular bed pathology. This limits its usefulness in detecting early or pre-clinical disease or differentiating PAH from left heart disease other causes of cardiac failure.

As a result of the limitations of existing biomarkers, there has been increasing interest in the identification and development of new, disease specific biomarkers for PAH.

Historically, biomarker discovery research has focussed upon a single molecule or pathway. However, the expectation that a single biomarker can unambiguously identify a disease appears increasingly simplistic. More recently, there has been a paradigm shift towards a ‘systems biology’ approach, which recognises that diseases involve the dysregulation of multiple gene regulatory networks, proteins, and metabolic processes (Chan and Loscalzo, 2012). Adopting this approach allows identification of multiple compounds that correlate with a disease state and characterise a disease ‘signature’. Thus, identification of different signatures within the spectrum of PAH may play an important future role in disease phenotyping.

1.14.2 Disease phenotyping and personalised medicine

The current clinical classification of pulmonary hypertension fails to adequately characterise the diversity of clinical phenotypes. This is particularly apparent in IPAH (see section 1.13). By nature of being a diagnosis of exclusion, the IPAH group is likely to represent a range of underlying disease subtypes.

There is considerable heterogeneity found in clinical practice. For example, the RV response to the presence of pulmonary hypertension differs between individuals with the same haemodynamic profile, with some quickly developing decompensated right heart failure and others being stable for many years (adaptive vs maladaptive clinical phenotype) (Dweik et al., 2014). A further example is the difference between older and younger patients with PAH- the older cohort being characterised by a higher incidence of LV diastolic dysfunction and vascular stiffness (Ling et al., 2012). Patients also vary considerably in terms of response to therapies (Humbert et al., 2010, Benza et al., 2010, Sitbon et al., 2005, Sitbon et al., 2002).

The ability to identify different phenotypes within this heterogeneous syndrome may be useful for prognostication and for customising treatment (Dweik et al., 2014, Brittain and Chan, 2016). Additionally, integration of data obtained using a number of different analytical strategies (for example genomics, proteomics and metabolomics) allows more extensive characterisation of phenotypes (‘deep phenotyping’), with further potential to advance mechanistic understanding of the disease and improve the targeting of therapies.

1.14.3 National Cohort Study of Idiopathic and Heritable PAH (COHORT)

The National Pulmonary Hypertension Centres of the UK and Ireland have established a research network to study factors which may be implicated in PAH development. The National Cohort Study of Idiopathic and Heritable PAH (COHORT) study involves longitudinal follow-up of patients and healthy relatives. As part of the study, participants undergo whole genome sequencing to look for genetic mutations which may be associated with PAH.

The aim of this is to provide a more complete understanding of the genetic contribution to PAH, and how genetic factors influence response to treatment and clinical outcomes. Additionally, this study will assess for environmental triggers which may be involved in the development of PAH. It is anticipated that in addition to identifying new genetic variants which contribute to PAH development, this research may identify genetic prognostic markers and new therapeutic targets.

1.15 Group 4 – CTEPH: Epidemiology, clinical presentation and natural history

Chronic thromboembolic pulmonary hypertension (CTEPH) is thought to result from failure of thrombus resolution in the pulmonary arteries with subsequent fibrosis and vascular remodelling (Lang et al., 2016).

CTEPH is an uncommon complication of pulmonary thromboembolism. The incidence of CTEPH following an acute pulmonary embolus (PE) is estimated to be 1–4.8% within the first 2 years after a symptomatic PE event (Pengo et al., 2004, Talati et al., 2016). Diagnosis is based on findings obtained after at least 3 months of effective anti-coagulation, in order to discriminate from 'subacute' PE. European registry data reported a history of previous acute PE in 74.8% of CTEPH patients (Pepke-Zaba et al., 2011). However, in a proportion of individuals, the disease may occur in the absence of a definite history of acute PE. It is unknown why some individuals develop chronic occlusion of the pulmonary vasculature after an acute pulmonary embolus, whereas others do not. However, a number of factors are known to predispose to abnormal thrombus resolution (Table 1.4) (Piazza and Goldhaber, 2011, Kim and Lang, 2012, Lang et al., 2013, Bonderman et al., 2009).

As with other types of pulmonary hypertension, persistently elevated pulmonary vascular resistance ultimately results in right heart failure. However, there is a curative treatment available for CTEPH-pulmonary endarterectomy. This involves surgical removal of the organised fibrous material from the lumen of proximal occluded vessels (Figure 1.4) along with resection of the neointima, whilst under cardiopulmonary bypass (Jenkins, 2015). In experienced centres, operative mortality is <5% (Jamieson et al., 2003) with very favourable long term clinical outcomes (Delcroix et al., 2016).

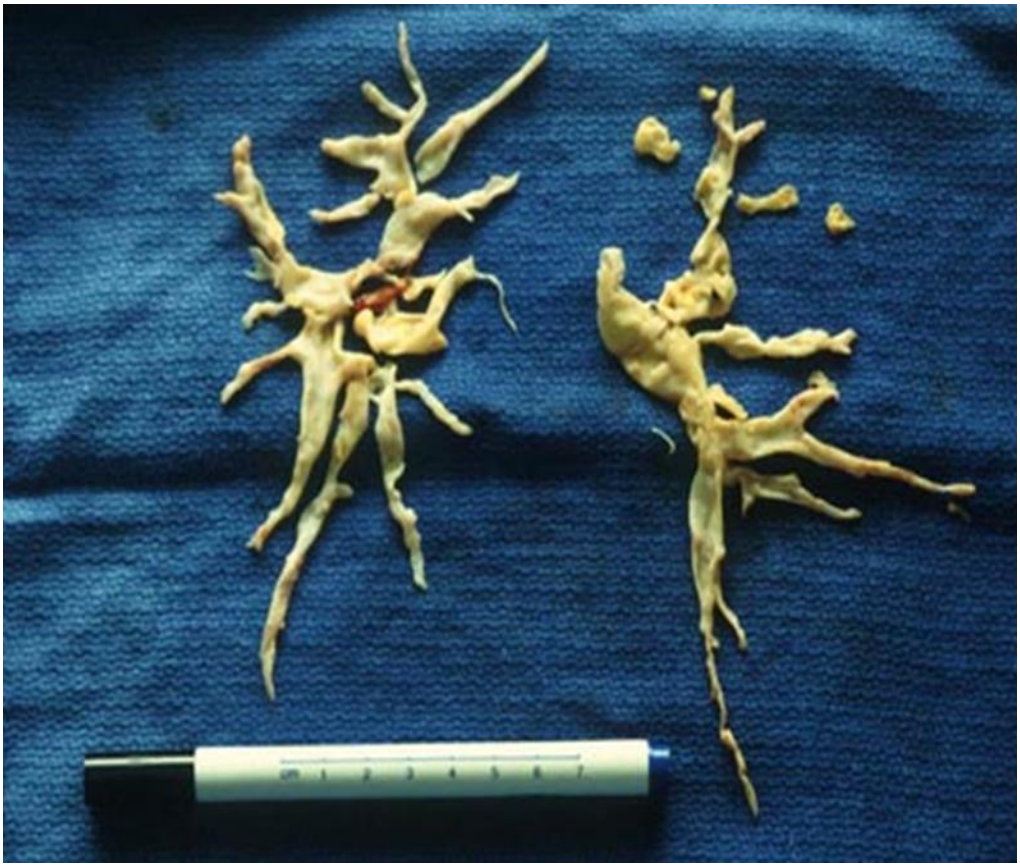


Figure 1.4- Pulmonary endarterectomy specimen. Organised fibrotic chronic vascular occlusion, removed from the pulmonary arteries during pulmonary endarterectomy. (Image courtesy of Mr David Jenkins, Papworth Hospital).

In inoperable cases where disease is in surgically inaccessible sites, patients may be treated with medical vasodilator therapies (Galiè et al., 2015). These therapies may also be used in cases where a patient is deemed unsuitable for surgery due to comorbidities or those in whom distal arteriopathy is thought to be the principal contributor to elevated pulmonary vascular resistance. More recently, the technique of balloon pulmonary angioplasty has been developed, providing an additional therapeutic option for selected CTEPH patients (Sato et al., 2016).

Table 1.4- CTEPH risk factors.

PE related factors
• Recurrent, unprovoked or idiopathic PE • Large perfusion defects when PE was detected • Younger or older age when PE was detected • PASP >50 mmHg at PE first manifestation
Haematological factors
• Lupus anticoagulant or antiphospholipid antibodies • Dysfibrinogenaemia • ABO blood groups other than O • Increased factor VIII • Increased lipoprotein(a) • Protein S and C deficiency • Anti-thrombin III deficiency • Activated Protein C resistance • Factor V Leiden mutation, prothrombin gene mutation
Other medical conditions
• Infected pacemaker or defibrillator leads, VA shunt • Splenectomy • Chronic inflammatory disorders (e.g. Inflammatory bowel disease, RA) • Hypothyroidism • Cancer

1.16 CTEPH pathobiology

The characteristic histological finding in CTEPH is occlusion of the proximal pulmonary arteries by eccentric, irregular intimal fibrosis and intravascular fibrous septa. Small areas of recanalisation in occluding organised thrombus may also be found, referred to as “colander” lesions (Wagenvoort, 1995, Lang et al., 2016, Simonneau et al., 2017).

In contrast with PAH, hyperplasia of the vessel media is not a significant feature (either mild or absent). However, it is recognised in addition to the occlusion of the large proximal pulmonary arteries in CTEPH, a distal arteriopathy is found in the arterioles and capillaries downstream. (Lang et al., 2016, Simonneau et al., 2017). These vessels show some similar changes to advanced PAH. Therefore, the increase in vascular resistance which occurs in CTEPH is not only due to the proximal fibrous occlusions, but also related to vascular dysfunction distal to the occluded arteries.

1.17 Chronic thromboembolic pulmonary vascular disease (CTED)

Chronic thromboembolic disease (CTED) is characterised by pulmonary vascular thromboembolic occlusions that persist despite anti-coagulation, without development of pulmonary hypertension.

Currently the natural history of CTED is poorly defined, but it is thought that CTED and CTEPH share the same underlying aetiology, and these two conditions may be considered as different ends of a spectrum of disease which results from failure of normal resolution of acute pulmonary emboli.

As routine follow up imaging after acute pulmonary embolism is not commonplace, the true prevalence of this condition is unknown. However, it is apparent that a significant proportion of individuals who suffer a symptomatic pulmonary embolus are left with persistent perfusion defects on imaging after at least 3 months of anticoagulation (Cosmi et al., 2011, Wartski and Collignon, 2000). The incidence may be as high as 50% (Nijkeuter et al., 2006), with larger initial clot burden increasing the likelihood of persistent perfusion defects (Alhadad et al., 2012). It is not clear why some patients with chronic unresolved pulmonary emboli develop PH and others do not, despite a similar burden of vascular occlusion seen on imaging.

Although many individuals with CTED will be asymptomatic, a proportion suffer symptomatic limitation, despite a mPAP below the diagnostic cut-off for PH and normal cardiac chamber size. These individuals have been shown to have impaired adaptation to exercise with delayed right ventricular relaxation and inefficient gas exchange on exertion (McCabe et al., 2013, McCabe et al., 2014). In contrast to CTEPH, pulmonary endarterectomy is not routinely recommended for patients with CTED. However, in carefully selected cohorts of CTED patients, pulmonary endarterectomy may result in improvement in symptoms and quality of life (Taboada et al., 2014).

1.18 The Immune Hypothesis: Inflammation and immunity in PAH

The association of PAH (particularly IPAH) with immune dysregulation has long been recognised (Rich et al., 1986, Asherson et al., 1984). However, the role of immune dysfunction in the disease pathogenesis remains poorly understood. Evidence of inflammation is frequently observed in the vascular lesions in PAH. It is uncertain whether this is of pathogenic importance or occurs as an epiphenomenon of the disease process. Other evidence indicating a role for inflammation and systemic immune disturbance in the disease process includes an association with connective tissue diseases (CTD) and other autoimmune conditions, increased frequency of circulating autoantibodies in individuals with PAH, increases in pro-inflammatory cytokines and abnormalities in circulating immune cells in peripheral blood. These aspects are further discussed in this section.

1.18.1 Local histological changes in the lung

Structural changes are found in PAH lungs consistent with an inflammatory mediated immune response. Changes within the arterial wall have been reported including elevated levels of RANTES (an important chemoattractant for monocytes and T-cells) and the presence of T lymphocytes (Dorfmüller et al., 2002), and increased numbers of dendritic cells in the arterial wall (Perros et al., 2007).

In addition to changes within the vessel wall, perivascular changes are also present. Infiltrates consisting of B and T lymphocytes and macrophages are found around the diseased vessels (Fig 1.5) (Tuder et al., 1994), with subsequent formation of tertiary lymphoid follicles (Fig 1.5) (Perros et al., 2012). Lymphoid neogenesis in the target organ is considered to be a hallmark of autoimmune diseases. For example, this feature is found in the joints and lungs in rheumatoid arthritis, the pancreas in autoimmune diabetes and salivary glands in Sjogren's syndrome (Pitzalis et al., 2014). This suggests that pulmonary lymphoid neogenesis in IPAH represents a local immune response to threat by pathogen or antigen in the lungs.

Additionally, in the peripheral lung of IPAH patients, 4-fold increases in the number of CD3+/CD8+ T lymphocytes have been reported, compared to healthy controls (Austin et al., 2010b). These changes were not limited to the areas of vascular remodelling, suggesting a more widespread process of immune activation in the lung.

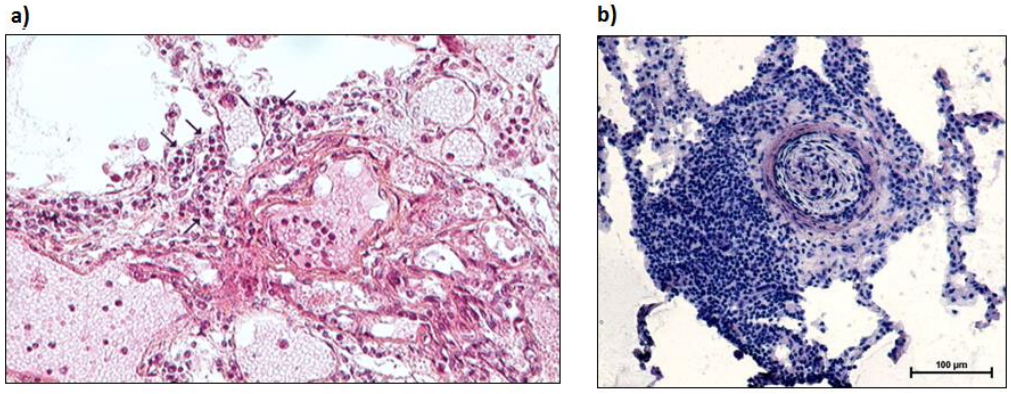


Figure 1.5- IPAH lung tissue histology. Haematoxylin and eosin examination of lung biopsy specimens from patients with IPAH; a) plexiform lesion with strong, mainly lymphocytic perivascular inflammatory infiltrate (Dorfmüller et al., 2003), b) lymphoid follicles adjacent to remodelled vessels (Perros et al., 2012).

1.18.2 Association with inflammatory states and autoimmune conditions

Pulmonary arterial hypertension occurs as a secondary complication to many connective tissue diseases such as systemic sclerosis where there is a reported prevalence of 8-14%, (Hachulla et al., 2005), systemic lupus erythematosus where prevalence is 0.5-14% (Haas, 2004) and others including mixed connective tissue disease (MCTD), polymyositis and primary Sjogren's syndrome. PAH is also associated with other immune-mediated conditions including HIV (Schwarze-Zander et al., 2015), and schistosomiasis (Mauad et al., 2014).

For over 30 years, the hypothesis that autoimmune mechanisms may be implicated in IPAH has been suggested (Holt et al., 1980), with speculation that IPAH is an autoimmune disease localised to the pulmonary vasculature. Indeed, there are a number of features in common between IPAH and autoimmune conditions. For example, both IPAH and autoimmune conditions traditionally show a female predominance and similar age distribution. The percentage of female IPAH patients is 60-83% according to various registries (McGoon et al., 2013). Additionally, there is a higher prevalence of autoimmune conditions in those with IPAH, suggesting a common immunogenetic susceptibility. In particular, there is a high prevalence of autoimmune thyroid disease in IPAH, which is 3-4 times greater than in age and sex matched cohorts (Wawrzyńska et al., 2004).

1.18.3 Circulating autoantibodies

Many autoantibodies are detected with increased frequency in IPAH, including anti-nuclear antibodies, anti-ssDNA, anti-phospholipid and anti-Ku antibodies (Rich et al., 1986). More recently, circulating autoantibodies directed against endothelial cells and fibroblasts have been detected in the serum of patients with IPAH and SSc-PAH (Tamby et al., 2005, Tamby et al., 2006), adding additional weight to the theory that autoimmune mechanisms are implicated in abnormal vascular remodelling.

1.18.4 Circulating immune cell abnormalities

1.18.4.1 Lymphopenia

A high prevalence of peripheral blood lymphopenia amongst various PH subtypes has been reported (Ulrich et al., 2006). However, the mechanism responsible for this and the role of lymphopenia in PAH pathobiology has not been explored. It is unclear whether lymphopenia may be implicated in the disease pathogenesis, whether it is secondary to heart failure, the treatment of the disease or whether it is an epiphenomenon of the chronic disease process.

Lymphopenia has recognised associations with immune-mediated conditions such as Sjogren's syndrome (Kirtava et al., 1995), SLE (Hochberg, 1997), Wegener's granulomatosis (Izzedine et al., 2002) and Rheumatoid Arthritis (Duquenne et al., 2015). In these diseases, peripheral blood lymphopenia may occur by a number of mechanisms including: reduced lymphocyte production due to bone marrow suppression, increased destruction through defective production or stress from unresolved antigenic stimulation, anti-lymphocyte antibodies and changes in lymphocyte distribution in intravascular and organ compartments.

Manipulations that generate functional T cell lymphopenia in animal models result in the development of a variety of autoimmune diseases (Barrett et al., 1995), thought to occur by allowing expansion of autoreactive T cells. Similarly, in an animal model of pulmonary hypertension, absence of T cells promotes development of the disease and vascular remodelling (Taraseviciene-Stewart et al., 2007), raising the question as to whether T cell deficiency may facilitate or potentiate development of pulmonary arterial hypertension.

1.18.4.2 T Lymphocytes

There has been particular focus on regulatory T cells in IPAH, which play an important role in maintaining self-tolerance and preventing inappropriate autoimmune responses by suppressing activation and expansion of self-reactive T cells. They are qualitatively and/or quantitatively deficient in many autoimmune conditions such as SLE, RA, graft versus host disease and multiple sclerosis (MS) (Bonelli et al., 2008). Increases in circulating regulatory T cells in the peripheral blood in IPAH have been reported in some studies (Ulrich et al., 2008a, Sada et al., 2016), whereas others have demonstrated deficiencies in Treg function (Huertas et al., 2012, Huertas et al., 2016).

Abnormalities in CD8+ T cells have also been reported, although results have been conflicting. Austin *et al.* reported significant increase in CD45RA+CCR7- cytotoxic effector memory cells and reduction in CD45+CCR7+ naïve CD8+ cells (Austin et al., 2010a). These findings were not replicated by Ulrich and colleagues who in fact found CD8+ T lymphocytes to be globally diminished in IPAH compared to controls (Ulrich et al., 2008a).

1.18.4.3 B lymphocytes, Natural Killer cells, monocytes and dendritic cells

Perturbations in other circulating immune cell subsets have also been reported, including altered gene expression by peripheral blood B lymphocytes suggesting B cell activation (Ulrich et al., 2008b), reduced activation of monocytes (Raychaudhuri et al., 2002a) and impaired natural killer cell phenotype and function (Ormiston et al., 2012).

1.18.4.4 Association with haematological conditions

PAH is found to occur in association with POEMS syndrome (Wang et al., 2017, Li et al., 2013) and multicentric Castleman's disease (Bull et al., 2003), raising the possibility of mechanistic links between these lymphoproliferative disorders and pulmonary vasculopathy. There has been some speculation that development of PAH in this setting may be a cytokine mediated process, which may be ameliorated by immunomodulatory therapy (Taniguchi et al., 2009).

1.18.5 Cytokines, chemokines and C- reactive protein

Cytokines in the lung are integral to the initiation and maintenance of immune and inflammatory responses. High levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNF alpha are found in patients with IPAH and CTD associated PAH (Humbert et al., 1995), and correlation between the levels of inflammatory cytokines and survival in IPAH and familial PAH has been demonstrated (Soon et al., 2010).

Elevated levels of inflammatory chemokines including CX3CL1 (Balabanian et al., 2002), MIP1α (Fartoukh et al., 1998) and CCL2 (Sanchez et al., 2007) have also been reported in IPAH. Additionally, levels of C reactive protein (CRP) are increased in PAH, with higher levels predicting worse response to therapy and decreased survival (Quarck et al., 2009).

1.18.6 Viral infection and PAH

A causal relationship between viral infection and development of PAH has not been established, however there is speculation that viral infection of pulmonary endothelial cells could be the trigger for initial apoptosis and subsequent angiogenic activity and proliferation (Cool et al., 2011). There are recognised associations between HIV infection and PAH, which develops in 0.5% individuals with HIV (Sitbon et al., 2008). It is plausible that viral infection may lead to exposure of epitopes not normally exposed and thereby trigger an autoimmune response, or that infection and associated inflammation provide a permissive environment for other initiating factors.

1.18.7 Therapeutic targeting of inflammation and immune dysfunction in PAH

There is some evidence to suggest that current drug therapies used in PAH treatment play a role in modulating the inflammatory components of PAH, in addition to their vasodilatory effects. Treprostinil has been demonstrated to inhibit inflammatory cytokine secretion and gene expression

by alveolar macrophages from healthy human volunteers (Raychaudhuri et al., 2002b) and in children, epoprostenol and bosentan have been found to reduce endothelial HLA-DR expression (Hall et al., 2009).

In animal models of PH, a number of anti-inflammatory and immunomodulatory treatments including glucocorticoids (Price et al., 2011), mycophenolate mofetil (Suzuki et al., 2006), rapamycin (Houssaini et al., 2013), triptolide (Faul et al., 2000) and the TNFα inhibitor etanercept (Sutendra et al., 2011) have been shown to attenuate development of the disease. Unfortunately, many agents which have shown promise in animal models do not result in similar success in humans with the disease. However, in humans there have been reports of significant improvement in PAH associated with CTD (particularly SLE) after immunosuppressive treatment, and in some cases even complete reversibility of PAH (Jais et al., 2008), demonstrating that effective treatment of the inflammatory condition can ameliorate the associated PAH.

Recent attention has been given to a potential role for tyrosine kinase inhibitors in attenuating pulmonary arterial remodelling. Similarly, despite promising effects in animal models (Schermuly et al., 2005), this success has not been replicated in human clinical trials. Imatinib as an add-on therapy in patients already on dual targeted therapy resulted in only a modest improvement in clinical outcomes and drug discontinuations were high, with significant serious adverse events (Hoeper et al., 2013a). Therefore, the search continues for new therapies which can effectively target immune and inflammatory mechanisms in PAH.

It is clear that inflammatory and immune responses contribute to the abnormal vascular remodelling which is central to development and progression of the disease. However, major questions remain unanswered, including: What triggers and propagates the abnormal host immune and inflammatory responses? Is autoimmunity implicated in this response? Why is the response localised to the pulmonary vasculature? Can we characterise immune profiles which identify patients who may respond to immunomodulatory treatment, allowing effective targeted therapy?

1.19 The Metabolic Hypothesis: Metabolic dysfunction in PAH

There is increasing evidence to suggest both local and systemic metabolic derangements in pulmonary arterial hypertension. However, it remains unclear as to whether metabolic abnormalities in PAH are fundamentally implicated in disease pathogenesis and progression or whether they are simply a marker of the disease process.

1.19.1 Changes in the metabolic function of the pulmonary circulation

Early evidence of impaired metabolic function of the pulmonary circulation in pulmonary hypertension was provided by Sole and colleagues (Sole et al., 1979) who demonstrated impaired clearance of noradrenaline in PH. They found that clearance by the pulmonary circulation was <2% in PH compared to ~25% in healthy individuals (Sole et al., 1979). This was followed by a number of studies demonstrating altered metabolism of substances which play a key role in the disease pathobiology, such as the vasoconstrictor endothelin which may undergo both excess synthesis and reduced clearance (Dupuis et al., 1998, Langleben et al., 2006). Additionally, studies have confirmed that this metabolic dysfunction can be ameliorated by PAH therapies (Langleben et al., 1999).

It must also be remembered that in addition to functional impairment of the vascular bed, the effects of raised pulmonary artery pressure and reduced cardiac output have consequences for distant organs. This includes 'back pressure' effects on liver and 'forward pressure' effects due to reduced cardiac output (thereby reduced perfusion of skeletal muscle, kidneys and other organs), which may also influence systemic metabolite profile. A key aspect in studying the metabolic function of the pulmonary circulation concerns the localisation of metabolic processes to the vasculature itself, which presents a number of challenges, which as discussed further in section 1.19.3.

1.19.2 Systemic metabolic abnormalities in PAH

Although pulmonary arterial hypertension is considered a disease localised to the lung vasculature, there has been recent research suggesting more widespread metabolic disturbances. This includes alterations in energy metabolism and evidence that metabolic factors are implicated the way that the right heart adapts to stress.

The hypothesis that PAH is a multi-organ metabolic disorder has recently emerged, with some researchers going as far as to suggest that global mitochondrial abnormalities may underpin the disease pathogenesis (Dromparis et al., 2010, Sutendra and Michelakis, 2014). This is supported by a number of studies in animal models, where metabolic changes predate development of elevated pulmonary pressures, and penetrance and severity of disease can be modulated by interventions against metabolic derangements (West et al., 2013, Rafikova et al., 2016, Michelakis et al., 2002).

1.19.2.1 Mitochondria in PAH

In health, cellular energy metabolism predominantly consists of ATP production by oxidation of pyruvate in the mitochondria. However, in PAH, mitochondria are hyperpolarised and their respiration is depressed, resulting in reduced mitochondrial ATP production and increased cytoplasmic glycolysis (Xu et al., 2007).

This 'glycolytic shift' in cellular energy metabolism is akin to the metabolic change observed in proliferating cancers, known as the Warburg effect (Warburg, 1956). This is manifest by predominant energy production via glycolysis followed by lactic acid fermentation in the cytosol. The Warburg effect is considered an adaptive mechanism exhibited by rapidly proliferating cells that allows for unrestrained growth. Additionally, mitochondria-dependent apoptosis is suppressed in PAH; a further similarity with cancer cells (Archer et al., 2008).

It is unclear whether the features observed are due to a primary intra-mitochondrial abnormality or a generalised extra-mitochondrial trigger that suppresses mitochondrial function, such as inflammation. In addition to being affected by systemic processes such as inflammation, the mitochondria themselves may have wider systemic effects besides those involved in energy metabolism by secretion of 'mitokines' and activation of the inflammasome NLPR3 (Kepp et al., 2011).

1.19.2.2 Insulin resistance and lipid profile in PAH

Dysregulated glucose metabolism and insulin resistance have well recognised effects in the systemic circulation, where this phenotype is associated with an inflammatory environment and endothelial dysfunction. It is now apparent that these factors may act as a modifier of pulmonary vascular disease, contributing to more severe disease. Individuals with PAH have been shown to have increases in insulin and HbA1c and abnormal lipid profile, biochemically resembling the metabolic syndrome, although they are neither obese nor diabetic (Zamanian et al., 2009).

1.19.2.3 Right ventricle metabolism

RV response to chronic pressure overload may result in adaptive RV hypertrophy with relatively preserved ejection fraction or maladaptive changes characterised by RV ischaemia, dilatation and hypokinesis. The structural changes which occur in the right ventricle are accompanied by a change in energy utilisation. Under normal circumstances, fatty acid oxidation is the primary cardiac energy source, whereas glucose metabolism is a secondary source. In health, the RV can switch its energy utilisation from fatty acids to glucose as needed. However, in dysfunctional RVH there is persistent

reliance on glucose metabolism, characterized by decreased expression of genes involved in fatty acid and glucose oxidation and impaired mitochondrial respiration (Gomez-Arroyo et al., 2013). This increase in glycolysis can be demonstrated by increased uptake of FDG-PET (Wang et al., 2016a).

Glycolysis in the context of ischaemia results in reduced contractility of RV myocytes, exacerbates RV impairment and worsens RV dysfunction, creating a vicious cycle. Therefore, in parallel with the altered cellular metabolism occurring in the PH pulmonary vasculature, right ventricular myocytes also develop an altered metabolic phenotype. (Piao et al., 2010). It is also recognised that systemic metabolic derangements such as insulin resistance and the metabolic syndrome influence right ventricle structure and function (Tadic et al., 2011, Zamanian et al., 2012). Additionally, presence of BMPR2 mutation in animal models has been shown to affects RV stress response, with impaired RV hypertrophy and lipid deposition in the ventricle (Hemnes et al., 2014).

1.19.3 Methods to assess the metabolic function of the pulmonary circulation

1.19.3.1 Measurement of transpulmonary gradient of substances

By comparing the nature and concentration of substances in blood prior to entering the pulmonary circulation with blood which has passed through the pulmonary circulation (e.g. pulmonary artery vs aorta), we can make inferences about the biological processes occurring in this circuit. The difference in the quantity of substances is referred to as the transpulmonary gradient. This technique has been used in assessing the gradient of a limited number of substances including endothelin, catecholamines and growth factors (Wilkens et al., 2003, Sole et al., 1979), and has been used to assess the effect of therapeutic agents on their target pathways (Langleben et al., 1999).

1.19.3.2 Indicator dilution method

This method is based upon detection of the amount of a particular substance which survives a single transpulmonary passage. It can be used to assess pulmonary endothelial ectozyme activity *in vivo*. It has predominantly been used to assess pulmonary endothelial ACE and endothelin receptor ETB activity. A radiolabelled substance is injected as rapid bolus into a central vein and arterial blood is simultaneously withdrawn by a peristaltic pump into a fraction collector. A ‘STOP’ solution is added to prevent further metabolic activity. The amount of radioactivity associated with the substrate which survived the transpulmonary passage is then quantified.

For example, pulmonary endothelial ACE activity can be assessed using a radiolabelled synthetic peptide specifically metabolised by ACE (3H-BPAP) (Orfanos et al., 1999). PCEB-ACE is uniformly

distributed along the luminal endothelial surface; therefore, its activity can be used to assess functional capillary surface area. Pulmonary endothelial ACE dysfunction has been shown to be an index of lung vascular injury (Orfanos et al., 2000).

1.19.3.3 Metabolic imaging- Detection of metabolic glycolytic shift using 18F-FDG PET

Positron Emission Tomography (PET) scanning has developed as a useful tool in investigation and diagnoses of malignancies through detection of increased glucose metabolism demonstrated by cancer cells. This imaging modality has more recently been applied in a number of vascular diseases such as atherosclerosis (Rudd et al., 2002, Rudd et al., 2007) and large vessel vasculitis (Bucerius, 2016), where inflammatory lesions display enhanced 18F-fluorodeoxyglucose (18F-FDG) uptake.

In cell culture, IPAH pulmonary artery endothelial cells display higher rates of glycolytic metabolism (Xu et al., 2007). A number of studies have investigated whether FDG PET could be useful in non-invasive assessment of the inflammatory remodelling and abnormal metabolism in PH. In animal models (murine monocrotaline and Sugen hypoxia PAH models), there is increased pulmonary 18F-FDG uptake, which occurs early and correlates with disease severity (Marsboom et al., 2012). However, studies in humans have highlighted a number of difficulties in clinical application of 18F-FDG PET scanning in pulmonary vascular disease. Although increased uptake of 18F-FDG tracer in IPAH lungs has been demonstrated, (Hagan et al., 2011) there is wide variability between subjects, heterogeneous distribution within the lungs and a lack of specificity for the lung vasculature (Hagan et al., 2011). Therefore, this strategy has not found practical clinical application in PAH.

1.19.3.4 Metabolomics

Traditionally, techniques to assess metabolism have been limited to a very narrow approach, usually targeted to a single substance or biochemical reaction. However, recent technological advances in the field of metabolomics now permit simultaneous assessment of thousands of metabolites in a tissue, organ or system. This technology can be used in a targeted fashion to study particular metabolites of interest, or in an untargeted fashion to assess the 'metabolome' of a system. It provides a snapshot of a multiple interconnected metabolic processes and can be used to assess the changes in metabolite milieu that occur in a disease or in response to an intervention.

Using an untargeted metabolomics approach, previously unrecognised metabolic derangements are being detected in many diseases. This is paving the way for advances in our understanding of complex disease processes, as well as aiding biomarker discovery, development of new diagnostic

tests, improved disease phenotyping and personalised therapies. For example, identification of hydroxybutyrate as an early biomarker for insulin resistance has resulted in development of the Quantose IR™ blood test, which is now used for clinical assessment, risk stratification and monitoring of patients and allows early targeting of disease modifying interventions (Milburn and Lawton, 2013) and metabolomics has been used to identify urinary metabolites which are sensitive markers of drug toxicity (Boudonck et al., 2009).

In PAH, several recent studies have used metabolomics technology to identify metabolites and pathways which may be important in the disease pathobiology. In cultured human pulmonary microvascular endothelial cells expressing BMPR2 mutations, increases in aerobic glycolysis, upregulation of the pentose phosphate pathway, increases in nucleotide salvage and polyamine biosynthesis, decreases in carnitine and fatty acid oxidation and impairment of the TCA cycle have been demonstrated (Fessel et al., 2012).

Metabolomic analysis of explanted lung tissue from patients with very advanced pulmonary arterial hypertension has also detected changes in glucose and fatty acid metabolism and the TCA cycle. Additionally, increased levels of multiple bile acid metabolites were found in lung tissue, leading the researchers to speculate that de-novo synthesis of bile acids may occur in the PAH lung (Zhao et al., 2014b). Metabolomic analysis of peripheral blood has recently provided further evidence of disrupted energy metabolism in PAH (Bujak et al., 2016), and has identified metabolites which distinguish IPAH and HPAH patients from healthy controls and metabolites which are prognostic (Rhodes et al., 2017). Metabolomic analysis of exhaled breath condensate has also identified metabolites which may distinguish patients with very severe IPAH from healthy controls (Mansoor et al., 2014).

By providing simultaneous assessment of multiple metabolic pathways, metabolomics technology is allowing us to more fully appreciate the extent the metabolic alterations in pulmonary vascular disease. However, it is unclear which of these metabolic alterations are attributable to pulmonary vascular endothelial dysfunction, changes in right heart metabolism or are indicative of systemic metabolic changes. Further research to address this question and to determine the mechanism of these metabolic changes is required.

1.20 The link between inflammatory and metabolic aspects of PAH pathogenesis

There is an evolving paradigm of PAH as an inflammatory disease in which there is immune dysregulation. This is supported by evidence of lung histological changes and disturbances in

circulating immune factors including cytokines, chemokines, autoantibodies and immune cells. Currently, it is unclear how inflammation may contribute to the pathogenesis of PAH. Indeed, it is possible that inflammation may initiate vascular remodelling (may be an “initial hit”), may be integral in its propagation (a “secondary hit”), or just be a reactive response to ongoing remodelling (“bystander” phenomenon).

It is likely that PAH develops as a consequence of multiple contributing factors including a permissive genotype, susceptible phenotype and exogenous triggers. In a susceptible individual, altered immune responses may result in transformation of a self-limited inflammatory response into a perpetuating injurious process. Given that vascular endothelium is distributed throughout the body, the question remains as to why the disease specifically affects the pulmonary circulation? Whether this is a reflection of a unique feature of the pulmonary circulation and its response to insult or injury or perhaps pathogen or autoantigen localisation to the lung circulation remains uncertain.

It is clear that immunological and inflammatory processes result in cellular metabolic changes. Therefore, immune and inflammatory disturbances are mechanistically linked to disturbances in metabolic processes. An example of this interplay is the induction of a PAH phenotype in PASMC via inhibition of pyruvate dehydrogenase activity by TNFα (Sutendra et al., 2011), demonstrating a link between excessive inflammation and altered PASMC glucose metabolism. Likewise, disturbances in metabolic function may not only act as a marker of abnormal vascular endothelial cell activity occurring as a consequence of the disease process, but metabolic factors such as insulin resistance may play an integral role in disease evolution and progression.

It is highly probable that the full extent of metabolic processes that occur in the pulmonary circulation is not yet appreciated, both in health and disease of this system. The nature of these metabolic processes and changes which reflect or influence disease pathology require further investigation and characterisation.

1.21 Fundamental concepts explored by this thesis

In pulmonary vascular disease, there are many routes by which abnormal endothelial cell responses and vascular remodelling may be initiated and propagated. There is an accumulating body of evidence linking both immune and metabolic derangements to pulmonary vascular disease. However, key questions remain as to how immunological and metabolic processes are a) implicated in and b) affected by the disease.

Additionally, the current ability to accurately phenotype disease subtypes within this heterogeneous syndrome is limited and advances in disease phenotyping are required for effective targeting of therapies.

Part 1 - Immunophenotyping

Using peripheral blood immunophenotyping, this thesis will explore the following questions:

1. Do patients with Idiopathic Pulmonary Arterial Hypertension have a peripheral blood immune cell 'signature' which differs from healthy controls?

2. Does the peripheral blood immune cell phenotype shed further light on the contribution of immunological mechanisms to the disease pathobiology?

Part 2 - Metabolomics

Using untargeted metabolomic profiling of circulating metabolites, including pre- and post- lung sampling, the following questions will also be explored:

1. Does the pulmonary circulation have previously unrecognised metabolic functions?

2. Is there a metabolic 'signature' which characterises pulmonary vascular diseases?

3. Does the metabolic phenotype shed further light on the contribution of metabolic mechanisms to the disease pathobiology?

2 Chapter 2- Study population, materials and methods

2.1 Part 1 - Immunophenotyping

2.1.1 Study population

Subjects were recruited prospectively from the Pulmonary Vascular Diseases Unit, Papworth Hospital, Cambridge UK. The unit is one of the 9 designated centres across the UK and Ireland providing specialist investigation and management for patients with pulmonary hypertension. All recruited patients provided written consent to participate under the Papworth Hospital tissue bank ethical approval (Donation for the collection and storage of human biological material for research; Cambridgeshire East Research Ethics Committee reference 08/H0304/56, tissue bank project number T01990). All recruited healthy controls provided written consent as per the Papworth Hospital pathology laboratory protocol (Version 1.1, 15th October 2013).

2.1.1.1 IPAH patients and healthy controls

28 IPAH patients and 28 healthy age and sex matched controls were recruited. IPAH patients enrolled met standard diagnostic criteria for IPAH, with presence of pulmonary arterial hypertension having been previously established by right heart catheterisation. This is defined by a mean pulmonary artery pressure (mPAP) ≥ 25mmHg, pulmonary capillary wedge pressure (PCWP) ≤ 15mmHg, pulmonary vascular resistance (PVR) > 3 Wood units and exclusion of other causes of pulmonary hypertension. Both incident and prevalent IPAH cases were recruited. Exclusion criteria included recent or active infection or use of medication known to alter immune cell profile.

Patients and healthy control subjects were matched according to age, gender, body mass index (BMI) and smoking status, as these are factors known to influence peripheral blood lymphocyte subsets (Santagostino et al., 1999). Controls were healthy staff members from Papworth Hospital. Control subjects were asked a series of screening questions to ensure no significant co-morbidity, immunomodulatory medication, active or recent infection or any known haematological disorder.

2.1.1.2 Other Pulmonary hypertension subgroups: HPAH, SSc-PAH and CTEPH

In addition to the IPAH patients recruited, 9 patients with HPAH, 12 patients with systemic sclerosis associated PAH (SSc-PAH) and 21 patients with chronic thromoboembolic pulmonary hypertension (CTEPH) were recruited. Both incident and prevalent cases were recruited. All patients recruited met

diagnostic criteria for pulmonary hypertension (Galiè et al., 2015). All IPAH and HPAH patients were also enrolled in the National Cohort Study of Idiopathic and Heritable PAH (COHORT). Therefore, HPAH patients had undergone whole genome DNA sequencing, with confirmation of the presence of a pathogenic mutation in the BMPR2 gene. SSc-PAH patients had disease confirmed according to the ACR/EULAR 2013 criteria for the classification of systemic sclerosis (van den Hoogen et al., 2013). CTEPH patients had a diagnosis made at the national surgical referral centre MDT with a minimum of two imaging modalities consistent with chronic thromboembolic pulmonary vascular occlusion with exclusion of other underlying causes of PH.

2.1.2 Peripheral blood immunophenotyping method

The immunophenotyping assay for this research was performed by Natalia Savinykh and Simon McCallum, National Institute for Health Research (NIHR) Biomedical Research Centre Immunophenotyping service, Department of Medicine, Addenbrooke's Hospital, Cambridge, UK.

2.1.2.1 Immunophenotyping - an overview

Immunophenotyping allows detection of specific cell subsets within a mixed population, according to their characteristic cell surface markers. Cells are stained and incubated with fluorescently labelled antibodies, designed to bind to the cell surface markers of interest. Labelled cells are then passed through a flow cytometer and subclasses detected based upon their size, internal complexity and the fluorescence emitted by the labelled antibodies bound by the cell [Figure 2.1].

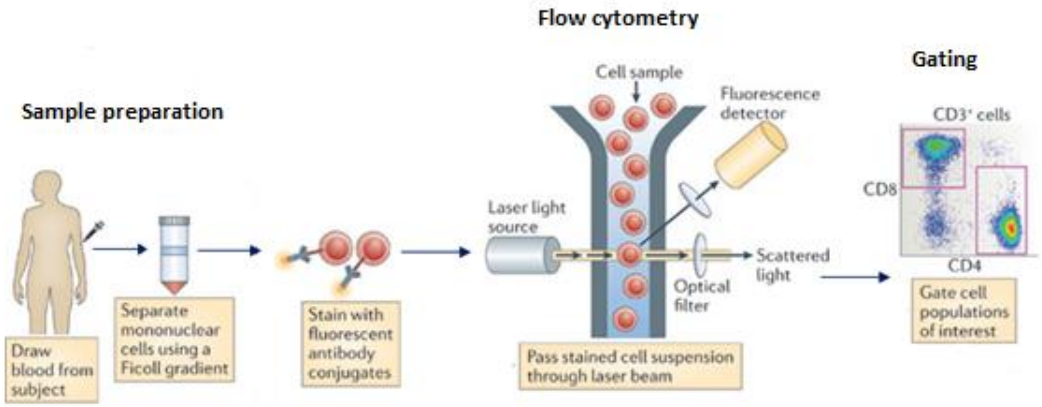


Figure 2.1- Overview of the Immunophenotyping process. Whole blood is drawn from the subject and PBMCs are separated from other blood components by density gradient separation. Cells are stained with fluorescently labelled antibodies and passed through a flow cytometer. Cell populations of interest are then identified according to the fluorescence emitted by the antibody conjugates bound to their cell surface markers (adapted from (Maecker et al., 2012)).

2.1.2.2 Blood sample collection

Whole blood was drawn from each subject by peripheral upper limb venepuncture. Samples from patient and control were taken within 1 hour of each other, to avoid effects which may be seen due to circadian variation in leukocyte profile. Blood was collected into S-Monovette® trisodium citrate blood tubes (Sarstedt AG & Co, Nümbrecht, Germany) through a 21-gauge needle. Fresh whole blood samples were processed as outlined below, to obtain peripheral blood mononuclear cells (PBMCs) which were then prepared for immediate immunophenotyping.

2.1.2.3 Peripheral blood mononuclear cell (PBMC) extraction

PBMCs were isolated from citrated whole blood by Histopaque 1077 (Sigma-Aldrich, Missouri, USA) density gradient separation. MACS (magnetic activated cell sorting) rinsing buffer was constituted by mixing 996mls 1 x phosphate buffered saline (PBS) with 4mL 0.5M ethylenediaminetetraacetic acid (EDTA). MACS running buffer was constituted by mixing 996mL 1 x PBS with 4mL 0.5M EDTA and 5g bovine serum albumin (BSA). Whole blood was diluted with MACS rinsing buffer to give a total volume of 150mL and was then mixed by inversion. The diluted blood was layered over the 15mL Histopaque in 4 x 50mL Falcon tubes (BD Biosciences, Oxford, UK), with an equal volume in each of the 4 tubes. The tubes were centrifuged at 700x*g* for 20 mins (ThermoScientific ST16R centrifuge, Waltham, USA) at room temperature.

Following centrifugation, 20-25mL of plasma was aspirated from each Falcon tube, then the PBMC interface was aspirated and transferred into a new Falcon tube. The PBMC interface from 2 tubes were then transferred into one Falcon tube, resulting in two PBMC-containing Falcon tubes. Rinsing buffer chilled to 4^{o}C was added to the two PBMC-containing Falcon tubes to give a volume of 50mL per tube, with mixing by inversion. This was followed by centrifugation at 700x*g* for 10 mins at 4^{o}C. Supernatant was poured off and cell pellets dispersed by flicking the tubes. Cells were resuspended in 50mL rinsing buffer chilled to 4^{o}C. This was followed by centrifugation at 200*xg* for 10 mins at 4^{o}C. Once again, supernatant was removed, cell pellets were dispersed by flicking the tubes and then were resuspended in 50mL running buffer, chilled to 4^{o}C.

Cell viability was then determined by trypan blue exclusion. A 5µL aliquot of cell suspension was removed and stained with trypan blue (Sigma Aldrich Ltd, Dorset, UK). The trypan blue treated cell suspension was then pipetted under the cover slip of a haemocytometer and live (unstained) cells and dead (stained) cells were counted to determine percentage viability. Samples included for analysis had a percentage viability of ≥80%. The PBMC suspension was again centrifuged for 8 mins at 4^{o}C and the supernatant was removed. PBMCs were divided into aliquots for each of the

immunophenotyping panels and resuspended in chilled running buffer at a concentration of 1x 10^6 cells/ml (1.5 x10^6 cells/mL for the B cell panel).

2.1.2.4 Immunofluorescence staining of peripheral blood mononuclear cells (PBMCs)

The immunophenotyping panels were adapted from the Human Immunology Project (Maecker et al., 2012). Antibodies were pipetted into polystyrene FACS tubes (one for each panel) [Table 2.1]. FcR blocking reagent (Miltenyi Biotec Ltd, Bisley, UK) was added to prevent non-specific antibody binding. Following vortexing, 1 x 10^6 PBMCs were added to each tube (1.5 x 10^6 PBMCs for the B cell panel).

The following anti-human monoclonal antibodies were obtained from:

eBioscience Inc. (San Diego, CA, USA); CD56 FITC, CD45RA PerCP-Cy5.5, CD123 PerCP-Cy5.5, CD27 PE-Cy7, CD16 APC, CD161 APC, PD1 APC, HLA-DR v450, CD3 NC605, CD14 NC605, CD4 APCeF780, CD20 APCeF780, CD19 APCeF780, CD62L APCeF780, CD8 NC650, CD3 NC650

Biolegend (San Diego, CA, USA); PECy7, CD4 v450

BD Biosciences (Oxford, UK); CXCR5 FITC, IgD FITC, CCR7 PE, CD25 PE, IgG PE, CD116 PE, CD24 PerCP-Cy5.5, CD38 APC, CD127 APC, CD19 v450

Miltenyi Biotec Ltd. (Bisley, UK); CCR4 PECy7, CD11c PECy7

R&D Systems Inc. (Minneapolis, MN, USA); CXCR3 FITC, CXCR5 PE

Cells were stained in the dark for 20 mins at 4^{o}C. The staining was then quenched by adding 2mL of chilled rinsing buffer to each tube. This was followed by centrifuging at 325x*g* for 8 minutes.

Table 2.1 - Immunophenotyping panels. Fluorochromes used for each panel and their corresponding cell surface markers are listed.

Panel	Fluorochromes								
	FITC	**PE**	**PerCP-Cy5.5**	**PE Cy7**	**AF647 APC**	**APC-eF780**	**PB v450**	**NC605**	**NC650**
T cell **1 x 10^6 PBMC**	CXCR3	CCR7	CD45RA	CCR6	CD38	CD4	HLA-DR	CD3	CD8
Tfh **1 x 10^6 PBMC**	CXCR3	CXCR5	CD45RA	CCR6	PD1	CD62L	CD4	CD3	CD8
Th17 **1 x 10^6 PBMC**	CXCR3	CCR7	CD45RA	CCR6	CD161	CD4	HLA-DR	CD3	CD8
Tregs **1 x 10^6 PBMC**	CXCR5	CD25	CD45RA	CCR4	CD127	CD4	HLA-DR	CD3	
B cells **1.5 x 10^6 PBMC**	IgD	IgG	CD24	CD27	CD38	CD20	CD19	CD3	
Myeloid **1 x 10^6 PBMC**	CD56	CD116	CD123	CD11c	CD16	CD19/20	HLA-DR	CD14	CD3

A live/dead stain, Zombie Aqua (Biolegend, San Diego, CA, USA) was included with each phenotyping panel. Single stain controls and unstained controls were run before each pair of samples were processed to obtain a fresh compensation matrix. Fluorescence minus one (FMO) controls and isotype controls were used when testing and validating the panels. Cells were analysed using a BD LSRFortessa™ analyser (BD Biosciences, Singapore) equipped with 4 lasers: blue 488nm, violet 405nm, red 640nm and yellow-green 561nm.

2.1.2.5 Flow cytometry gating

Data analysis was performed using FlowJo software (Version 10.0.7, Ashland, Oregon). The gating strategy was based upon The Human Immunophenotyping Consortium standards. This was carried with the assistance of by National Institute for Health Research (NIHR) Biomedical Research Centre Immunophenotyping service, Department of Medicine, Addenbrooke's Hospital, Cambridge, and by Miss Emily Groves, Morrell Laboratory, University of Cambridge.

A time vs side scattered light area (SSC-A) plot of all events was firstly used to check for even flow during the run and allow for identification of any artefacts caused by poor flow. A plot of SSC-A vs forward scattered light area (FSC-A) was used to distinguish lymphocytes and FSC-A vs forward scatter width (FSC-W) was used to eliminate doublets. A live-dead stain vs SSC-A plot was used to eliminate dead cells. Subsequently, sequential gating was carried out to distinguish subpopulations of T lymphocytes, B lymphocytes, natural killer (NK) cells, monocytes and dendritic cells were using bivariate dot plots based on cell surface marker expression, as described below, for each of the six panels; B cell, T cell, Tfh, Treg, Th17 and myeloid [Figure 2.2-2.7]. Programmed cell death 1 protein (CD279) expression by CD4+ T cell and CD8+ T cell subpopulations was also assessed.

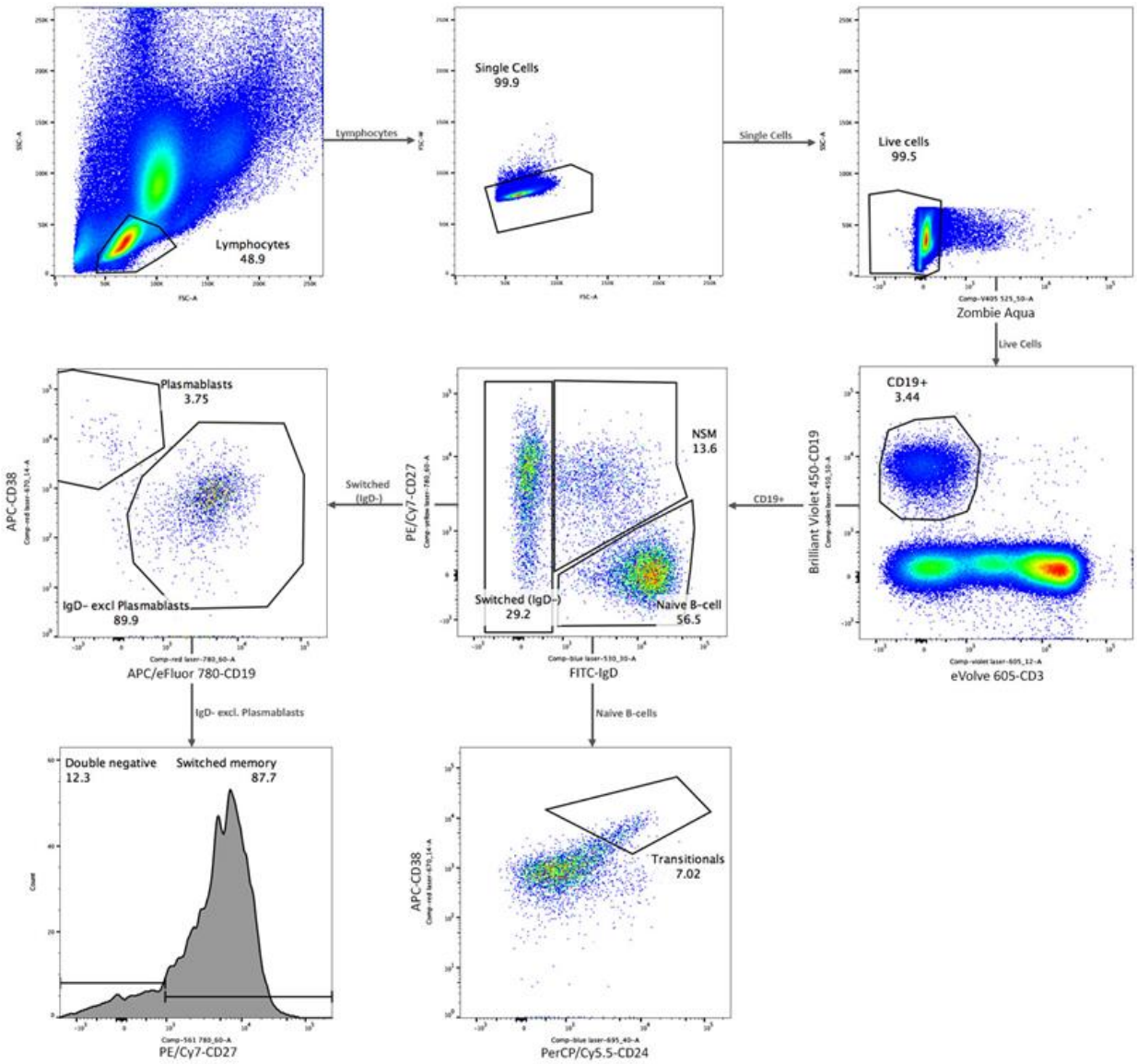


Figure 2.2- B cell panel gating strategy. FSC (A) vs SSC (A) was used to distinguish lymphocytes. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. B cells were identified from the lymphocyte population as CD3-CD19+ cells. The CD19+ cells were then divided according to expression of CD27 and IgD into the following subpopulations; non-switched memory cells (IgD+ CD27+), naïve B cells (IgD+ CD27-) and class switched (IgD-) cells. Class switched (IgD-) B cells were then further subdivided into plasmablasts (CD20- CD38+) and non-plasmablasts (CD20+ CD38-). The non-plasmablasts (CD20+CD38-IgD- B cells) were then gated into double negative (CD27-IgD-) and switched memory (CD27+IgD-) subsets. Naïve B cells (IgD+) were further analysed to distinguish transitional B cells (CD24hi CD38hi).

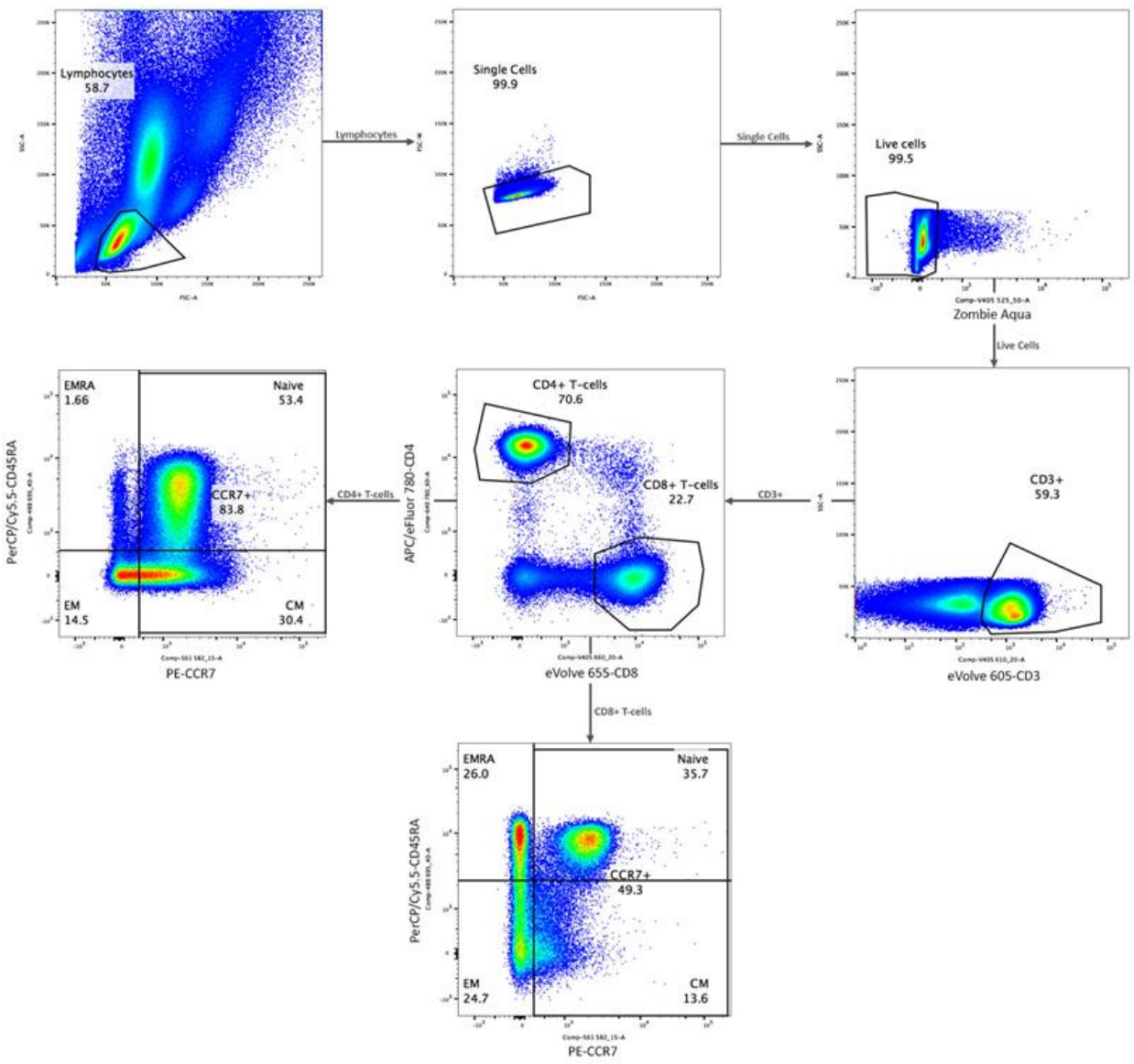


Figure 2.3- T cell panel gating strategy. FSC (A) vs SSC (A) was used to distinguish lymphocytes. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. T cells were distinguished from the lymphocyte population as CD3+ cells. T cells were then separated into CD4+ and CD8+ T cell subclasses. CCR7 and CD45 expression were used to identify effector memory RA (CCR7- CD45RA+), effector memory (CCR7-CD45RA-), naïve (CCR7+CD45RA+) and central memory (CCR7+CD45RA-) subsets.

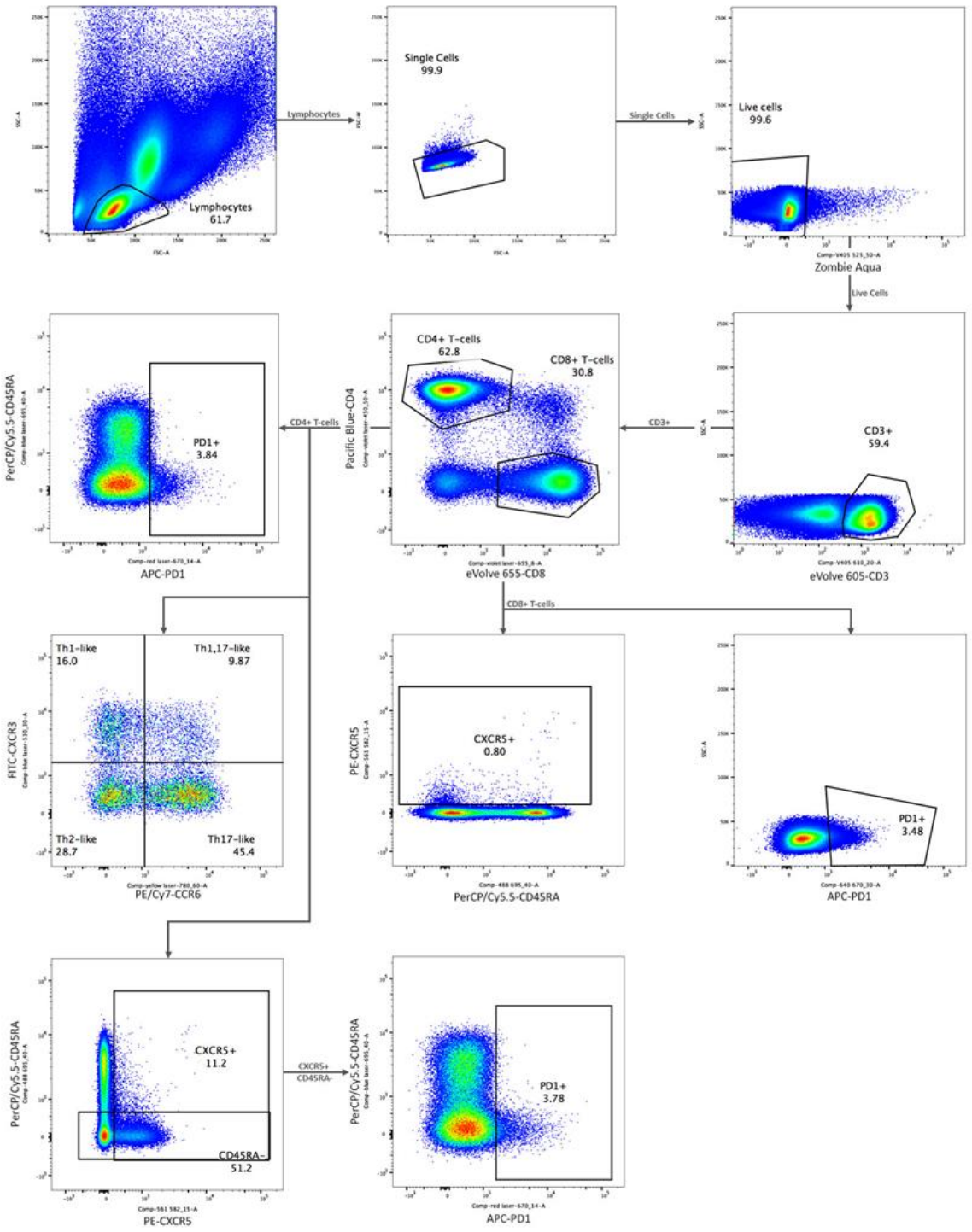


Figure 2.4- Tfh cell panel gating strategy. FSC (A) vs SSC (A) was used to distinguish lymphocytes. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. T cells were distinguished from the lymphocyte population as CD3+ cells. T cells were then separated into CD4+ and CD8+ T cell subclasses according to cell surface marker expression. Within the CD4+ T cell population, Tfh (CXCR5+ CD45RA-), Th1-like (CXCR3+ CCR6-), Th2-like (CXCR3- CCR6-), Th17-like (CXCR3- CCR6+) and Th1, Th17-like (CXCR3+ CCR6+) cells were identified. Within the CD8+ T cell population, CXCR5+ T cells were identified. Programmed cell death 1 protein (CD279) expressing Tfh and CXCR5+ CD8+ T cells were also subsequently identified.

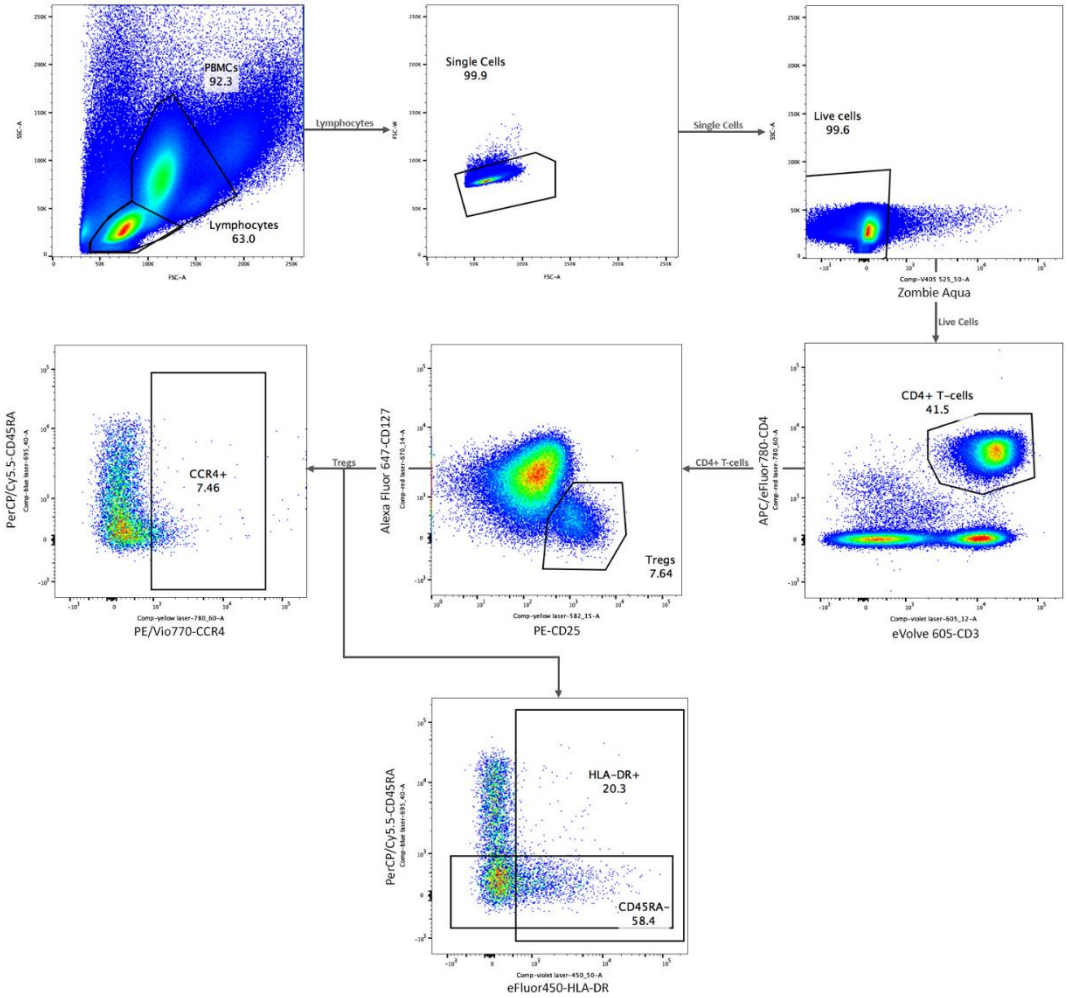


Figure 2.5- Treg cell panel gating strategy. FSC (A) vs SSC (A) was used to distinguish lymphocytes. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. T cells were distinguished from the lymphocyte population as CD3+ cells. CD4+ T cell expression of CD25, CD127 and was used to identify Tregs (CD25+ CD127low CCR4+). HLA-DR and CD45RA expression were used to distinguish memory Tregs (CD45RA+), naïve Tregs (CD45RA-) and to identify activated T regs (HLA DR+).

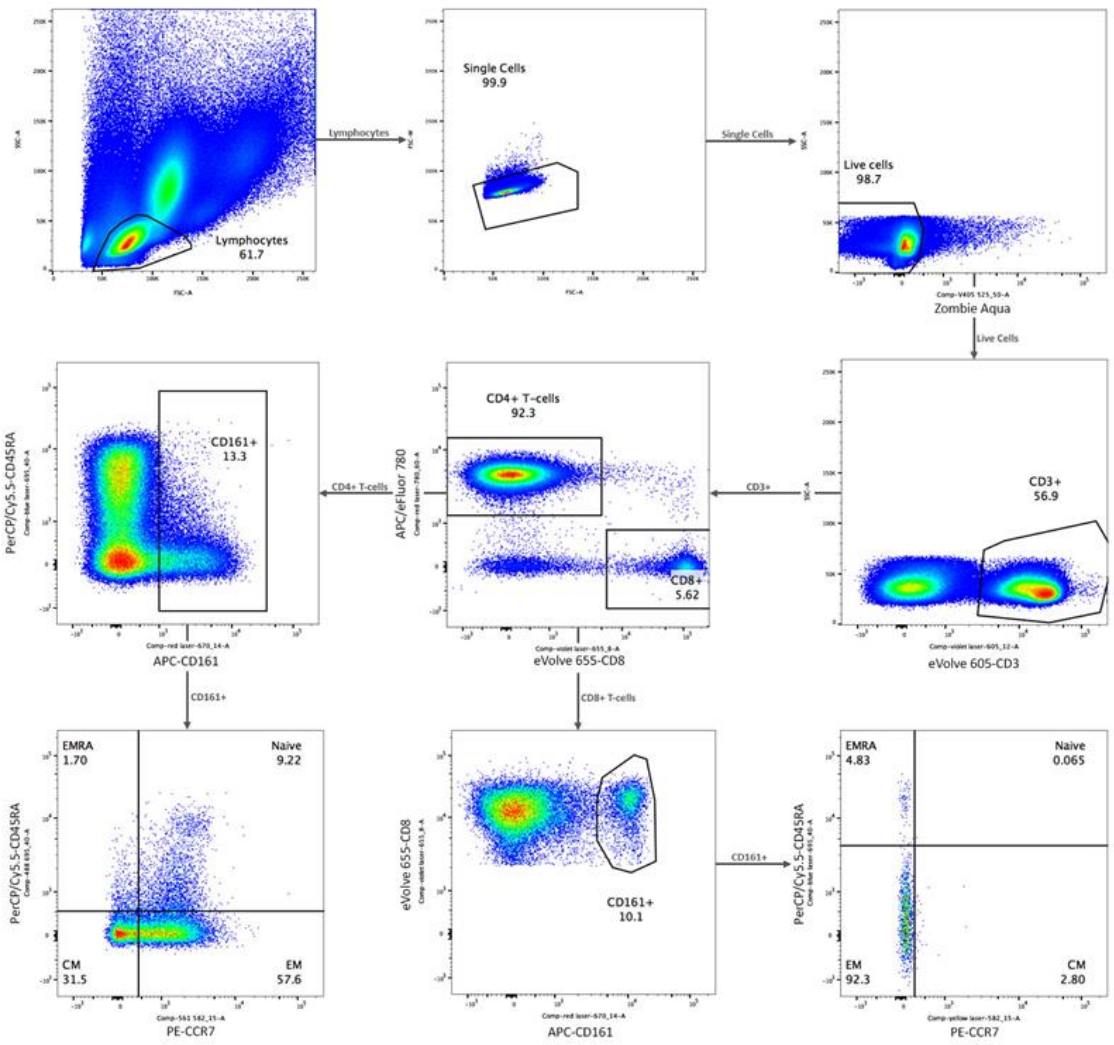


Figure 2.6- Th17 cell panel gating strategy. FSC (A) vs SSC (A) was used to distinguish lymphocytes. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. T cells were distinguished from the lymphocyte population as CD3+ cells. T cells were then separated into CD4+ and CD8+ T cell subclasses according to cell surface marker expression. Within the CD4+ and CD8+ T cell populations, cells expressing CD161 were gated. Subpopulations within the CD161+ population were then identified; effector memory RA (CCR7- CD45RA+), effector memory (CCR7-CD45RA-) naïve (CCR7+CD45+) and central memory (CCR7+CD45-).

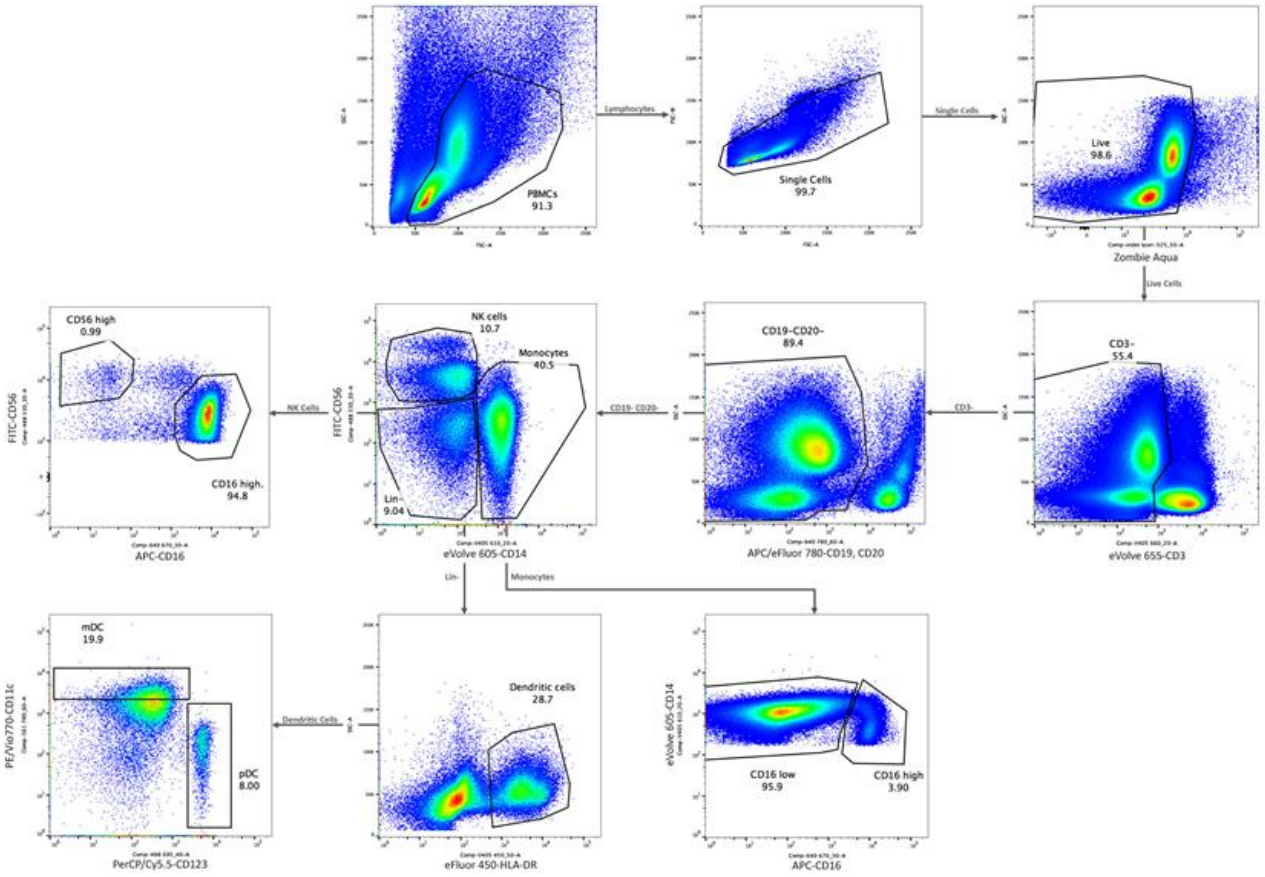


Figure 2.7- Myeloid cell panel gating strategy. FSC (A) vs SSC (A) was used to identify PBMCs. FSC (A) vs FSC (W) was used to eliminate doublets. A live-dead stain vs SSC (A) plot was used to eliminate dead cells. Myeloid cells were identified from the PBMC population as CD3-CD19-CD20- cells. These cells were then gated according to CD14 and CD56 expression. This allowed monocytes (CD14+) and NK cells (CD14-CD56+) to be distinguished. CD3-CD19-CD20-CD14-CD56- cells were classed as lineage negative and the population expressing HLA-DR identified as dendritic cells, with subsequent division of myeloid dendritic cells (CD11c+) and plasmacytoid dendritic cells (CD123+). NK Cells were further subdivided into CD56 hi and CD16 hi populations. Monocytes were further subdivided into CD16 low and CD16 hi populations.

2.1.3 Measurement of serum immunoglobulin concentration

Immunoglobulin subclasses IgA, IgM, total IgG, IgG1, IgG2, IgG3 and IgG4 were analysed in serum from patients and age and sex matched controls. Serum samples were obtained by upper limb venepuncture as described above. Samples were analysed by the Cambridge Biomedical Campus Pathology Partnership Immunology laboratory, Addenbrooke's Hospital, Cambridge.

Serum IgA, IgM and total IgG were quantified using polyethylene glycol (PEG) enhanced immunoturbidometric assay. This was done using the ADVIA 2400 Chemistry system analyser (Siemens. Camberley, Surrey), according to manufacturer's protocol. Via an automated process, diluted antigen solution was combined with serum containing the corresponding immunoglobulin, resulting in the formation of immunoprecipitates (accelerated by the use of polyethylene glycol). The effect of precipitate formation on the transmission of infrared light through the sample was then measured. Transmitted light intensity was detected by the analyser photodiode and by constructing a standard curve from the transmitted light intensity of known standards, the concentration of each immunoglobulin in the serum was automatically determined.

Serum IgG subclasses IgG1, IgG2, IgG3 and IgG4 were quantified using nephelometry. This was also a fully automated process, using the BN™ II System analyser (Siemens, Camberley, Surrey), equipped with an infrared light source. Similarly, a solution containing antigen specific to each IgG subclass was combined with the serum, resulting in the formation of immunoprecipitates. Scattered light intensity of the sample was detected by a photodiode and IgG subclass concentration automatically determined by comparison with the scattered light intensity of known standards.

2.1.4 Measurement of serum Interleukin 21 (IL-21) concentration

Serum IL-21 was measured in stored serum from 45 IPAH patients and 60 healthy controls by a 'sandwich' enzyme linked immunosorbent assay (ELISA), using a capture antibody and a biotin conjugated IL-21 detection antibody. This was carried out by Emily Groves, Department of Medicine, University of Cambridge, using the affymetrix human IL-21 ELISA Ready set GO! kit, 2nd generation (eBioscience, San Diego, CA, USA).

The ELISA plate was coated with 100µL per well of capture antibody in coating buffer (10X phosphate buffered saline (PBS)), the plate sealed and incubated overnight at 2-8°C. Following incubation, the wells were aspirated and then washed with wash buffer (1x PBS, 0.05% Tween-20). One part 5X ELISA/ELISPOT diluent was diluted with 4 parts deionised (DI) water and 200µL was added to each well, followed by incubation at room temperature for 1 hour. Reference standards were prepared by serial dilution of the ELISA/ELISPOT diluent, constructing a standard curve for a total of 8 points. 100µL of serum was added to each of the test wells, the plate sealed and incubated at room temperature for 2h. This was followed by aspiration and washing of the wells.

100µL of detection antibody (diluted in 1X ELISA/ELISPOT diluent) was then added to each well and the plate was incubated at room temperature for 1h, followed by aspiration and washing of the wells. 100µL/well of the detection enzyme Avidin-HRP (diluted in 1X ELISA/ELISPOT diluent) was then added to each well. The plate was sealed and incubated at room temperature for 30 mins. Aspiration of the wells and further washes were carried out.

100µL of chromogenic substrate solution (1X tetramethylbenzidine (TMB)) was then added to each well and the plate was incubated at room temperature for 15minutes. 50µL of stop solution (1M

H_3PO_4) was added to each well. The plate was read at a wavelength of 450 nm and absorbance compared to the standard curve to quantify IL-21 level.

2.1.5 Statistical analysis

2.1.5.1 IPAH compared to healthy age and sex matched controls

The D'Agostino-Pearson test was used to assess whether data were normally distributed for each parameter or population. Unpaired t tests (with Welch's correction if unequal standard deviation) were used to compare data which were normally distributed, and the Mann Whitney U test was used to analyse data which did not conform to a normal distribution. A p value <0.05 was considered statistically significant. False discovery rate correction for multiple testing was subsequently applied to each of the six panels [Appendix table 1.1].

2.1.5.2 Correlation with clinical markers of disease severity

Pearson's correlation coefficient (for normally distributed data) or Spearman's rank correlation (for data which did not conform to a normal distribution) was used to assess correlation between immune cell subsets and clinical parameters which are used to assess disease severity; mean pulmonary artery pressure (mPAP), pulmonary vascular resistance (PVR), N-terminal-pro-brain natriuretic peptide (NTproBNP) and cardiac index (CI). Cell subsets were assessed relative to clinical deterioration from time of diagnosis (time to clinical worsening), to explore whether the immune cell phenotype observed was associated with clinical disease progression.

Time to clinical worsening (TTCW) was defined as either disease progression (based on a ≥15% decrease in 6-minute walk distance, plus either worsening of functional class or need for additional PAH therapy), hospitalisation for worsening PAH, need for atrial septostomy or lung transplant or the introduction of parenteral prostacyclin therapy.

2.1.5.3 Other PH subtypes

Identification of leukocyte subpopulations in which statistically significant differences were present between IPAH and controls facilitated a targeted analysis of selected leukocyte subpopulations between the disease groups. Tfh cells, B cell subpopulations, regulatory T cells and PD1+ CD8+ T cells were compared. The Kruskal-Wallis test or ANOVA (depending on normality of distribution of cell subpopulations) was used to assess for statistically significant differences between the groups.

The data were also analysed comparing each disease subgroup separately to IPAH and to the healthy controls using t tests with Welch's correction or the Mann Whitney U test (depending on normality of distribution of cell subpopulations).

2.2 Part 2- Metabolomics

2.2.1 Study Population

Participants aged 18 to 80 years were recruited prospectively from the Pulmonary Vascular Diseases Unit, Papworth Hospital, Cambridge UK. All recruited participants provided written consent. Ethical approval for the study was obtained from the Research Ethics Committee, East of England, Cambridge South (reference REC EE/15/0201).

2.2.1.1 Pulmonary vascular disease patients

A total of 60 patients with pulmonary vascular disease were recruited. Patients were recruited from the following disease groups;

1. **Chronic thromboembolic pulmonary vascular occlusions** (n=48)
   This included patients with Chronic Thromboembolic Pulmonary Hypertension (CTEPH) and chronic thromboembolic vascular occlusions without pulmonary hypertension (chronic thromboembolic disease- 'CTED').
2. **Idiopathic Pulmonary Arterial Hypertension (IPAH)** (n=9)

Patients recruited met standard diagnostic criteria as defined by the ESC/ERS Guidelines 2015 (Galiè et al., 2015). Both incident and prevalent cases were recruited. Principal exclusion criteria included the following:

1. Cognitive or psychiatric impairment affecting capacity to give informed consent

2. Co-existing lung disease (e.g. obstructive airways disease, parenchymal lung disease). This was assessed by review of medical history, thoracic radiological imaging and pulmonary function tests. Subjects with significantly abnormal pulmonary function tests (including FEV1 <80% predicted or with KCO (transfer coefficient) <60% predicted) were excluded

3. Chronic Kidney Disease stage 4 or 5

4. Left heart disease, including pulmonary capillary wedge pressure (PCWP) >15mmHg and/or left sided valvular heart disease or ventricular impairment on echocardiogram

5. Peripheral arterial vascular disease which would preclude radial arterial blood sampling

6. Active infection

7. Known hepatic cirrhosis, liver failure or history of alcohol excess

8. Current illicit substance use

Demographic and clinical data collected from all participants included; age, gender, ethnicity, body mass index (BMI), body surface area (BSA), World Health Organisation (WHO) functional class, comorbidities, medications, smoking status, alcohol consumption and haemodynamics at the time of right heart catheterisation. Additionally, biochemical data (full blood count, urea and electrolytes, liver function tests and NTproBNP), pulmonary function test parameters, 6-minute walk distance, echocardiographic parameters and Cambridge Pulmonary Hypertension Outcome Review score (CAMPHOR) were recorded.

2.2.1.2 Healthy controls

27 individuals without pulmonary vascular disease were recruited. These control subjects were healthy staff members from Papworth Hospital. The control subjects were asked a series of screening questions to ensure no significant co-morbidity, prior to recruitment to the study.

2.2.2 Blood sample collection and initial processing

All blood samples were obtained in the morning, from non-fasted individuals.

In the healthy controls, a single 5mL blood sample was drawn from a vein in the antecubital fossa using a 23-gauge needle and 10mL syringe.

In pulmonary vascular disease patients, blood samples were collected during elective right heart catheterisation procedures (described further in section 2.2.3), allowing simultaneous blood sample collection and measurement of haemodynamics.

Blood samples in the patient group were collected from three anatomical sites; the superior vena cava (via the catheter device), pulmonary artery (via the catheter device) and radial artery (via peripheral arterial puncture). A 5mL sample of blood was collected from each site. The three samples were obtained within 10 mins.

Firstly, the superior vena cava and pulmonary artery blood samples were collected. Immediately after this, the peripheral arterial sample was obtained from the radial artery. Radial artery puncture was carried out using a 23-gauge needle attached to a syringe. 1 hour prior to arterial sampling, topical local anaesthetic gel was applied (4% w/w tetracaine) to the overlying skin to reduce patient discomfort during blood sampling.

All blood samples were collected in plastic K2 EDTA BD Vacutainer blood tubes (Becton, Dickinson and Company, New Jersey, USA), with each tube inverted several times to ensure mixing with anticoagulant. Samples were immediately placed on ice to arrest ongoing metabolic activity and transferred to a ThermoScientific ST16R centrifuge (ThermoFisher Scientific, Waltham, USA) which had been pre-chilled to 4 degrees Celsius. Blood samples were centrifuged at 1000x*g* for 10 mins for plasma separation. Plasma samples were aliquoted into chilled Sarstedt Cryopure cryovials (Sarstedt AG & Co, Nümbrecht,Germany) and immediately flash frozen in liquid nitrogen. All samples were then stored at -80^{o}C. Within 6 months from the time of collection, all frozen samples were shipped on dry ice Metabolon Laboratories (Durham, North Carolina, USA) and analysed as a single batch. Untargeted, semi-quantitative metabolic profiling of samples was conducted using the Metabolon Discovery HD4 platform, as described in Section 2.2.5.

2.2.3 Right heart catheterisation

Cardiopulmonary haemodynamic data and blood samples for analysis were collected during right heart catheterisation. Quadruple lumen 6 French fluid-filled Swan-Ganz catheters [Figure 2.8], (Edwards Lifesciences, Irvine, CA, USA) connected to a Philips Haemosphere cardiac catheterisation monitor (Philips Medical Systems, Surrey, UK) were used for the procedures.

Patients were non-fasted and had a standard light hospital breakfast on the morning of the procedure. Height and weight for each patient was recorded. All catheterisation measurements were taken with the patient in the supine position, at rest, breathing room air. Study participants were non-sedated and no haemodynamically altering medications were given prior to sample collection. The catheter was inserted into a central vein (internal jugular or femoral vein) under local anaesthetic, using ultrasound guidance. The catheter was floated through the right atrium and right ventricle into the proximal pulmonary circulation. Characteristic pressure waveforms seen at the different anatomical locations were used to establish catheter position, with correct position also confirmed using fluoroscopy.

The following pressure measurements were recorded: mean right atrial pressure (RAP), right ventricular systolic pressure (RVSP), right ventricular end diastolic pressure (RVEDP), systolic pulmonary artery pressure (sPAP), diastolic pulmonary artery pressure (dPAP), mean pulmonary artery pressure (mPAP) and pulmonary capillary ‘wedge’ pressure (PCWP). All pressure measurements were taken during breath holding at end-expiration.

Heart rate (HR), systemic blood pressure (BP) and peripheral oxygen saturations were recorded non-invasively using electrocardiography, an electronic blood pressure monitor and fingertip pulse oximeter. Cardiac rhythm during the procedure was also noted. Cardiac output was measured by thermodilution method using a Datex Ohmeda S/5 machine (Datex-Ohmeda Inc., WI, USA) and was also calculated using the Fick method.

Pulmonary artery oxygen saturation and mixed venous oxygen saturation were recorded using an Avoximeter 1000E machine (Accriva diagnostics CA, USA). Transpulmonary pressure gradient and pulmonary vascular resistance were calculated using standard haemodynamic formulae.

Figure 2.8- Quadruple lumen Swan-Ganz catheter used for right heart catheterisation. The catheter is inserted into a central vein. The balloon is then inflated and used to float the catheter into the right heart and proximal pulmonary vessels. Blood samples are taken and fluid injected via the catheter ports. The intravascular pressure at the catheter tip is recorded by a pressure transducer. Connection to a thermistor allows cardiac output assessment by the thermodilution method.

2.2.4 Global metabolomic analysis- an overview

Metabolomic profiling involves the identification of multiple metabolites (principally small molecules with a molecular weight <1500 Da) within a biological sample.

The process of analysing a biological sample involves [Figure 2.9]:

1. Separation of metabolites within a complex mixture- usually using chromatography

2. Detection and identification of metabolites using mass spectrometry

3. Data pre-processing, metabolite and biochemical pathway analysis

4. Biological interpretation

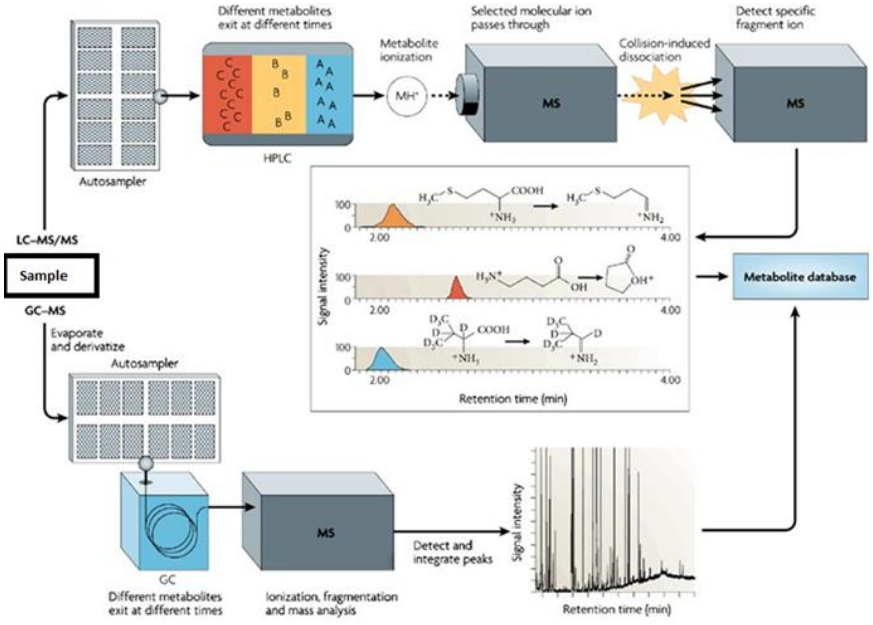


Figure 2.9 - Overview of Metabolomic sample processing and analysis. Metabolites are separated by liquid chromatography or gas chromatography. Following ionisation, mass spectrometry is used to detect metabolites and their ion fragments and subsequently identify them according to retention time and mass-to-charge ratio (adapted from (Last et al., 2007)).

2.2.5 Metabolomic profiling- sample processing and analysis

Sample preparation for metabolomic analysis, metabolite separation and detection was carried out by Metabolon Inc. (Durham, NC, USA) using the Metabolon HD4 Discovery platform. A total of 209 plasma samples were analysed.

2.2.5.1 Sample preparation

Sample preparation was carried out using the automated MicroLab STAR system (Hamilton Company, Reno, NV, USA). Several recovery standards were added to the samples prior to extraction, allowing monitoring and verification of the extraction process.

Proteins were removed by precipitation with methanol, with vigorous shaking using a SPEX SamplePrep 2000 Geno Grinder (Elvatech, Kiev, Ukraine), followed by centrifugation. Organic solvent was removed using a Zymark TurboVap (SOTAX AG, Aesch, Switzerland). The resulting extract was divided into aliquots which were prepared for analysis using solvents compatible with the different separation and detection methods described below. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency.

2.2.5.2 Metabolite separation and detection

Several different methods were used for metabolite separation and detection, in order to maximise the number of metabolites identified. Samples were divided into 4 aliquots and analysed using ultra high-performance liquid chromatography methods. Three aliquots were analysed using reversed phase ultra-high-performance liquid chromatography with tandem mass spectrometry (RP UHPLC-MS/MS). Electrospray ionisation (ESI) was used to reduce ion fragmentation. For two of the RP UHPLC aliquots, positive ion mode electrospray ionisation (ESI) was used. For the third aliquot, negative ion mode ESI was used for one aliquot.

The fourth aliquot was analysed using hydrophilic interaction liquid chromatography (HILIC UPLC-MS/MS) with negative ion mode ESI. Liquid chromatography was performed using Waters ACQUITY UPLC systems (Waters Corporation, Milford, MA, USA). 4 separation conditions were used, optimised for different metabolite species [Table 2.2].

Table 2.2- Liquid chromatography columns used for metabolite extraction.

Chromatography method	**Column**	**Constituents**
Reversed phase liquid chromatography	C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm)	1. Methanol, water, 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA).
		2. Methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA
		3. Methanol, water, 6.5mM ammonium bicarbonate at pH 8.
Hydrophilic interaction liquid chromatography	HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm)	4. Water, acetonitrile with 10mM ammonium formate, pH 10.8.

Mass spectrometry was performed using a ThermoScientific Q-Exactive mass spectrometer with heated electrospray ionisation (HESI-II) and Orbitrap mass analyser operated at 35,000 mass resolution (ThermoFisher Scientific, MA, USA). Following detection by mass spectrometry, chemical identity of the molecules was determined.

2.2.5.3 Metabolite identification

All molecular ions, fragments and adducts were searched against a reference library of >14,000 compounds, based on authenticated standards. This was used to identify molecules based upon the retention time/index (RI), mass to charge ratio (m/z), and fragment ion spectra.

Metabolite identification criteria included a retention index within a narrow window of the proposed metabolite and accurate mass match to the library +/- 10 ppm. Probability based MS/MS forward and reverse scores were also used to control against false positive rates. Any ions which could not be definitively identified were given a numerical designation. Molecules matched to the reference library by software were manually confirmed by chemical spectral analysts, along with removal of artefacts, mis-assignments and background noise.

Metabolite concentration was determined by area under the curve analysis. The peak area of the metabolite in the sample was compared with the peak area of the standard of a known concentration in order to determine metabolite concentration.

2.2.5.4 Quality assurance and quality control measures

Samples analysed in each batch were randomised and interspersed with a number of controls:

1. Ultra-pure water samples served as 'blanks'

2. A sample consisting of solvents used in the extraction process was used to identify any contamination induced by the extraction process

3. A pooled matrix sample was produced by taking a small amount of each sample in the batch. This was used as a technical replicate throughout the batch in order to help distinguish biological variability from process variability

4. A pooled matrix sample not derived from the batch being analysed was also used. This pooled matrix sample was developed from a large pool of human plasma which has been extensively characterised. Standards in this pool were chosen on the basis that they were known not to interfere with measurement of endogenous compounds. Each sample in the batch being analysed was spiked with this QC sample allowing instrument performance monitoring.

The median relative standard deviation (RSD) of the internal standards that were added to each sample was used to assess instrument variability. The median RSD for all endogenous metabolites in the pooled matrix sample was used to assess overall process variability.

2.2.5.5 Data pre-processing

Raw data of metabolite concentrations were pre-processed and reported in the form of standardised intensities. Each metabolite in original scale was then rescaled to set the median equal to 1 (by dividing each metabolite concentration by the median for that metabolite). This provided the concentration of the metabolite in each sample, relative to the median of all the samples processed as part of the study. Where a metabolite was not detected, standardised intensity was set as the minimum detected value for that compound.

2.2.5.6 Statistical analysis

Data analysis was conducted using R package for statistical analysis (Version 3.3.1, R Core Team 2013, Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.ref), Excel 2016 (Version 16.0, Microsoft, Redmond, WA, USA) and GraphPad Prism for Windows (Version 7, GraphPad Software Inc, La Jolla, CA, USA).

The concentration (median scaled standardised intensity) of each metabolite within the total batch of 209 samples was assessed to determine whether each metabolite was normally distributed, using the D'Agostino-Pearson test. The concentration of the majority of metabolites detected did not follow a Gaussian distribution. Therefore, non-parametric tests were used in all subsequent analyses.

Analysis 1- Comparison of metabolite concentration in disease venous samples and healthy control venous samples

The Mann Whitney U test was used to compare metabolite concentration in the disease group with healthy controls. False discovery rate adjustment for multiple testing was applied, using the Benjamini-Krieger-Yekutieli method (Q=0.05).

Metabolites in which there was a significant difference between disease and control were then grouped according to super pathway (eg. lipids, amino acids, carbohydrate) and subpathway (e.g. phospholipids, sphingolipids, long chain fatty acids). For each metabolite, calculation of the ratio of metabolite concentration between disease and control was used to indicate the direction of change in metabolite concentration between disease and control and allowed identification of groups and subgroups of metabolites with consistent unidirectional perturbations. Over-representation analysis of the metabolites which were found to be significantly different between disease and control also performed. This was calculated using the formula:

$$\textbf{Enrichment value} = \frac{k/m}{N/n}$$

k= number of significant metabolites in pathway; m= number of detected metabolites in pathway; N= total number of significant metabolites in the experiment; n= total number of detected metabolites in the experiment

Analysis 2- Comparison of metabolite concentration in paired samples from different anatomical sites

In those individuals in whom multisite samples were obtained, differences in metabolite concentration between paired samples from the different sites (SVC and PA, PA and ART, ART and SVC) were assessed using the Wilcoxon matched pairs signed rank test. False discovery rate adjustment for multiple testing was applied, using the Benjamini-Krieger-Yekutieli method.

3 Chapter 3- Immunophenotyping IPAH and healthy controls

3.1 Introduction and objectives

There is increasing evidence of an association between Idiopathic Pulmonary Arterial Hypertension (IPAH) and immune dysregulation. This includes the presence of perivascular immune cell infiltrates and pulmonary lymphoid neogenesis in IPAH lungs, increased frequencies of circulating autoantibodies and a recognised association with autoimmune diseases (Chapter 1, 1.18).

However, there has been only limited study of circulating immune cell populations in IPAH, primarily focussed on T cells (Ulrich 2008, Austin 2010, Huertas 2012). We set out to undertake a more detailed phenotyping of leukocyte subsets in the peripheral blood of IPAH patients, to further characterise circulating immune cell alterations in the disease.

Primary objectives

- To phenotype circulating peripheral blood leukocytes in patients with IPAH and compare this immune cell phenotype to healthy age and sex matched controls.
- To measure serum immunoglobulin concentration in patients with IPAH and compare this to healthy controls

Hypothesis 1- Patients with IPAH have a different peripheral blood immune cell profile to healthy individuals.

Hypothesis 2- The concentration of serum immunoglobulins differs between patients with IPAH and healthy individuals.

Hypothesis 3- IPAH patients with more severe clinical markers of disease will have a more deranged immune cell profile than those with milder disease.

3.2 Study population and methods

Peripheral blood leukocytes from 28 patients with IPAH were compared to 28 healthy matched controls. Patients and controls were matched according to age, sex, smoking status and body mass index. Whole blood was drawn from each subject by peripheral upper limb venepuncture. Following this, the blood was processed to separate out the peripheral blood mononuclear cells (PBMCs) and immunophenotyping was carried out, as described in chapter 2.

In brief, PBMCs were isolated from citrated whole blood by Histopaque 1077 density gradient separation. Cells were surface stained with antibodies and their conjugate fluorochromes. A standardised flow cytometry panel for cell surface markers of leukocyte sub-populations was used, adapted from the Human Immunology Project (Maecker et al., 2012). Subpopulations of T and B lymphocytes and myeloid cells were distinguished using bivariate dot plots based on cell surface marker expression. Additionally, immunoglobulin (Ig) subclasses in serum from IPAH patients and controls were analysed by PEG enhanced immunoturbidometric assay and nephelometry, and serum IL-21 levels were measured by enzyme linked immunosorbent assay (ELISA), using a capture antibody and a biotin conjugated IL-21 detection antibody.

3.3 Data analysis

The D'Agostino-Pearson test was used to assess whether data were normally distributed for each parameter or population. Unpaired t tests (with Welch's correction if unequal standard deviation) were used to compare normally distributed data and the Mann Whitney U test was used to analyse data which did not conform to a normal distribution. A p value < 0.05 was considered statistically significant. A total of 52 cell subpopulations were compared between IPAH patients and controls [appendix table 1]. False discovery rate adjustment for multiple testing was then applied to each of the 6 panels.

Subsequently, Pearson's correlation coefficient (for normally distributed data) or Spearman's rank correlation (for data which did not conform to a normal distribution) was used to assess correlation between immune cell subsets and clinical parameters.

3.4 Subject demographics

28 patients with IPAH and 28 healthy age and sex matched controls were recruited. Study population demographics are summarised in Table 3.1 and Table 3.2.

Mean IPAH patient age was 42 years. Mean control age was 42 years. 82% of subjects recruited were female, in keeping with the female predominance typically seen in IPAH populations (McGoon et al., 2013). IPAH haemodynamic characteristics included a mean pulmonary arterial pressure (mPAP) of 51.3±12.5mmHg, Cardiac index (CI) of 2.47±0.79 L/min/m^2 and pulmonary vascular resistance (PVR) of 9.7± 4.4 Wood units. Patients recruited were in World Health Organisation (WHO) functional class I-III at the time of sampling. Both incident IPAH cases and prevalent IPAH cases were recruited.

Consistent with the reported high prevalence of co-existing thyroid disease (Wawrzyńska et al., 2004), 7 IPAH patients had a past history of autoimmune thyroid disease. 4 IPAH patients had previously been treated for Graves thyroid disease (autoimmune thyrotoxicosis) and 3 patients had a history of hypothyroidism. All patients were euthyroid at the time of recruitment.

Patients recruited were receiving a range of targeted therapies for pulmonary arterial hypertension including 17 (61%) on phosphodiesterase 5 inhibitors, 14 (50%) on prostanoid therapy and 10 (36%) on endothelin receptor antagonists. 2 patients were newly diagnosed and treatment naïve. 21 patients (75%) were treated with a combination of pulmonary hypertension therapies.

Table 3.1-Study population demographics - IPAH and healthy controls. Populations were matched according to age, sex, BMI and smoking status. Results are presented as mean ± standard deviation, except where stated otherwise.

	IPAH	**Control**
Number of subjects	28	28
Age (years)	41.8 ± 10.5	42 ± 12.1
Sex (female:male ratio)	23:5	23:5
Caucasian (%)	86%	93%
BMI	27.8 ± 6.1	24.2 ± 3.5
Current smoker (number)	1/28	1/28

Table 3.2-IPAH population clinical characteristics. Results are presented as mean ± standard deviation, except where stated otherwise.

Clinical parameter	**IPAH patients**
WHO Class (I/II/III/IV)	4/10/14/0
Age at diagnosis (years)	35.5 ± 13.1
Time from diagnosis to sampling (years-mean, range)	6.7 (0-17)
Haemodynamics RAP (mmHg) mPAP (mmHg) PCWP (mmHg) PVR (Wood units) CI (L/min/m^2)- thermodilution method	 8.7 ± 3.4 51.3 ± 12.5 10.7± 3.1 9.7± 4.4 2.47± 0.79

6 minute walk distance (metres)	461± 109
Serum NTproBNP levels (pg/ml)	547± 922
Pulmonary hypertension therapy	26 (93%)
Nil/monotherapy/combination therapy	2/5/21
PDE5 inhibitor	17
Endothelin receptor antagonist	10
Prostanoid	14
Calcium channel blocker	5
sGC stimulator	1

3.5 Results

Results of all populations and subpopulations analysed are summarised in appendix Table 1.

3.5.1 Lymphocytes

Total lymphocyte count (cells per million PBMCs) was reduced in IPAH compared to controls (p=0.0042) [Figure 3.1]. The relative reduction in lymphocyte count was attributable to reduction of T lymphocytes in IPAH (p=0.0253) [Figure 3.2A]. B lymphocyte count did not differ significantly between IPAH and controls [Figure 3.2B]. Although both mean CD4+ and mean CD8+ T lymphocyte count were lower in IPAH, the reduction noted was not statistically significant [Figure 3.3].

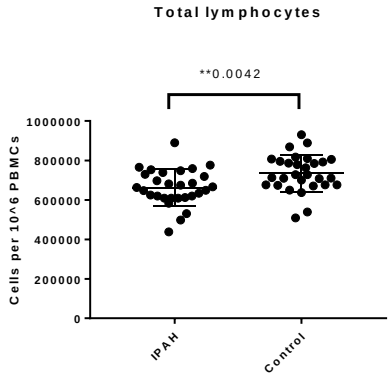


Figure 3.1- Total lymphocyte count (cells per 10^6 PBMCs) in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). Plots show cell frequencies with mean ± SD for each group.

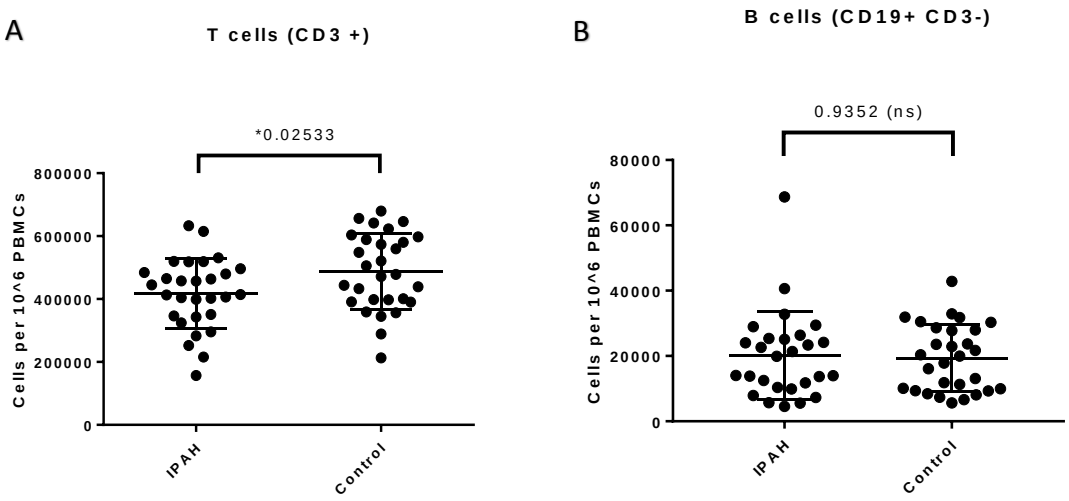


Figure 3.2- T and B lymphocyte count (cells per 10^6 PBMCs) in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). (A) T cells were identified as CD3+ lymphocytes, (B) B cells were identified as CD19+ CD3- lymphocytes. Plots show cell frequencies with mean ± SD for each group.

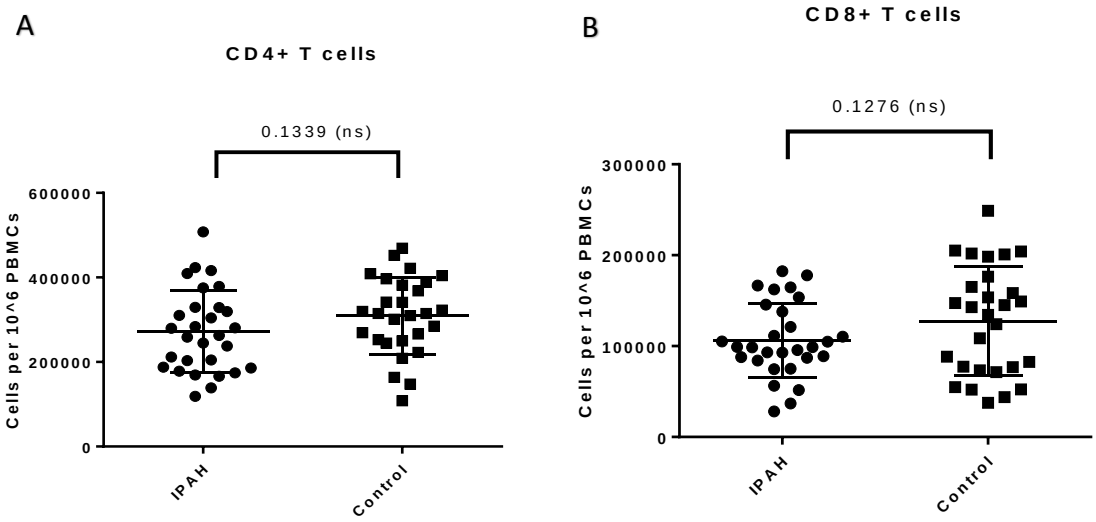


Figure 3.3- CD4+ and CD8+ T lymphocyte count (cells per 10^6 PBMCs) in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes, and subsequently subdivided according to; (A) CD4 expression and (B) CD8 expression. Plots show cell frequencies with mean ± SD for each group.

3.5.2 B cells

The most striking differences were observed in B cell subpopulations, with a significant decrease in both switched memory B cells (p= 0.0143) [Figure 3.4A] and non-switched memory B cells (p= 0.0026) [Figure 3.4B] and a significant increase in plasmablasts (p= 0.0099) [Figure 3.4C] and 'double negative' (CD27- IgD-) B cells in IPAH (p= 0.0143) [Figure 3.4D].

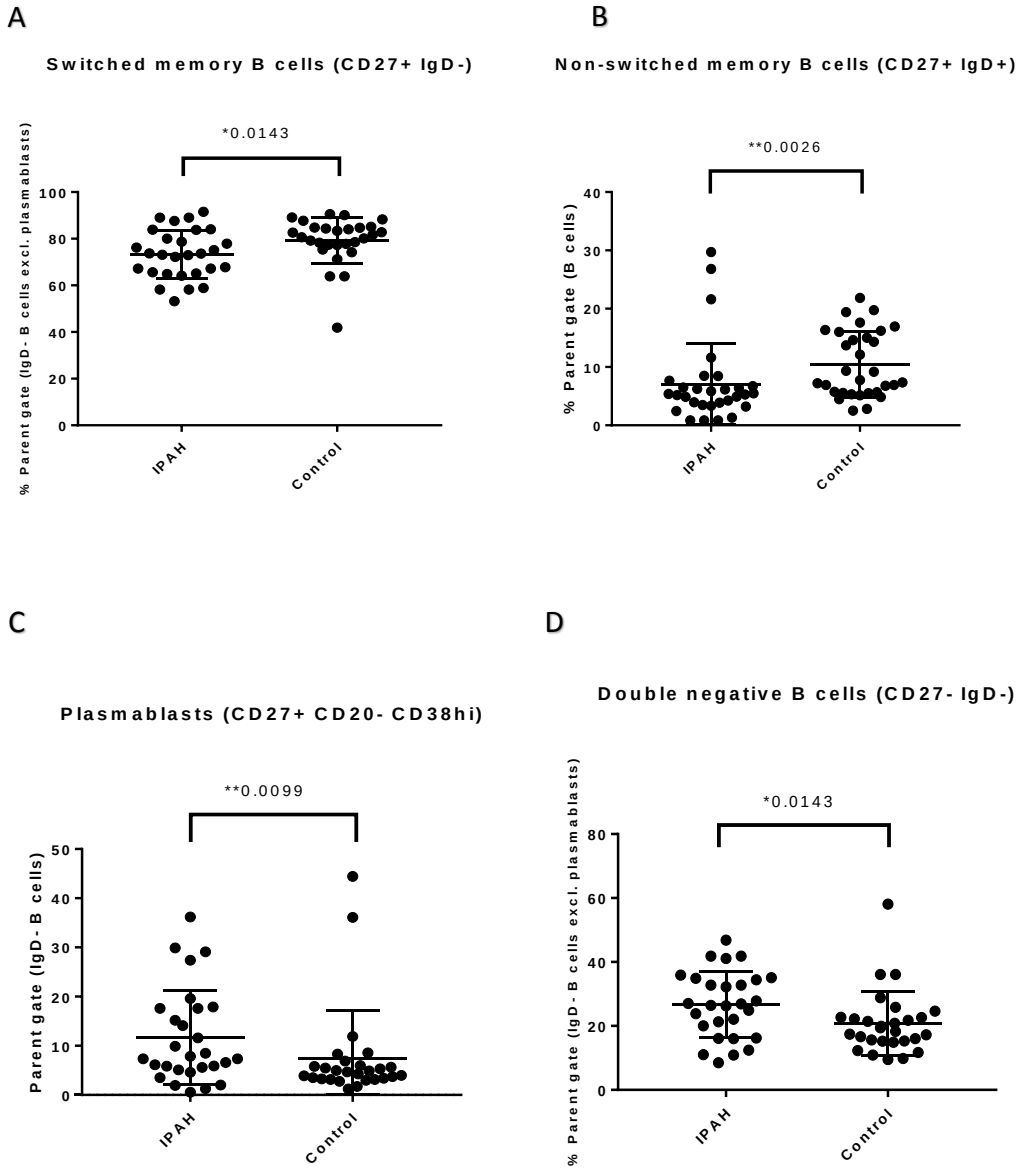


Figure 3.4- B cell subpopulations in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). B cells were identified as CD19+ CD3- lymphocytes. Sequential gating was used to identify; (A) switched memory B cells (CD27+ IgD-), (B) non-switched memory B cells (CD27+ IgD+), (C) plasmablasts (CD27+ IgD- CD20- CD38+) and (D) double negative B cells (CD27- IgD-). Plots show cell frequencies with mean ± SD.

3.5.3 CD4+ T cells

The frequencies of CD4+ T cells (T helper cells) and CD4+ naïve, effector memory, central memory and effector memory RA subpopulations were not significantly different between disease and control. However, there was a significant increase in T follicular helper (Tfh) cells (p=0.0111) [Figure 3.5A] and Th2-like Tfh cells (p=0.0212) [Figure 3.5B].

Numbers of regulatory T cells (Tregs) did not differ significantly between IPAH patients and controls (p =0.3308) [Figure 3.6A]. Analysis of activated Tregs (HLA-DR+) also did not demonstrate any significant difference between disease and control (p =0.7345) [Figure 3.6B].

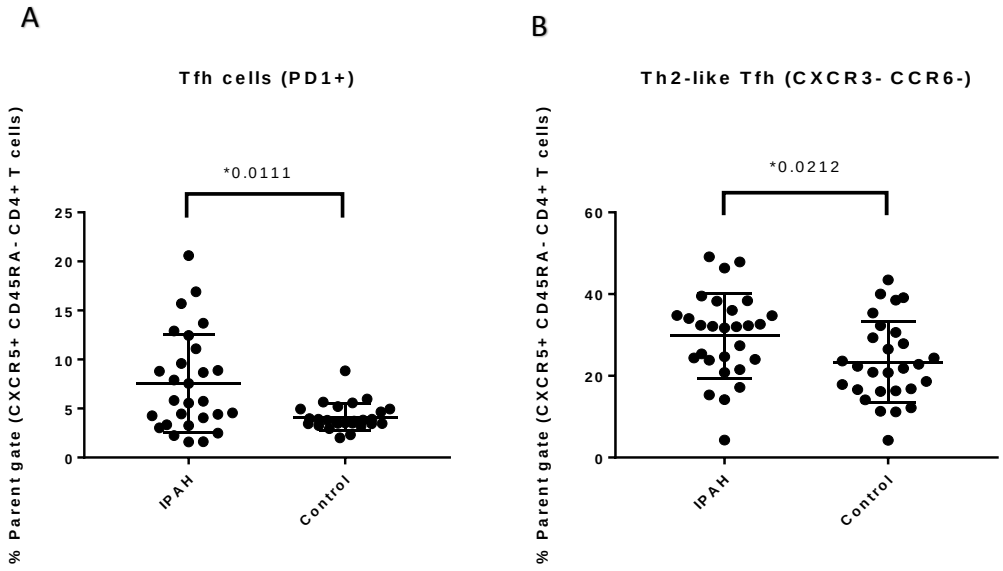


Figure 3.5- Tfh cells in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes. Sequential gating was used to identify; (A) Tfh cells (CXCR5+ CD45RA- PD1+) and (B)Th2-like Tfh cells (CXCR3- CCR6-). Plots show cell frequencies with mean ± SD.

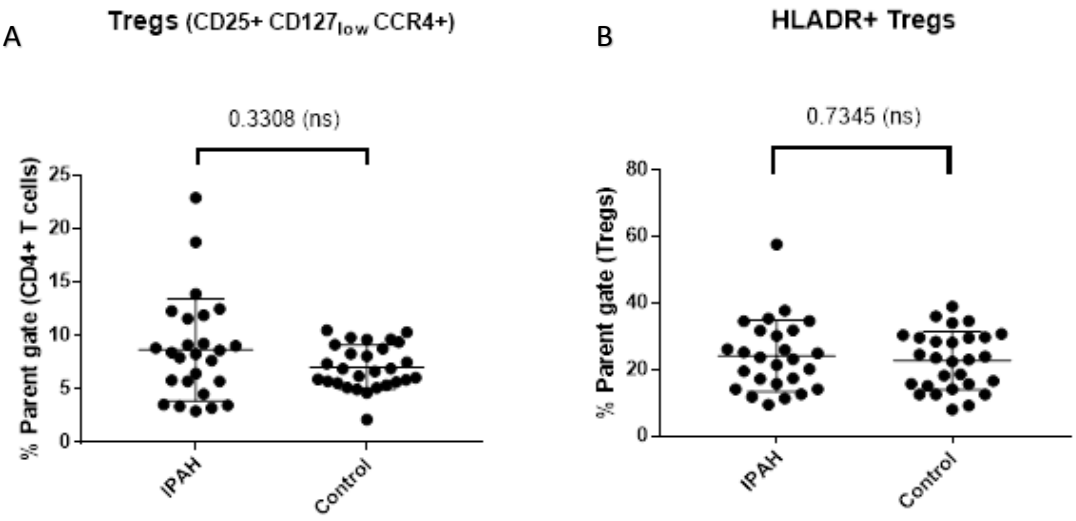


Figure 3.6-Regulatory T cells (Tregs) in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes. Sequential gating identified; (A) Tregs (CD25+ CD127low CCR4+) and (B) activated Tregs (HLA-DR+ Tregs). Plots show cell frequencies with mean ± SD.

3.5.4 CD8+ T cells

The frequencies of CD8+ T cells [Figure 3.7A] and CD8+ naïve, effector memory, central memory and effector memory RA subpopulations were not significantly different between disease and control. However, there was a significant increase in PD1+ CD8+ T cells in IPAH (p= 0.0332) [Figure 3.7B].

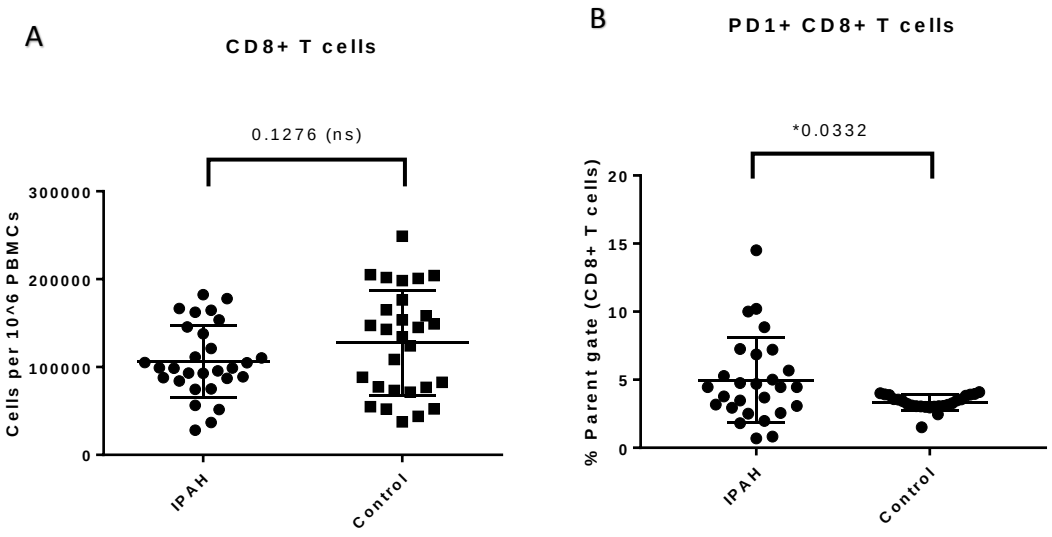


Figure 3.7- CD8+ T cells in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A) and T cells were identified as CD3+ lymphocytes. Sequential gating was used to identity; (A) CD8+ T cells and (B) CD8+ PD1+ T cells. Plots show cell frequencies with mean ± SD.

3.5.5 Th17 cells

CD4+ and CD8+ cell naive, central memory, effector memory and effector memory subpopulations expressing CD16 were compared between IPAH and control. There were no significant differences between IPAH and control in any Th17 (CD16 expressing) T cell population.

3.5.6 Natural killer, dendritic cells and monocytes

Dendritic cells, monocytes and their subsets were not significantly different between IPAH and control. Although the natural killer cell population as a whole did not differ between disease and control [Figure 3.8A], there was altered balance in NK cell subsets in IPAH, with a significant reduction in $CD16^{hi}$ natural killer cells in IPAH (p =0.12) [Figure 3.8B]. Although CD56 NK cell mean was higher in IPAH, this was not statistically significant (p= 0.5).

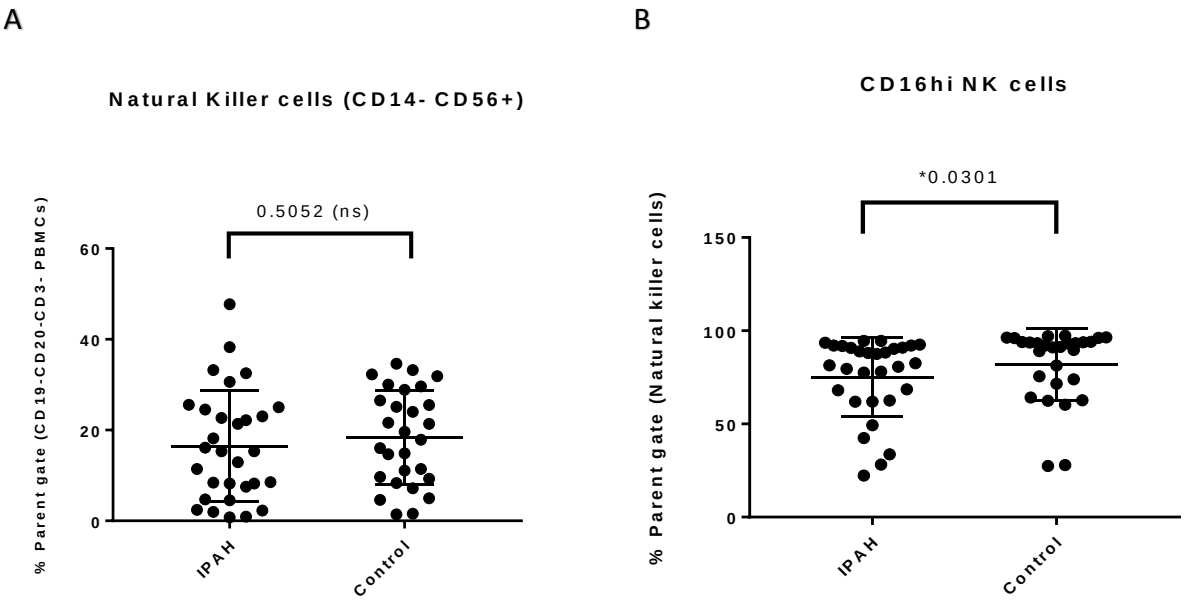


Figure 3.8- Total Natural Killer cell and CD16hi Natural Killer cells in IPAH and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Using flow cytometry, myeloid cells were identified from the PBMC population as CD3-CD19-CD20- cells. Sequential gating was used to identify; (A) NK cells (CD14-CD56+) and (B) CD16 hi NK cells. Plots show cell frequencies with mean ± SD.

3.5.7 Serum immunoglobulins

Serum IgA, IgM, total IgG and IgG subclasses 1-4 were quantified in 27 patients and 27 age and sex matched controls. No significant differences in immunoglobulin concentration were present in IPAH compared to controls [Figure 3.9, Figure 3.10].

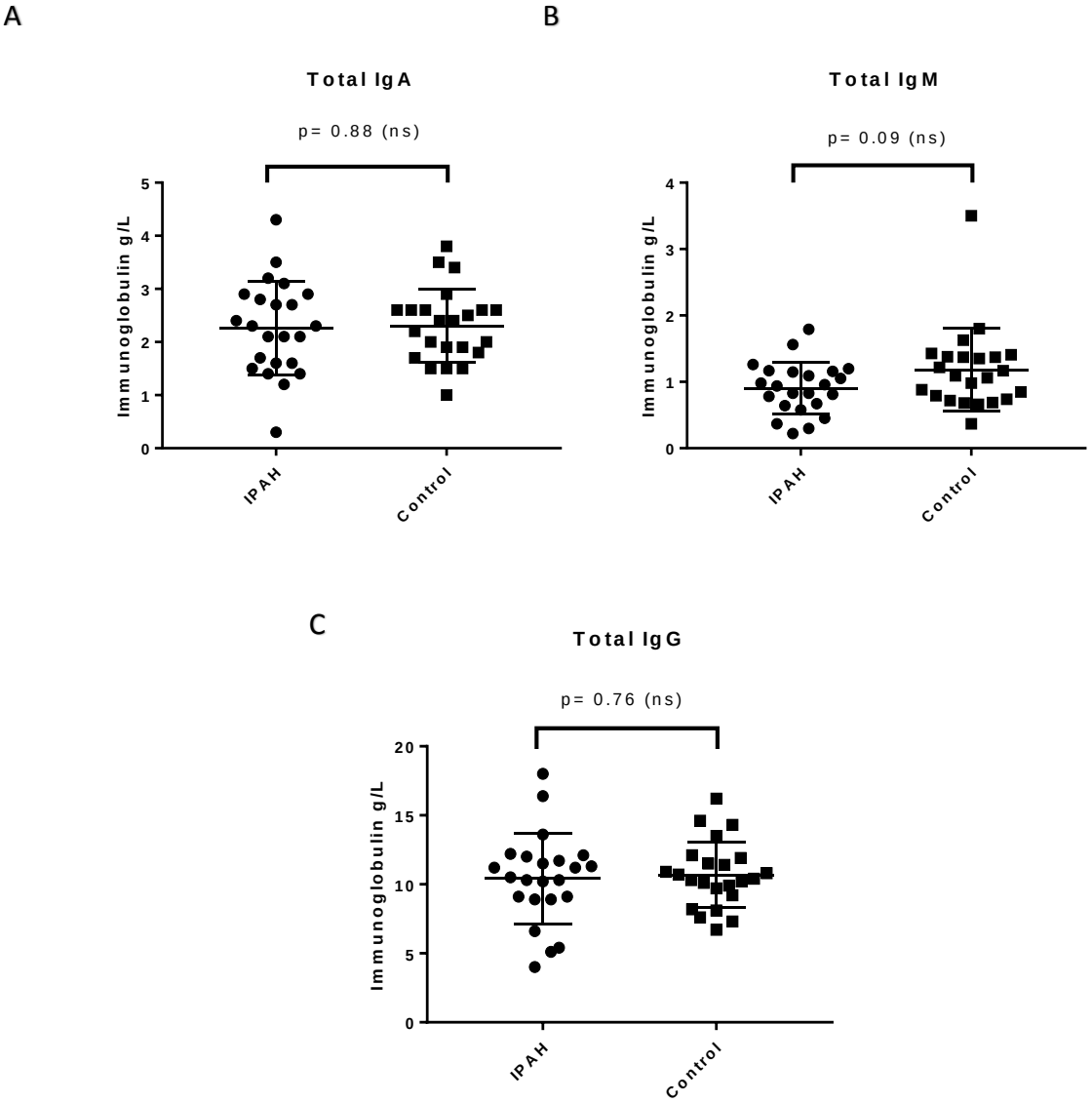


Figure 3.9- Serum immunoglobulin concentration in IPAH and healthy controls. Immunoglobulin subclasses (A) IgA, (B) IgM and (C) IgG were measured in serum from 27 IPAH patients and 27 age and sex matched healthy controls using polyethylene glycol (PEG) enhanced immunoturbidometric assay. Plots show immunoglobulin concentration with mean ± SD.

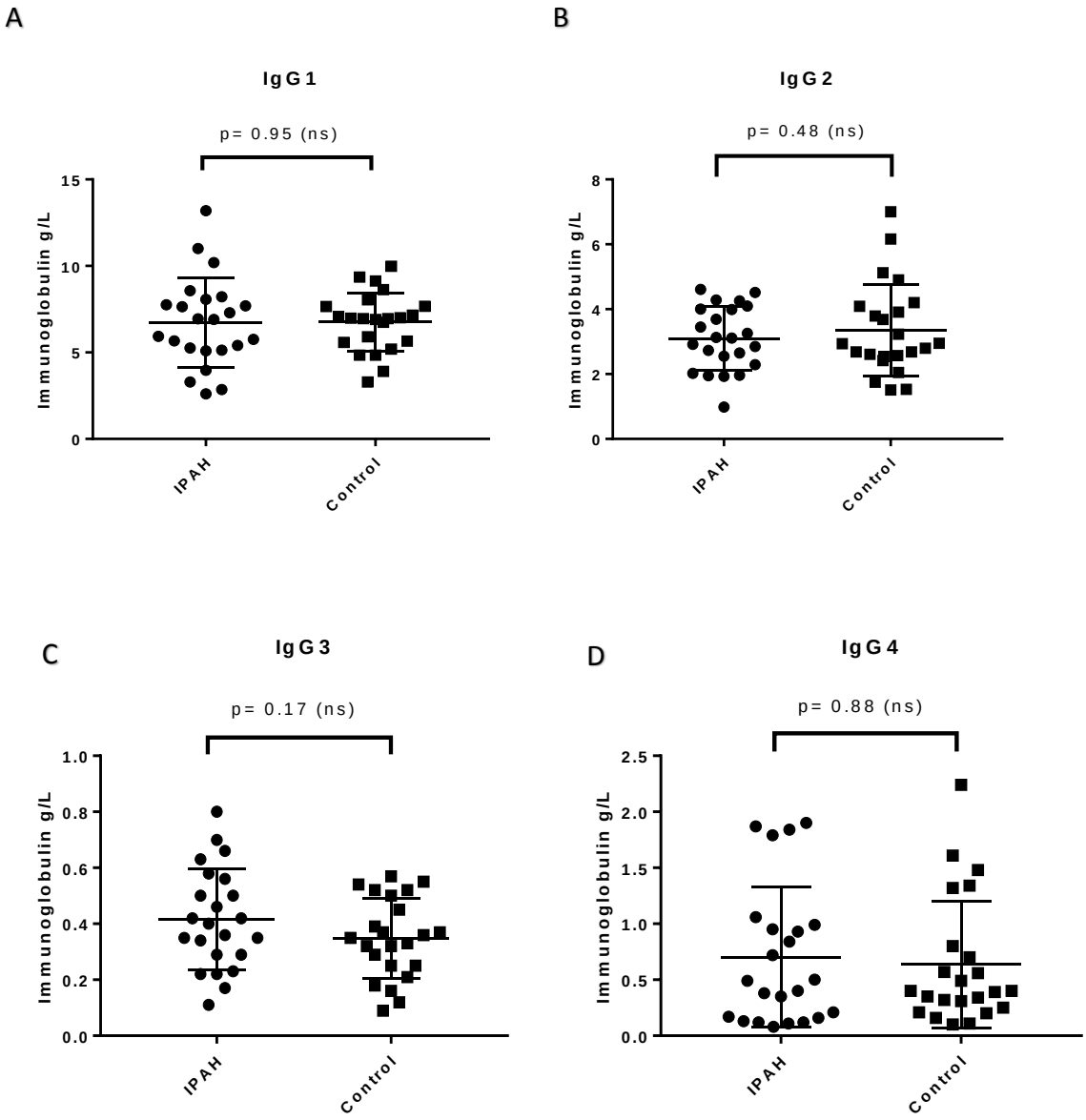


Figure 3.10- Serum immunoglobulin concentration in IPAH and healthy controls. Immunoglobulin G subclasses IgG1, IgG2, IgG3 and IgG4 (A-D) were analysed in serum from 27 IPAH patients and 27 age and sex matched healthy controls using nephelometry. Plots show immunoglobulin concentration with mean ± SD.

3.5.8 Serum IL-21

Serum IL-21 was subsequently measured in stored serum from 45 IPAH patients and 60 healthy controls by enzyme linked immunosorbent assay (ELISA), using a biotin conjugated IL-21 detection antibody. Serum IL-21 levels were significantly higher in the IPAH group (p= 0.0024) [Figure 3.11].

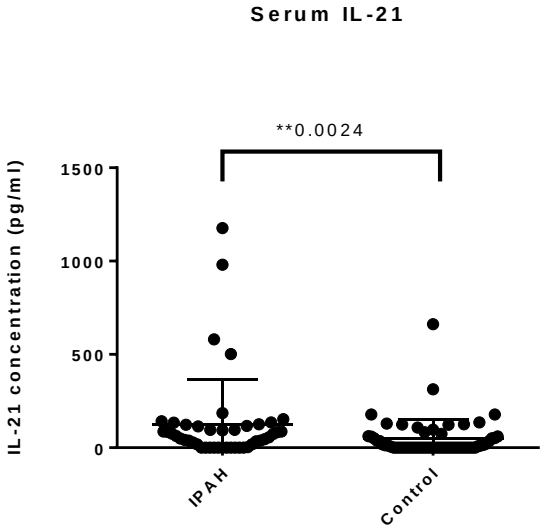


Figure 3.11- Serum IL-21 concentration in IPAH and healthy controls. Serum IL-21 was measured in stored serum from 45 IPAH patients and 60 healthy controls by a 'sandwich' enzyme linked immunosorbent assay (ELISA), using a capture antibody and a biotin conjugated IL-21 detection antibody. Plots show IL-21 concentration with mean ± SD.

3.5.9 Clinical parameters

The correlation was assessed between immune cell subsets and clinical parameters which are used to assess disease severity; mean pulmonary artery pressure (mPAP), pulmonary vascular resistance (PVR), N-terminal-pro-brain natriuretic peptide (NTproBNP) and cardiac index (CI).

The immune cell subsets assessed were plasmablasts, memory B cells, double negative B cells, Tfh cells, Th2-like Tfh and PD1-expressing CD8+ T cells. These cell subsets were also assessed relative to TTCW (time to clinical worsening, as defined in 2.5.2), to explore whether the immune cell phenotype observed was associated with clinical disease progression. Plasmablasts showed a moderate positive correlation with mean pulmonary artery pressure, but did not correlate with PVR, cardiac index or NTproBNP. Conversely, both Tfh and PD1+ CD8+ T cells showed an inverse correlation with mPAP [Figure 3.12].

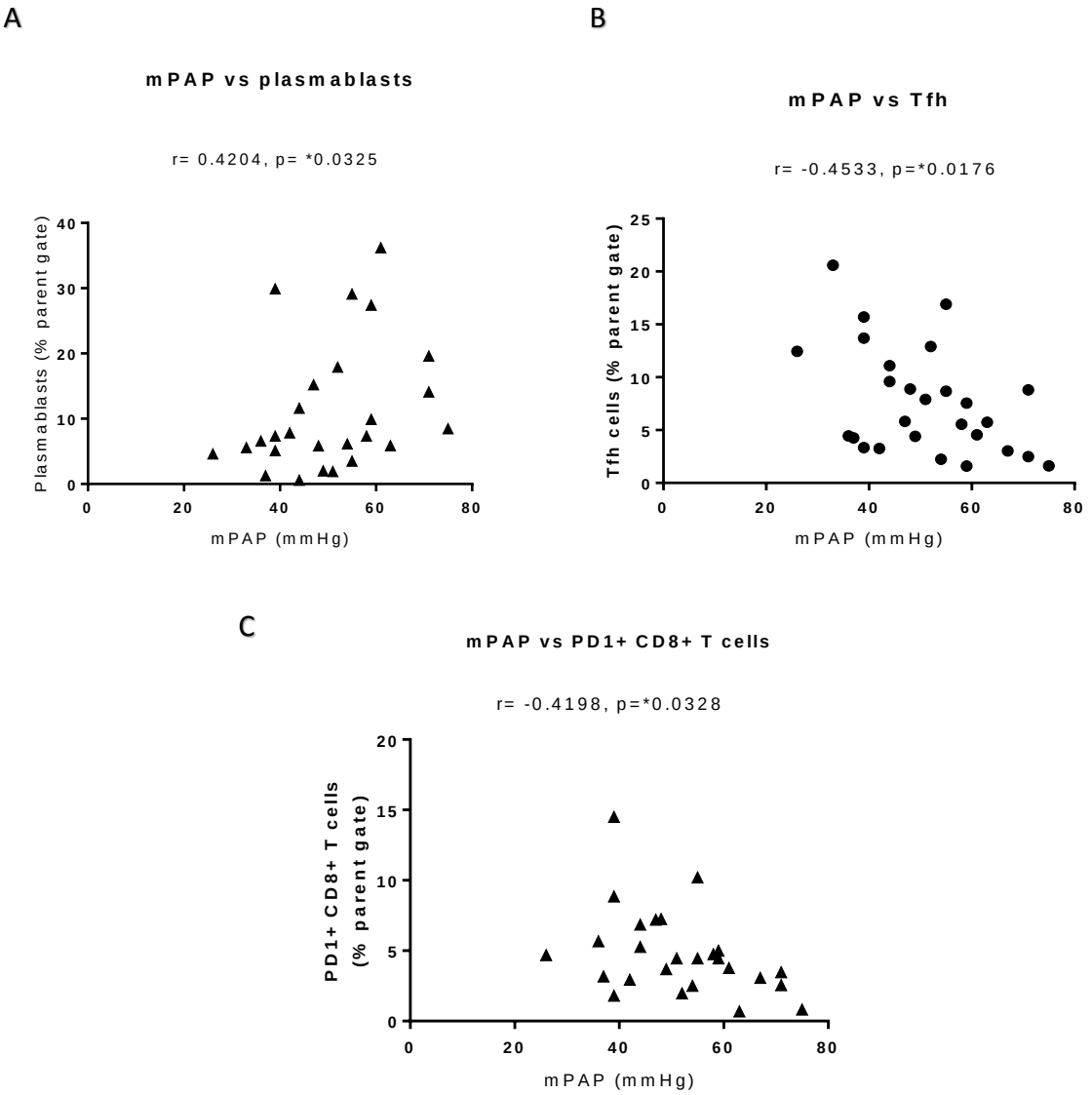


Figure 3.12- Correlation of cell subpopulations with clinical markers of disease severity. Pearson's correlation coefficient or Spearman's rank correlation was used to assess correlation between immune cell subsets and clinical parameters which are used to assess disease severity. Shown above are; (A) mPAP vs plasmablast frequency (B) mPAP vs Tfh frequency (C) PD1+ CD8+ T cell frequency vs mPAP.

There were no statistically significant correlations between TTCW and the immune cell subpopulations assessed. 16 out of 28 patients had a clinical worsening event between the time of diagnosis and the time of sampling. The IPAH group was subsequently divided into 2 groups according to clinical evidence of significant disease progression, defined as follows:

1. 'Deteriorating'- patients who met the criteria for at least 1 clinical and worsening event since diagnosis
2. 'Stable'-patients who had not had a clinical worsening event since diagnosis.

Although there was a trend towards higher plasmablasts and Tfh cells [Figure 3.13] and lower memory B cells in those who had a disease worsening event, these differences were not statistically significant.

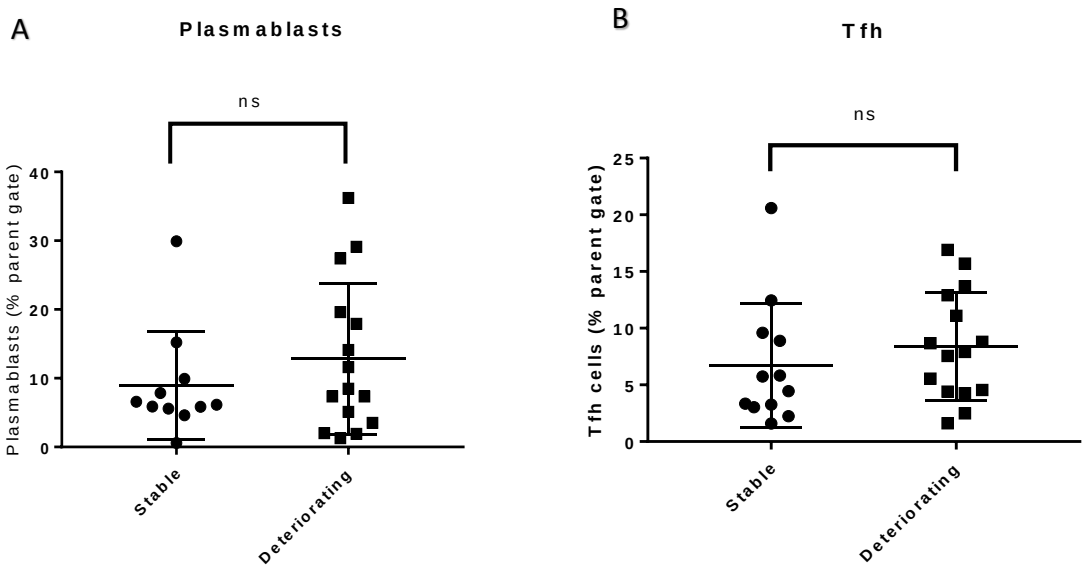


Figure 3.13- Plasmablast and Tfh cell frequency in IPAH, according to clinical worsening. For each IPAH patient, clinical records were reviewed to determine time to clinical worsening (TTCW). TTCW was defined as either disease progression (based on a ≥15% decrease in 6-minute walk distance, plus either worsening of functional class or need for additional PAH therapy), hospitalisation for worsening PAH, need for atrial septostomy or lung transplant or the introduction of parenteral prostacyclin therapy. Patients were divided into 2 groups; those who had not had a clinical worsening event since the time of diagnosis ('stable') and those who had 1 or more clinical worsening events ('deteriorating') and the frequency of (A) plasmablasts and (B) Tfh cells were compared between the 2 groups. Plots show cell frequency with mean ± SD.

3.6 Discussion

3.6.1 Total lymphocytes

My results confirm the presence of a relative lymphopenia in IPAH, compared to age and sex matched healthy controls. The potential mechanisms for lymphopenia in IPAH are multiple.

Lymphopenia is a recognised feature of a number of inflammatory conditions (Núñez et al., 2011), and chronic diseases such as renal failure (Pernice et al., 2006). Depletion in lymphocytes also occurs in heart failure, as a consequence of neurohormonal activation, oxidative stress and increased gastrointestinal lymphatic pressure (Weng et al., 2014).

As both inflammation and neurohormonal activation are present in IPAH, it is possible that these mechanisms could lead to lymphopenia. Additionally, the progressive right sided heart failure which develops during the disease, with consequent increase in oxidative stress and increased GI lymphatic pressure, may predispose to lymphocyte loss in IPAH.

It is also plausible that inflammation in IPAH may result in lymphopenia through a redistribution of circulating lymphocytes towards areas of disease activity, whereby lymphocytes migrate into lung tissue, depleting the circulating lymphocyte pool. This concept is supported by the presence of lymphocytic perivascular lung infiltrates in IPAH (Tuder et al., 1994).

Notably, the depletion in lymphocytes in IPAH is not global in nature, but is localised to the CD4+ T lymphocyte population. This selective depletion suggests that the lymphopenia observed in IPAH is not simply attributable to systemic physiological stress, generalised bone marrow or thymic suppression or non-selective gastrointestinal losses. This contrasts with previous findings of Ulrich et al (Ulrich et al., 2008a), who found a depletion of CD8+ rather than CD4+ T cells. The mechanisms underlying the CD4+ T cell depletion I have detected are unclear and require further investigation.

T cell lymphopenia has recognised associations with autoimmunity and immune dysfunction. For example, lymphopenia is one of the clinical and immunologic criteria used in the diagnosis and classification of SLE (Hochberg, 1997), and correlates with disease severity (Vilá et al., 2006). Anti-lymphocyte antibodies are commonly detected in this disease (Chun Li 2014). Other examples include Sjogren's syndrome, which is associated with severe lymphopenia in 5% of patients (Kirtava et al., 1995), Wegener's granulomatosis (Izzedine et al., 2002) and treatment naive rheumatoid arthritis (Duquenne et al., 2015).

The presence of T cell lymphopenia in IPAH and the recognised increased autoantibody production in IPAH (Rich et al., 1986) are features in common with autoimmune conditions. It is plausible that there may be shared mechanisms in these disease processes, raising the question as to whether anti-lymphocyte antibodies may be implicated in IPAH pathobiology.

A further consideration is whether the lymphopenia observed in some T lymphocyte subpopulations may be responsible for expansion of other T lymphocyte subpopulations. Lymphopenia induced proliferation (LIP) is recognised as a homeostatic mechanism to maintain a constant number of T cells in the circulation (Rocha et al., 1989). In a T cell deficient environment, T cells are able to proliferate without the presence of antigen, in an attempt to restore T cell numbers (Min et al., 2005, Rocha et al., 1989).

LIP is not only of importance in normal physiological situations, but may also be important in diseases where lymphopenia occurs, including autoimmune disease. There is some evidence to suggest that in susceptible individuals, LIP may promote development of autoimmune disease (Baccala and Theofilopoulos, 2005). In these individuals, a higher background frequency of potentially autoreactive T cells, coupled with frequent or chronic lymphopenia, promotes expansion of these autoreactive cells. Therefore, it is possible that dysregulated immune responses may not be caused simply by the absence of a T cell subpopulation, but potentially by the disturbed T cell repertoire which forms in attempt to maintain homeostasis of total T cell numbers.

3.6.2 B cells

My research has detected novel and striking differences in B cell subpopulations in IPAH compared to healthy controls. Although other immunophenotyping studies have assessed T lymphocytes in IPAH (Huertas et al., 2012, Austin et al., 2010a, Ulrich et al., 2008a), investigation of B lymphocytes has been largely neglected. There have been no reported studies characterising B lymphocyte subpopulations. However, a very small study of 5 patients, which analysed the RNA expression profile of peripheral blood B lymphocytes suggested the presence of B cell activation (Ulrich et al., 2008b).

Activation of naïve B cells occurs within the germinal centres of secondary lymphoid organs, where B cells undergo further antigen dependent maturation. After proliferation and affinity maturation, follicular B cells receive a final differentiation signal and exit the germinal centre as either an

antibody producing plasma cell or memory B cell. This final stage of maturation occurs under the influence of cytokines secreted by T helper cells.

The finding of increased plasmablasts in IPAH, accompanied by a decrease in memory B cells (both switched and non-switched) provides evidence of altered B cell differentiation in IPAH, with deviation towards antibody production rather than immunological memory. In addition to being found in the context of both acute and chronic infection, increased circulating plasmablasts are present in autoimmune conditions such as SLE and RA and decreased non-switched memory B cells have been reported in systemic sclerosis and SLE (Wang et al., 2013).

The increase in plasmablasts present in IPAH is also accompanied by an increase in Tfh cells. This is consistent with stimulation of a pathway which usually leads to B cell antibody production, and matches the immune cell phenotype seen in SLE and Sjogrens syndrome (Szabó et al., 2016, Bohnhorst et al., 2001a, Bohnhorst et al., 2001b). These findings, along with a decrease in memory B cells suggest presence of B cell activation, which may be driven by an unresolved antigenic stimulus in IPAH, which in turn may lead to a humoral immune response. The presence of B cell activation in is also supported by evidence of upregulation of multiple B cell RNA transcripts in IPAH (Ulrich et al., 2008b).

The reduction in classical memory B cells (IgD- CD27+) in IPAH was also accompanied by an increase in 'double negative' (IgD- CD27-) B cells. These cells have short telomeres and low replicative ability and characteristics of cell senescence. Expansion of this cell subpopulation is found with advancing age (Bulati et al., 2011), and has been reported in SLE (Wei et al., 2007) and Alzheimer's disease (Bulati et al., 2015). Increases in this cell subpopulation in disease have been speculated to occur as a result of chronic inflammation, leading to state of premature biological ageing of the immune system (Bulati et al., 2011). This hypothesis could equally be applied to IPAH, where there is clear evidence of inflammation in the disease pathobiology (as discussed in Chapter 1, section 1.18).

3.6.3 T cells

After development in the bone marrow, followed by selection in the thymus, 'naïve' forms of CD4+ and CD8+ T lymphocytes circulate in the periphery. After priming of a naïve T cell by encounter with antigen, the cell receives signals which direct it to proliferate and differentiate. Some of the progeny become short lived effector cells and some form long lived memory T cells that will survive in a quiescent state until they re-encounter the same antigen, reactivate and provide an accelerated immune response.

3.6.3.1 CD4+ T cells

CD4+ T cell lineages

CD4+ (T helper cells) develop into one of a number of lineages; Tfh, Th1, Th2, Th17, Th9 or Treg. The differentiation pathway the cell takes depends on the cytokine milieu, concentration of antigens, type of antigen presenting cells and costimulatory molecules present.

Th1 and Th2 cells

Th1 cells are involved in cell mediated inflammatory reactions and secrete IL-2, granulocyte-macrophage colony-stimulating factor and the inflammatory cytokines IFN-y and tumour necrosis factor. They are involved in elimination of intracellular pathogens and are associated with organ-specific autoimmunity and delayed hypersensitivity reactions (Annunziato et al., 2015).

Th2 cells mount the immune response to extracellular parasites, including helminths and produce cytokines such as IL-4 and IL-5 that help B cells to survive, proliferate and differentiate and are associated with humoral-type immune responses. Th2 cytokines particularly encourage IgE responses which play a major role in induction and persistence of allergic diseases (Cohn et al., 2004).

Notably, alterations in the Th1/Th2 balance have been identified in a number of autoimmune diseases and can have an impact on the outcome of autoimmune responses (Ogawara et al., 2003). In IPAH, there was an increase in Th2 cells but not Th1 cells. It may be that cytokine production by this subpopulation provides further stimulus to B cell proliferation in IPAH.

Th17

Th17 cells are defined by their ability to produce IL-17 cytokines, which are pro-inflammatory with roles in defensive immunity against bacteria and fungi, particularly at mucosal sites such as lung, gut and the oral cavity (Guglani and Khader, 2010). Increases in CD161+ CD4+ cells have been linked to a number of different inflammatory, immune and autoimmune related diseases including multiple sclerosis, psoriasis, Crohn's disease and RA, as well as allograft rejection (Annibali et al., 2011, Martin et al., 2013). However, CD161 expressing T cell subsets were not altered in IPAH, suggesting that these do not play a major role in established disease.

Tfh cells (CD4+ CXCR5+ PD1+)

Despite overall depletion in the CD4+ T cell compartment, IPAH patients demonstrated significantly increased populations of T follicular helper (Tfh) cells. Tfh are critical in the selection and survival of B cells, therefore play a key role in regulating antigen-specific humoral immunity. They direct somatic hypermutation and isotype switching of follicular B cells and within the germinal centres continue to provide B cell help, facilitating formation of antibody producing plasma cells and memory B cells.

Diseases in which there is chronic unresolved antigenic stimulation are associated with increases in Tfh cells. For example, increases are found in systemic lupus erythematosus (SLE), Sjogrens syndrome, rheumatoid arthritis (RA), autoimmune thyroid disease and myasthenia gravis (Arroyo-Villa et al., 2014). This increase usually correlates with clinical disease severity.

Tfh are also increased in human immunodeficiency virus (HIV) infection and their number positively correlates with plasma viraemia (Cubas et al., 2013, Lindqvist et al., 2012, Perreau et al., 2013). This occurs despite the progressive decline in total CD4+ T cells, and is accompanied by a decrease in memory B cells and an increase in plasma cells, similar to the findings in the IPAH group studied.

In summary, the expansion of Tfh in IPAH is a feature in common with other diseases where there is immune dysfunction, and is consistent with activation of a pathway which is directed towards humoral immune responses by B cells. This hypothesis is supported by the accompanied expansion of plasmablasts in IPAH (see section 3.6.2) and the increase in IL-21 (see section 3.6.4).

Tfh subsets

Tfh cells ($CXCR5^{+}CD4^{+}$ T cells) may be further classified into three subsets; Tfh1 ($CXCR3^{+}CCR6^{-}$), Tfh2 ($CXCR3^{-}CCR6^{-}$) and Tfh17 ($CXCR3^{-}CCR6^{+}$) (Morita et al., 2011). Skewed distribution of circulating memory Tfh subsets have been reported in some autoimmune diseases. For example, higher levels of Th2 and Th17 over Th1 have been reported in SLE (Le Coz et al., 2013), juvenile dermatomyositis (Morita et al., 2011) and Guillan-Barre syndrome (Che et al., 2016). There is evidence to suggest that Tfh2 and Tfh17 but not Tfh1 promote differentiation of B cells towards antibody-producing cells (Morita et al., 2011) via secretion of IL-21.

My results demonstrate an increase in Th2-like Tfh cells in IPAH, paralleling the changes seen in immune disorders. It is possible that Tfh2 may play a role in the pathogenesis of IPAH, yielding more antibody-producing cells and subsequent antibody-mediated humoral immune response. This is

supported by the differences noted in IPAH B cell subpopulations and elevated IL-21 levels found in IPAH.

Regulatory T cells (Tregs)

Regulatory T cells play an important role in immune homeostasis, self-tolerance and prevention of autoimmunity. They maintain the balance between an appropriate degree of immune activation required to respond to noxious stimuli, whilst preventing excessive tissue damage. Therefore, reactive increases in regulatory T cell number may be seen in response to a threat to self-tolerance and regulatory T cell depletion may result in failure of immune homeostasis (Vignali et al., 2008, Sakaguchi et al., 2008).

The role of Tregs in autoimmune disease is well demonstrated in animal models where depletion of Tregs results in a range of autoimmune diseases, and repletion results in reversal of the disease (Sakaguchi and Sakaguchi, 2005). Similarly, in an animal model of PAH, the importance of regulatory T cells in protecting against the development of PAH has been demonstrated (Tamosiuniene et al., 2011). Treg deficits in number and/or function have been noted in a number of human autoimmune diseases including multiple sclerosis (MS) (Viglietta et al., 2004), RA (Lawson et al., 2006) and SLE (Valencia et al., 2007), suggesting that Treg quantitative or functional deficiencies may be implicated in these diseases.

Contrary to previously published studies in IPAH where increases in Treg number were reported (Austin et al., 2010a, Ulrich et al., 2008a), I did not find any significant differences in Tregs in IPAH patients compared to controls. However, it must be noted that the markers used to define the Treg population were different. The marker FoxP3 was used in previous studies. However, it is now known that FoxP3 expression is not restricted to Tregs (Gavin et al., 2006, Wang et al., 2007). In my research, Tregs were instead defined as CD25+ $CD127^{low}$ CCR4+ CD4+ T cells (Maecker et al., 2012).

Treg functional deficiency in PAH has also been reported, despite the Treg population being normal in size (Huertas et al., 2016). It is feasible that in response to vascular injury, a functional deficiency in Tregs may result in failure of these cells to ensure appropriate injury resolution, which may then result in inappropriate inflammation and exuberant vascular remodelling. However, further functional study of Tregs are needed to establish their role in IPAH pathobiology.

3.6.3.2 CD8+ T cells

Following activation by interaction with antigen presenting cells in the presence of CD4+ T help, CD8+ cells undergo clonal expansion and can travel throughout the body in search of antigen positive cells. Upon encounter with antigen, they are then able to carry out direct cytotoxic functions.

Within the CD8+ T cell group, there was a reduction in CD8+ CCR7 T cells which was attributable to the naïve CD8+ T cell population (CCR7+CD45RA+). This is consistent with previous findings of Austin et al (Austin et al., 2010a).

In contrast to the depletion of naïve T cells detected, I identified a significant increase in PD1-expressing CD8+ T cells in IPAH. This finding is consistent with increased T cell activation. However, this may also indicate CD8+ T cell exhaustion. T cell exhaustion refers to a state whereby T cells become progressively less effective and eventually undergo apoptosis (Yi et al., 2010). The phenomenon has been described in situations of chronic antigenic stimulation such as malignancy, chronic infections including HIV, hepatitis B and hepatitis C and autoimmune diseases (Jiang et al., 2015). In the presence of antigen, under normal circumstances naïve T cells are stimulated to differentiate into effector T cells and after clearance of antigen they develop into memory T cells. When there is persistence of antigenic stimulus, T cells do not transit to memory phenotype. Instead, these T cells progressively lose effector function (Wherry et al., 2003) and there is sustained expression of inhibitory receptors, culminating in T cell anergy and finally apoptosis. In particular, PD1 (Programmed Death 1), is a hallmark of CD8+ T cell exhaustion.

Antigen and CD4+ T cell help strongly influence CD8+ T cell exhaustion; as antigen increases and/or CD4+ help decreases, virus-specific T cells become more exhausted. In chronic viral infections, the severity of T cell exhaustion correlates with viral load and or the number of epitopes presented, longer duration of infection and loss of CD4+ T cell help (Wherry et al., 2003, Day et al., 2006). It has been postulated that block the PD1–PDL pathway may have therapeutic potential in ameliorating T cell exhaustion (Barber et al., 2006).

However, PD1 expression is not necessarily synonymous with terminal differentiation to ‘exhaustion’ and inevitable decline and death. An alternative view is now emerging, whereby some authors have challenged the notion that this phenomenon represents a dysfunctional state in overwhelmed T cells, suggesting instead that increased PD1 expression may in fact represent an adaptive state. (Utzschneider et al., 2013, Hong et al., 2013, Paley et al., 2012, Duraiswamy et al., 2011). For example, Utzscheneider et al. (2013) have proposed that during persistent infection, effector T cells

may stably differentiate into this state to allow viral replication to be limited without causing overwhelming immunological pathology.

This concept may also apply to autoimmune diseases in which there is persistent antigenic stimulation. In a range of autoimmune diseases, presence of CD8 T cell 'exhaustion' has been found to be associated with a better prognosis (McKinney et al., 2015). Notably, in the IPAH group I studied, PD1+ CD8+ T cells showed an inverse correlation with mean pulmonary artery pressure. However, the true significance of this finding is uncertain.

In summary, the reduction in naïve CD8+ T cells and increase in PD1-expressing T cells in IPAH is consistent with an activated CD8+ T cell population, indicating a response to the presence of an antigenic stimulus. Whether PD1 expression constitutes solely activation, whether it is indicative of deleterious 'exhaustion' or whether it represents an adaptive mechanism to limit immunological damage is unclear. This could be further explored by analysis of other cell surface inhibitory markers and studies of CD8+ T cell function.

3.6.4 IL-21

Serum IL-21 was increased in IPAH patients compared to healthy controls. Interleukin-21 (IL-21) is predominantly produced by $CD4^+$ T cell populations. The highest production is by Tfh cells and Th17 cells. The IL-21 receptor is broadly expressed on many lymphoid and myeloid cell populations allowing a diverse range of actions.

IL-21 triggers a primarily proliferative response in CD4+ T cell, Tfh, Th17, NK, NKT, B cell and to a lesser extent, CD8+ t cell populations and enhances macrophage phagocytosis, whereas it inhibits generation and survival of Treg populations (Attridge et al., 2012) and inhibits mast and dendritic cell immune and inflammatory responses. It plays a major role in B cell immunoglobulin responses. In the context of a co-stimulatory T cell signal, IL-21 induces differentiation of naïve B cells to form plasma cells and has been implicated in the promotion of autoimmune disease (Gharibi et al., 2016, Tangye, 2015). GWAS studies have identified variants of the IL-21 gene (along with IL-2) as susceptibility locus for SLE (Hughes et al., 2011), type 1 diabetes (Asano et al., 2007) and inflammatory bowel disease (Márquez et al., 2009). Serum IL-21 levels are raised in SLE and RA (Wang et al., 2014, Rasmussen et al., 2010), in conjunction with increased Tfh number, and levels correlate with disease severity.

Similarly, IL-21 has been implicated in other immune-mediated diseases including autoimmune hepatitis (Abe et al., 2016), psoriasis (Caruso et al., 2009, He et al., 2012) and allergic disorders (Chao

et al., 2015, Xiao et al., 2015). It also plays an important role in the context of malignancies where it can induce antitumor responses through activation of T and NK cells (Bhatt et al., 2015).

Elevation of IL-21 in IPAH, in conjunction with an increase in the Tfh cells known to secrete this cytokine adds further evidence to suggest Tfh-mediated immune activation in IPAH. This is also supported by evidence of infiltration of IL21+ cells in vascular lesions in the lungs of IPAH patients (Hashimoto-Kataoka et al., 2015), prominence of these cells in tertiary lymphoid follicles which develop in IPAH lung (Perros et al., 2012) and IL-21 potentiation of the development of PAH in mice (Hashimoto-Kataoka et al., 2015).

3.6.5 Myeloid cells

Amongst myeloid cells, altered NK cell differentiation was observed. These cells play a role in both innate and adaptive immune responses. $CD16^{hi}$ NK cells ($CD56^{dim}$) are considered to be the most cytotoxic NK subset (Poli et al., 2009), facilitating both antibody-dependent cellular cytotoxicity and direct cytotoxicity independent of antibody (Mandelboim et al., 1999). This subpopulation was reduced in IPAH.

The reduction in $CD16^{hi}$ NK cells was accompanied by a relative increase in $CD56^{hi}$ NK cells, although this was not statistically significant. The $CD56^{hi}$ subtype usually comprises a minority of NK cells in PBMC (<10%) but is the major NK subtype in tissues and secondary lymphoid organs. $CD56^{hi}$ NK cells are considered to be more proliferative, to have a higher capacity for cytokine production after stimulation and to have poor cytotoxic effector activity at rest, compared with $CD56^{dim}$ cells (Poli et al., 2009).

Impaired natural killer cell phenotype and function has previously been reported in PAH patients (Ormiston et al., 2012). In contrast to my findings, the study found that the $CD16^{hi}$ ($CD56^{low}$) population was expanded, but functionally defective. Another study has suggested that deficiencies in NK cells may be associated with an increased risk of death in PAH patients (Edwards et al., 2013). However, whether these cells play a role in the disease pathobiology remains uncertain.

3.7 Summary and Conclusion

Within the group of IPAH patients studied, a peripheral blood signature suggesting immune dysregulation is found. Increases in T follicular helper cells (particularly Th2 like Tfh) and plasmablasts are consistent with activation of a pathway which usually culminates in a humoral immune response. This is supported by the elevation in IL-21 which is secreted by activated Tfh and stimulates B cell differentiation. Additionally, an increase in PD1-expressing CD8 T cells is consistent with T cell activation in response to presence or persistence of antigen.

Notably, the peripheral blood immune cell phenotype detected in IPAH is similar to the profile found in a number of autoimmune diseases. This supports the hypothesis that in some patients with IPAH, the disease may be mediated by shared mechanisms. Although there were no significant differences in immunoglobulin quantity between IPAH patients and healthy controls, the potential role of antibodies in the disease process should not be discounted. The analysis presented has only explored immunoglobulin quantity rather than function and thereby does not shed light on immunoglobulin specificity or self-reactivity. Further research to explore these functional aspects are warranted.

This analysis also revealed statistically significant correlations between some key clinical markers of disease severity and B cell abnormalities observed. However, this correlation was not statistically significant for all markers of disease severity. This may be explained by the fact that a number of clinical variables (such as haemodynamics) are only measured infrequently due to their invasive nature. Because of this, their value may not accurately represent the clinical state at the time of blood sampling, particularly in patients who have undergone recent changes to their treatment. Sampling at the time of right heart catheterisation in all patients would provide immediate pairing of haemodynamics with immunophenotype, thereby providing more robust evidence of correlation with haemodynamics.

It must also be noted that IPAH is a clinically heterogeneous disease and these findings should not be generalised to all IPAH patients. Disease heterogeneity is particularly notable amongst older IPAH patients when compared with younger IPAH patients (Chapter 1, Section 1.13). Therefore, we would not necessarily expect the immune signature I have identified to be a universal feature of IPAH. In this cohort studied, few older individuals were recruited, due to the presence of comorbidity in these patients and lack of availability of healthy age-matched controls.

Due to the nature of the clinical presentation of IPAH, often the disease is diagnosed at an advanced stage. The lack of early detection makes it difficult to study early disease and to establish at which point in the disease development the immune cell alterations become evident. Studies in animal models of the disease may provide further insight into this process. Additionally, longitudinal assessment of immune cell profile in IPAH patients paired with haemodynamic and biochemical clinical data are required to establish whether immune profile correlates with disease activity and whether therapeutic strategies modulate both immune cell profile and clinical outcomes. Together these findings provide evidence of immune dysregulation in IPAH which requires further exploration.

4 Chapter 4- Immunophenotyping of other pulmonary hypertension subgroups

4.1 Introduction and objectives

Immunophenotyping of peripheral blood leukocytes has demonstrated distinct differences in IPAH immune cell profile compared to healthy individuals (Chapter 3). This supports the growing body of evidence for the role of immune responses in the disease pathobiology.

I sought to further explore these findings by characterising the immune cell phenotypes of other pulmonary hypertension subgroups: 1. Chronic thromboembolic pulmonary hypertension (CTEPH), 2. Systemic sclerosis associated PAH (SSc-PAH) and 3. Heritable pulmonary arterial hypertension (HPAH).

The purpose of this was to determine whether the findings in IPAH indicate immune dysfunction which is specific to IPAH disease pathobiology, or whether these findings are a universal feature shared by the disease subtypes. Therefore, IPAH immune cell phenotypes were compared with a type of pulmonary hypertension which does not have strong associations with autoimmunity (CTEPH), a type of pulmonary hypertension with a strong autoimmune basis (systemic sclerosis associated PAH) and a type of pulmonary hypertension where a genetic mutation in the BMPR2 gene plays a role in the disease pathogenesis.

Pulmonary hypertension subgroups

1. CTEPH

CTEPH is thought to occur as a result of failure of thrombus resolution in the pulmonary arteries, leading to chronic vascular occlusion coupled with dysfunction of the distal vasculature (Lang et al., 2016). In contrast to IPAH and CTD associated PAH, it does not show a female predominance or have strong associations with immune dysfunction. However, there is a recognised association with chronic inflammatory states, such as chronic infection and malignancy, which are thought to confer risk of developing the disease by causing impairment of thrombus resolution (Lang et al., 2013).

2. SSc-PAH

SSc-PAH refers PAH which occurs as a secondary complication of the autoimmune connective tissue disease systemic sclerosis. Although PAH is associated with a number of connective tissue diseases,

it is particularly prevalent in systemic sclerosis and is the leading cause of death in this disease (Chaisson and Hassoun, 2013). As in IPAH, immune cell infiltrates have been identified in pulmonary arterial vascular lesions in systemic sclerosis-PAH (Dorfmüller et al., 2007). Additionally, anti-fibroblast and anti-endothelial antibodies have been identified, which may be implicated in vascular injury and remodelling (Tamby et al., 2005, Tamby et al., 2006).

3. HPAH

Heritable pulmonary arterial hypertension (HPAH) refers to pulmonary arterial hypertension occurring due to mutations in predisposing genes or in a familial context. Mutations in the BMPR2 gene remain the most common genetic abnormality implicated in heritable PAH (Chapter 1, Section 1.10). However, disease penetrance is low, and therefore other factors must be implicated in disease development. These factors are currently not known.

Objective

To phenotype circulating leukocytes in patients with CTEPH, SSc-PAH and HPAH to determine whether the immune cell 'signature' found in IPAH is specific to this disease or whether it is common amongst pulmonary hypertension subtypes.

Hypothesis

Peripheral blood leukocyte phenotype differs between IPAH and other PH subtypes, reflecting their different disease pathoaetiologies.

4.2 Study population

Subjects were recruited prospectively from the Pulmonary Vascular Diseases Unit, Papworth Hospital, Cambridge UK, as described in section 2.1. 21 patients with CTEPH, 12 patients with SSc-PAH and 9 patients with HPAH were recruited. All patients recruited met diagnostic criteria as described in 2.1.2. An additional 6 controls were added to the 28 controls originally recruited. These disease subgroups and expanded control group were compared to the group of 28 IPAH patients detailed in chapter 3.

4.3 Subject demographics

Subject demographics and clinical parameters are summarised in Table 4.1 and Table 4.2.

Similar to the IPAH group studied, patients in all groups were predominantly prevalent cases and the majority were receiving medical treatment for pulmonary arterial hypertension.

The mean age in the SSc-PAH and CTEPH groups was higher than the other groups studied. In all IPAH, HPAH and SSc-PAH, there was a marked predominance of females, reflecting the known female predilection for development of these diseases.

In each disease group, patients were on a range of pulmonary hypertension therapies, although notably prostanoid therapy was much more common in the IPAH group, compared to the other disease subgroups studied.

Table 4.1- Subject demographics: Healthy controls and pulmonary hypertension sub-groups.

Clinical parameter	**Controls**	**IPAH**	**CTEPH**	**SSc-PAH**	**HPAH**
Number of subjects	34	28	21	12	9
Age (years)	41.7 ± 12.4	41.8 ± 10.5	63.1± 14.2	64.6 ± 12.5	49 ± 15.1
Sex (Female:Male)	29:5	23:5	11:10	9:3	8:1
BMI (kg/m^2)	24.6 ± 3.6	27.8 ± 6.1	29.3 ± 7.5	24 ± 5.4	28.4 ± 4.4

Table 4.2- Pulmonary hypertension subgroup clinical parameters.

Clinical parameter	**IPAH**	**CTEPH**	**SSc-PAH**	**HPAH**
Number of subjects	28	21	12	9
WHO Class (I/II/III/IV)	4/10/14/0	1/10/10/0	0/2/10/0	0/4/5/0
RAP (mmHg)	8.7 ± 3.4	8.9 ± 4.5	8.3 ± 4.4	9.7 ± 4.2
mPAP (mmHg)	51.3 ± 12.5	41.4 ± 9.7	40 ± 12	58 ± 15.7
PCWP (mmHg)	10.7 ± 3.1	11 ± 4.5	10 ± 4	10.6 ± 4.4
PVR (Wood units)	9.7 ± 4.4	7.5 ± 3.1	7.5 ± 3.1	17.2 ± 10

CI (L/min/m^2)- Thermodilution method	2.5 ± 0.8	2.2 ± 0.5	2.5 ± 0.5	1.8 ± 0.5
6 minute walk distance (metres)	461 ± 109	316 ± 133	326 ± 116	365 ± 106
Serum NTproBNP levels (pg/mL)	547 ± 922	1033 ± 1345	2027 ± 2822	1085 ± 891
Pulmonary hypertension therapy				
Nil/monotherapy/combination	2/5/21	5/10/6	2/2/8	0/1/8
PDE5 inhibitor	17	12	9	6
Endothelin receptor antagonist	10	6	2	7
Prostanoid	14	0	3	5
sGC stimulator	1	4	0	0

4.4 Methods

Whole blood was drawn from each subject by peripheral upper limb venepuncture. PBMC isolation and immunophenotyping of fresh blood samples was carried out immediately, as described in section 2.2.

4.5 Data analysis

Previous identification of leukocyte subpopulations in which statistically significant differences were present between IPAH and controls guided a targeted analysis of cell subpopulations. This included analysis of Tfh cells, B cell subpopulations and PD1-expressing CD8+ T cells. In view of regulatory T cell abnormalities previously identified by other studies (Austin et al., 2010a), this cell subpopulation was also assessed in all groups. Firstly, the Kruskal-Wallis test or ANOVA (depending on whether the cell populations conformed to a normal distribution) were used to assess for statistically significant differences between the groups. Secondly, to further characterise differences between the subgroups, each disease subgroup was in turn compared to each of the other groups using t tests with Welch's correction (for normally distributed data) or the Mann Whitney U test (when the data did not conform to a normal distribution).

4.6 Results

4.6.1 Lymphocytes

Total lymphocyte count (cells per million PBMCs) differed between the 5 groups (p< 0.0001), [Figure 4.1]. In addition to the lymphopenia detected in IPAH compared to controls, total lymphocyte count was reduced in both SSc-PAH and CTEPH relative to control (p= 0.0039 and p < 0.0001 respectively). The reduction in total lymphocytes detected in all of these groups was attributable to a reduction in T cells [Figure 4.2A]. Total B cell count did not differ significantly between the 5 groups (p= 0.3) [Figure 4.2B].

In SSc-PAH, both CD4+ and CD8+ T cells were depleted relative to the age-matched controls (p = 0.0103 and p = 0.0253 respectively). In HPAH, although total lymphocytes were not significantly reduced when compared with controls (p = 0.0940), there was a significant reduction in CD4+ T cells (p = 0.0368). Conversely, in CTEPH there was significant reduction in CD8+ T cells only (p= 0.0168) [Figure 4.3]. These comparisons are summarised in Table 4.3.

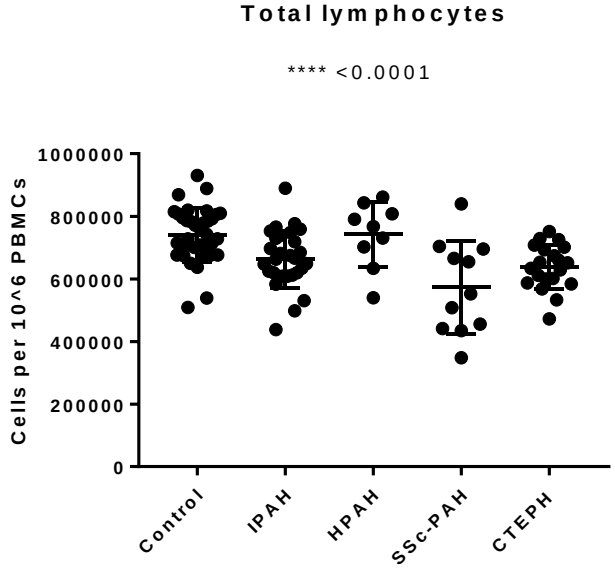


Figure 4.1- T and B lymphocyte count (cells per 10^6 PBMCs) in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). Plot shows cell frequencies with mean ± SD.

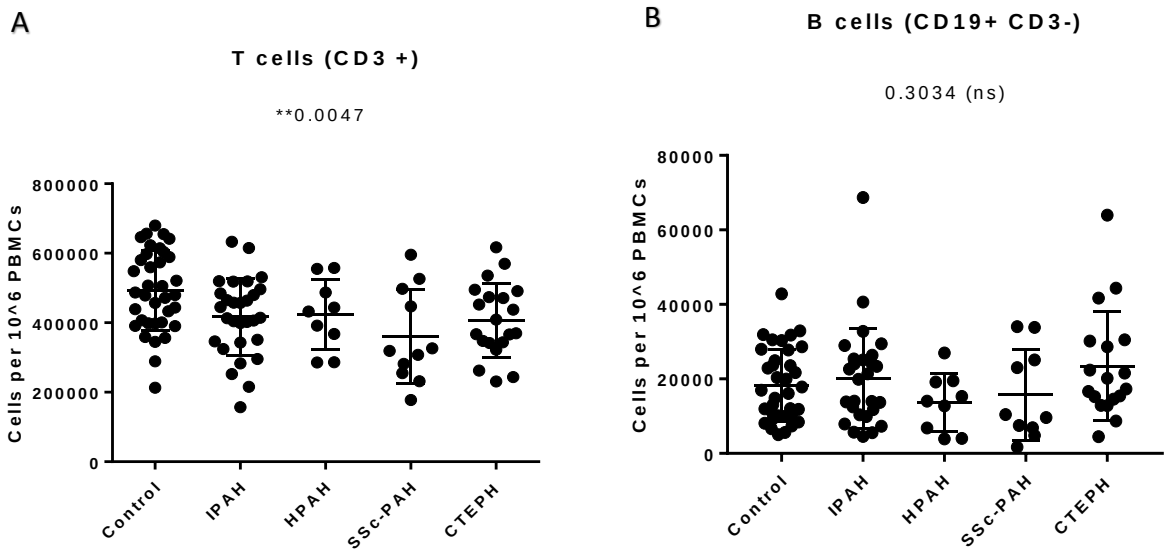


Figure 4.2- T and B lymphocyte count (cells per 10^6 PBMCs) in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). (A) T cells were identified as CD3+ lymphocytes, (B) B cells were identified as CD19+ CD3- lymphocytes. Plots show cell frequencies with mean ± SD.

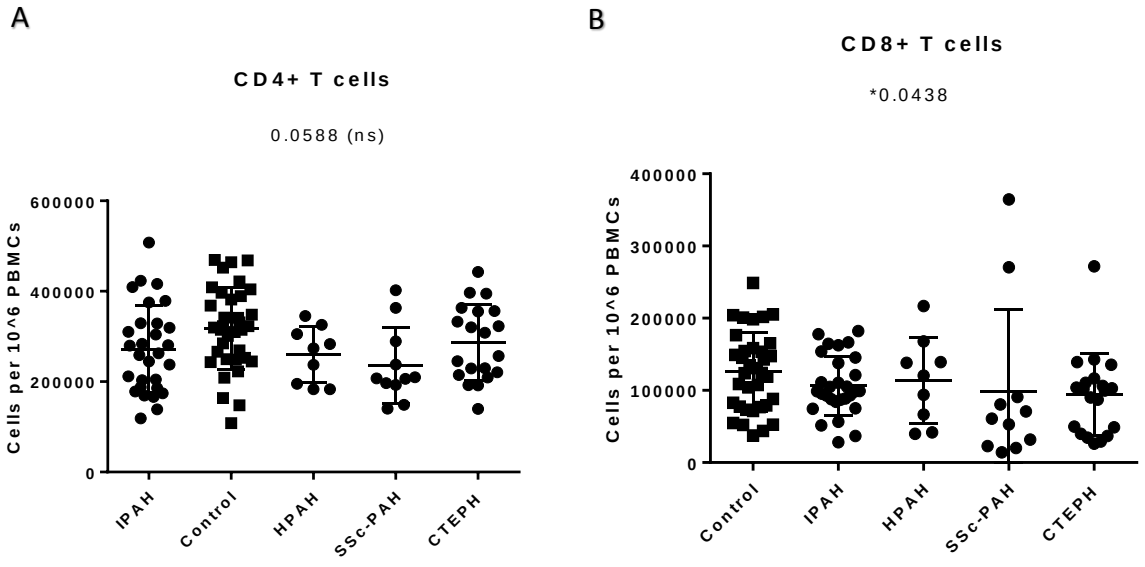


Figure 4.3- CD4+ and CD8+ T lymphocyte count (cells per 10^6 PBMCs) in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes, and subsequently subdivided according to (A) CD4 expression and (B) CD8 expression. Plots show cell frequencies with mean ± SD.

Table 4.3- Comparison of total lymphocytes, total T cells, CD4+ T cell and CD8+ T cell populations between individual subgroups. p values for unpaired t test or Mann Whitney U test. Significant p values (p <0.05) are highlighted.

Comparison	T cell subpopulation			
	Total lymphocytes	T cells	CD4+	CD8+
IPAH vs HPAH	0.0631	0.8814	0.6428	0.7382
Control vs HPAH	0.9903	0.0940	*0.0368	0.6021
IPAH vs SSc	0.0837	0.2347	0.2548	*0.0254
Control vs SSc	**0.0039	*0.0103	*0.0126	*0.0253
IPAH vs CTEPH	0.2913	0.7607	0.5888	0.3271
Control vs CTEPH	****<0.0001	**0.0079	0.2052	*0.0168
SSc vs CTEPH	0.1946	0.3352	0.1231	0.1968
SSc vs HPAH	**0.0083	0.2527	0.4843	0.1754
HPAH vs CTEPH	*0.0191	0.7092	0.3437	0.2534

4.6.2 B cells

Memory B cells (both class switched and non-switched) and double negative B cells showed significant differences between the groups [Figure 4.4a, Figure 4.4b]. Subsequent comparisons are summarised in Table 4.4. Although SSc-PAH and CTEPH groups also showed differences in these B cell subpopulations relative to controls, the increase in plasmablasts seen in both IPAH and HPAH was not found in SSc-PAH or CTEPH [Table 4.4].

Notably, in all four B cell subpopulations assessed, HPAH patients showed a significant difference compared to control. Similar to IPAH patients, those with HPAH showed a reduction in switched and non-switched memory B cells, an increase in plasmablasts and increase in double negative B cells [Figure 4.4, Table 4.4].

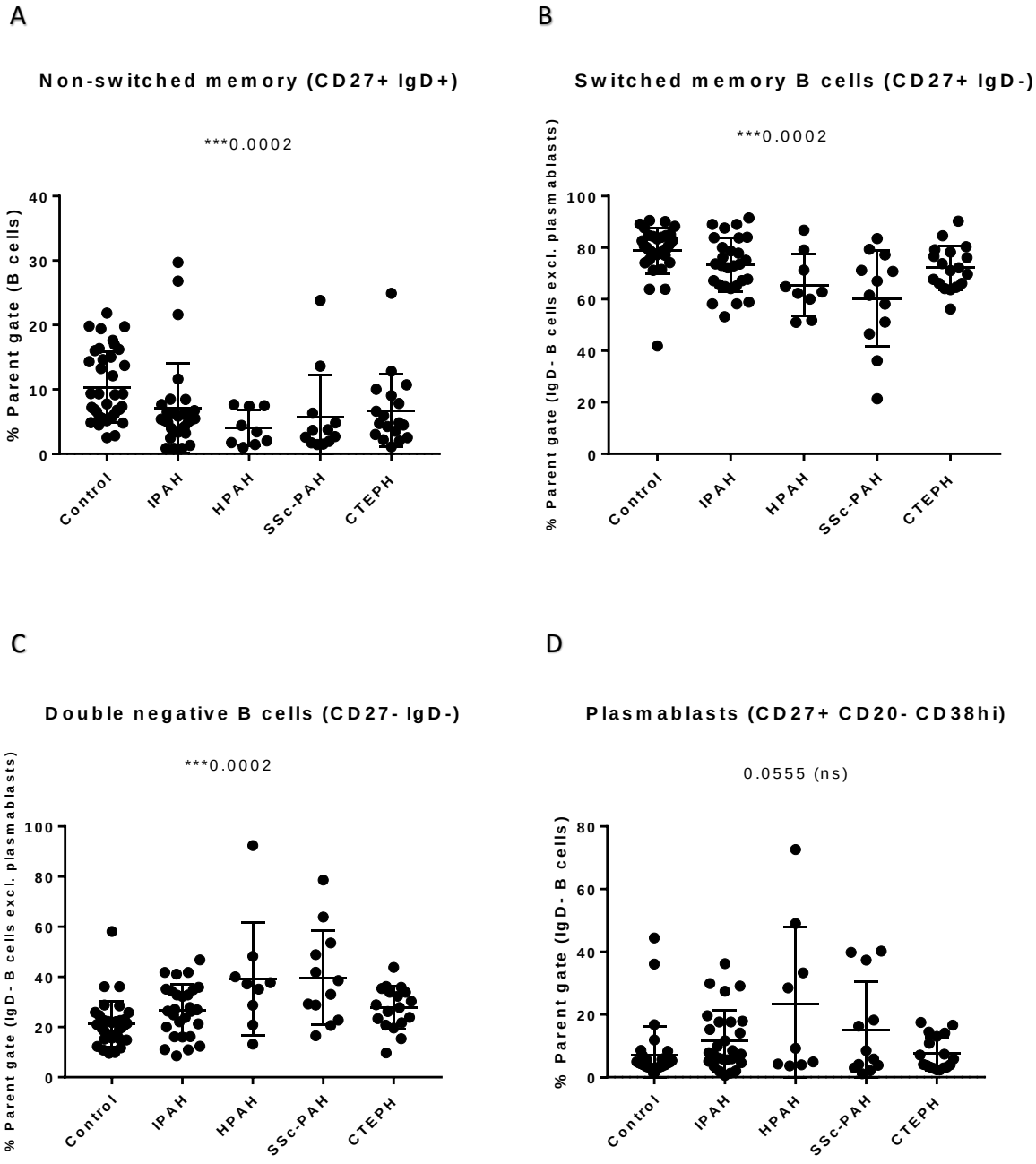


Figure 4.4- B cell subpopulations in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). B cells were identified as CD19+ CD3- lymphocytes. Sequential gating was used to identify; (A) switched memory B cells (CD27+ IgD-), (B) non-switched memory B cells (CD27+ IgD+), (C) plasmablasts (CD27+ CD20- CD38+ IgD-) and (D) double negative B cells (CD27- IgD-). Plots show cell frequencies with mean ± SD.

Table 4.4- Comparison of B cell subpopulations between individual subgroups. p values for unpaired t test or Mann Whitney U test. Significant p values ($p < 0.05$) are highlighted.

Comparison	B cell subpopulation			
	Non-switched memory	Switched memory	Plasmablasts	Double negative
IPAH vs HPAH	0.2993	0.1030	0.5148	0.0562
Control vs HPAH	**0.0014	**0.0027	*0.0463	**0.0024
IPAH vs SSc-PAH	0.1534	**0.0076	0.9369	*0.0401
Control vs SSc-PAH	***0.0007	*0.0230	0.2920	***0.0002
IPAH vs CTEPH	>0.99	0.7256	0.1370	0.7138
Control vs CTEPH	**0.0055	**0.0029	0.6994	**0.0029
SSc-PAH vs CTEPH	0.2154	0.0567	0.1306	0.0565
SSc-PAH vs HPAH	0.9170	0.4434	0.3958	0.8078
HPAH vs CTEPH	0.1450	0.1540	0.0938	0.0827

4.6.3 T cells

Comparisons between the groups are summarised in Table 4.5. Tfh cells and Th2-like Tfh cells showed a significant difference in frequency between the five disease groups [Figure 4.5]. In HPAH, Tfh and Th2-like Tfh were elevated relative to controls (p= 0.0423 and p= 0.002 respectively), similar to the previous findings in IPAH. In contrast, in the other disease groups, Tfh frequencies were not significantly different to controls.

Frequencies of PD1-expressing CD8+ T cells did not differ significantly between the five groups (p= 0.2230), [Figure 4.6]. However, it is evident that the proportion of PD1+ CD8+ T cells showed much greater variability in the disease groups compared to controls. Regulatory T cells did not show any significant difference between the five groups (p= 0.3686) [Figure 4.7].

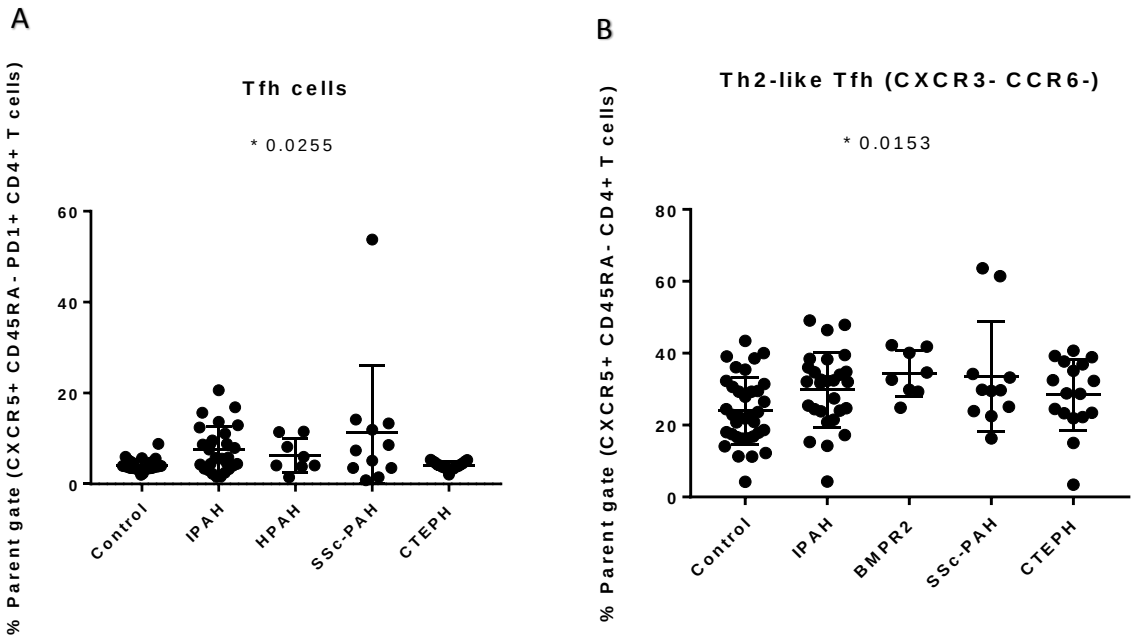


Figure 4.5- Tfh cells in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes. Sequential gating was used to identify (A) Tfh cells (CXCR5+ CD45RA- PD1+) and (B) Th2-like Tfh cells (CXCR3- CCR6-). Plots show cell frequencies with mean ± SD.

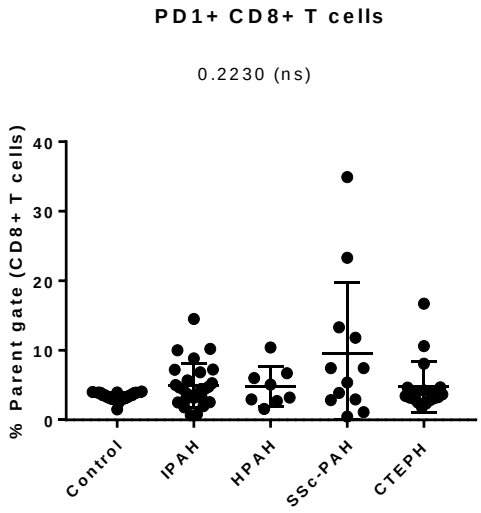


Figure 4.6- PD1+ CD8+ T cells in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained Peripheral blood samples from 28 IPAH patients and 28 age and sex matched controls healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A) and T cells were identified as CD3+ lymphocytes. Sequential gating was used to identity CD8+ T cells. Plot shows cell frequencies with mean ± SD.

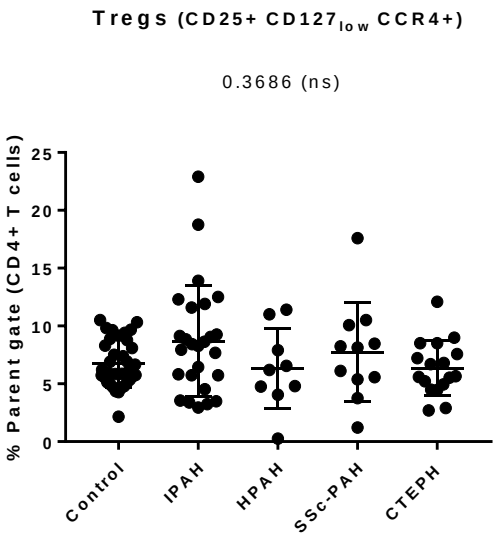


Figure 4.7- Regulatory T cells (Tregs) in healthy controls, IPAH, HPAH, SSc-PAH and CTEPH. Peripheral blood samples from 34 healthy controls, 28 IPAH patients, 21 CTEPH, 12 SSc-PAH and 9 HPAH patients were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. Lymphocytes were identified from the PBMC population by flow cytometry by gating of FSC (A) vs SSC (A). T cells were identified as CD3+ lymphocytes. Sequential gating was used to identify Tregs (CD25+ CD127low CCR4+). Plot shows cell frequencies with mean ± SD.

Table 4.5- Comparison of T cell subpopulations between individual subgroups. Significant p values ($p < 0.05$) are highlighted.

Comparison	**T cell subpopulation**			
	CD8+ PD1+	**Tfh**	**Th2 like Tfh**	**Treg**
IPAH vs HPAH	0.9570	0.4580	0.1454	0.2363
Control vs HPAH	0.9531	*0.0423	**0.0020	0.7339
IPAH vs SSc	0.7192	0.9388	0.4659	0.5839
Control vs SSc	0.6428	0.0815	0.0707	0.4757
IPAH vs CTEPH	0.6517	**0.0012	0.6714	0.0593
Control vs CTEPH	0.7890	0.5944	0.1262	0.555
SSc vs CTEPH	0.2665	0.1469	0.3425	0.3432
SSc vs HPAH	0.3944	0.6422	0.8732	0.4274
HPAH vs CTEPH	0.8919	0.1340	0.0896	0.9850

4.6.4 Overview of similarities and differences between groups

4.6.4.1 CTEPH

Similar to the other PH subgroups studied, patients with CTEPH showed a reduction in switched memory B cells and increase in double negative B cells relative to controls. However, in contrast to the difference detected between IPAH and healthy controls (Chapter 3), increased frequencies of Tfh, Th2-like Tfh and plasmablasts were not present in CTEPH. Comparisons are summarised in Table 4.6.

Table 4.6- Summary of comparisons between CTEPH and other groups.

Comparison	**Sig. diff.**	**Higher in CTEPH**	**p value**	**Lower in CTEPH**	**P value**
CTEPH vs control	YES	Double negative B cells	0.0029	Total lymphocytes T cells CD8+ T cells Switched memory B	< 0.0001 0.0079 0.0168 0.0029
CTEPH vs IPAH	YES			Tfh	0.0012
CTEPH vs HPAH	YES			Total lymphocytes	0.0191
CTEPH vs SSc-PAH	NO				

4.6.4.2 SSc-PAH

The immune cell phenotype of SSc-PAH also shows a reduction in memory B cells and increased double negative B cells relative to healthy controls. Consistent with activation of humoral mediated immunity which is a known feature of SSc-PAH, mean levels of both plasmablasts and Tfh were higher in SSc-PAH than controls [Table 4.7]. However, these differences were not statistically significant. Comparison are summarised in Table 4.8.

Table 4.7- Summary of Tfh and plasmablast frequencies in groups studied. Median, mean and standard deviation are shown.

Tfh	**IPAH**	**Control**	**HPAH**	**SSc-PAH**	**CTEPH**
Median	5.78	3.81	5.03	7.37	4.09
Mean	7.54	4.11	6.31	11.24	4.08
SD	5.02	1.23	3.7	14.86	0.84
Plasmablasts					
Median	7.60	4.56	9.2	7.19	4.97
Mean	11.65	7.16	23.27	15.03	7.53
SD	9.59	8.94	24.69	15.48	5.36

Table 4.8- Summary of comparisons between SSc-PAH and other groups.

Comparison	Sig. diff.	Higher in SSc-PAH	p value	Lower in SSc-PAH	p value
SSc-PAH vs control	YES	Double negative B cells	0.0002	Total lymphocytes T cells CD4+ T cells CD8+ T cells Switched memory B Non-switched mem. B	0.0039 0.0103 0.0126 0.0253 0.0230 0.0007
SSc-PAH vs IPAH	YES	Double negative B cells	0.0401	CD8+ T cells Switched memory B	0.0254 0.0076
SSc-PAH vs HPAH	YES			Total lymphocytes	0.0083
SSc-PAH vs CTEPH	NO				

4.6.4.3 HPAH

In HPAH, differences in immune cell phenotype are found compared to healthy controls, which parallel those seen in IPAH (Chapter 3). In the T cell subpopulations assessed, this included increases in Tfh and Th2-like Tfh, as in IPAH. In the B cell subpopulations assessed, this included increases in plasmablasts and double negative B cells and a reduction in switched and non-switched memory B cells, as in IPAH. The derangements in some immune cell subsets was even more pronounced in HPAH than IPAH. For example, non-switched memory B cells were lower in HPAH than in any other group studied. Comparisons are summarised in Table 4.9.

Table 4.9- Summary of comparisons between HPAH and other groups.

	Sig. diff.	Higher in HPAH	p value	Lower in HPAH	p value
HPAH vs control	YES	Plasmablasts Double negative B Tfh Th2-like Tfh	0.0463 0.0024 0.0423 0.0020	Switched memory B Non-switched mem. B	0.0027 0.0014
HPAH vs IPAH	NO				
HPAH vs SSc-PAH	YES	Lymphocytes	0.0083		
HPAH vs CTEPH	YES	Lymphocytes	0.0191		

4.7 Discussion

Previously, there have been no published immunophenotyping studies in the CTEPH population and there has been only limited study of SSc-PAH and HPAH, focused predominantly on functional aspects of regulatory T cells (Huertas et al., 2012). Therefore, my research has provided the most comprehensive immunophenotyping assessment to date not only in IPAH, but also in these disease subgroups.

My results demonstrate both a number of differences and a number of similarities in immune cell phenotype in between PH disease subgroups, and between disease subgroups and healthy controls. In all PH subgroups except HPAH, there were depletions in total numbers of T lymphocytes, when compared with the healthy controls. In SSc-PAH and CTEPH, there was a global T cell depletion. In contrast, in both IPAH and HPAH compared to healthy controls, there was a selective reduction in CD4+ T cells. Therefore, it is possible that different mechanisms may be responsible for the different patterns of lymphopenia observed in these subgroups (potential mechanisms are previously discussed in Chapter 3, Section 3.5.1).

Notably, the increases in Tfh, Th2-like Tfh and plasmablasts found in IPAH and HPAH compared to healthy controls are not found in CTEPH. This immunophenotypic signature which characterises IPAH and HPAH has also been reported in immunoinflammatory diseases including multiple sclerosis (Romme Christensen et al., 2013), active rheumatoid arthritis (Arroyo-Villa et al., 2014), and graft versus host disease (Forcade et al., 2016). Therefore, my findings support the hypothesis that immuno-inflammatory mechanisms are implicated in the pathogenesis of IPAH and HPAH but not CTEPH.

Although antibody-mediated immune activation is a recognised feature of active SSc, surprisingly I did not detect a statistically significant increase in plasmablasts or Tfh in the SSc-PAH population. This may be partly due to the small sample size and also due to the fact that SSc patients who develop PAH usually have advanced disease, where much of the immune-mediated damage may have occurred earlier in the disease process. Further study in a larger population may clarify this.

Interestingly, a reduction in memory B cells and an increase in double negative B cells are features of all PH subgroups studied, regardless of disease pathoaetiology. The fact that these B cell abnormalities are present in the different disease subgroups raises the question as to whether these changes are attributable to the presence of the abnormal haemodynamics, chronic heart failure, inflammation associated with vascular remodelling or medication used to treat the disease.

An increase in 'double negative' B cells has been reported as a feature of ageing and chronic inflammatory states (Colonna-Romano et al., 2009), whereas reduction in memory B cells has previously been reported both in chronic heart failure (Seeger et al., 2013) and in a range of immunologically mediated diseases including HIV (De Milito et al., 2001), systemic sclerosis (Simon et al., 2016), hashimoto's thyroiditis (Liu et al., 2017) and ulcerative colitis (Wang et al., 2016b). However, the mechanism responsible for these B cell changes shared by the PH subtypes is unclear and requires further investigation.

Intriguingly, there were no significant differences in immune cell phenotype between IPAH and HPAH. Clinically these PAH subtypes closely resemble one another, although HPAH patients tend to present at a younger age and have a more rapid disease progression (Sztrymf et al., 2008). Studies of animal models and pulmonary artery smooth muscle cells (PASMCs) suggest that loss of BMPR2 gene function promotes an exaggerated inflammatory response in response to antigen, hypoxia and inflammatory stimuli, thereby predisposing to the development of pulmonary hypertension (Park et al., 2013). However, the exact mechanisms leading to the development of PAH in BMPR2 mutation carriers is not fully understood. It is plausible that the presence of the mutation acts as a susceptibility factor for disease or potentiates disease development, and that the underlying mechanism (and perhaps the initial trigger(s) for the disease) are shared in IPAH and HPAH, which is the reason that these diseases exhibit a shared immunophenotype.

Limitations

My data provide a small-scale study of peripheral blood immunophenotype in different PH subtypes, with novel findings which warrant further exploration. It should be acknowledged that the subjects recruited in the CTEPH, SSc-PAH and HPAH disease populations were not as closely matched as the initial IPAH and control cohorts studied in Chapter 3. Of particular note, both CTEPH and SSc-PAH patients were older, and the CTEPH group contained a higher proportion of male subjects. Therefore, age-related changes may have had some potential influence on immune cell phenotype in these groups (Apoil et al., 2017, Montecino-Rodriguez et al., 2013, Stervbo et al., 2015). Additionally, although care was taken to recruit individuals who did not have other diseases, older individuals are more likely to have had undiagnosed comorbidity. Additionally, samples collected from the CTEPH, SSc-PAH and HPAH groups may be influenced by circadian and seasonal factors (Mazzoccoli et al., 2011, Kirsch et al., 2012, Paglieroni and Holland, 1994, Lévi et al., 1988) as they were not collected at the same time as the initial IPAH and control samples.

Within the disease subgroups, most patients were receiving treatment for their disease, although the nature of treatment differed. In particular, intravenous prostanoid therapy was common amongst the IPAH group but not CTEPH. Both treatment of the disease and differences in the type of treatment received could potentially have influences on immune cell phenotype. However, immunophenotyping studies which assess treatment effect are lacking. These potential influences could be further studied by immunophenotyping a greater number of treatment naïve patients and subsequently monitoring immune phenotype in response to initiation of treatment. Longitudinal monitoring of immunophenotype would also provide insights into the potential role of circulating immune cell phenotype in disease progression and whether immunophenotyping has a prognostic value.

4.8 Conclusion

In comparison to healthy controls, the immune cell phenotype in IPAH and HPAH has a number of features which are not found in CTEPH, including an increase in Tfh cells and an increase in plasmablasts. This suggests that these abnormalities are a feature of IPAH and HPAH disease pathobiology, rather than secondary to the presence of abnormal haemodynamics or medication used to treat the disease. This adds to the growing body of evidence implicating immuno-inflammatory dysfunction in the pathobiology of IPAH, and also sheds new light on the potential shared mechanisms in HPAH.

In addition to the differences found between subgroups, I have also demonstrated that reduction in memory B cells and an increase in double negative B cells is a feature common to all PH subgroups studied. These findings are present despite different disease pathoaetiologies. However, it is unclear as to whether these abnormalities are the result of altered cardiopulmonary haemodynamics common to all pulmonary hypertension subgroups, whether it is a reflection of treatment of the disease, or whether there are other mechanisms responsible for this B cell derangement. Further research is required to elucidate the mechanisms behind the derangements in immune cell phenotype that I have detected and to explore the functional consequences of these abnormalities.

5 Chapter 5- Circulating blood metabolite profile in pulmonary vascular disease

5.1 Introduction

Historically, assessment of metabolism in pulmonary vascular disease has been limited to a narrow approach, usually targeted to a single substance or biochemical reaction. However, recent advances in metabolomics now permit simultaneous assessment of thousands of metabolites in a tissue, organ or system. Several studies have used metabolomics technology to identify metabolites and pathways which may be important in the pathobiology of PAH by studying cultured human pulmonary microvascular endothelial cells (Fessel et al., 2012), explanted lung tissue (Zhao et al., 2014a) and exhaled breath condensate (Mansoor et al., 2014). Few studies have assessed metabolite profile of circulating blood in humans (Bujak et al., 2016, Rhodes et al., 2017).

It is unknown whether the metabolic changes identified by these studies are a marker of pulmonary vascular bed dysfunction associated with abnormal vascular remodelling and loss of functional vascular surface area, whether they are markers of right heart strain or whether they reflect a more widespread metabolic dysfunction in the disease. Additionally, there have been no studies which have characterised the circulating blood metabolome in CTEPH and CTED. Therefore, it is also unknown whether the metabolic abnormalities detected in PAH are unique to this disease subtype, or whether they are a shared feature in other disease processes affecting the pulmonary vasculature.

I sought to explore this further, by profiling circulating metabolites in patients with a spectrum of pulmonary vascular diseases, including IPAH, CTEPH and CTED. Firstly, I set out to establish whether there are significant differences in the metabolite profile of venous blood between the disease population and healthy controls. I then sought to determine whether blood metabolite profile differs between the disease subgroups, and whether there is a correlation between metabolite concentration and cardiopulmonary haemodynamics.

5.2 Objectives and hypotheses

Objective 1: To profile circulating metabolites in venous blood samples from patients with pulmonary vascular disease and healthy controls and identify metabolites which show a difference in concentration between disease and control.

Hypothesis 1: Metabolites in venous blood of patients with pulmonary vascular disease will differ from healthy individuals.

Objective 2: To identify circulating metabolites which show a difference in concentration between disease subtypes; IPAH, CTEPH and CTED.

Hypothesis 2: Metabolite concentration in venous blood will differ between patients with IPAH, CTEPH and CTED.

Objective 3: To identify metabolites which correlate with abnormal pulmonary haemodynamics and with markers of cardiac dysfunction.

Hypothesis 3: Metabolic abnormalities detected in patients with pulmonary vascular disease are associated with abnormal pulmonary haemodynamics and with markers of cardiac dysfunction.

5.3 Methods

Blood samples were collected as described in Chapter 2. In summary, blood samples from patients were collected at the time of right heart catheterisation. Blood samples from healthy controls were collected by peripheral upper limb venepuncture. After collection in K2 EDTA tubes, the samples were immediately placed on ice and processed to obtain plasma (as described in section 2.2.2). Samples were frozen and stored at -80˚C and later processed in a single batch by Metabolon Inc. (Durham, NC, USA). Untargeted metabolic profiling was carried out using the Metabolon DiscoveryHD4™ platform (as described in section 2.2.5).

The concentration of each metabolite (expressed as median scaled standardised intensity) was compared between venous samples from patients with pulmonary vascular disease and healthy controls using the Mann Whitney U test, with false discovery rate (FDR) adjustment for multiple testing. Subsequently, the concentration of each metabolite (expressed as median scaled standardised intensity) was compared between venous samples from patients with different subtypes of pulmonary vascular disease, using the Mann Whitney U test, with false discovery rate adjustment for multiple testing.

For metabolites which showed a significant difference in concentration between disease and control, correlation of the median scaled metabolite concentration with the following clinical parameters was assessed; pulmonary vascular resistance (PVR), mean pulmonary artery pressure (mPAP), cardiac index (CI) and N-terminal pro brain natriuretic peptide (NTproBNP) level. A two-tailed test was conducted, using Spearman's rank correlation. Metabolite concentration is expressed as relative standardised intensity (RSI). Spearman r is quoted with 95% confidence interval.

5.4 Study population

The study population included 57 patients with pulmonary vascular disease and 27 healthy controls [Table 5.1]. The disease group consisted of 9 patients with IPAH and 48 patients with chronic thromboembolic pulmonary vascular occlusions [Table 5.2]. Within the chronic thromboembolic pulmonary vascular occlusions group, there were patients both with and without pulmonary hypertension [Table 5.3].

There were some patients who had been treated with pulmonary endarterectomy, with varying degrees of haemodynamic improvement. The heterogeneous nature of the disease population was intended to firstly reflect the disease population in clinical practice, secondly to allow comparisons to be made between patients with and without pulmonary hypertension, and thirdly to assess whether metabolic changes may potentially be reversed by pulmonary endarterectomy.

Table 5.1- Demographics of the disease group and the control group.

	All pulmonary vascular disease	**All control**
Number of individuals	57	27
Gender (% male)	58	56
Age (mean, range)	56.3 (22-79)	44.6 (19-75)
Body Mass Index	29.7	26
Ethnicity (% Caucasian)	91%	93%

Table 5.2- Clinical characteristics of the disease group.

	Chronic vascular occlusions	IPAH
Number of individuals	48	9
Gender (% male)	63	33
Age (mean, range)	57.7 (22-79)	49 (28-77)
Body Mass Index	30	28
Ethnicity (% Caucasian)	90%	100%
RAP (mmHg)	8	8
mPAP (mmHg)	32	48
PVR (Wood units)	5.9	12.3
CI (L/min/m2)	2.1	1.9
Creatinine (µmol/L)	98	92
Bilirubin (µmol/L)	12	40
NTproBNP (ng/L)	839	384

Table 5.3- Chronic pulmonary vascular occlusions subgroup characteristics.

Chronic pulmonary vascular occlusions subgroup	**Number of patients**
PULMONARY HYPERTENSION	**27**
Proximal CTEPH- treatment naïve	13
Previous pulmonary endarterectomy for proximal CTEPH, residual PH	11
Distal CTEPH	3
NO PULMONARY HYPERTENSION	**21**
Chronic thromboembolic vascular occlusions without PH	8
Previous pulmonary endarterectomy for proximal CTEPH, no residual PH	13

5.5 Results

5.5.1 Comparison of disease with controls

The concentration of metabolites in SVC blood samples from 57 patients with pulmonary vascular disease was compared to peripheral venous blood samples from 27 healthy controls. A total of 1375 metabolites were detected [shown in appendix Table 2], of which 430 metabolites showed a significant difference between disease and control after FDR adjustment for multiple testing [Table 5.4]. This consisted of 283 endogenous metabolites, 27 xenobiotics and 120 unidentified compounds. (Using the more stringent Bonferroni adjustment, 69 metabolites showed a significant difference between disease and control). Of the endogenous metabolites, this comprised of 166 lipid metabolites, 63 amino acids, 24 peptides, 16 nucleotides, 5 carbohydrate metabolites, 5 cofactors and vitamins and 4 TCA cycle metabolites. Enrichment analysis confirmed that amongst the metabolites which showed a significant difference between disease and control, 42 sub-pathways were over-represented [Table 5.5].

Amongst the metabolites which showed a significant difference between disease and control, fold-change in mean metabolite concentration between disease and control was calculated. Subgroups of metabolites with consistent unidirectional perturbations were then identified. Key findings are shown in Figure 5.1.

Table 5.4- Metabolite pathways in which there was a significant difference in concentration between venous blood samples from disease and controls. Metabolite concentration expressed as median scaled standardised intensity was used and false discovery rate adjustment for multiple testing was applied. * indicates metabolites sub-pathways which retain statistical significance after Bonferroni method adjustment.

SUPER_PATHWAY	Number of metabolites	SUB_PATHWAY	Number of metabolites
Amino Acid	63	Leucine, Isoleucine and Valine Metabolism*	10
		Methionine, Cysteine, SAM and Taurine Metabolism*	10
		Tryptophan Metabolism*	7
		Histidine Metabolism*	6
		Phenylalanine and Tyrosine Metabolism*	6
		Lysine Metabolism*	5
		Glutamate Metabolism*	4

		Polyamine Metabolism*	4
		Urea cycle; Arginine and Proline Metabolism*	4
		Alanine and Aspartate Metabolism*	3
		Glutathione Metabolism	2
		Glycine, Serine and Threonine Metabolism	2
Carbohydrate	5	Aminosugar Metabolism*	3
		Fructose, Mannose and Galactose Metabolism	1
		Glycogen Metabolism	1
Cofactors and Vitamins	5	Ascorbate and Aldarate Metabolism	3
		Vitamin A Metabolism*	2
Energy	4	TCA Cycle	4
Lipid	166	Lysolipid*	38
		Steroid*	24
		Phospholipid Metabolism*	19
		Plasmalogen*	15
		Fatty Acid, Monohydroxy	9
		Long Chain Fatty Acid*	9
		Sphingolipid Metabolism	9
		Fatty Acid Metabolism (Acyl Carnitine)*	8
		Lysoplasmalogen*	6
		Medium Chain Fatty Acid*	5
		Polyunsaturated Fatty Acid (n3 and n6)	4
		Fatty Acid, Dicarboxylate	3
		Glycerolipid Metabolism*	3
		Lyso-phospho-ether*	3
		Fatty Acid Metabolism (Acyl Choline)	2
		Fatty Acid Metabolism (Acyl Glycine)	2
		Fatty Acid Metabolism (Acyl Glutamine)*	1
		Fatty Acid, Amide	1
		Fatty Acid, Amino	1

		Fatty Acid, Branched	1
		Ketone Bodies	1
		Mevalonate Metabolism	1
		Sterol	1
Nucleotide	16	Purine Metabolism, Adenine containing	5
		Pyrimidine Metabolism, Uracil containing	3
		Purine Metabolism, Guanine containing*	2
		Pyrimidine Metabolism, Cytidine containing	2
		Pyrimidine Metabolism, Thymine containing	2
		Purine Metabolism, (Hypo)Xanthine/Inosine containing*	1
		Pyrimidine Metabolism, Orotate containing	1
Peptide	24	Dipeptide*	17
		Gamma-glutamyl Amino Acid	4
		Acetylated Peptides	2
		Polypeptide	1

Table 5.5- Metabolite sub-pathways over-represented in disease compared to control. Of the metabolite pathways in which significant differences were identified between disease and controls, a number of sub-pathways which were over-represented (enrichment value >1).

Sub-pathway	**Compounds in pathway**		**Enrichment value**
	significant	**detected**	
Polyamine Metabolism	4	4	3.20
Vitamin A Metabolism	2	2	3.20
Lysoplasmalogen	6	6	3.20
Glycerolipid Metabolism	3	3	3.20
Lyso-phospho-ether	3	3	3.20
Fatty Acid Metabolism (Acyl Choline)	2	2	3.20
Mevalonate Metabolism	1	1	3.20
Pyrimidine Metabolism, Thymine containing	2	2	3.20
Ascorbate and Aldarate Metabolism	3	4	2.40
Steroid	24	32	2.40

Medium Chain Fatty Acid	5	7	2.28
Fatty Acid Metabolism(Acyl Glycine)	2	3	2.13
Purine Metabolism, Guanine containing	2	3	2.13
Plasmalogen	15	23	2.09
Fatty Acid, Monohydroxy	9	14	2.06
Long Chain Fatty Acid	9	14	2.06
Lysolipid	38	60	2.03
Purine Metabolism, Adenine containing	5	8	2.00
Methionine, Cysteine, SAM and Taurine Metabolism	10	20	1.60
Histidine Metabolism	6	12	1.60
Aminosugar Metabolism	3	6	1.60
Glycogen Metabolism	1	2	1.60
Fatty Acid Metabolism (Acyl Glutamine)	1	2	1.60
Fatty Acid, Amide	1	2	1.60
Fatty Acid, Amino	1	2	1.60
Ketone Bodies	1	2	1.60
TCA Cycle	4	9	1.42
Fatty Acid Metabolism (Acyl Carnitine)	8	18	1.42
Alanine and Aspartate Metabolism	3	7	1.37
Pyrimidine Metabolism, Uracil containing	3	7	1.37
Leucine, Isoleucine and Valine Metabolism	10	24	1.33
Lysine Metabolism	5	12	1.33
Dipeptide	17	41	1.33
Glutathione Metabolism	2	5	1.28
Pyrimidine Metabolism, Cytidine containing	2	5	1.28
Acetylated Peptides	2	5	1.28
Glutamate Metabolism	4	11	1.16
Tryptophan Metabolism	7	20	1.12
Polyunsaturated Fatty Acids	4	12	1.07
Fatty Acid, Branched	1	3	1.07
Pyrimidine Metabolism, Orotate containing	1	3	1.07
Polypeptide (bradykinin)	1	3	1.07

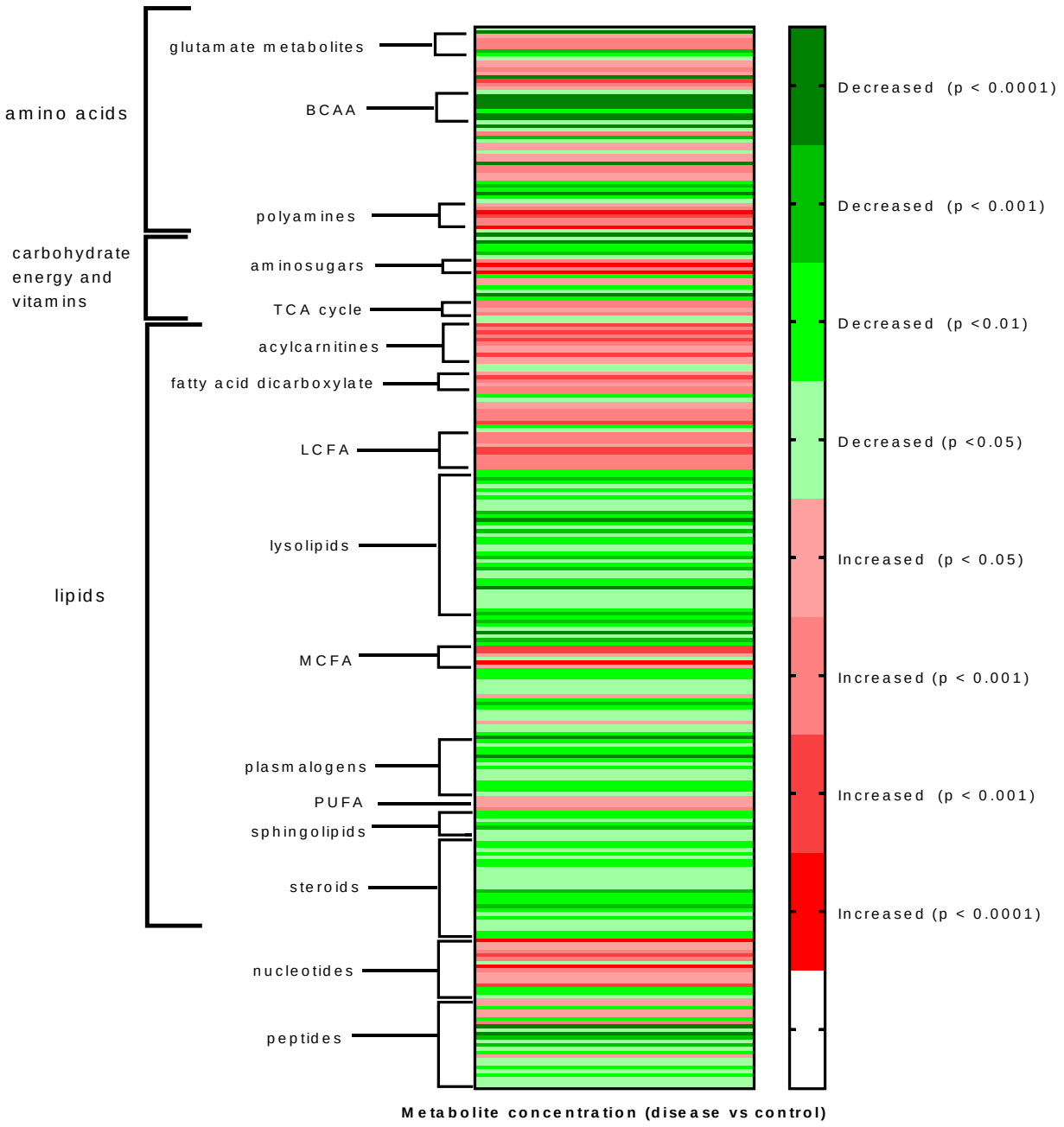


Figure 5.1- Heat map displaying key groups of endogenous metabolites which showed a significant difference in concentration between disease and control venous plasma. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease and 27 healthy controls was carried out using the Metabolon DiscoveryHD4™ platform. The concentration of each metabolite (median scaled standardised intensity) was compared between venous samples from patients with pulmonary vascular disease and healthy controls using the Mann Whitney U test, with FDR adjustment. Metabolites which were increased in the disease group compared to healthy controls are shown in red, metabolites which were decreased in the disease group compared to healthy controls are shown in green. BCAA-branched chain amino acids, TCA- tricarboxylic acid, LCFA-long chain fatty acid, MCFA-medium chain fatty acid, PUFA-polyunsaturated fatty acid.

5.5.2 Disease subgroup analysis

Chronic thromboembolic vascular occlusions: CTEPH vs CTED

228 metabolites showed a significant difference between CTEPH and CTED prior to FDR adjustment for multiple testing. However, after FDR adjustment, only one metabolite, the diacylglycerol linoleoyl-linolenoyl-glycerol (18:2/18:3) showed a significant difference between CTEPH and CTED. The concentration of this metabolite was higher in those without pulmonary hypertension [Figure 5.2].

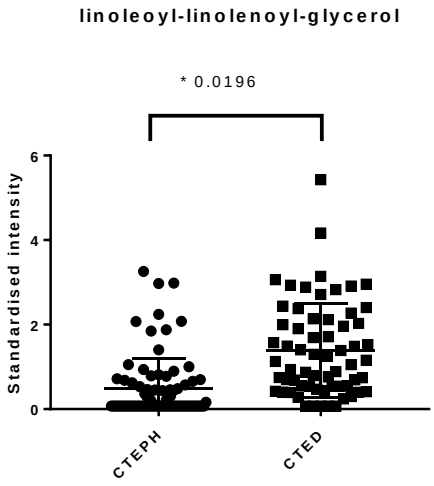


Figure 5.2- Linoleoyl-linolenoyl-glycerol (18:2/18:3) concentration in CTEPH compared to CTED patients. The concentration of each metabolite (median scaled standardised intensity) was compared between venous plasma samples from 27 patients with CTEPH and 21 patients with CTED, using the Mann Whitney U test with FDR adjustment. Linoleoyl-linolenoyl-glycerol (18:2/18:3) was the only metabolite to show a significant difference in concentration between the groups.

2. CTEPH vs IPAH

130 metabolites showed a significant difference between CTEPH and IPAH prior to FDR adjustment for multiple testing. However, none retained statistical significance after FDR adjustment.

3. IPAH vs CTED

After FDR adjustment, only one metabolite, the lysolipid 1-lignoceroyl-GPC (24:0) showed a significant difference between IPAH and CTED. The concentration of this metabolite was higher in the CTED group than the IPAH group [Figure 5.3].

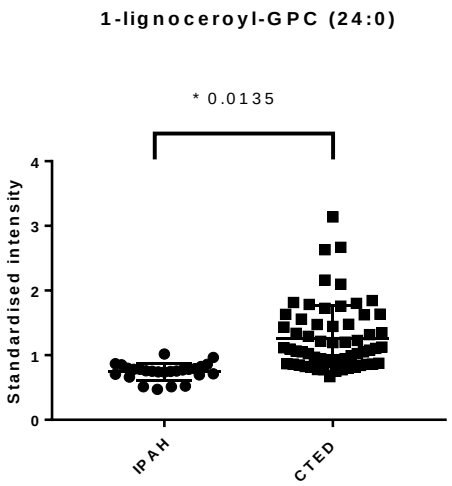


Figure 5.3- 1-lignoceroyl-GPC (24:0) in IPAH compared to CTED patients. The concentration of each metabolite (median scaled standardised intensity) was compared between venous plasma samples from 27 patients with CTEPH and 21 patients with CTED, using the Mann Whitney U test with FDR adjustment. 1-lignoceroyl-GPC (24:0) was the only metabolite to show a significant difference in concentration between the groups.

5.5.3 Clinical correlations

Many metabolites which showed a significant difference in concentration between disease and control were correlated with clinical markers of disease severity, providing evidence of an increasingly severe metabolic derangement in patients with more advanced disease. A number of key correlations are illustrated below [Figure 5.4]. Full results of all correlations assessed are detailed in appendix 3.

Polyamines and catecholamine metabolites were positively correlated with PVR, mPAP and NTproBNP level, and were negatively correlated with CI, indicating an association between these metabolites all 4 markers of disease severity. TCA cycle metabolites and histidine metabolites also showed a positive correlation with PVR mPAP and NTproBNP. Aminosugars such as N-acetylglucosaminylasparagine showed a positive correlation with PVR and NTproBNP. Arginine was negatively correlated with PVR, mPAP and BNP whereas associated metabolites dimethylarginine and urea were positively correlated. Modified nucleotides such as N1-methyladenosine, N1-methylinosine and N2, N2-dimethylguanosine were also significantly correlated with markers of disease severity.

Fatty acid metabolites (including medium and long chain fatty acids and PUFAs) show a significant correlation with NTproBNP and CI, indicating an association between these metabolites and impaired cardiac function. However, fatty acid metabolites did not show significant correlation with pulmonary haemodynamics (mPAP and PVR). In contrast, acylcarnitine concentration shows a positive correlation with both PVR, mPAP and BNP. Diacylglycerols were negatively correlated with PVR, mPAP and BNP. Steroid hormones such as DHEA were positively correlated with cardiac index.

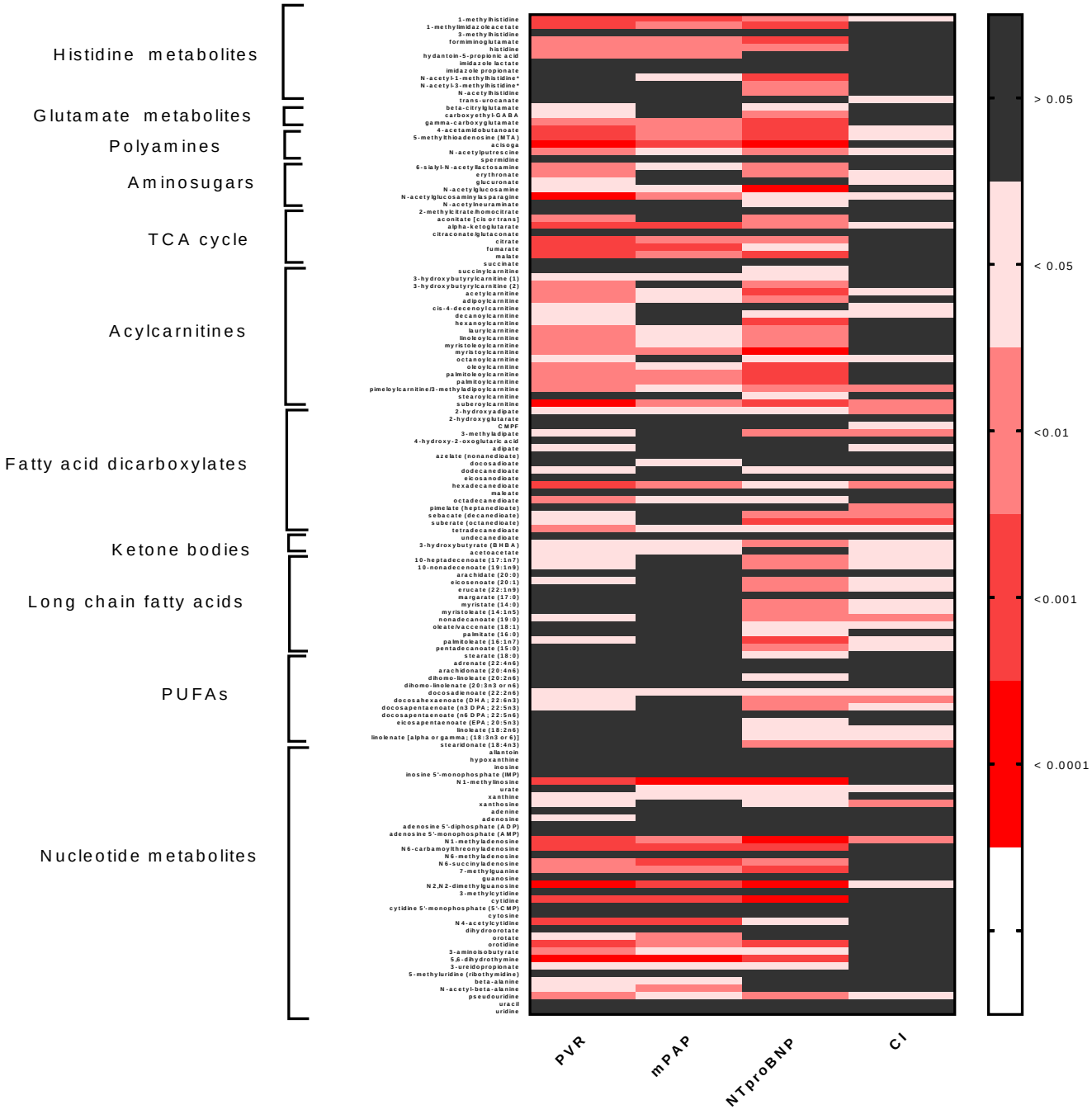


Figure 5.4- Heat map showing correlation of metabolite subgroups with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. For metabolites which showed a significant difference in concentration between disease and control, correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Metabolites which showed a significant correlation with clinical markers of disease severity are shown in red scale. Those which did not show a significant correlation are shown in black. TCA- tricarboxylic acid, PUFA-polyunsaturated fatty acid.

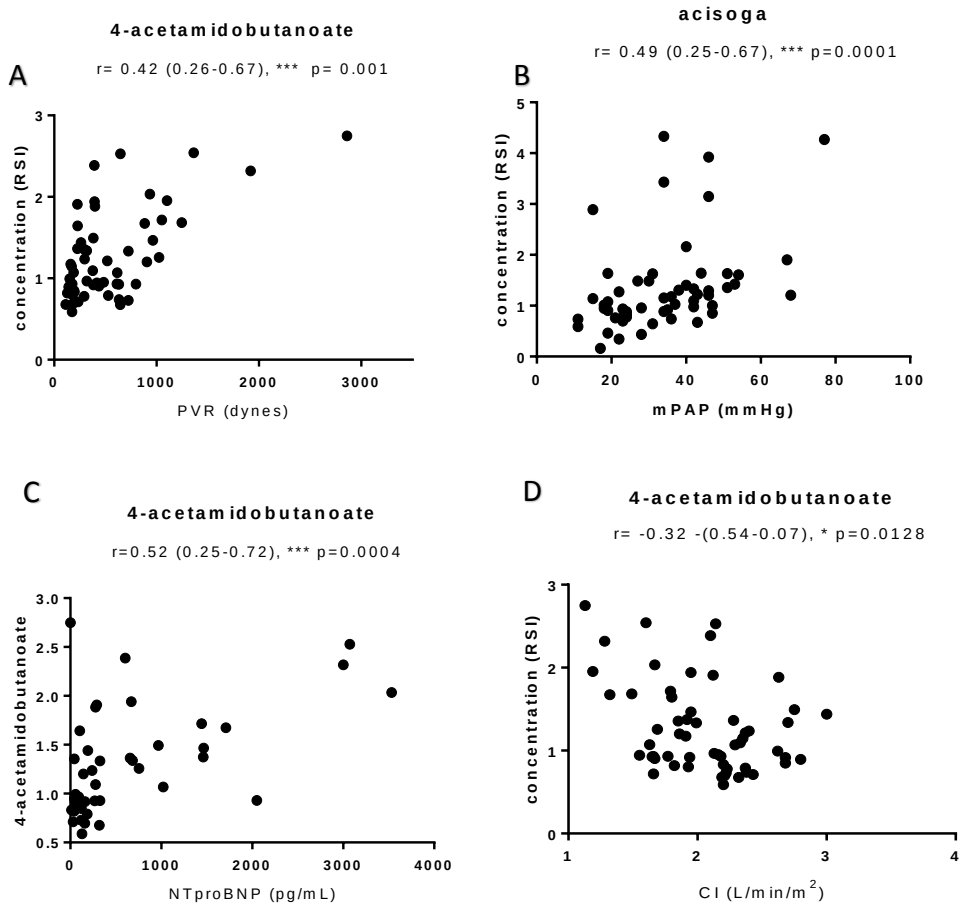


Figure 5.5-Correlation of polyamine metabolites with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) 4-acetamidobutanoate vs PVR (B) acisoga vs mPAP (C) 4-acetamidobutanoate vs NTproBNP (D) 4-acetamidobutanoate vs CI.

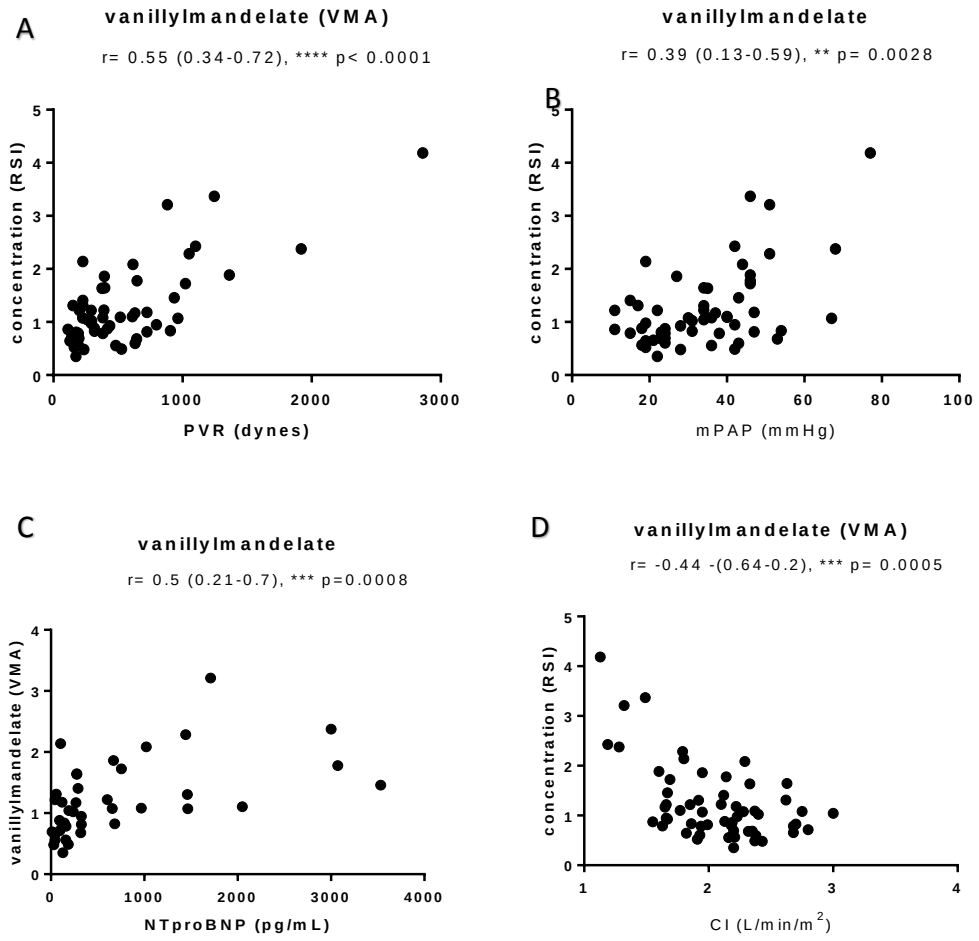


Figure 5.6- Correlation of vanillylmandelate with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) vanillylmandelate vs PVR (B) vanillylmandelate vs mPAP (C) vanillylmandelate vs NTproBNP (D) vanillylmandelate vs CI.

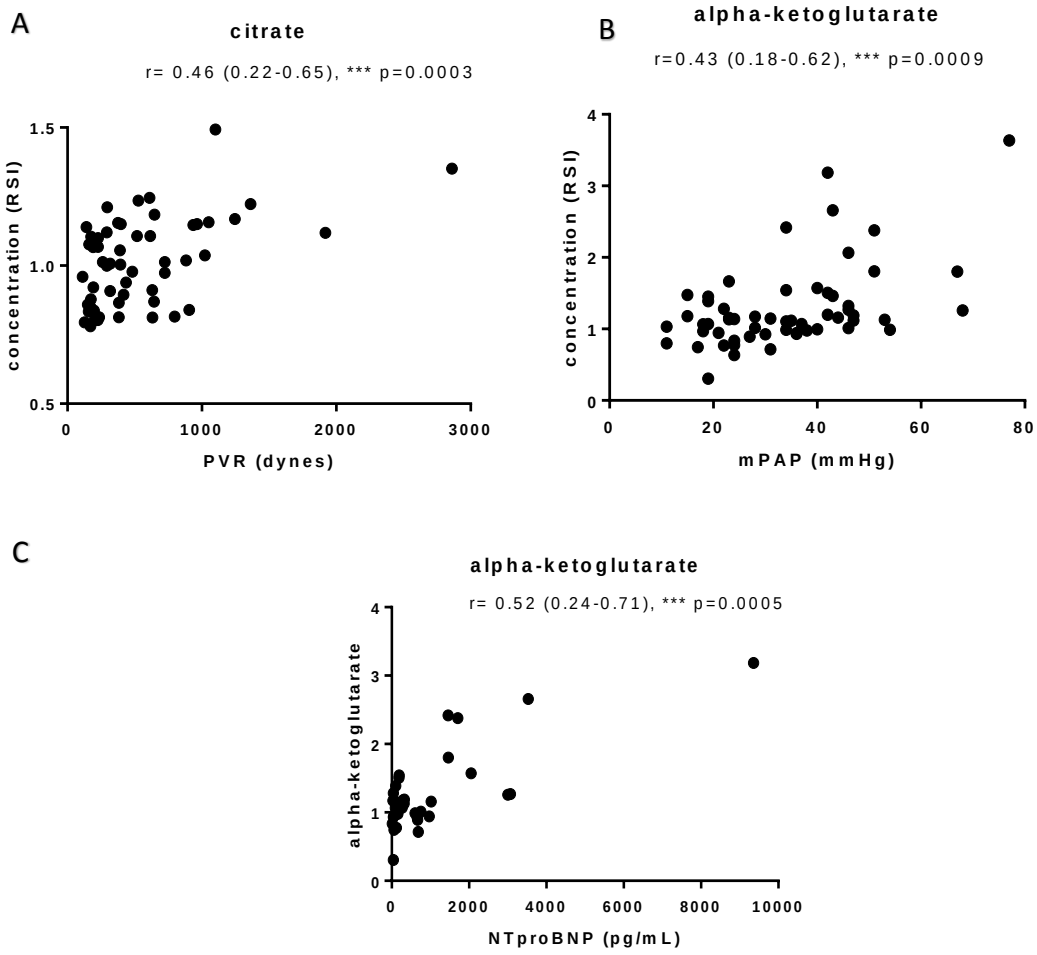


Figure 5.7- Correlation of TCA cycle metabolites with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) citrate vs PVR (B) alpha-ketoglutarate vs mPAP (C) alpha-ketoglutarate vs NTproBNP.

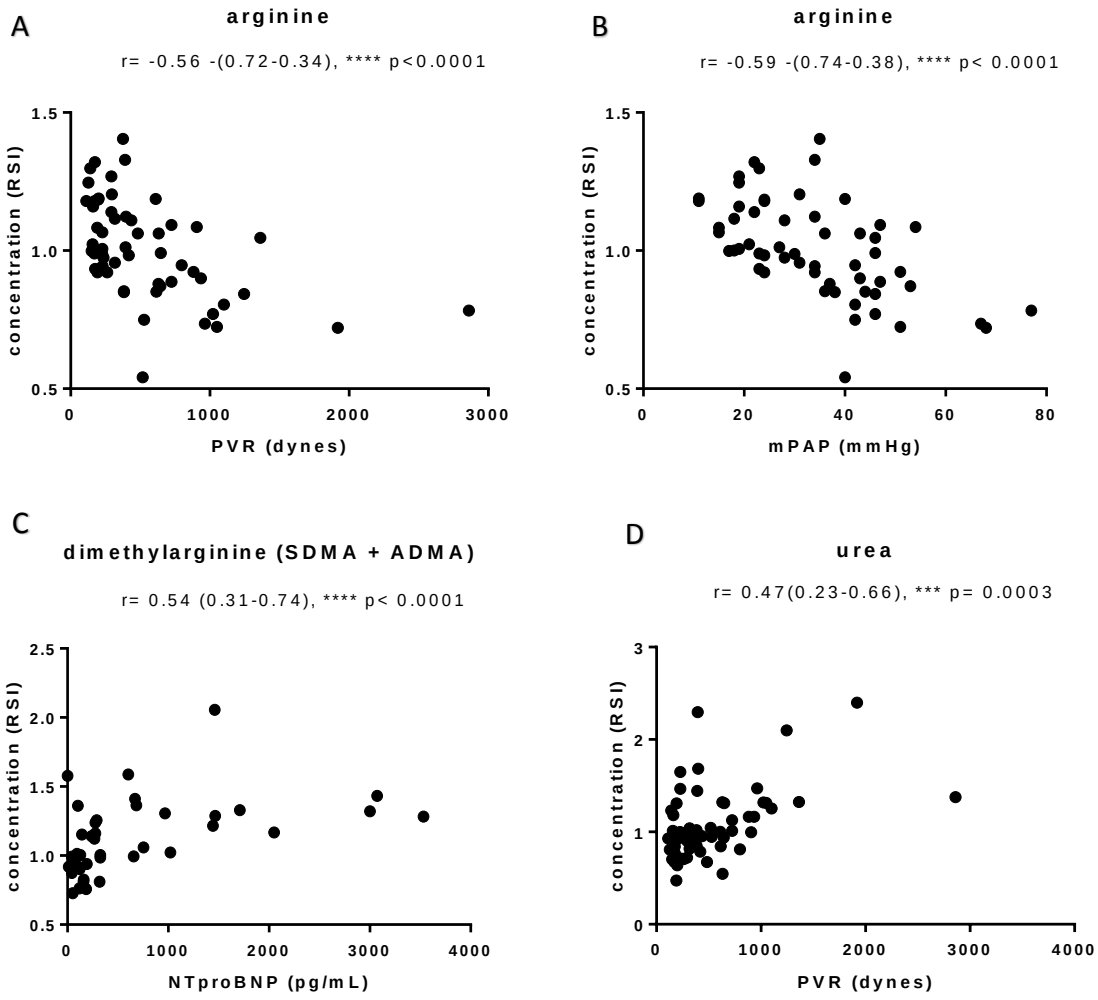


Figure 5.8- Correlation of arginine metabolites with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) arginine vs PVR (B) arginine vs mPAP (C) dimethylarginine vs NTproBNP (D) urea vs PVR.

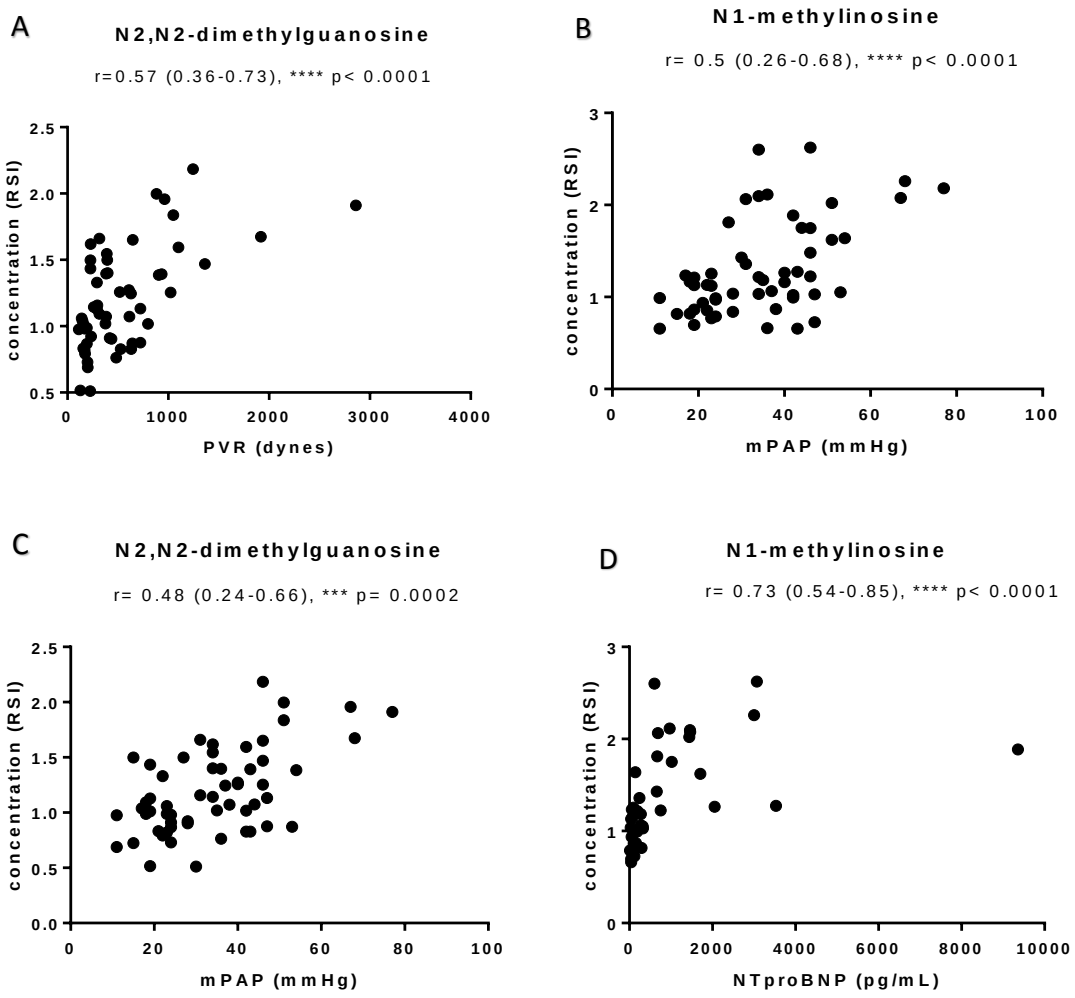


Figure 5.9- Correlation of modified nucleotides with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) N2,N2-dimethylguanosine vs PVR (B) N1-methylinosine vs mPAP (C) N2,N2-dimethylguanosine vs mPAP (D) N1-methylinosine vs NTproBNP.

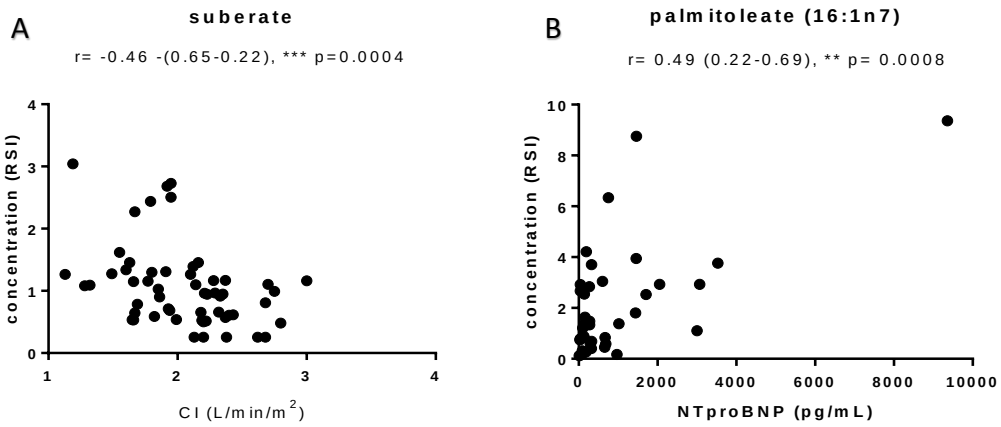
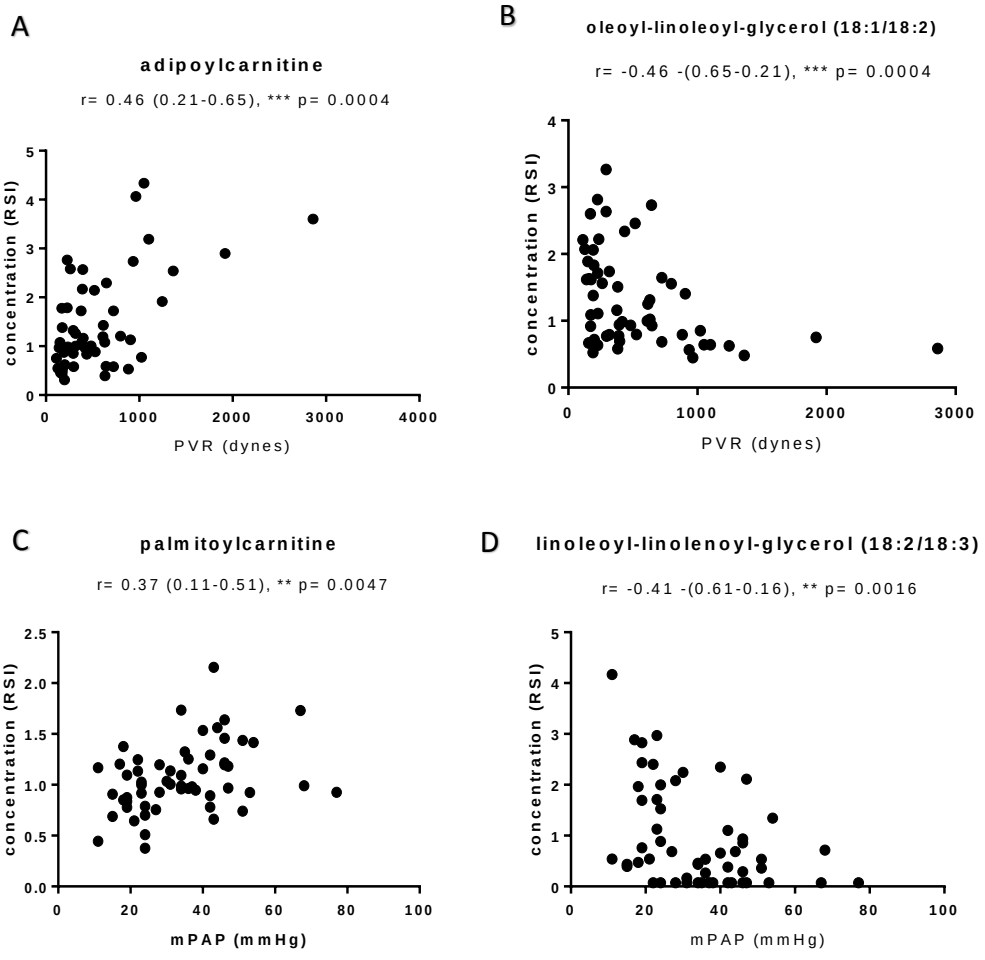


Figure 5.10-Correlation of fatty acid concentration with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) suberate vs CI (B) palmitoleate vs NTproBNP.



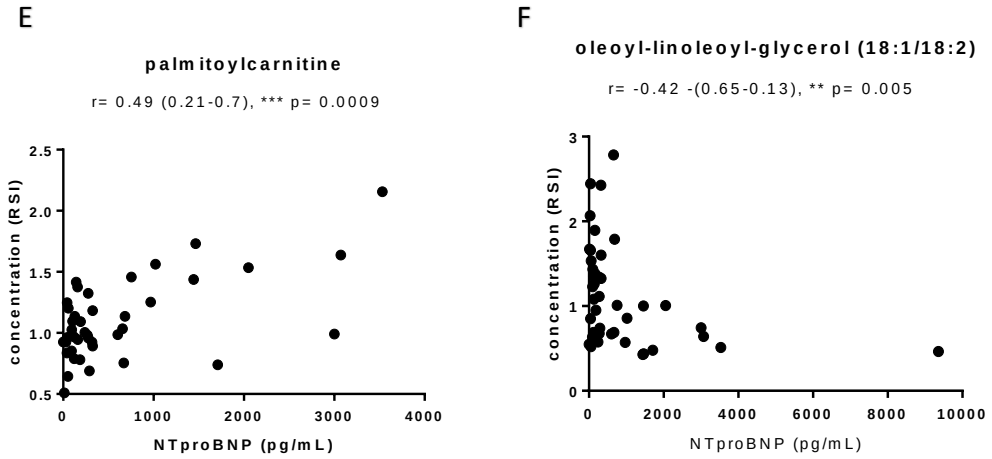


Figure 5.11- Correlation of acylcarnitines with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) adipoylcarnitine vs PVR (B) oleoyl-linoleoyl-glycerol vs PVR (C) palmitoylcarnitine vs mPAP (D) linoleoyl-linolenoyl-glycerol vs mPAP (E) palmitoylcarnitine vs NTproBNP (F) oleoyl-linoleoyl-glycerol vs NTproBNP.

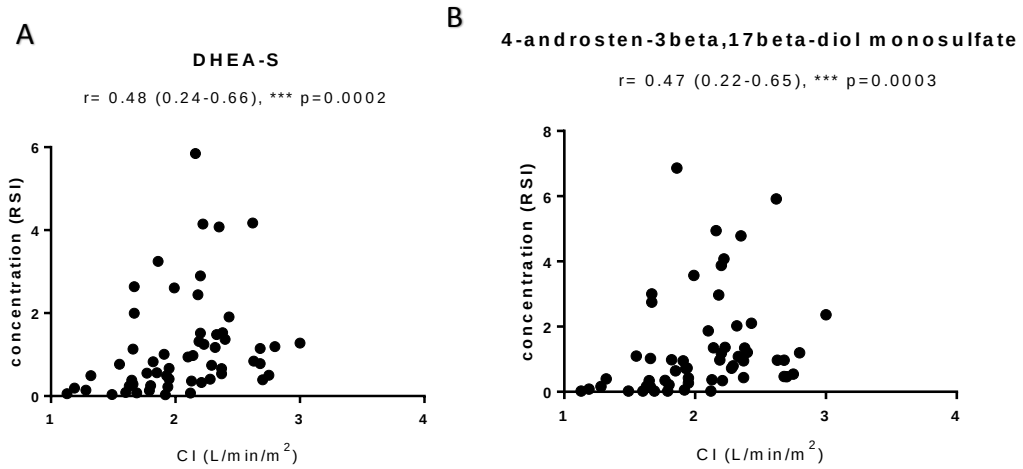


Figure 5.12- Correlation of steroid hormones with markers of disease severity. Metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease was carried out using the Metabolon DiscoveryHD4™ platform. Correlation of metabolite concentration (RSI) with PVR, mPAP, CI and NTproBNP level was assessed. A two-tailed test was conducted, using Spearman's rank correlation. Representative plots above show correlation of; (A) DHEA-S vs CI (B) 4-androsten-3beta,17beta-diol monosulfate vs CI.

5.6 Discussion

My findings provide evidence of disrupted energy metabolism in PVD. In particular, there is evidence of disrupted fatty acid metabolism. Metabolic changes consistent with increased cellular proliferation and a reduction in antioxidant metabolites were also apparent in my dataset. These findings are further discussed below.

5.6.1 Evidence of disrupted fatty acid metabolism

Long chain fatty acids and acylcarnitines

The concentration of both long chain fatty acids (LCFA) and acylcarnitines is increased in PVD, compared with healthy controls. In health, LCFA are the primary cardiac energy source. Acylcarnitines play an important role in metabolism of LCFA, facilitating their transport into the mitochondria for beta oxidation and may accumulate when fatty acid beta oxidation is impaired (Koves et al., 2008).

Increases in LCFA and acylcarnitines have been previously found in the blood of patients with PAH, compared to controls (Brittain et al., 2016), and in lung tissue of PAH patients (Zhao et al., 2014a). Additionally, abnormalities in fatty acid metabolism have been demonstrated in a number of experimental models of PAH (Sutendra et al., 2010). Decreased RV fatty acid use may lead to myocardial lipid accumulation, exacerbating RV dysfunction (Brittain et al., 2016). Additionally, accumulation of acylcarnitines may promote cardiac dysfunction via pro-arrhythmogenic effects (Bonnet et al., 1999).

My findings of increased plasma LCFA and acylcarnitines, combined with the increased myocardial glucose uptake and elevated myocardial lipid content reported in other studies, suggests a switch in cardiac substrate utilisation from fatty acids to glucose in PVD. Whether this metabolic switch is a result of impaired mitochondrial ability to utilise fatty acids, or whether this occurs due to preferential use of glucose remains uncertain.

The concentration of the majority of fatty acids showed a significant correlation with cardiac haemodynamics, but did not correlate with pulmonary haemodynamics. This supports the hypothesis that myocardial failure to utilise fatty acids drives fatty acid accumulation (Sutendra et al., 2010). Acylcarnitine concentration, however, correlated with both cardiac and pulmonary markers of disease severity and therefore may be a more general feature of altered metabolism in the disease.

Fatty acid dicarboxylates and acylglycines

The increased levels of fatty acid dicarboxylates and acylglycines detected in PVD provides further evidence to that fatty acid beta oxidation is impaired in the disease.

Usually, direct mitochondrial beta oxidation is the preferred route for fatty acid oxidation. However, omega oxidation in the endoplasmic reticulum is upregulated when beta oxidation is defective (Wanders et al., 2011, Sanders et al., 2006). In addition to the increase concentration observed in circulating blood, increased levels of fatty acid dicarboxylates have previously been reported in PAH lung tissue (Zhao et al., 2014a). Acylglycines are normal intermediates of amino acid and fatty acid metabolism. However, elevated levels of these metabolites have also been found in the urine and blood of patients with various fatty acid oxidation disorders (Bonafé et al., 2000, Kimura and Yamaguchi, 1999).

5.6.2 Other perturbations in energy metabolism

TCA cycle

A number of previous studies have suggested that TCA cycle dysfunction exists in PAH, as part of a shift towards glycolytic metabolism (Bujak et al., 2016). However, I did not detect any significant increase in glycolytic intermediates in the disease group studied. Key TCA cycle intermediates including citrate, malate, alpha-ketoglutarate and aconitate were increased in our PVD group, suggesting upregulation of the TCA cycle, perhaps in an attempt to meet increased bionenergetic demands. These findings are consistent with those found by Zhao in lung tissue of PAH patients (Zhao et al., 2014a). However, this contrasts with findings of a depletion of TCA intermediates in cultured BMPR2 mutant pulmonary microvascular endothelial cells (Fessel et al., 2012). It is possible that the lack of concordance in findings may be due to the different metabolic environment in cell culture compared to tissue and circulating blood.

Nucleotides

Adenine containing nucleotides such as adenosine monophosphate (AMP), adenosine diphosphate (ADP) and N1-methyladenosine were increased in the disease group. This was accompanied by an increase in cytidine containing nucleotides. In addition to their role in energy metabolism, both adenine and cytosine are required for DNA and RNA synthesis. Increases in these nucleotides may therefore be reflective of both increased bioenergetic and biosynthetic demands in the disease group.

Ketone bodies

Elevation of ketone bodies often represents a state in which there is insufficient carbohydrate supply to meet bioenergetic demands. However, ketones also become an increasingly important cardiac fuel source in the context of heart failure where myocardial capacity for oxidising fatty acids is deficient and they are elevated in proportion to the severity of cardiac dysfunction (Aubert et al., 2016).

An increase in ketones is also observed in the metabolic syndrome phenotype where insulin resistance results in low glucose uptake by cells despite glucose being plentiful (Cotter et al., 2013). A number of studies have identified an association between insulin resistance and PAH (Heresi et al., 2017, Grinnan et al., 2016, West et al., 2013, Pugh et al., 2011), therefore, insulin resistance may also contribute to the increase in ketones we detected in the disease group.

Another situation in which ketone formation may occur is when the rate of fatty acid oxidation exceeds TCA cycle capacity. When fatty acid oxidation produces more acetyl CoA than the TCA cycle can utilise, the acetyl CoAs are used to generate ketone bodies. My findings suggest that fatty acid oxidation may be impaired in the disease group, therefore the increase in ketones detected is more likely to be due to reduced capacity for oxidising fatty acids rather than exceeded TCA cycle capacity.

5.6.3 Evidence of increased cellular proliferation

Polyamines

A significant increase in polyamine metabolites was detected in the disease group. This is consistent with findings in cell culture, human lung tissue and animal models of pulmonary hypertension (Fessel et al., 2012, Barnes et al., 2015, Olson et al., 1984, Orlinska et al., 1988, Atkinson et al., 1987). Polyamines have a diverse range of functions including modulation of chromatin structure, gene transcription and translation, DNA stabilisation, signal transduction, cell growth, proliferation and migration, membrane stability, functioning of ion channels and receptor-ligand interactions (Pegg, 2009, Casero and Marton, 2007). The lung exhibits a higher polyamine uptake than any other major organ (Hoet and Nemery, 2000).

The most marked synthesis and accumulation of polyamines occurs in rapidly growing tissues. Conversely, a reduction in polyamines results in cell growth arrest or senescence. Polyamine content is increased in many cancers, where increased levels are coupled with decreased apoptosis, increased tumour invasion and increased metastasis (Gerner and Meyskens, 2004, Milovic and Turchanowa, 2003, Schipper et al., 2003, Xu et al., 2016, Takahashi et al., 2015, Huang et al., 2015)

and levels are also increased in other hyperproliferative diseases, such as psoriasis (Broshtilova et al., 2013, Tierney et al., 1985).

It is plausible that the increased polyamine levels in PVD are reflective of cellular proliferation and remodelling of the pulmonary vasculature (Morrison and Seidel, 1995, Barnes et al., 2015). Suppression of polyamine biosynthesis in animal models of PH has been shown to inhibit the pathological vascular remodelling found in the disease (Olson et al., 1986).

Nitric oxide is a potent inhibitor of polyamine synthesis (Hillary and Pegg, 2003), therefore, reduced nitric oxide bioavalibility which has been demonstrated in PAH (Tonelli et al., 2013) may also be implicated in the elevation of polyamines observed. I found that higher levels of polyamines are correlated with both adverse cardiac and adverse pulmonary haemodynamics. As discussed previously, polyamines play an important role in supporting tissues with high cell turnover. Therefore, their concentration is likely to increase in association with increasing biosynthetic demands of both pulmonary vasculature and myocardium in the context of increasing disease severity.

Glutamate metabolites

In the PVD group, there was also a reduction in glutamine, accompanied by increases in glutamate and its metabolites, consistent with glutaminolysis. Glutaminolysis is a feature of proliferating cells and plays an important role in replenishing metabolic intermediates (Dang, 2010, Yang et al., 2017). This finding provides further evidence of a metabolic shift to enable cellular proliferation. Recent studies have also implicated glutaminolysis in right ventricular maladaptive changes in PAH (Piao et al., 2013, Bertero et al., 2016).

Sphingolipids, lysolipids (hydrolysed phospholipids) and acylcholines

Sphingolipids are key components of cell membranes, involved in cellular protection, cell recognition and signalling (Bartke and Hannun, 2009, Hannun and Obeid, 2008, El Alwani et al., 2006). In pulmonary vascular disease, depletion in sphingolipids may represent increased utilisation for cell membranes in the context of vascular remodelling. However, it has recently been revealed that sphingolipid metabolites such as ceramide and sphingosine-1-phosphate have many other functions which may be relevant in the disease pathogenesis, as they have been shown to mediate cellular apoptosis, proliferation, differentiation, stress responses and inflammation (Maceyka and Spiegel, 2014).

Lysophospholipids and acylcholines are also an important constituent of cell membranes. Therefore, depletion of lysophospholipids and acylcholines may also reflect increased cell turnover associated with vascular remodelling. However, lysophospholipids also play a role in cell signalling, proliferation and differentiation, cell morphological changes and regulation of gap junctions (Lundbaek and Andersen, 1994, Morris et al., 2009). They are involved in regulation of endothelial cell function and phenotypic modulation of vascular smooth muscle cells (Hayashi et al., 2001). Therefore, depletion of these metabolites may be implicated in disordered vascular endothelial and smooth muscle cell behaviour in PVD.

Aminosugars

There was a significant increase in aminosugars, such N-acetylglucosamine and glucoronate in PVD. These metabolites are important building blocks for glycoproteins, glycosaminoglycans, glycolipids and polysaccharides and are required for cellular proliferation, wound healing and repair. Additionally, glucuronic acid is required for waste removal and detoxification (Perreault et al., 2013).

Post-translational modification of proteins by O-linked N-acetylglucosamine is associated with cellular stress responses (Wende, 2016) where it is thought to serve as an autoprotective mechanism, promoting cell survival (Zachara et al., 2004). This attachment to cardiac proteins is increased in cardiovascular disease and heart failure (Medford and Marsh, 2014, Dassanayaka and Jones, 2014, Laczy et al., 2009). It is possible that this process may also occur in PVD, leading to a reactive increase in N-acetylglucosamine, conversely, this process may be impaired, leading to accumulation in the plasma. I propose that the increase in aminosugars in PVD may be a reactive phenomenon, to support increase cellular biosynthetic demands associated with vascular remodelling, attempted cardiac auto-protection and the increased need for elimination of waste products.

5.6.4 Reduction in antioxidant metabolites

A reduction in metabolites with antioxidant roles, including glutathione, vitamin A metabolites and plasmalogens was found in PVD, which may represent increased consumption and/or deficiency of these metabolites. Glutathione deficiency contributes to oxidative stress (Wu et al., 2004), and a reduction in glutathione biosynthesis has previously been observed in the monocrotaline rat model of PAH (Rafikova et al., 2016). Therefore, the reduced levels of glutathione we have identified may potentiate development and progression of PVD.

Retinol and 4-oxo retinoic acid, in addition to their importance in photoreceptor function and vision, have antioxidant effects and more diverse metabolic roles including involvement in immune cell function, hormone synthesis, haematopoiesis and modulation of iron metabolism (Guo and Nolle, 2013, Brown et al., 2015, Raverdeau and Mills, 2014, Hall et al., 2011, Mendes et al., 2016).

In the monocrotaline rat model of PAH, administration of all trans retinoic acid has been shown to attenuate pulmonary vascular remodelling (Xin et al., 2015, Qin et al., 2001). Additionally, IPAH patients have previously been found to have reduced retinoic acid levels, and retinoic acid treatment has been shown to inhibit pulmonary artery smooth muscle cell growth *in vitro* (Preston et al., 2005). Therefore, reduction in vitamin A metabolites may potentiate the development of pulmonary hypertension by permitting proliferation of vascular smooth muscle cells.

Plasmalogens are a subtype of cell membrane glycerophospholipids, enriched in the brain, heart, skeletal muscle, lung, spleen and peripheral blood lymphocytes and neutrophils (Braverman and Moser, 2012, Nagan and Zoeller, 2001). They are recognised to have antioxidant effects, with plasmalogen deficient endothelial cells being more sensitive to hypoxia and reactive oxygen species (Zoeller et al., 2002, Engelmann, 2004, Lessig and Fuchs, 2009). Therefore, it is possible that the decreased plasmalogen concentration we have detected in the disease group may exacerbate vascular injury. Reduced plasmalogen levels have also been identified in association with coronary artery disease and myocardial ischaemia (Scherrer and Gross, 1989, Sutter et al., 2015, Sutter et al., 2016).

5.6.5 Other notable metabolic changes in PVD

5.6.5.1 Polyunsaturated fatty acids (PUFAs)

Docosadienoate and linoleate metabolites were increased in the pulmonary vascular disease group. These N6 polyunsaturated fatty acids (PUFAs) are precursors for the synthesis of arachidonic acid and prostaglandins and play a role in mediating both pro and anti-inflammatory effects (Russo, 2009). PUFAs are essential fatty acids (must be ingested and cannot be synthesized *de novo*), therefore the increase in PUFA metabolites detected in the disease group may simply represent differences in dietary intake.

5.6.5.2 Steroids

Many sex steroids, including dehydroepiandrosterone-sulfate (DHEA-S) and pregnenalone were depleted in the pulmonary vascular disease group. Depletion in sex steroids as a consequence of

chronic neurohormonal activation is a recognised feature of chronic heart failure (Jankowska et al., 2006, Güder et al., 2010) and chronic inflammatory diseases (Imrich, 2002, Straub et al., 2000). Lower levels of DHEA-S have previously been reported in men with PAH (Ventetuolo et al., 2016). DHEA is known to have a vasodilatory effect on the pulmonary circulation (Patel et al., 2014, Oka et al., 2007) and in animal models has been shown to prevent or ameliorate pulmonary hypetension associated with hypoxia (Bonnet et al., 2003, Hampl et al., 2003, Dumas de la Roque et al., 2013).

The concentration of steroid hormones was found to be lower in those with a lower cardiac output, consistent with the known depletion of these hormones in the context of heart failure (Jankowska et al., 2006).

5.6.5.3 Branched chain amino acids

Branched chain amino acids (leucine, isoleucine and valine) act as a precursor for keto acids, and are also a precursor for muscle protein synthesis. In conditions where there is protein or muscle loss (eg. starvation, trauma, sepsis, cancer, rheumatic conditions), supplementation of BCAAs promotes protein synthesis and reduces protein catabolism (Tsien et al., 2015, Yoshikawa et al., 2017). Sarcopenia and reduced muscle strength is observed in PAH (Mainguy et al., 2010, Batt et al., 2014) and whether supplementation of BCAA in this setting is of clinical benefit is unknown.

In addition to their role in anabolism, BCAAs also play an important role in immunity and are required for lymphocyte growth and proliferation (Calder, 2006). Supplementation of depleted BCAAs has been shown to increase lymphocyte proliferation, modify the pattern of cytokine production and shift of the immune response from Th2 to Th1 (Bassit et al., 2002). However, excessive levels may in fact promote inflammation and oxidative stress (Zhenyukh et al., 2017, Zhang et al., 2017). Therefore, BCAA depletion may have other implications in PVD, beyond protein metabolism.

5.6.5.4 Bradykinin

Bradykinin is an inflammatory mediator and endothelium dependent vasodilator. Binding of bradykinin to endothelial B2-receptors results in release of NO and formation of prostaglandins, thereby exerting vasodilator, anti- ischemic, and anti-proliferative effects (Golias et al., 2007). Therefore, decreased bradykinin levels detected in the disease group may have multifaceted adverse effects. Conversely, administration of a bradykinin agonist has been shown to reduce pulmonary

artery pressure and RV hypertrophy in animal models of pulmonary hypertension (Taraseviciene-Stewart et al., 2002, Taraseviciene-Stewart et al., 2005).

5.6.5.5 Others

Several other metabolites including vanillylmandelate, arginine and histidine metabolites and the modified nucleosides N2, N2- dimethylguanosine and N1-methylinosine showed a significant correlation with both pulmonary and cardiac haemodynamic indices. Vanillylmandelate is an end-stage metabolite of the catecholamines, epinephrine, and norepinephrine. Increased sympathetic nervous system activation is present in PH, therefore, it is unsurprising that vanillylmandelate levels are increased in the disease group, and show a strong correlation with disease severity.

Arginine, which is required to support nitric oxide synthesis was found to be negatively correlated with PVR, mPAP and BNP, consistent with reduced nitric oxide bioavailibity known to be implicated in pulmonary hypertension (Tonelli et al., 2013). Conversely, levels of arginine metabolites (such as dimethylarginines) which have been implicated in inhibition of NO synthesis (Franceschelli et al., 2013) were positively correlated with disease severity.

Increased levels of N1-methylinosine and N2, N2-dimethylguanosine have been reported to distinguish PAH patients from symptomatic patients without pulmonary hypertension and also correlate with prognosis (Rhodes C, 2016). Increases in the concentration of these post-transcriptional modifications of tRNA may reflect upregulation of the translational apparatus due to high tRNA turnover associated with oxidative stress and cell damage. However, further study is required to determine the role of these metabolites in the disease process.

5.6.6 Disease subgroup analysis

I hypothesised that there would be differences in circulating metabolite concentration between pulmonary hypertension subtypes with a different pathological basis (IPAH vs CTEPH) and between patients with chronic thromboembolic vascular occlusions with and without associated pulmonary hypertension (CTEPH vs CTED).

Unfortunately, subgroup analysis was limited by small sample size and underpowered to detect differences between disease subgroups. Only 2 metabolites showed a statistically significant difference between disease subgroups. However, the potential for metabolite profile to differentiate between disease subgroups should not be discounted. Future analysis with an increased sample size may help to identify additional metabolic differences between the subgroups.

5.7 Conclusions

Comparison of circulating metabolites in pulmonary vascular disease with healthy controls has demonstrated differences in multiple metabolic pathways. My data suggest altered bioenergetic metabolism in the disease. Firstly, elevated long chain fatty acids, acylcarnitines and acylglycines are in keeping with a decrease in lipid beta oxidation. The accompanying increase in fatty acid dicarboxylates, formed by omega oxidation, suggests that beta oxidation may be impaired or inhibited. The elevation of ketone bodies detected in the disease group may also be a consequence of impaired capacity for oxidising fatty acids, necessitating the use of alternative energy sources by the myocardium.

Secondly, increases in TCA cycle intermediates suggest upregulation of this cycle, in keeping with the increased energy requirements associated with vascular remodelling and increased right ventricular workload. The concept of increased requirement for substrate to support cellular proliferation and vascular remodelling is supported by the finding of increased polyamines and aminosugars in the disease group.

My data also suggest that in pulmonary vascular disease there is depletion of substances important in the response to oxidative stress, including vitamin A metabolites, plasmalogens and glutathione metabolites.

These findings demonstrate that metabolic abnormalities in pulmonary vascular disease are more wide-ranging than previously recognised, and are present not only in PAH but are also present in CTEPH and CTED. Further research is required to determine the mechanism of these metabolic changes and to establish their role in disease evolution and progression.

6 Chapter 6- Assessment of the gradient of circulating metabolites between anatomical sites

6.1 Introduction

I have demonstrated that the concentration of many metabolites in venous blood differs between patients with pulmonary vascular disease and healthy controls (Chapter 5). However, the source of these metabolic disturbances is unknown. It is unclear whether the metabolic changes detected in PVD are the result of alterations in metabolism which are localised to the pulmonary circulation or right heart, or whether they represent systemic metabolic dysfunction in the disease process.

Comparison of the metabolite concentration in circulating blood sampled from different anatomical sites may allow localisation of the metabolic changes and provide further insight into their role in the disease process. For example, comparison of paired blood samples from the superior vena cava (SVC) and pulmonary artery (PA) may provide insight into the right heart metabolism and its contribution to the circulating metabolite profile. Similarly, comparison of paired pulmonary artery and radial artery (RA) samples may be used to make inferences about changes in metabolite concentration which occur during transpulmonary passage, and paired radial artery (RA) and superior vena cava (SVC) samples may be used to make inferences about changes in metabolite concentration which occur across the systemic circulation.

6.2 Objective and hypothesis

Objective: To assess the gradient of circulating metabolites between anatomical sites, by comparing metabolite concentration in paired blood samples:

1. Superior vena cava (SVC) to pulmonary artery (PA)

2. Pulmonary artery (PA) to radial artery (RA)

3. Radial artery (RA) to superior vena cava (SVC)

Hypothesis: Metabolic abnormalities that we have identified in the disease population are due to local metabolic dysfunction of the pulmonary vasculature or right heart. Therefore, a significant difference in the concentration of metabolites will be detected between anatomical sites.

6.3 Study population

The disease population consisted of 57 patients with pulmonary vascular disease; 9 patients with IPAH and 48 patients with chronic thromboembolic pulmonary vascular occlusions [Chapter 5, table 5.2].

6.4 Methods

Blood samples were collected at the time of right heart catheterisation, as described in Section 2.2. Metabolite concentration (expressed as median scaled relative standard intensity) was compared between paired samples taken from the different anatomical sites using Wilcoxon matched pairs signed rank test. False discovery rate adjustment for multiple testing was applied. Metabolite groups in which significant differences were detected between the 2 sites were subsequently included in over-representation analysis.

6.5 Results

6.5.1 Transcardiac metabolite gradients

Amongst 1375 metabolites detected, 79 showed a significant difference in concentration between the SVC and PA site. Of these 79 metabolites, there were 65 endogenous metabolites, 13 unidentified metabolites and 1 xenobiotic. The endogenous metabolites are shown in Table 6.1.

Amongst the metabolite subgroups in which a significant change in concentration was detected between SVC and PA blood samples, 24 sub-pathways were over-represented [Table 6.2].

Multiple lipid groups showed a significant reduction in concentration in PA compared to SVC blood, including long chain fatty acids, medium chain fatty acids and polyunsaturated fatty acids. There was also a significant reduction in the concentration of TCA cycle metabolites in PA compared to SVC blood. Additionally, there was reduction in the concentration of n-acetylputrescine, 1-methylnicotinamide and a number of nucleotides.

Conversely, there was a significant increase in glutamate, 4-hydroxyglutamate and isoleucylglutamate PA compared to SVC blood, accompanied by depletion in glutamine. There was also an increase in ketones, creatine, phenylacetylcarnitine and 3- phosphoglycerate. These changes are summarised in Figure 6.1.

Table 6.1-Endogenous metabolites which showed a significant difference in concentration between paired SVC and PA samples. Blood samples were collected at the time of right heart catheterisation. Metabolite concentration (expressed as median scaled relative standard intensity) was compared between paired samples using the Wilcoxon matched pairs signed rank test with FDR adjustment.

BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY	p value
Aspartate	Amino Acid	Alanine and Aspartate Metabolism	0.0280
Creatine	Amino Acid	Creatine Metabolism	0.0077
Creatinine	Amino Acid	Creatine Metabolism	0.0466
4-hydroxyglutamate	Amino Acid	Glutamate Metabolism	0.0037
S-1-pyrroline-5-carboxylate	Amino Acid	Glutamate Metabolism	0.0054
Glutamine	Amino Acid	Glutamate Metabolism	0.0163
Glutamate	Amino Acid	Glutamate Metabolism	<0.0001
imidazole lactate	Amino Acid	Histidine Metabolism	0.0194
trans-urocanate	Amino Acid	Histidine Metabolism	0.0394
Isovalerylcarnitine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0044
Isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0054
Leucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0181
2-hydroxybutyrate/2-hydroxyisobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0194
N-acetylmethionine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0330
alpha-ketobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0416
N-acetylputrescine	Amino Acid	Polyamine Metabolism	0.0002
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0204
Citrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0335
3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.0114
Glucose	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	<0.0001
1-methylnicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	<0.0001
alpha-ketoglutarate	Energy	TCA Cycle	0.0003
Succinate	Energy	TCA Cycle	0.0013
Citrate	Energy	TCA Cycle	0.0017
aconitate [cis or trans]	Energy	TCA Cycle	0.0018
Fumarate	Energy	TCA Cycle	<0.0001
Malate	Energy	TCA Cycle	<0.0001
oleoyl ethanolamide	Lipid	Endocannabinoid	0.0258
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	0.0019
3-hydroxylaurate	Lipid	Fatty Acid, Monohydroxy	0.0005
9-hydroxystearate	Lipid	Fatty Acid, Monohydroxy	0.0119
2-hydroxypalmitate	Lipid	Fatty Acid, Monohydroxy	0.0370
3-hydroxydecanoate	Lipid	Fatty Acid, Monohydroxy	0.0418
Glycerol	Lipid	Glycerolipid Metabolism	0.0245
Acetoacetate	Lipid	Ketone Bodies	0.0005
3-hydroxybutyrate (BHBA)	Lipid	Ketone Bodies	<0.0001
myristoleate (14:1n5)	Lipid	Long Chain Fatty Acid	0.0002
pentadecanoate (15:0)	Lipid	Long Chain Fatty Acid	0.0002
10-heptadecenoate (17:1n7)	Lipid	Long Chain Fatty Acid	0.0004
palmitoleate (16:1n7)	Lipid	Long Chain Fatty Acid	0.0026

palmitate (16:0)	Lipid	Long Chain Fatty Acid	0.0038
oleate/vaccenate (18:1)	Lipid	Long Chain Fatty Acid	0.0155
10-nonadecenoate (19:1n9)	Lipid	Long Chain Fatty Acid	0.0370
myristate (14:0)	Lipid	Long Chain Fatty Acid	<0.0001
caprate (10:0)	Lipid	Medium Chain Fatty Acid	0.0178
laurate (12:0)	Lipid	Medium Chain Fatty Acid	0.0417
5-dodecenoate (12:1n7)	Lipid	Medium Chain Fatty Acid	<0.0001
Choline	Lipid	Phospholipid Metabolism	0.0007
linolenate [alpha or gamma; (18:3n3 or 6)]	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0002
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0002
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0008
arachidonate (20:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0013
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0018
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0034
docosapentaenoate (n3 DPA; 22:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0051
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0304
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	<0.0001
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	Lipid	Sterol	0.0012
Xanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0013
7-methylguanine	Nucleotide	Purine Metabolism, Guanine containing	0.0009
Orotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0005
5,6-dihydrothymine	Nucleotide	Pyrimidine Metabolism, Thymine containing	0.0010
Phenylacetylcarnitine	Peptide	Acetylated Peptides	<0.0001
Isoleucylalanine	Peptide	Dipeptide	0.0006
Isoleucylglutamate	Peptide	Dipeptide	<0.0001

Table 6.2 -Over-representation analysis of metabolite subgroups which showed a significant difference in concentration between paired SVC and PA samples. Metabolite subgroups which showed an enrichment value >1 are listed.

SUPER PATHWAY	SUB PATHWAY	ENRICHMENT VALUE
Lipid	Ketone Bodies	17.41
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	13.05
Amino Acid	Creatine Metabolism	11.60
Energy	TCA Cycle	11.60
Lipid	Long Chain Fatty Acid	9.95
Nucleotide	Pyrimidine Metabolism, Thymine containing	8.70
Lipid	Medium Chain Fatty Acid	7.46
Amino Acid	Glutamate Metabolism	6.33
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	5.80
Lipid	Glycerolipid Metabolism	5.80
Nucleotide	Purine Metabolism, Guanine containing	5.80
Nucleotide	Pyrimidine Metabolism, Orotate containing	5.80
Lipid	Fatty Acid, Monohydroxy	4.97
Amino Acid	Polyamine Metabolism	4.35
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	3.48
Lipid	Endocannabinoid	3.48
Peptide	Acetylated Peptides	3.48
Amino Acid	Histidine Metabolism	2.90
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2.61
Amino Acid	Alanine and Aspartate Metabolism	2.49
Lipid	Sterol	2.49
Amino Acid	Leucine, Isoleucine and Valine Metabolism	2.18
Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	2.18
Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.93

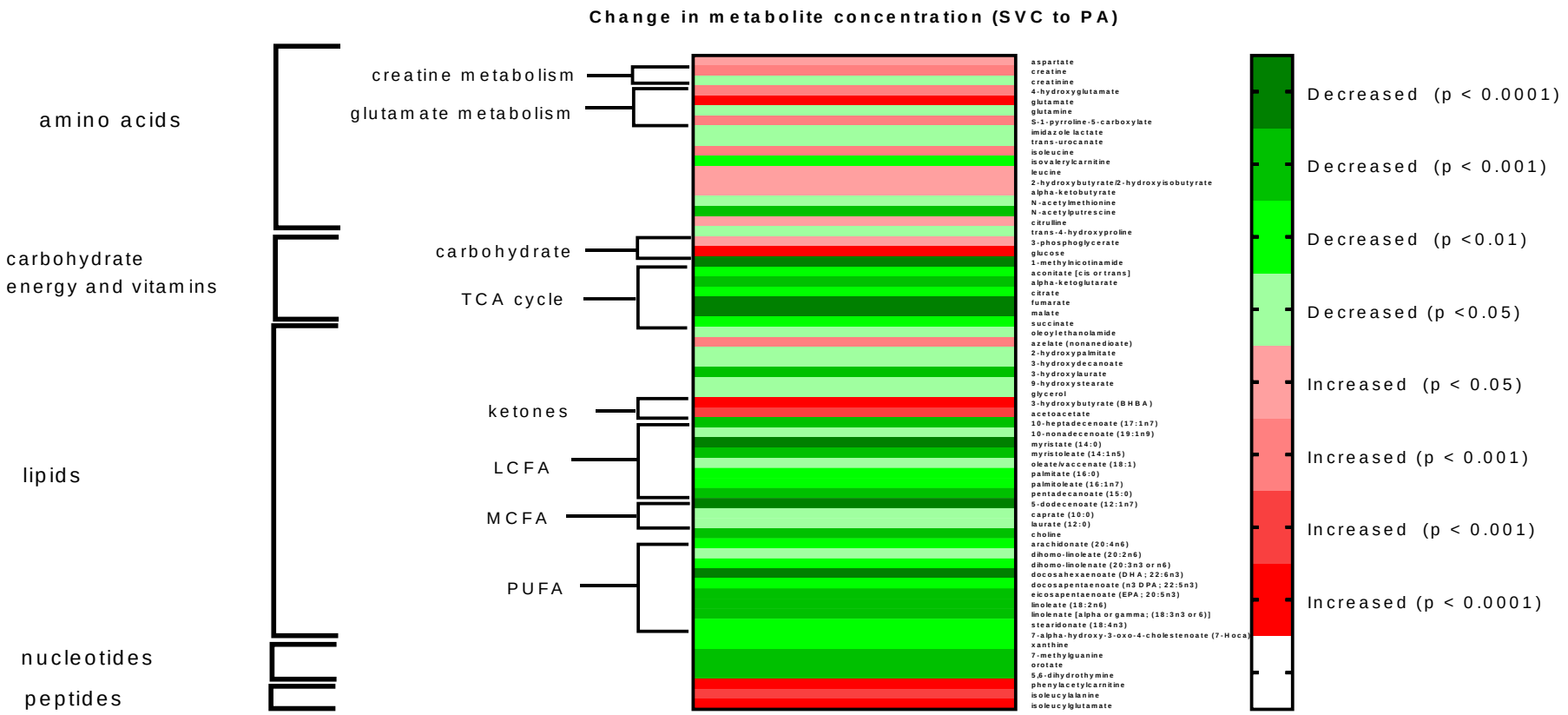


Figure 6.1- Heat map displaying endogenous metabolites which showed a significant difference in concentration between SVC and PA plasma in patients with pulmonary vascular disease. Paired blood samples were taken from the SVC and PA sites during right heart catheterisation of 57 patients with pulmonary vascular disease. Untargeted metabolic profiling of the plasma was carried out using the Metabolon DiscoveryHD4™ platform. The concentration of each metabolite (median scaled standardised intensity) was compared between SVC and PA samples using the Wilcoxon matched pairs signed rank test with FDR adjustment. Metabolites which were increased in the PA compared to SVC samples are shown in red, metabolites which were decreased in the PA compared to SVC samples are shown in green. SVC-superior vena cava, PA-pulmonary artery, TCA- tricarboxylic acid, LCFA-long chain fatty acid, MCFA-medium chain fatty acid, PUFA-polyunsaturated fatty acid.

6.5.2 Transpulmonary metabolite gradients

Amongst 1375 metabolites, 268 showed a significant difference in concentration between the paired pulmonary artery (PA) and radial artery (RA) blood samples, after false discovery rate adjustment. Of these 268 metabolites, 208 were endogenous metabolites, 42 were unidentified metabolites and 18 were xenobiotics. Of the xenobiotics, 1 was a drug (lignocaine), the others were food components and chemicals. The endogenous metabolites are shown in Table 7.3.

42 sub-pathways were over-represented. This included carbohydrate metabolites such as disaccharides, glycogen products and aminosugars, TCA cycle metabolites and multiple lipid groups including acylcholines, lysolipids and fatty acids, amongst others [Table 7.4].

There was predominant reduction in the concentration of many metabolites in RA compared to PA blood samples. These changes are summarised in Figure 6.2.

6.5.2.1 Amino acids

There was depletion of amino acids in RA compared to paired PA samples. This included amino acids involved in many different sub-pathways, such as alanine and aspartate metabolism, glutamate metabolism and tryptophan metabolism. However, an exception to this was sarcosine, which showed a significant increase in concentration in RA samples.

6.5.2.2 Carbohydrates

Carbohydrates showed a decrease in concentration in the RA compared to PA sample, including glucose, fructose and galactose metabolites, and glucose precursors maltose and maltotriose. Aminosugars such as glucuronate and N-acetylneuraminate were also depleted.

6.5.2.3 Cofactors and vitamins

There was a significant increase in haem concentration in the RA compared to PA sample. Conversely, there was depletion of ascorbate and aldarate metabolites. There were differences in the concentration of nicotinate metabolites, with a reduction in nicotinamide concentration, accompanied by an increase in 1-methylnicotinamide.

6.5.2.4 TCA cycle

There was an increase in citrate concentration in RA compared to PA blood. However, there was a depletion of other TCA cycle metabolites such as aconitate and malate.

6.5.2.5 Lipids

Numerous lipids showed a significant decrease in concentration in RA compared to PA blood, including acylcholines, the eicosanoid 12-Hydroxyeicosatetraenoic acid (12-HETE) and other lipid groups including lysolipids, plasmalogens, medium chain and polyunsaturated fatty acids. Amongst glycerolipids, there was a decrease in glycerophosphoglycerol, but an increase in glycerol 3 phosphate. Acylcarnitines showed a significant increase in concentration in RA compared to PA blood. This was accompanied by a decrease in carnitine.

6.5.2.6 Peptides

There was a predominant decrease in concentration of peptides in the RA compared to PA sample, such as leucyleucine and histadylphenylalanine. However, there was increase in phenylacetylcarnitine. Additionally, there was an increase in concentration of fibrinogen cleavage peptides, although these metabolites were only detected in a small proportion of patients.

6.5.2.7 Nucleotides

Nucleotide metabolites such as adenine, cytidine, IMP and ADP showed a significant decrease in concentration in RA samples compared to PA samples. Only dihydroorotate showed an increase in concentration between PA and RA.

Table 6.3- Endogenous metabolites which showed a significant difference in concentration between paired RA and PA samples. Blood samples were collected at the time of right heart catheterisation. Metabolite concentration (expressed as median scaled relative standard intensity) was compared between paired samples using the Wilcoxon matched pairs signed rank test with FDR adjustment.

BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY	p value
asparagine	Amino Acid	Alanine and Aspartate Metabolism	0.0005
N-acetylasparagine	Amino Acid	Alanine and Aspartate Metabolism	0.0014
N-carbamoylalanine	Amino Acid	Alanine and Aspartate Metabolism	0.0073
aspartate	Amino Acid	Alanine and Aspartate Metabolism	< 0.0001
creatine	Amino Acid	Creatine Metabolism	0.0003
pyroglutamine	Amino Acid	Glutamate Metabolism	0.0002
N-acetylglutamate	Amino Acid	Glutamate Metabolism	0.0003
N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism	0.0003
gamma-carboxyglutamate	Amino Acid	Glutamate Metabolism	0.0146
beta-citrylglutamate	Amino Acid	Glutamate Metabolism	< 0.0001
S-1-pyrroline-5-carboxylate	Amino Acid	Glutamate Metabolism	< 0.0001
glutamate	Amino Acid	Glutamate Metabolism	< 0.0001
N-acetylglycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0018
N-acetylserine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0034
glycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0370
sarcosine	Amino Acid	Glycine, Serine and Threonine Metabolism	< 0.0001
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	0.0116
hydantoin-5-propionic acid	Amino Acid	Histidine Metabolism	0.0049
ethylmalonate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0011
3-hydroxyisobutyrate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0185
isoleucine	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0229
N2-acetyllysine	Amino Acid	Lysine Metabolism	0.0002
glutarate (pentanedioate)	Amino Acid	Lysine Metabolism	0.0110
pipecolate	Amino Acid	Lysine Metabolism	0.0498
cysteine sulfinic acid	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0001
methionine sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0041
methionine sulfone	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0220
S-adenosylhomocysteine (SAH)	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0260
N-methyltaurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0359
cystine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0498
taurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	< 0.0001
hypotaurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	< 0.0001
xanthurenate	Amino Acid	Tryptophan Metabolism	0.0040
C-glycosyltryptophan	Amino Acid	Tryptophan Metabolism	0.0072
thioproline	Amino Acid	Tryptophan Metabolism	0.0136

tryptophan	Amino Acid	Tryptophan Metabolism	0.0205
serotonin	Amino Acid	Tryptophan Metabolism	< 0.0001
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0024
N-methylproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0130
homocitrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0133
arginine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0194
N-acetylglucosamine/N-acetylgalactosamine	Carbohydrate	Aminosugar Metabolism	0.0010
glucuronate	Carbohydrate	Aminosugar Metabolism	0.0033
N-acetylneuraminate	Carbohydrate	Aminosugar Metabolism	< 0.0001
erythronate	Carbohydrate	Aminosugar Metabolism	< 0.0001
sucrose	Carbohydrate	Disaccharides and Oligosaccharides	0.0381
mannitol/sorbitol	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0018
mannose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0139
galactonate	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0194
fructose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	< 0.0001
maltose	Carbohydrate	Glycogen Metabolism	< 0.0001
maltotriose	Carbohydrate	Glycogen Metabolism	< 0.0001
glucose	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.0017
glycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	< 0.0001
arabonate/xylonate	Carbohydrate	Pentose Metabolism	0.0002
arabitol/xylitol	Carbohydrate	Pentose Metabolism	0.0443
ribonate	Carbohydrate	Pentose Metabolism	< 0.0001
threonate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0002
oxalate (ethanedioate)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0014
gulonate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0340
heme	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	0.0003
bilirubin (E,E)	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	0.0105
quinolinate	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0002
1-methylnicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0049
nicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	< 0.0001
citrate	Energy	TCA Cycle	0.0003
aconitate [cis or trans]	Energy	TCA Cycle	0.0005
malate	Energy	TCA Cycle	0.0027
2-methylcitrate/homocitrate	Energy	TCA Cycle	0.0370
alpha-ketoglutarate	Energy	TCA Cycle	0.0406
carnitine	Lipid	Carnitine Metabolism	0.0260

oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	Lipid	Diacylglycerol	0.0014
oleoyl-arachidonoyl-glycerol (18:1/20:4) [2]	Lipid	Diacylglycerol	0.0047
oleoyl-oleoyl-glycerol (18:1/18:1) [2]	Lipid	Diacylglycerol	0.0143
12-HETE	Lipid	Eicosanoid	0.0024
oleoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	0.0002
palmitoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	< 0.0001
methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.0035
suberoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0019
3-hydroxybutyrylcarnitine (1)	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0022
linoleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0157
myristoleoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0290
acetylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	< 0.0001
3-hydroxybutyrylcarnitine (2)	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	< 0.0001
2-hydroxyglutarate	Lipid	Fatty Acid, Dicarboxylate	0.0130
adipate	Lipid	Fatty Acid, Dicarboxylate	0.0149
2-hydroxystearate	Lipid	Fatty Acid, Monohydroxy	0.0113
2-hydroxypalmitate	Lipid	Fatty Acid, Monohydroxy	< 0.0001
glycerol 3-phosphate	Lipid	Glycerolipid Metabolism	0.0008
glycerophosphoglycerol	Lipid	Glycerolipid Metabolism	< 0.0001
myo-inositol	Lipid	Inositol Metabolism	0.0016
chiro-inositol	Lipid	Inositol Metabolism	0.0418
2-stearoyl-GPI (18:0)	Lipid	Lysolipid	0.0003
2-oleoyl-GPE (18:1)	Lipid	Lysolipid	0.0003
1-nonadecanoyl-GPC (19:0)	Lipid	Lysolipid	0.0004
2-arachidonoyl-GPC (20:4)	Lipid	Lysolipid	0.0009
1-dihomo-linolenoyl-GPE (20:3n3 or 6)	Lipid	Lysolipid	0.0009
1-palmitoyl-GPC (16:0)	Lipid	Lysolipid	0.0014
1-oleoyl-GPS (18:1)	Lipid	Lysolipid	0.0015
1-myristoyl-GPC (14:0)	Lipid	Lysolipid	0.0016
1-pentadecanoyl-GPC (15:0)	Lipid	Lysolipid	0.0019
1-oleoyl-GPE (18:1)	Lipid	Lysolipid	0.0031
1-erucoyl-GPC (22:1)	Lipid	Lysolipid	0.0047
1-docosapentaenoyl-GPC (22:5n6)	Lipid	Lysolipid	0.0049
1-palmitoleoyl-GPI (16:1)	Lipid	Lysolipid	0.0067
2-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	0.0073
2-oleoyl-GPC (18:1)	Lipid	Lysolipid	0.0116
2-linoleoyl-GPC (18:2)	Lipid	Lysolipid	0.0224
1-arachidonoyl-GPE (20:4n6)	Lipid	Lysolipid	0.0232
1-palmitoyl-GPE (16:0)	Lipid	Lysolipid	0.0324
1-dihomo-linoleoyl-GPC (20:2)	Lipid	Lysolipid	< 0.0001
1-eicosenoyl-GPC (20:1)	Lipid	Lysolipid	< 0.0001
1-palmitoyl-GPI (16:0)	Lipid	Lysolipid	< 0.0001
1-adrenoyl-GPC (22:4)	Lipid	Lysolipid	< 0.0001
1-dihomo-linolenoyl-GPC (20:3n3 or 6)	Lipid	Lysolipid	< 0.0001

1-linoleoyl-GPI (18:2)	Lipid	Lysolipid	< 0.0001
1-arachidonoyl-GPC (20:4n6)	Lipid	Lysolipid	< 0.0001
1-arachidonoyl-GPI (20:4)	Lipid	Lysolipid	< 0.0001
1-arachidoyl-GPC (20:0)	Lipid	Lysolipid	< 0.0001
1-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	< 0.0001
1-linoleoyl-GPC (18:2)	Lipid	Lysolipid	< 0.0001
1-eicosapentaenoyl-GPC (20:5)	Lipid	Lysolipid	< 0.0001
1-stearoyl-GPE (18:0)	Lipid	Lysolipid	< 0.0001
1-oleoyl-GPC (18:1)	Lipid	Lysolipid	< 0.0001
1-stearoyl-GPI (18:0)	Lipid	Lysolipid	< 0.0001
1-linolenoyl-GPC (18:3)	Lipid	Lysolipid	< 0.0001
1-oleoyl-GPI (18:1)	Lipid	Lysolipid	< 0.0001
1-margaroyl-GPC (17:0)	Lipid	Lysolipid	< 0.0001
1-stearoyl-GPC (18:0)	Lipid	Lysolipid	< 0.0001
1-docosapentaenoyl-GPC (22:5n3)	Lipid	Lysolipid	< 0.0001
1-palmitoleoyl-GPC (16:1)	Lipid	Lysolipid	< 0.0001
1-stearyl-GPC (O-18:0)	Lipid	Lyso-phospho-ether	< 0.0001
1-palmityl-GPC (O-16:0)	Lipid	Lyso-phospho-ether	< 0.0001
1-palmityl-GPE (O-16:0)	Lipid	Lyso-phospho-ether	< 0.0001
1-(1-enyl-stearoyl)-GPC (P-18:0)	Lipid	Lysoplasmalogen	0.0016
1-(1-enyl-oleoyl)-GPE (P-18:1)	Lipid	Lysoplasmalogen	< 0.0001
1-(1-enyl-palmitoyl)-GPE (P-16:0)	Lipid	Lysoplasmalogen	< 0.0001
1-(1-enyl-stearoyl)-GPE (P-18:0)	Lipid	Lysoplasmalogen	< 0.0001
1-(1-enyl-palmitoyl)-GPC (P-16:0)	Lipid	Lysoplasmalogen	< 0.0001
1-(1-enyl-oleoyl)-GPC (P-18:1)	Lipid	Lysoplasmalogen	< 0.0001
laurate (12:0)	Lipid	Medium Chain Fatty Acid	0.0025
heptanoate (7:0)	Lipid	Medium Chain Fatty Acid	0.0370
3-hydroxy-3-methylglutarate	Lipid	Mevalonate Metabolism	< 0.0001
1-arachidonylglycerol (20:4)	Lipid	Monoacylglycerol	0.0396
1-stearoyl-2-oleoyl-GPS (18:0/18:1)	Lipid	Phosphatidylserine (PS)	0.0003
1-stearoyl-2-arachidonoyl-GPS (18:0/20:4)	Lipid	Phosphatidylserine (PS)	0.0049
1-stearoyl-2-adrenoyl-GPE (18:0/22:4)	Lipid	Phospholipid Metabolism	0.0007
1-stearoyl-2-docosapentaenoyl-GPE (18:0/22:5n3)	Lipid	Phospholipid Metabolism	0.0025
docosahexaenoylcholine	Lipid	Phospholipid Metabolism	0.0026
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	Lipid	Phospholipid Metabolism	0.0030
1-stearoyl-2-docosahexaenoyl-GPS (18:0/22:6)	Lipid	Phospholipid Metabolism	0.0034
1-palmityl-2-stearoyl-GPC (O-16:0/18:0)	Lipid	Phospholipid Metabolism	0.0133
1-arachidoyl-2-arachidonoyl-GPC (20:0/20:4)	Lipid	Phospholipid Metabolism	0.0214
1-stearyl-2-docosapentaenoyl-GPC (O-18:0/22:5n3)	Lipid	Phospholipid Metabolism	0.0370
glycerophosphoethanolamine	Lipid	Phospholipid Metabolism	< 0.0001
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	< 0.0001
phosphoethanolamine	Lipid	Phospholipid Metabolism	< 0.0001
glycerophosphoinositol	Lipid	Phospholipid Metabolism	< 0.0001
arachidonoylcholine	Lipid	Phospholipid Metabolism	< 0.0001

choline phosphate	Lipid	Phospholipid Metabolism	< 0.0001
dihomo-linolenoyl-choline	Lipid	Phospholipid Metabolism	< 0.0001
cytidine 5'-diphosphocholine	Lipid	Phospholipid Metabolism	< 0.0001
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	Lipid	Plasmalogen	0.0003
1-(1-enyl-stearoyl)-2-docosapentaenoyl-GPE (P-18:0/22:5n3)	Lipid	Plasmalogen	0.0007
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	Lipid	Plasmalogen	0.0013
1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)	Lipid	Plasmalogen	0.0028
1-(1-enyl-stearoyl)-2-docosahexaenoyl-GPE (P-18:0/22:6)	Lipid	Plasmalogen	0.0066
1-(1-enyl-stearoyl)-2-oleoyl-GPE (P-18:0/18:1)	Lipid	Plasmalogen	0.0181
1-(1-enyl-palmitoyl)-2-docosahexaenoyl-GPE (P-16:0/22:6)	Lipid	Plasmalogen	0.0227
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0001
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0011
docosapentaenoate (n3 DPA; 22:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0041
arachidonate (20:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
sphinganine-1-phosphate	Lipid	Sphingolipid Metabolism	0.0001
sphinganine	Lipid	Sphingolipid Metabolism	0.0002
sphingosine	Lipid	Sphingolipid Metabolism	0.0014
N-stearoyl-sphingosine (d18:1/18:0)	Lipid	Sphingolipid Metabolism	0.0453
sphingosine 1-phosphate	Lipid	Sphingolipid Metabolism	< 0.0001
campesterol	Lipid	Sterol	0.0189
allantoin	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0002
inosine 5'-monophosphate (IMP)	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0003
adenosine 5'-diphosphate (ADP)	Nucleotide	Purine Metabolism, Adenine containing	0.0001
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	< 0.0001
adenine	Nucleotide	Purine Metabolism, Adenine containing	< 0.0001
cytidine 5'-monophosphate (5'-CMP)	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.0027
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	< 0.0001
orotidine	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0001
dihydroorotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0003
orotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0084
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0110
3-ureidopropionate	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0136
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0146
phenylacetylcarnitine	Peptide	Acetylated Peptides	0.0007
phenylacetylglutamine	Peptide	Acetylated Peptides	0.0464
phenylalanyltryptophan	Peptide	Dipeptide	0.0019
tryptophylleucine	Peptide	Dipeptide	0.0022
phenylalanylarginine	Peptide	Dipeptide	0.0029
isoleucylglutamate	Peptide	Dipeptide	0.0030

isoleucylalanine	Peptide	Dipeptide	0.0035
serylalanine	Peptide	Dipeptide	0.0043
cyclo(pro-val)	Peptide	Dipeptide	0.0260
prolylproline	Peptide	Dipeptide	< 0.0001
histidylphenylalanine	Peptide	Dipeptide	< 0.0001
leucylleucine	Peptide	Dipeptide	< 0.0001
DSGEGDFXAEGGGVR	Peptide	Fibrinogen Cleavage Peptide	0.0240
ADSGEGDFXAEGGGVR	Peptide	Fibrinogen Cleavage Peptide	0.0354
gamma-glutamyl-alpha-lysine	Peptide	Gamma-glutamyl Amino Acid	0.0209

Table 6.4- Over-representation analysis of metabolites which showed a significant difference in concentration between paired SVC and PA samples. Metabolites which showed an enrichment value >1 are listed.

SUPER PATHWAY	SUB PATHWAY	ENRICHMENT VALUE
Carbohydrate	Disaccharides and Oligosaccharides	5.13
Carbohydrate	Fructose, Mannose and Galactose Metabolism	5.13
Carbohydrate	Glycogen Metabolism	5.13
Lipid	Eicosanoid	5.13
Lipid	Fatty Acid Metabolism (Acyl Choline)	5.13
Lipid	Inositol Metabolism	5.13
Lipid	Lyso-phospho-ether	5.13
Lipid	Lysoplasmalogen	5.13
Lipid	Mevalonate Metabolism	5.13
Lipid	Phosphatidylserine (PS)	5.13
Nucleotide	Pyrimidine Metabolism, Orotate containing	5.13
Peptide	Fibrinogen Cleavage Peptide	5.13
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	3.85
Carbohydrate	Aminosugar Metabolism	3.42
Lipid	Glycerolipid Metabolism	3.42
Lipid	Lysolipid	3.33
Amino Acid	Glutamate Metabolism	3.26
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	3.08
Amino Acid	Alanine and Aspartate Metabolism	2.93
Energy	TCA Cycle	2.85
Carbohydrate	Pentose Metabolism	2.57
Lipid	Carnitine Metabolism	2.57
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	2.57
Nucleotide	Pyrimidine Metabolism, Uracil containing	2.20
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	2.14
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2.05
Nucleotide	Pyrimidine Metabolism, Cytidine containing	2.05
Peptide	Acetylated Peptides	2.05
Nucleotide	Purine Metabolism, Adenine containing	1.92

Amino Acid	Glycine, Serine and Threonine Metabolism	1.87
Amino Acid	Creatine Metabolism	1.71
Amino Acid	Guanidino and Acetamido Metabolism	1.71
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.71
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	1.71
Lipid	Plasmalogen	1.56
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	1.47
Lipid	Medium Chain Fatty Acid	1.47
Amino Acid	Lysine Metabolism	1.28
Amino Acid	Tryptophan Metabolism	1.28
Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	1.28
Peptide	Dipeptide	1.25
Amino Acid	Urea cycle; Arginine and Proline Metabolism	1.14

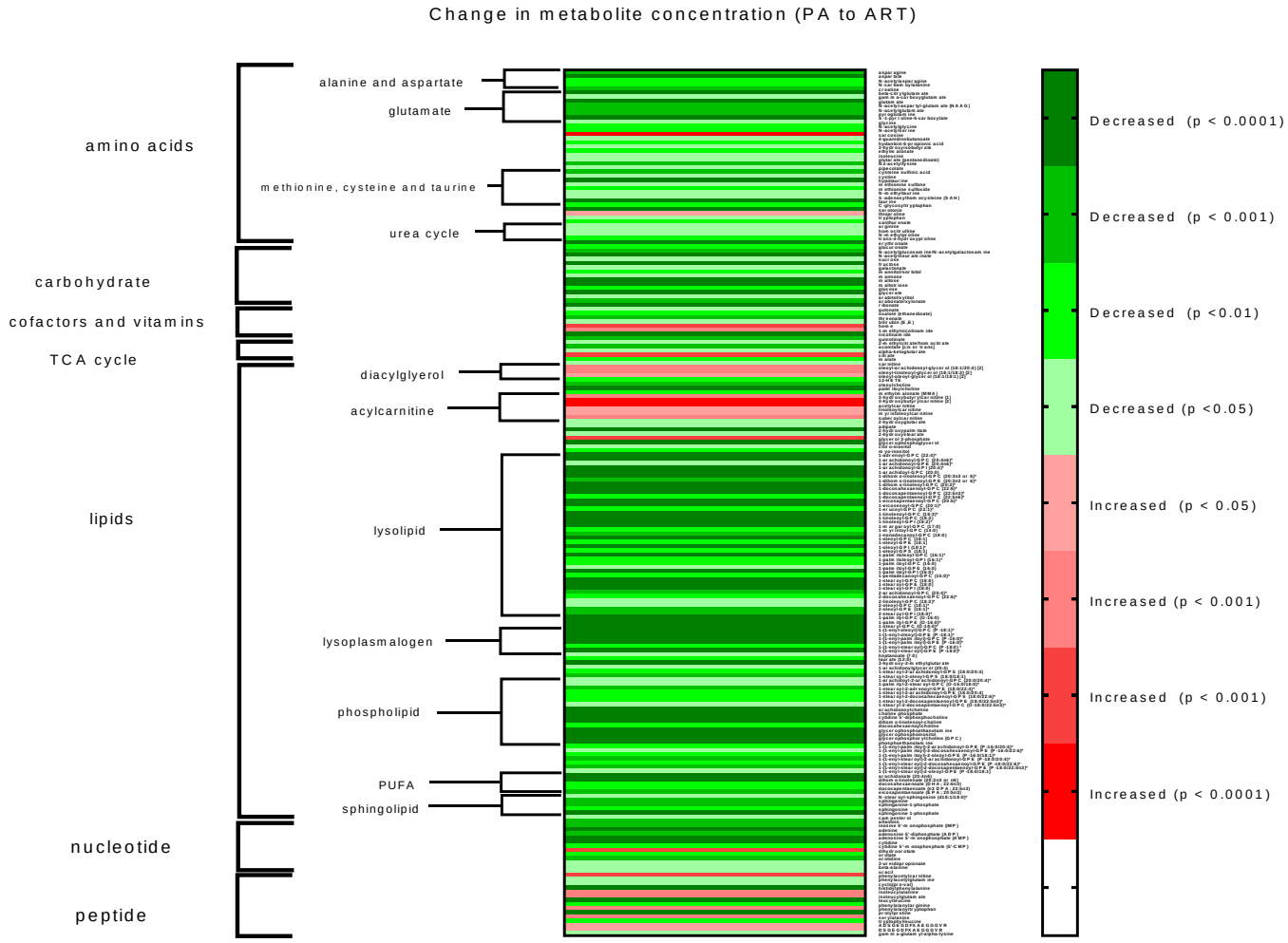


Figure 6.2- Heat map displaying endogenous metabolites which showed a significant difference in concentration between PA and ART plasma in patients with pulmonary vascular disease. Paired blood samples were taken from the PA and ART sites during right heart catheterisation of 57 patients with pulmonary vascular disease. Untargeted metabolic profiling of the plasma was carried out using the Metabolon DiscoveryHD4™ platform. The concentration of each metabolite (median scaled standardised intensity) was compared between PA and ART samples using Wilcoxon matched pairs signed rank test with FDR adjustment. Metabolites which were increased in the ART compared to PA samples are shown in red, metabolites which were decreased in the PA compared to SVC samples are shown in green. PA-pulmonary artery, ART-radial artery, PUFA-polyunsaturated fatty acid.

6.5.3 Systemic metabolite gradients

Amongst 1375 metabolites, 341 showed a significant difference in concentration between the paired radial artery (RA) and superior vena cava (SVC) blood samples, after false discovery rate adjustment. Of these 341 metabolites, 266 were endogenous, 58 were unidentified metabolites and 17 were xenobiotics. The endogenous metabolites are shown in Table 6.5. 49 sub-pathways were over-represented [Table 6.6].

In SVC samples compared to RA samples, there was a predominant increase in concentration of carbohydrate, energy, lipid and nucleotide metabolites, including glycogen metabolites, TCA cycle intermediates and fatty acids. There were also increases in acylcholines, phosphatidylserines and polyamines. A reduction in glutamate was observed, accompanied by an increase in glutamate metabolites, and acylcarnitine concentration was also reduced. These findings are summarised in Figure 6.3.

Table 6.5- Endogenous metabolites which showed a significant difference in concentration between paired RA and PA samples. Blood samples were collected at the time of right heart catheterisation. Metabolite concentration (expressed as median scaled relative standard intensity) was compared between paired samples using the Wilcoxon matched pairs signed rank test with FDR adjustment.

BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY	p value
asparagine	Amino Acid	Alanine and Aspartate Metabolism	0.0001
N-acetylasparagine	Amino Acid	Alanine and Aspartate Metabolism	0.0208
N-carbamoylalanine	Amino Acid	Alanine and Aspartate Metabolism	0.0376
guanidinoacetate	Amino Acid	Creatine Metabolism	0.0014
creatinine	Amino Acid	Creatine Metabolism	0.0023
beta-citrylglutamate	Amino Acid	Glutamate Metabolism	0.0001
N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism	0.0001
glutamate	Amino Acid	Glutamate Metabolism	0.0010
gamma-carboxyglutamate	Amino Acid	Glutamate Metabolism	0.0010
pyroglutamine	Amino Acid	Glutamate Metabolism	0.0048
N-acetylglutamine	Amino Acid	Glutamate Metabolism	0.0049
glutamine	Amino Acid	Glutamate Metabolism	0.0081
N-acetylglutamate	Amino Acid	Glutamate Metabolism	< 0.0001
5-oxoproline	Amino Acid	Glutathione Metabolism	0.0051
cys-gly, oxidized	Amino Acid	Glutathione Metabolism	0.0401
N-acetylserine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0011
threonine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0100
glycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0210
N-acetylglycine	Amino Acid	Glycine, Serine and Threonine Metabolism	0.0336
sarcosine	Amino Acid	Glycine, Serine and Threonine Metabolism	< 0.0001
4-guanidinobutanoate	Amino Acid	Guanidino and Acetamido Metabolism	0.0002

imidazole lactate	Amino Acid	Histidine Metabolism	0.0028
hydantoin-5-propionic acid	Amino Acid	Histidine Metabolism	0.0065
formiminoglutamate	Amino Acid	Histidine Metabolism	0.0068
1-methylimidazoleacetate	Amino Acid	Histidine Metabolism	0.0097
N-acetyl-3-methylhistidine	Amino Acid	Histidine Metabolism	0.0246
ethylmalonate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0002
2-methylbutyrylcarnitine (C5)	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0025
3-hydroxyisobutyrate	Amino Acid	Leucine, Isoleucine and Valine Metabolism	0.0159
N2-acetyllysine	Amino Acid	Lysine Metabolism	0.0010
glutarate (pentanedioate)	Amino Acid	Lysine Metabolism	0.0023
hypotaurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0001
taurine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0006
cysteine sulfinic acid	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0008
alpha-ketobutyrate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0018
cystine	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0085
methionine sulfoxide	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0155
methionine sulfone	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0294
cysteine s-sulfate	Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	0.0337
phenylalanine	Amino Acid	Phenylalanine and Tyrosine Metabolism	0.0472
N-acetylputrescine	Amino Acid	Polyamine Metabolism	0.0028
4-acetamidobutanoate	Amino Acid	Polyamine Metabolism	0.0467
kynurenine	Amino Acid	Tryptophan Metabolism	0.0035
3-hydroxykynurenine	Amino Acid	Tryptophan Metabolism	0.0047
serotonin	Amino Acid	Tryptophan Metabolism	0.0068
C-glycosyltryptophan	Amino Acid	Tryptophan Metabolism	0.0130
indolelactate	Amino Acid	Tryptophan Metabolism	0.0387
ornithine	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0130
homocitrulline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	0.0163
trans-4-hydroxyproline	Amino Acid	Urea cycle; Arginine and Proline Metabolism	< 0.0001
glucuronate	Carbohydrate	Aminosugar Metabolism	0.0001
N-acetylneuraminate	Carbohydrate	Aminosugar Metabolism	0.0019
erythronate	Carbohydrate	Aminosugar Metabolism	< 0.0001
mannitol/sorbitol	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0007
fructose	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0017
galactonate	Carbohydrate	Fructose, Mannose and Galactose Metabolism	0.0484
maltose	Carbohydrate	Glycogen Metabolism	< 0.0001
maltotriose	Carbohydrate	Glycogen Metabolism	0.0011
3-phosphoglycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.0326
glycerate	Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	< 0.0001

arabonate/xylonate	Carbohydrate	Pentose Metabolism	0.0001
ribitol	Carbohydrate	Pentose Metabolism	0.0002
arabitol/xylitol	Carbohydrate	Pentose Metabolism	0.0074
ribonate	Carbohydrate	Pentose Metabolism	< 0.0001
threonate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0002
oxalate (ethanedioate)	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0017
gulonate	Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	0.0092
heme	Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	0.0003
quinolinate	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0001
1-methylnicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0020
nicotinamide	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0044
trigonelline (N'-methylnicotinate)	Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	0.0326
alpha-ketoglutarate	Energy	TCA Cycle	0.0001
succinate	Energy	TCA Cycle	0.0004
2-methylcitrate/homocitrate	Energy	TCA Cycle	0.0048
citrate	Energy	TCA Cycle	0.0270
fumarate	Energy	TCA Cycle	< 0.0001
malate	Energy	TCA Cycle	< 0.0001
aconitate [cis or trans]	Energy	TCA Cycle	< 0.0001
deoxycarnitine	Lipid	Carnitine Metabolism	0.0047
oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	Lipid	Diacylglycerol	0.0078
oleoyl-arachidonoyl-glycerol (18:1/20:4) [2]	Lipid	Diacylglycerol	0.0140
oleoyl-oleoyl-glycerol (18:1/18:1) [2]	Lipid	Diacylglycerol	0.0337
12-HETE	Lipid	Eicosanoid	0.0165
oleoyl ethanolamide	Lipid	Endocannabinoid	0.0016
palmitoyl ethanolamide	Lipid	Endocannabinoid	0.0048
oleoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	< 0.0001
palmitoylcholine	Lipid	Fatty Acid Metabolism (Acyl Choline)	< 0.0001
methylmalonate (MMA)	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.0004
propionylcarnitine	Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	0.0044
3-hydroxybutyrylcarnitine (2)	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0001
3-hydroxybutyrylcarnitine (1)	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0018
suberoylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	0.0090
acetylcarnitine	Lipid	Fatty Acid Metabolism(Acyl Carnitine)	< 0.0001
malonate	Lipid	Fatty Acid Synthesis	0.0016
pimelate (heptanedioate)	Lipid	Fatty Acid, Dicarboxylate	0.0014
2-hydroxyglutarate	Lipid	Fatty Acid, Dicarboxylate	0.0015
adipate	Lipid	Fatty Acid, Dicarboxylate	0.0044
azelate (nonanedioate)	Lipid	Fatty Acid, Dicarboxylate	0.0228
2-hydroxystearate	Lipid	Fatty Acid, Monohydroxy	0.0001

9-hydroxystearate	Lipid	Fatty Acid, Monohydroxy	0.0002
3-hydroxylaurate	Lipid	Fatty Acid, Monohydroxy	0.0022
3-hydroxydecanoate	Lipid	Fatty Acid, Monohydroxy	0.0379
2-hydroxypalmitate	Lipid	Fatty Acid, Monohydroxy	< 0.0001
glycerophosphoglycerol	Lipid	Glycerolipid Metabolism	0.0002
glycerol	Lipid	Glycerolipid Metabolism	0.0005
glycerol 3-phosphate	Lipid	Glycerolipid Metabolism	0.0044
myo-inositol	Lipid	Inositol Metabolism	0.0004
3-hydroxybutyrate (BHBA)	Lipid	Ketone Bodies	0.0014
palmitate (16:0)	Lipid	Long Chain Fatty Acid	0.0001
10-heptadecenoate (17:1n7)	Lipid	Long Chain Fatty Acid	0.0002
palmitoleate (16:1n7)	Lipid	Long Chain Fatty Acid	0.0002
eicosenoate (20:1)	Lipid	Long Chain Fatty Acid	0.0019
10-nonadecenoate (19:1n9)	Lipid	Long Chain Fatty Acid	0.0046
oleate/vaccenate (18:1)	Lipid	Long Chain Fatty Acid	0.0082
nonadecanoate (19:0)	Lipid	Long Chain Fatty Acid	0.0167
stearate (18:0)	Lipid	Long Chain Fatty Acid	0.0249
myristate (14:0)	Lipid	Long Chain Fatty Acid	< 0.0001
myristoleate (14:1n5)	Lipid	Long Chain Fatty Acid	< 0.0001
pentadecanoate (15:0)	Lipid	Long Chain Fatty Acid	< 0.0001
1-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	0.0001
1-oleoyl-GPC (18:1)	Lipid	Lysolipid	0.0001
1-eicosapentaenoyl-GPC (20:5)	Lipid	Lysolipid	0.0001
1-nonadecanoyl-GPC (19:0)	Lipid	Lysolipid	0.0001
1-linolenoyl-GPC (18:3)	Lipid	Lysolipid	0.0001
1-oleoyl-GPI (18:1)	Lipid	Lysolipid	0.0002
1-docosapentaenoyl-GPC (22:5n3)	Lipid	Lysolipid	0.0002
1-stearoyl-GPC (18:0)	Lipid	Lysolipid	0.0002
1-docosapentaenoyl-GPC (22:5n6)	Lipid	Lysolipid	0.0002
1-margaroyl-GPC (17:0)	Lipid	Lysolipid	0.0002
1-arachidoyl-GPC (20:0)	Lipid	Lysolipid	0.0003
1-stearoyl-GPE (18:0)	Lipid	Lysolipid	0.0003
2-arachidonoyl-GPC (20:4)	Lipid	Lysolipid	0.0004
1-palmitoleoyl-GPC (16:1)	Lipid	Lysolipid	0.0005
2-docosahexaenoyl-GPC (22:6)	Lipid	Lysolipid	0.0011
2-stearoyl-GPI (18:0)	Lipid	Lysolipid	0.0022
1-oleoyl-GPS (18:1)	Lipid	Lysolipid	0.0032
1-palmitoyl-GPC (16:0)	Lipid	Lysolipid	0.0044
2-linoleoyl-GPC (18:2)	Lipid	Lysolipid	0.0044
1-palmitoleoyl-GPI (16:1)	Lipid	Lysolipid	0.0066
1-myristoyl-GPC (14:0)	Lipid	Lysolipid	0.0074
2-oleoyl-GPE (18:1)	Lipid	Lysolipid	0.0080
2-oleoyl-GPC (18:1)	Lipid	Lysolipid	0.0081
1-palmitoleoyl-GPE (16:1)	Lipid	Lysolipid	0.0085

1-pentadecanoyl-GPC (15:0)	Lipid	Lysolipid	0.0249
1-dihomo-linolenoyl-GPE (20:3n3 or 6)	Lipid	Lysolipid	0.0326
1-oleoyl-GPE (18:1)	Lipid	Lysolipid	0.0337
1-erucoyl-GPC (22:1)	Lipid	Lysolipid	0.0339
1-lignoceroyl-GPC (24:0)	Lipid	Lysolipid	0.0437
1-palmitoyl-GPI (16:0)	Lipid	Lysolipid	< 0.0001
1-arachidonoyl-GPI (20:4)	Lipid	Lysolipid	< 0.0001
1-linoleoyl-GPI (18:2)	Lipid	Lysolipid	< 0.0001
1-adrenoyl-GPC (22:4)	Lipid	Lysolipid	< 0.0001
1-stearoyl-GPI (18:0)	Lipid	Lysolipid	< 0.0001
1-dihomo-linoleoyl-GPC (20:2)	Lipid	Lysolipid	< 0.0001
1-dihomo-linolenoyl-GPC (20:3n3 or 6)	Lipid	Lysolipid	< 0.0001
1-arachidonoyl-GPC (20:4n6)	Lipid	Lysolipid	< 0.0001
1-eicosenoyl-GPC (20:1)	Lipid	Lysolipid	< 0.0001
1-linoleoyl-GPC (18:2)	Lipid	Lysolipid	< 0.0001
1-stearyl-GPC (O-18:0)	Lipid	Lyso-phospho-ether	0.0001
1-palmityl-GPC (O-16:0)	Lipid	Lyso-phospho-ether	< 0.0001
1-palmityl-GPE (O-16:0)	Lipid	Lyso-phospho-ether	< 0.0001
1-(1-enyl-stearoyl)-GPE (P-18:0)	Lipid	Lysoplasmalogen	0.0001
1-(1-enyl-palmitoyl)-GPC (P-16:0)	Lipid	Lysoplasmalogen	0.0001
1-(1-enyl-palmitoyl)-GPE (P-16:0)	Lipid	Lysoplasmalogen	0.0001
1-(1-enyl-stearoyl)-GPC (P-18:0)	Lipid	Lysoplasmalogen	0.0002
1-(1-enyl-oleoyl)-GPC (P-18:1)	Lipid	Lysoplasmalogen	< 0.0001
1-(1-enyl-oleoyl)-GPE (P-18:1)	Lipid	Lysoplasmalogen	< 0.0001
caprate (10:0)	Lipid	Medium Chain Fatty Acid	0.0001
laurate (12:0)	Lipid	Medium Chain Fatty Acid	< 0.0001
5-dodecenoate (12:1n7)	Lipid	Medium Chain Fatty Acid	< 0.0001
3-hydroxy-3-methylglutarate	Lipid	Mevalonate Metabolism	< 0.0001
1-arachidonylglycerol (20:4)	Lipid	Monoacylglycerol	0.0026
1-stearoyl-2-oleoyl-GPS (18:0/18:1)	Lipid	Phosphatidylserine (PS)	0.0035
1-stearoyl-2-arachidonoyl-GPS (18:0/20:4)	Lipid	Phosphatidylserine (PS)	0.0479
choline	Lipid	Phospholipid Metabolism	0.0004
1-oleoyl-2-linoleoyl-GPC (18:1/18:2)	Lipid	Phospholipid Metabolism	0.0018
1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6)	Lipid	Phospholipid Metabolism	0.0022
phosphoethanolamine	Lipid	Phospholipid Metabolism	0.0033
1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	Lipid	Phospholipid Metabolism	0.0051
cytidine 5'-diphosphocholine	Lipid	Phospholipid Metabolism	0.0052
1-myristoyl-2-linoleoyl-GPC (14:0/18:2)	Lipid	Phospholipid Metabolism	0.0063
1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)	Lipid	Phospholipid Metabolism	0.0067
phosphatidylcholine (15:0/18:1, 17:0/16:1)	Lipid	Phospholipid Metabolism	0.0083
1-stearoyl-2-docosapentaenoyl-GPC (18:0/22:5n6)	Lipid	Phospholipid Metabolism	0.0085
1-stearoyl-2-adrenoyl-GPE (18:0/22:4)	Lipid	Phospholipid Metabolism	0.0095
1-palmitoyl-2-eicosapentaenoyl-GPC (16:0/20:5)	Lipid	Phospholipid Metabolism	0.0121
1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	Lipid	Phospholipid Metabolism	0.0121

1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)	Lipid	Phospholipid Metabolism	0.0124
1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)	Lipid	Phospholipid Metabolism	0.0124
1-stearoyl-2-docosapentaenoyl-GPE (18:0/22:5n3)	Lipid	Phospholipid Metabolism	0.0124
1-linoleoyl-2-arachidonoyl-GPE (18:2/20:4)	Lipid	Phospholipid Metabolism	0.0155
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	Lipid	Phospholipid Metabolism	0.0208
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	Lipid	Phospholipid Metabolism	0.0212
choline phosphate	Lipid	Phospholipid Metabolism	0.0216
1-stearoyl-2-docosahexaenoyl-GPS (18:0/22:6)	Lipid	Phospholipid Metabolism	0.0228
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	Lipid	Phospholipid Metabolism	0.0238
1-palmitoyl-2-stearoyl-GPE (16:0/18:0)	Lipid	Phospholipid Metabolism	0.0269
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	Lipid	Phospholipid Metabolism	0.0273
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	Lipid	Phospholipid Metabolism	0.0294
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)	Lipid	Phospholipid Metabolism	0.0337
phosphatidylcholine (18:0/20:2, 20:0/18:2)	Lipid	Phospholipid Metabolism	0.0371
1-pentadecanoyl-2-docosahexaenoyl-GPC (15:0/22:6)	Lipid	Phospholipid Metabolism	0.0484
glycerophosphorylcholine (GPC)	Lipid	Phospholipid Metabolism	< 0.0001
arachidonoylcholine	Lipid	Phospholipid Metabolism	< 0.0001
glycerophosphoinositol	Lipid	Phospholipid Metabolism	< 0.0001
glycerophosphoethanolamine	Lipid	Phospholipid Metabolism	< 0.0001
dihomo-linolenoyl-choline	Lipid	Phospholipid Metabolism	< 0.0001
docosahexaenoylcholine	Lipid	Phospholipid Metabolism	< 0.0001
1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)	Lipid	Plasmalogen	0.0112
1-(1-enyl-stearoyl)-2-docosapentaenoyl-GPE (P-18:0/22:5n3)	Lipid	Plasmalogen	0.0119
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	Lipid	Plasmalogen	0.0148
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	Lipid	Plasmalogen	0.0191
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0001
docosadienoate (22:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	0.0072
arachidonate (20:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
docosapentaenoate (n3 DPA; 22:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
linolenate [alpha or gamma; (18:3n3 or 6)]	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)	< 0.0001
sphinganine	Lipid	Sphingolipid Metabolism	0.0005
sphingosine	Lipid	Sphingolipid Metabolism	0.0023
sphinganine-1-phosphate	Lipid	Sphingolipid Metabolism	0.0061
sphingosine 1-phosphate	Lipid	Sphingolipid Metabolism	0.0080
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	Lipid	Sterol	0.0005
N1-methylinosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0004
xanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0017

inosine 5'-monophosphate (IMP)	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0021
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0054
allantoin	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.0130
adenosine 5'-monophosphate (AMP)	Nucleotide	Purine Metabolism, Adenine containing	0.0001
adenosine	Nucleotide	Purine Metabolism, Adenine containing	0.0049
adenosine 5'-diphosphate (ADP)	Nucleotide	Purine Metabolism, Adenine containing	0.0130
adenine	Nucleotide	Purine Metabolism, Adenine containing	< 0.0001
7-methylguanine	Nucleotide	Purine Metabolism, Guanine containing	0.0001
N2,N2-dimethylguanosine	Nucleotide	Purine Metabolism, Guanine containing	0.0159
N4-acetylcytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	0.0458
cytidine	Nucleotide	Pyrimidine Metabolism, Cytidine containing	< 0.0001
orotidine	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0005
dihydroorotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	0.0127
orotate	Nucleotide	Pyrimidine Metabolism, Orotate containing	< 0.0001
5,6-dihydrothymine	Nucleotide	Pyrimidine Metabolism, Thymine containing	0.0081
uracil	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0004
3-ureidopropionate	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0105
beta-alanine	Nucleotide	Pyrimidine Metabolism, Uracil containing	0.0458
phenylacetylcarnitine	Peptide	Acetylated Peptides	< 0.0001
leucylleucine	Peptide	Dipeptide	0.0001
tryptophylleucine	Peptide	Dipeptide	0.0007
serylalanine	Peptide	Dipeptide	0.0026
phenylalanylarginine	Peptide	Dipeptide	0.0049
histidylphenylalanine	Peptide	Dipeptide	0.0244
phenylalanyltryptophan	Peptide	Dipeptide	0.0262
isoleucylglutamate	Peptide	Dipeptide	< 0.0001
isoleucylalanine	Peptide	Dipeptide	< 0.0001
prolylproline	Peptide	Dipeptide	< 0.0001
gamma-glutamylglutamine	Peptide	Gamma-glutamyl Amino Acid	0.0010
gamma-glutamyl-alpha-lysine	Peptide	Gamma-glutamyl Amino Acid	0.0015
gamma-glutamylmethionine	Peptide	Gamma-glutamyl Amino Acid	0.0023
gamma-glutamylhistidine	Peptide	Gamma-glutamyl Amino Acid	0.0097
gamma-glutamyltyrosine	Peptide	Gamma-glutamyl Amino Acid	0.0163
gamma-glutamylalanine	Peptide	Gamma-glutamyl Amino Acid	0.0213
gamma-glutamyl-epsilon-lysine	Peptide	Gamma-glutamyl Amino Acid	0.0233
gamma-glutamylglycine	Peptide	Gamma-glutamyl Amino Acid	0.0484

Table 6.6- Over-representation analysis of metabolites which showed a significant difference in concentration between paired RA and SVC samples. Metabolites which showed an enrichment value >1 are listed.

SUPER PATHWAY	SUB PATHWAY	ENRICHMENT VALUE
Carbohydrate	Glycogen Metabolism	5.17
Lipid	Eicosanoid	5.17
Lipid	Fatty Acid Metabolism (Acyl Choline)	5.17
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	5.17
Lipid	Glycerolipid Metabolism	5.17
Lipid	Lyso-phospho-ether	5.17
Lipid	Lysoplasmalogen	5.17
Lipid	Mevalonate Metabolism	5.17
Lipid	Phosphatidylserine (PS)	5.17
Nucleotide	Pyrimidine Metabolism, Orotate containing	5.17
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	4.31
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	4.14
Lipid	Long Chain Fatty Acid	4.06
Energy	TCA Cycle	4.02
Carbohydrate	Fructose, Mannose and Galactose Metabolism	3.88
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	3.88
Amino Acid	Glutamate Metabolism	3.76
Amino Acid	Creatine Metabolism	3.45
Carbohydrate	Pentose Metabolism	3.45
Nucleotide	Purine Metabolism, Guanine containing	3.45
Lipid	Lysolipid	3.36
Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	3.23
Peptide	Gamma-glutamyl Amino Acid	2.76
Amino Acid	Polyamine Metabolism	2.58
Carbohydrate	Aminosugar Metabolism	2.58
Lipid	Carnitine Metabolism	2.58
Lipid	Fatty Acid Synthesis	2.58
Lipid	Inositol Metabolism	2.58
Lipid	Ketone Bodies	2.58
Nucleotide	Purine Metabolism, Adenine containing	2.58
Nucleotide	Pyrimidine Metabolism, Thymine containing	2.58
Amino Acid	Glycine, Serine and Threonine Metabolism	2.35
Amino Acid	Alanine and Aspartate Metabolism	2.22
Lipid	Medium Chain Fatty Acid	2.22
Nucleotide	Pyrimidine Metabolism, Uracil containing	2.22
Amino Acid	Histidine Metabolism	2.15
Amino Acid	Glutathione Metabolism	2.07
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2.07
Lipid	Endocannabinoid	2.07
Nucleotide	Pyrimidine Metabolism, Cytidine containing	2.07
Lipid	Phospholipid Metabolism	1.87

Lipid	Fatty Acid, Monohydroxy	1.85
Amino Acid	Guanidino and Acetamido Metabolism	1.72
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1.72
Amino Acid	Tryptophan Metabolism	1.29
Lipid	Fatty Acid Metabolism(Acyl Carnitine)	1.15
Lipid	Fatty Acid, Dicarboxylate	1.15
Peptide	Dipeptide	1.13
Peptide	Acetylated Peptides	1.03

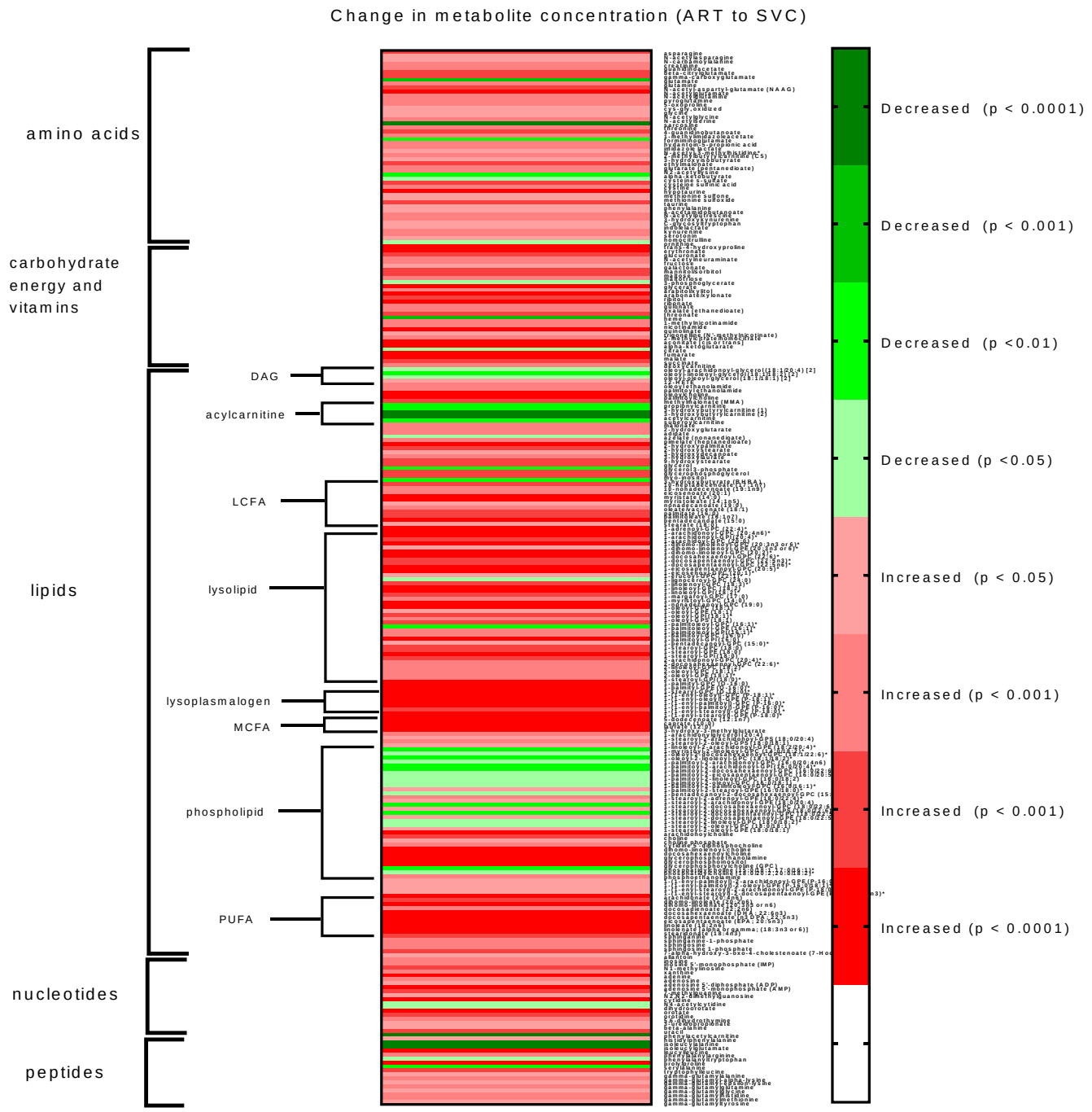


Figure 6.3- Heat map displaying endogenous metabolites which showed a significant difference in concentration between ART and SVC plasma in patients with pulmonary vascular disease. Paired blood samples were taken from the ART and SVC sites during right heart catheterisation of 57 patients with pulmonary vascular disease. Untargeted metabolic profiling of the plasma was carried out using the Metabolon DiscoveryHD4™ platform. The concentration of each metabolite (median scaled standardised intensity) was compared between ART and SVC samples using Wilcoxon matched pairs signed rank test with FDR adjustment. Metabolites which were increased in the SVC compared to ART samples are shown in red, metabolites which were decreased in the SVC compared to ART samples are shown in green. ART-radial artery, SVC-superior vena cava, DAG-diacyclglycerol, LCFA-long chain fatty acids, MCFA-medium chain fatty acid, PUFA-polyunsaturated fatty acid.

6.6 Summary of key metabolic changes

Table 6.7- Summary of key metabolic differences between disease and control and between anatomical sites.

Metabolite subgroup	Concentration in disease v control	Across right heart (SVC-PA)	Across pulm circ, (PA-ART)	Across systemic circ, (ART-SVC)
Acylcarnitine	↑		↑	↓
Long chain fatty acids	↑	↓		↑
Polyamines	↑	↓		↑
Ketone bodies	↑	↑		↑
Aminosugars	↑			↑
TCA cycle	↑	↓	↓	↑
PUFA	↑	↓	↓	↑
Adenine nucleotides	↑		↓	↑
Acylcholines	↓		↓	↑
Lysolipid	↓		↓	
Lysophosphoether	↓			↑
Lysoplasmalogen	↓		↓	↑
Plasmalogen	↓		↓	↑

6.7 Discussion

6.7.1 Transcardiac metabolite gradients

The products of cardiac metabolism and cardiac dysfunction may be released into blood circulating through the heart. Therefore, changes in metabolite profile between paired blood samples pre- right heart transit (SVC) and post- right heart transit (PA) can be expected to reflect right heart metabolism. The changes in metabolite concentration detected between SVC and PA confirm that right heart metabolism contributes to alterations in circulating metabolite profile. The metabolite changes detected are primarily reflective of cardiac bioenergetic demand. This is evidenced by transcardiac changes in glutamate metabolites, ketones and TCA cycle metabolites. As would be expected, fatty acids, which are the preferred cardiac energy source, were universally depleted in PA blood. This is likely to reflect myocardial consumption. Additionally, TCA cycle metabolites were depleted, again suggesting myocardial utilisation. The depletion of fatty acids observed suggests that

there remains ongoing myocardial fatty acid utilisation, despite evidence of altered fatty acid metabolism that I and others have detected in PVD (Section 5.6.1).

The increase in glutamate metabolites, in conjunction with the depletion of glutamine suggests glutaminolysis. This process can provide alpha ketoglutarate which can then enter the TCA cycle, providing additional energy to cardiac muscle in the context of increased myocardial bioenergetic demand. Recent studies have implicated this process in right ventricular maladaptive changes in PAH (Piao et al., 2013).

There was also an increase in ketones, which are recognised as an important energy source in heart failure (Aubert et al., 2016, Bedi et al., 2016) and an increase in the glycolytic intermediate 3-phosphoglycerate, suggesting that there is also active glycolytic cardiac metabolism. Creatine showed an increase across the right heart. Creatine is abundant in both skeletal muscle and myocardial cells, and is known to increase in the blood in response to insults to the myocardium (Zervou et al., 2016). Therefore, the increase in creatine observed may reflect release by the failing heart.

Additionally, the polyamine metabolite n-acetyl putrescine was depleted. Polyamines have been shown to be associated with cardiac remodelling (Meana et al., 2016, Giordano et al., 2012) and therefore may play a role in altered cardiac structure and function in PVD. Large increases in myocardial polyamine content have been demonstrated in a mouse model of cardiac remodelling (Sansbury et al., 2014) and polyamine supplementation may also have potential cardioprotective effects (Eisenberg et al., 2016). Therefore, the depletion observed may represent myocardial uptake to support cardiac remodelling. Amongst other metabolite changes detected included depletion of 1-methylnicotinamide across the right heart. This metabolite of nicotinamide has anti-inflammatory properties, and may activate prostacyclin production (Chlopicki S, 2007), therefore may play a role in PVD pathobiology.

6.7.2 Transpulmonary metabolite gradients

A predominant reduction in metabolite concentration across the pulmonary circulation was found, suggesting uptake, metabolism or biotransformation of these metabolites.

6.7.2.1 Amino acid

The lungs are known to be involved in regulation of circulating amino acids, which are taken up by active transport (Hughes et al., 2001). Therefore, evidence of depletion of multiple amino acid groups across the pulmonary circulation is unsurprising.

Despite depletion of other amino acid metabolites across the pulmonary circulation, sarcosine showed an increase in concentration. Sarcosine (n-methylglycine) is an intermediate in the metabolism of choline to glycine.

A number of studies have suggested that sarcosine is an oncometabolite. Elevated levels have been reported in association with metastatic prostate and breast cancer (Heger et al., 2016, Lucarelli et al., 2013, Cha et al., 2014), administration of sarcosine has been reported to induce an invasive phenotype in benign cells and sarcosine inhibition has been reported to suppress tumour growth (Khan AP 2013). Higher expression of sarcosine related proteins has also been associated with worse cancer prognosis (Cha et al., 2014, Yoon et al., 2014). Sarcosine may also promote angiogenesis, by modulating expression of angiogenic growth factors (Sudhakaran et al., 2014).

Dysregulated angiogenesis appears to play a role in the evolution of CTEPH (Alias et al., 2014) and PAH (Voelkel and Gomez-Arroyo, 2014, Tuder et al., 2001). Therefore, the increase in sarcosine concentration I have detected across the pulmonary circulation could perhaps be or a marker or a modulator of angiogenesis in pulmonary vascular disease.

6.7.2.2 Carbohydrate and TCA cycle

The major fuel source for the metabolic processes of the lung is glucose. As gluconeogenesis does not occur in lung tissue and glycogen stores are limited, the lung relies upon the pulmonary circulation for its glucose requirement (Fisher, 1984). Therefore, the observed depletion of glucose and associated metabolites is a feature of normal lung metabolism. A number of previous studies that have demonstrated accentuated glucose uptake in PAH lungs (Zhao et al., 2013, Hagan et al., 2011). It has been suggested that the increased glucose uptake reflects high metabolic demands associated with inflammation and vascular remodelling. To confirm whether this is the case in the population that I studied, comparison of the glucose uptake in disease with healthy individuals is required.

Glucose, fatty acids or amino acids can all be metabolised to produce acetyl CoA, which fuels the TCA cycle. The conversion of this acetyl Co-A to citrate is the key ‘entry point’ into the cycle. When TCA cycle intermediates such as malate are consumed, a reactive increase in citrate may occur to sustain the cycle. Therefore, the increase in citrate across the pulmonary circulation, coupled with the depletion of other components of the TCA cycle, may represent TCA cycle upregulation in the lung. Conversely, this could represent failure of citrate utilisation (hence accumulation), therefore reduction in TCA cycle activity.

6.7.2.3 Cofactors and vitamins

There was a significant increase in haem in RA compared to PA samples. This could represent a degree of intravascular haemolysis during red cell transit through the dysfunctional microvasculature. Chronic intravascular haemolysis is known to be associated with endothelial dysfunction (Reiter et al., 2002, Rother et al., 2005), and free haemoglobin released by haemolysis may result in abnormally high levels of nitic oxide consumption, impaired vasodilatation (Reiter et al., 2002, Minneci et al., 2005) and a pro-coagulant state (Ataga et al., 2007, Cappellini, 2007).

Alternatively, the increase in haem observed may represent increased haemolysis associated with arterial sampling procedures. Radial arterial samples were obtained from a small calibre vessel, which may increase the potential for haemolysis. Additionally, the higher flow rates in the systemic circulation may also increase the potential for erythrocyte shear stress. Although I would have ideally liked to sample from a proximal arterial site, a compromise was required in order for the study to be practically and ethically feasible.

A significant difference in the concentration of ascorbate and aldarate metabolites between PA and RA sites was also observed. Ascorbate itself was not detected in any of the samples, likely due to its short half-life in the circulation. However, ascorbate metabolites such as threonate were detectable. Ascorbate acts as an antioxidant, and supplementation has potential benefit in a range of conditions including emphysema, sepsis and malignancy (Gupta et al., 2016, Mikirova et al., 2012, Han et al., 2010). Therefore, depletion of ascorbate metabolites in the context of PVD may represent consumption by the 'injured' pulmonary circulation.

There were also differences in the concentration of nicotinate metabolites across the pulmonary circulation, with a reduction in nicotinamide concentration, accompanied by an increase in the concentration of its primary metabolite, 1-methylnicotinamide. 1 methylnicotinamide is recognised to have anti-inflammatory and antithrombotic properties, with actions mediated by a prostacyclin (PGI2)-dependent mechanism (Biedroń et al., 2008, Bartuś et al., 2008, Tanaka et al., 2015, Mateuszuk et al., 2009). Vascular inflammation is associated with the upregulation of nicotinamide N-methyltransferase activity and subsequent increase in plasma 1 methylnicotinamide levels (Mateuszuk et al., 2009) and data suggests that in animal models, treatment with 1-methylnictonamide can prevent the impairment of NO-dependent endothelial function (Bartuś et al., 2008).

Activation of the nicotinamide pathway has recently been demonstrated in pulmonary hypertension in rats and humans (Fedorowicz et al., 2016) with progressive increases in plasma 1-

methylnicotinamide occurring as the disease progresses. Given the protective role of 1-methylnicotinamide in the context of vascular inflammation, this may play a compensatory, protective role in pulmonary vascular disease.

6.7.2.4 Lipids

Although pulmonary eicosanoid metabolism is well characterised (Bakhle and Ferreira, 2011), the fate of many circulating lipids during transpulmonary passage in health has not been previously reported. My research provides novel evidence to suggest that a wide variety of lipids undergo pulmonary circulation uptake or metabolism.

Fatty acids, phospholipids, lysolipids

Numerous lipids showed a significant decrease in concentration in RA compared to PA blood, including acylcholines, the eicosanoid 12-HETE and others including lysolipids, plasmalogens, medium chain and polyunsaturated fatty acids.

Lipids form 90% of the constituents of surfactant, in particular, phosphatidylcholine and phosphatidylglycerol molecules predominate (Goerke, 1998). Additionally, eicosanoids are formed from fatty acids. Therefore, depletion of fatty acids, phospholipids and groups such as acylcholines (which are a constituent of phospholipids) across the pulmonary circulation, may represent uptake of these molecules by the lung for synthesis of surfactant, eicosanoids and cell membranes.

Acylcarnitines

In contrast to all other groups of lipids, there was a significant increase in acylcarnitines across the pulmonary circulation. I previously demonstrated an increase in acylcarnitines in patients with PVD compared to controls (Chapter 5). The transpulmonary increase in these metabolites suggests that the increased acylcarnitine levels in PVD may be due to a metabolic disturbance localised to the pulmonary circulation.

As described previously, (Chapter 5), acylcarnitines accumulate when fatty acid oxidation is impaired. These molecules have been shown to accumulate in the lungs of mice that lack the fatty acid oxidation enzyme long-chain acyl-CoA dehydrogenase (LCAD). This accumulation then inhibits pulmonary surfactant and thereby predisposes to lung injury (Otsubo et al., 2015). Therefore, defective pulmonary mitochondrial fatty acid oxidation in PVD may result in increased acylcarnitine production. This is supported by a number of studies which demonstrate the presence of mitochondrial dysfunction in PAH (Xu et al., 2007, Archer et al., 2008). Whether mitochondrial

dysfunction is also a feature of CTEPH is unknown. In turn, acylcarnitines, via effects on surfactant, may increase the risk of lung injury when exposed to a harmful stimulus.

Eicosanoids

In my study, only one eicosanoid was detected, 12-HETE, which showed a decrease in concentration across the pulmonary circulation. 12-HETE has pro-inflammatory properties and has been implicated in the pathogenesis of vasculopathies in diabetes mellitus and Churg-Strauss syndrome (Szczeklik et al., 2012, Al-Shabrawey et al., 2011, Issan et al., 2013) and hyperproliferative conditions such as cancer and psoriasis (Nguyen et al., 2016, Hussain et al., 1994). It has been shown to promote neovascularisation and angiogenesis. Increased levels have been detected In PAH and higher levels have been found to be associated with worse survival (Al-Naamani et al., 2016, Ross et al., 2015). My results suggested clearance of 12-HETE during transpulmonary passage, therefore increased levels of 12-HETE may be a marker of impaired pulmonary vascular bed metabolism.

6.7.2.5 Peptides

Fibrinogen cleavage peptides were only detected in a small proportion of patients, in whom there was an increase in concentration across the pulmonary circulation, in-keeping with active conversion to fibrin. Additionally, phenylacetylcarnitine concentration was increased, which may reflect carnitine accumulation secondary to impairment of fatty acid oxidation.

6.7.2.6 Nucleotides

The pulmonary vascular endothelium is known to remove > 95% of adenosine and its derivatives from the pulmonary circulation (Hughes et al., 2001). As expected, there was predominant reduction in the concentration of circulating nucleotides across the pulmonary circulation. The exception was an increase in the orotate precursor dihydroorotate. This pathway is important in pyrimidine synthesis, and dihydroorotate dehydrogenase inhibition has been suggested as a therapeutic target in autoimmune and inflammatory diseases and cancers (Vyas and Ghate, 2011). However, the role of this pathway in PVD is unclear.

6.7.3 Systemic metabolite gradients

The difference in metabolite concentration between paired radial artery and superior vena cava samples (RA vs SVC) may reflect systemic metabolic processes and/or dietary intake. However, this does not allow localisation of systemic metabolic processes to any particular organ or tissue.

There was an increase in glycogen metabolites across the systemic circulation, consistent with glycogenolysis which occurs in the liver and muscle tissues. Additionally, there were increases in metabolites associated with energy metabolism including carbohydrates and TCA cycle intermediates. There was also a large increase in the concentration of short and medium chain fatty acids (which are absorbed directly into the blood) and long chain fatty acids (which require fragmentation and absorption via the lymphatic pathway). There were particularly marked increases in acylcholines and phosphatidylserines which are key components of cell membranes and are important in cell signalling.

The increase in fatty acids detected was accompanied by reduction in acylcarnitine concentration. This may indicate acylcarnitine utilisation for fatty acid transport into the mitochondria, facilitating mitochondrial fatty acid utilisation for energy.

A reduction in glutamate was also observed in SVC compared to ART samples, accompanied by an increase in glutamate metabolites, which may reflect systemic consumption of glutamate. Glutamate is used to fuel the TCA cycle (via conversion to alpha-ketoglutarate, which was increased in SVC samples), but also may be used for processes such as aminosugar metabolism, protein biosynthesis, pyrimidine and porphyrin metabolism.

An increase in polyamines was also detected. Polyamines may be absorbed from dietary intake or synthesised endogenously via the amino acids L-methionine and L-ornithine. Although ornithine concentration was reduced in SVC samples, methionine concentration was found to be increased. It is unclear whether the increase in polyamines observed reflects *de novo* synthesis or dietary absorption.

6.8 Study limitations

My research has provided novel insights into metabolic perturbations in pulmonary vascular disease compared to health. Furthermore, by comparing metabolite concentration between different anatomical sites, this study has provided insight into the contribution of different components of the circuit to the circulating metabolite profile.

However, there are a number of limitations to this study, which are discussed below.

6.8.1.1 Study population and sample size

Firstly, there was a relatively small number of subjects included in the study, especially relative to the number of metabolites assessed. Secondly, the cohort of patients recruited was heterogeneous. Although representative of the PVD population seen in our clinical practice, it is unclear whether there are distinct metabolic differences between disease subgroups as the study was underpowered for subgroup analysis.

6.8.1.2 Comparisons with health

Remarkably, to date, the metabolic function of the pulmonary circulation has not been fully investigated in health, let alone disease. The fate of many metabolites during transpulmonary passage is not well documented in the literature. Although there are a greater number of studies describing cardiac metabolism, again, published data remains limited. The studies which do exist are largely based on animal models and are usually focussed on a single molecule of a limited array of molecules. Therefore, there is a lack of published data in health with which we can compare these findings. In order to fully establish the extent to which circulating metabolite gradients are abnormal in PVD, comparison of metabolite concentration between anatomical sites is required in a healthy population.

As part of this research, I was able to obtain paired blood samples from the SVC, PA and RA sites from 5 healthy individuals. However, due to this very small sample size, there were no statistically significant differences in the concentration of metabolites between the anatomical sites (even prior to FDR adjustment for multiple testing). In order to make meaningful comparisons between health and disease, a larger sample size of healthy individuals is required. However, invasive sampling in a truly healthy comparator population has ethical implications and implications for study recruitment.

6.8.1.3 Untargeted Metabolomics approach

The untargeted metabolomics approach is both a strength and a weakness of this study. By assessing all identifiable metabolites, whether expected or unexpected, pathways which have not previously been implicated in the disease pathobiology have been highlighted as avenues for further exploration. However, the untargeted approach does not provide truly unbiased profiling- in this study, the metabolites which could be identified were limited to those contained in the Metabolon reference library. Many ‘unknown’ metabolites were detected, for which standards for identification or quantification are lacking, resulting in a fragmented picture of the metabolome.

Another disadvantage of the untargeted metabolomics approach is the lack of absolute quantification of metabolite concentration. Lack of absolute quantification means that metabolite concentration in my study population cannot easily be compared to existing clinical reference ranges or other studies.

6.8.1.4 Alternative sources of metabolite variation

The differences I have identified between health and disease need to be interpreted with caution. There are many exogenous factors which may influence circulating metabolite profile, including diet, medication, stress, physical activity and circadian rhythms (Yin et al., 2015a, Salvagno et al., 2017, Ang et al., 2012, Berton et al., 2017, Lewis et al., 2010, Yan et al., 2009, Winnike et al., 2009). In this study, where possible, steps were taken to minimise unwanted sources of variation. However, the effects of these potential confounders must be considered. Additionally, sampling at a single point in time provides only a 'snapshot' of metabolism at that time point and may not necessarily be indicative of the overall metabolic phenotype of the disease. Therefore, longitudinal data is required to determine the relationship of metabolic changes to the natural history of the disease.

6.8.1.5 Biological interpretation

Existing knowledge about the function of many metabolites and their biological interactions is lacking. Thus, their true role in metabolism and their relevance in the context of disease cannot be fully established. Many metabolites play a role in several pathways and are the product or substrate of many different enzymes or processes. Thus, it is a challenge to pinpoint an altered metabolite to a specific pathway or enzyme, and alterations in metabolite profile must be considered in the context of the wider matrix of its biological interactions.

Additionally, I have suggested that variation in plasma metabolite concentration between anatomical sites is the result of local metabolic processes. However, it cannot be assumed that plasma levels necessarily reflect tissue or cellular metabolism. For example, mitochondrial metabolite concentration may be very different from the cytoplasmic concentration, which may be very different from plasma concentration. Furthermore, time lags are involved between organ changes and plasma changes. Therefore, inferences relating changes in plasma to cellular, tissue and organ processes should be treated with caution.

6.9 Future directions

This study forms a 'discovery cohort'. However, these findings need to be confirmed in an independent 'validation cohort'. Following confirmation of my findings, metabolites of interest could then be further studied using a targeted approach, with absolute quantification of metabolite concentration. This may allow identification and quantification of metabolites which discriminate health from disease, differentiate disease subgroups and provide an indicator of disease severity and prognosis. However, for this approach to provide a potential biomarker for pulmonary vascular diseases, a number of factors need to be considered.

Identification of disease specific biomarkers may be hampered by dynamic fluctuations in metabolite concentration and exogenous factors which may influence metabolite profile. The differences in metabolite concentration we have detected between different anatomical sites also has implications for clinical testing. Therefore, the variability in the concentration of a potential metabolite biomarker, both within and between individuals, would need to be considered. As disease changes not only one metabolite, but entire metabolic pathways, a multi-marker panel, made up of several metabolites may be a more robust biomarker than a single metabolite.

Translation of a metabolic biomarker into a clinically useful test must also take into account the potential effects of pre-analytical variation. For example, factors such as collection technique and sample storage have the potential to significantly affect many metabolites (Yin et al., 2015a). Therefore, adherence to standardised protocols for sample collection, handling and storage would be important to preserve sample stability.

In addition to discrimination of health from disease, it would be important to ensure that pulmonary vascular diseases could be distinguished from other disease states. This could be further explored by studying a number of disease comparator groups. Radiolabelling of metabolites of interest could also provide further insight into site specific metabolism. Additionally, integration of metabolomic data with immunophenotyping data, proteomics, transcriptomics and genomics may provide a deeper biological understanding of the disease process.

6.10 Conclusions

Transcardiac metabolite gradients in PVD are consistent with myocardial bioenergetic demand, with consumption of fatty acids and TCA cycle metabolites, accompanied by an increase in glutamate metabolites and ketones. Depletion of polyamines and 1-methylnicotinamide are also present, which may be implicated in maladaptive cardiac remodelling.

Transpulmonary metabolite gradients show a predominant depletion of metabolites across the pulmonary circulation. My results are the first to characterise the fate of many circulating metabolites upon transpulmonary passage, demonstrating the importance of the pulmonary circulation in wide ranging metabolic processes. In particular, I have provided novel insight into the flux of many circulating lipid species. Additionally, I have identified a number of metabolites which show an increase in concentration across the pulmonary circulation, including acylcarnitines, sarcosine and 1- methylnicotinamide. These metabolites show plausible links to the disease pathogenesis and highlight areas for further research.

In contrast to transpulmonary metabolite gradients, across the systemic circulation there was a predominant increase in circulating metabolite concentration. Some of these changes, for example the increase in glutamate metabolites are likely to reflect systemic organ metabolism. Other changes, such as the increase in carbohydrates and fatty acids are likely to represent systemic dietary absorption of metabolites required for bioenergetic processes. Confirmation of these findings is required in an independent ‘validation cohort’ as well as a comparison of metabolite gradients in disease with appropriately matched healthy individuals.

7 Chapter 7- Final summary and concluding remarks

7.1 Summary of major findings and conclusions

The work in this thesis was undertaken to investigate and better understand systemic features of pulmonary vascular diseases by utilising unbiased metabolomics an immunophenotyping platforms. The primary aims were to characterise the circulating blood immune cell phenotype and metabolite profile and to identify differences between disease and healthy controls, and differences between disease subtypes.

The findings presented in this thesis may guide future developments in disease phenotyping, which may ultimately facilitate tailored therapy and improved prognostication. Additionally, these findings may inform the future development of disease specific biomarkers to assist diagnosis and monitoring. My main findings are summarised below.

7.1.1 Peripheral blood immunophenotyping

My study provides the most comprehensive assessment of peripheral blood immune cell phenotype in patients with IPAH to date. It is also the first to assess peripheral blood immunophenotype in CTEPH and HPAH.

I have identified significant differences between IPAH and healthy controls in peripheral blood. This includes evidence of altered B cell differentiation in IPAH, with an increase in plasmablasts, accompanied by a decrease in memory B cells, indicating a shift towards B cell activation and effector function. My research has also demonstrated alterations in T cell subsets in IPAH, characterised by an increase in Tfh cells and PD1-expressing CD8+ T cells and a reduction in naïve CD8+ T cells. Together, the increase in plasmablasts, Tfh cells and IL-21 detected in IPAH is consistent with stimulation of a pathway which usually results in a humoral immune response.

Importantly, this immunophenotype was not found in CTEPH, suggesting that immunological changes reflect fundamental differences in pathophysiology between disease subgroups, and are not simply the consequence of altered pulmonary haemodynamics or disease-associated heart failure. Surprisingly, HPAH patients showed an immune cell phenotype which did not differ significantly from IPAH patients. Similar to IPAH, HPAH patients showed a significant reduction in memory B cells, increase in plasmablasts, increase in double negative B cells and increase in Tfh, when compared with healthy controls. This suggests shared immunological mechanisms may exist in IPAH and HPAH.

This supports the hypothesis that mutation of the BMPR2 receptor acts as a susceptibility factor for disease or potentiates disease development, and that second hit mechanisms (and perhaps initial triggers for the disease) are shared in IPAH and HPAH.

Conclusions: In IPAH, a peripheral blood signature suggesting immune dysregulation is found, with evidence of both B cell and T cell aberrations. These findings support the hypothesis that dysfunctional immune activation may be implicated in the pathobiology of IPAH.

Peripheral blood immune cell phenotype did not differ significantly between IPAH and HPAH associated with BMPR2 mutation, suggesting these subtypes may have a shared immunopathological mechanisms.

Further research to determine the mechanisms responsible for the derangements in immune cell phenotype detected is required, and to explore the functional consequences of these abnormalities. Additionally, longitudinal assessment of immune cell phenotype, paired with haemodynamic, biochemical and clinical data is also required to establish whether immune profile correlates with disease activity and whether immunomodulation may improve disease outcomes.

7.1.2 Metabolomic profiling of circulating blood

My research has identified metabolic abnormalities in pulmonary vascular disease which are more wide-ranging than previously recognised. The findings I have presented confirm that a number of metabolic abnormalities which have been previously reported in PAH are also present in CTEPH and CTED.

My findings provide evidence of altered energy metabolism in pulmonary vascular disease, in particular, the presence of impaired fatty acid beta oxidation. I have also identified metabolic changes consistent with increased cellular proliferation, such as increases in polyamines and aminosugars, accompanied by depletion of metabolites important in the response to oxidative stress, including vitamin A metabolites and glutathione. These metabolic changes may potentiate disease development and progression.

Additionally, I have provided novel insight into metabolite flux between different anatomical sites.

I have identified metabolic changes which do not appear to be localised to a particular site, and are therefore likely to be due to global rather than site specific metabolic changes. However, I have also identified metabolic changes which localise to sub-compartments of the circulation, including a set of metabolites which are pulmonary-specific.

My analysis of transcardiac metabolite gradients showed depletion of energy substrates between SVC and PA, consistent with myocardial bioenergetic demand.

Transpulmonary metabolite gradients showed predominant clearance of a diverse range of metabolites, illustrating the extensive function of the pulmonary circulation in metabolite uptake and biotransformation. In particular, my findings provide novel insight into the fate of many classes of lipids, which have not previously been described and suggest that a wide variety of lipids undergo pulmonary circulation uptake or metabolism.

In contrast to all other lipid-associated metabolites, acylcarnitines showed a significant increase in concentration across the pulmonary circulation. This suggests that impaired pulmonary fatty acid metabolism may be the primary source for the increased levels of acylcarnitines detected in the disease group. This finding is shared amongst disease subtypes.

Additionally, a number of other metabolites including sarcosine (a promoter of angiogenesis), and 1-methylnicotinamide (which is associated with vascular inflammation) showed a localised increase across the pulmonary circulation. The potential role of these metabolites in the disease requires exploration.

Most importantly, further study of metabolite gradients between anatomical sites in healthy individuals is also required, to allow comparisons of site specific metabolism between health and disease.

Conclusion: My findings demonstrate that both local and systemic metabolic dysfunction are present in PVD, involving numerous complex and interconnected pathways.

Alterations in energy metabolism are a shared feature amongst different disease subgroups including PAH, CTEPH and CTED, suggesting that therapies targeting this aspect of metabolism may potentially have benefits across the spectrum of disease. Additional research is required to determine how these pathways may be manipulated for therapeutic benefit.

Ultimately, in order to fully understand the ways in which circulating metabolites are altered in PVD, studies which assess transcardiac and transpulmonary gradients in a healthy population are required.

7.2 Relationship between metabolic and immunological mechanisms

My research has demonstrated clear evidence of both immunological and metabolic aberrations in patients with pulmonary vascular disease compared with healthy individuals.

However, it is unclear how immunological and metabolic alterations are linked, whether they are both a reactive phenomenon in the disease pathology, or whether they are intrinsic to the disease initiation, and whether they occur in parallel, or in sequence.

It is well recognised that host metabolic state affects immune cell function, differentiation and ability to respond to threat (Gerriets and MacIver, 2014, Cohen et al., 2017). Conversely, inflammation and immune activation are involved in the pathogenesis of 'metabolic' diseases such type 2 diabetes (Pickup, 2004, Keane et al., 2017), and diseases which are traditionally thought of as immunoinflammatory, such as rheumatoid arthritis and HIV are associated with metabolic complications (Chimenti et al., 2015, Kerekes et al., 2014, Nguyen et al., 2017, Hemkens and Bucher, 2014).

In the context of PVD, regardless of disease subtype, disease initiation may be triggered by vascular injury (whether this is due to infection, thrombus, autoantibodies, toxins or other noxious stimuli), resulting in an inflammatory response and immune activation. During immune activation, lymphocytes switch from a resting state to a highly active state. This is associated with a shift in the metabolism of these cells towards high rates of glycolysis and reduced mitochondrial fatty acid oxidation (Pearce et al., 2013, Rhoads et al., 2017, Frauwirth and Thompson, 2004, Doughty et al., 2006, Sukumar et al., 2013, Cham et al., 2008). Conversely, cells associated with immune quiescence, such as memory T cells and Tregs, rely predominantly on fatty acid uptake and oxidation (Michalek et al., 2011, Angelin et al., 2017, Howie et al., 2017, Pearce et al., 2009, van der Windt and Pearce, 2012).

Therefore, it is plausible that impaired mitochondrial fatty acid oxidation in PVD (and consequent increased glycolysis) may promote the function of pro-inflammatory, activated immune cells and may hinder the generation and function of regulatory and memory cell subsets.

7.3 Therapeutic targeting of the immunometabolic axis

As metabolic substrate provision is integral to immune cell activation, differentiation and function, selective metabolic inhibitors may have a role in therapeutic immunomodulation. By inhibiting the appropriate pathway, or targeting several pathways simultaneously, this approach may allow immune cell subset specific blockade (Patel and Powell, 2017, Bettencourt and Powell, 2017, Lee and Tian, 2015, Lee et al., 2015, Yin et al., 2015b, Shriver and Manchester, 2011, Byersdorfer et al., 2013).

Conversely, using immunomodulatory treatments which suppress exuberant immune activation may have beneficial effects on metabolic state in disease processes (Larsen et al., 2007, Cugno et al., 2010, Bhargava et al., 2012).

The results I have presented in this thesis have demonstrated that in pulmonary vascular diseases a number of immunological aberrations are shared amongst disease subtypes, for example, reduction in memory B cells. Similarly, a number of metabolic abnormalities are shared between disease subtypes, such as increases in long chain fatty acids and acylcarnitines. However, my findings also demonstrate features which differ between disease subtypes. For example, increased Tfh cells and increased plasmablasts are found in IPAH but not in CTEPH. Therefore, I have identified a number of pathways which may be therapeutic targets for all disease subtypes, but also pathways which may be targeted in specific subgroups. Future advances in disease phenotyping may therefore facilitate tailored therapy. Further research is required to establish whether altered metabolic and immune fingerprints may also be useful in predicting the development of PVD as well as the response to the therapy.

7.4 Conclusions

There is evidently a complex interplay between metabolic factors, inflammation and immunity in pulmonary vascular diseases. There is increasing appreciation that immune cells affect important non-immune functions, including metabolism. Conversely, the behaviour of immune cells is influenced by metabolic factors.

Further studies which increase our understanding of the immunological–metabolic crosstalk in PVD are required. In particular, studies to determine the extent to which metabolic changes are instructive vs responsive during changes in immune cell function and to determine whether altered immune cell mitochondrial metabolism influences lymphocyte activation and differentiation.

In addition, the differing response of pulmonary vascular and systemic vascular mitochondria to noxious stimuli require further investigation to determine whether differing pulmonary and systemic mitochondrial properties are implicated in disease localisation to the pulmonary vasculature.

This research has also highlighted the wider role of the pulmonary circulation in multiple metabolic processes. These functions have not been comprehensively studied in health. Further research is required to characterise the full extent of normal pulmonary vascular metabolic functions in health, to allow us to more fully understand the ways in which metabolism is altered in disease, and to determine whether this may be targeted for therapeutic benefit.

Appendices

Table 1. Summary of 52 peripheral blood mononuclear cell subpopulations compared between IPAH patients and healthy controls. Peripheral blood samples from 28 IPAH patients and 28 age and sex matched healthy controls were obtained. PBMCs were isolated and stained with fluorescently labelled antibodies. A standardised flow cytometry panel for cell surface markers of leukocyte sub-populations was used. Subpopulations of T and B lymphocytes and myeloid cells were distinguished using bivariate dot plots based on cell surface marker expression. Cell frequencies were compared between IPAH and control for each of the 52 subpopulations.

Panel	Population	Defined by
B cell	B-cells	CD3- CD19 +
	Naive	CD3- CD19+ CD27- IgD+
	Transitional	CD3- CD19+ CD27- IgD+ CD24hi CD38hi
	NSM	CD3- CD19+ CD27+ IgD+
	Switched	CD3- CD19+ IgD-
	IgD- excluding plasmablasts	CD3- CD19+ IgD-, excluding plasmablasts
	Double neg	CD3- CD19+ CD27- IgD-, excluding plasmablasts
	Switched mem	CD3- CD19+ CD27+ IgD-, excluding plasmablasts
	Plasmablasts	CD3- CD19 + CD20- IgD- CD38hi
T cell	T cell	CD3+
	CD4+ T-cells	CD3+ CD4+
	CD4+ CM	CD3+ CD4+ CCR7+ CD45RA-
	CD4+ EM	CD3+ CD4+ CCR7- CD45RA-
	CD4+ EMRA	CD3+ CD4+ CCR7- CD45RA+
	CD4+ Naive	CD3+ CD4+ CCR7+ CD45RA+
	CD8+ T-cells	CD3+ CD8+
	CD8+ CCR7+	CD3+ CD8+ CCR7+
	CD8+ CM	CD3+ CD8+ CCR7+ CD45RA-
	CD8+ EM	CD3+ CD8+ CCR7- CD45RA-
	CD8+ EMRA	CD3+ CD8+ CCR7- CD45RA+
	CD8+ Naive	CD3+ CD8+ CCR7+ CD45RA+
Tfh	CD4+ CD45RA-	
	CD4+ CD45RA- CXCR5+	
	CD4+ PD1+	

	Tfh	CD45RA- CXCR5+ PD1+
	Th1,17-like Tfh	CCR6+, CXCR3+
	Th1-like Tfh	CCR6- CXCR3+
	Th2-like Tfh	CXCR3- CCR6-
	Th17-like Tfh	CCR6+ CXCR3-
	CD8+ CXCR5+	
T regs	Treg parent	CD3+ CD4+ CD25+ CD127 low
	Treg	CD3+ CD4+ CD25+ CD127 low, CCR4+
	Naïve Treg	CD3+ CD4+ CD25+ CD127 low, CCR4+ CD45RA-
	Memory Treg	CD3+ CD4+ CD25+ CD127 low, CCR4+ CD45+
	Activated Treg	CD3+ CD4+ CD25+ CD127 low, CCR4+ HLA DR+
Th17	Th17	CD3+ CD161+
	CD4+ CD161+	CD3+ CD4+ CD161+
	CD8+ CD161+	CD3+ CD8+ CD161+
	CD4+ CCR7+	CD3+ CD4+ CD161+ CCR7+
	CD8+ CCR7+	CD3+ CD8+ CD161+ CCR7+
Myeloid	Non T cells	CD3-
	Non T non B cells	CD3- CD19- CD20-
	Lineage negative	CD3- CD19- CD20- CD14- CD56-
	Dendritic cells	CD3- CD19- CD20- CD14- CD56- HLA-DR+
	Plasmacytoid DC	CD3- CD19- CD20- CD14- CD56- HLA-DR+ CD123+
	Myeloid DC	CD3- CD19- CD20- CD14- CD56- HLA-DR+ CD11c+
	Monocytes	CD3- CD19- CD20- CD14 +
	CD16 high Monocytes	CD3- CD19- CD20- CD14 +
	CD16 low Monocytes	CD3- CD19- CD20- CD14 +
	NK cells	CD14- CD56+
	CD16 high NK cells	CD14- CD56+ CD16hi

Table 2. Metabolites detected in venous plasma from patients with pulmonary vascular disease and healthy controls. Untargeted metabolic profiling of venous plasma from 57 patients with pulmonary vascular disease and 27 healthy controls was carried out using the Metabolon DiscoveryHD4™ platform. 1375 metabolites were detected. X denotes metabolites which could not be definitively identified according to the Metabolon biochemical reference library.

BIOCHEMICAL	SUPER PATHWAY	SUB PATHWAY
alanine	Amino Acid	Alanine and Aspartate Metabolism
asparagine	Amino Acid	Alanine and Aspartate Metabolism
aspartate	Amino Acid	Alanine and Aspartate Metabolism
N-acetylalanine	Amino Acid	Alanine and Aspartate Metabolism
N-acetylasparagine	Amino Acid	Alanine and Aspartate Metabolism
N-acetylaspartate (NAA)	Amino Acid	Alanine and Aspartate Metabolism
N-carbamoylalanine	Amino Acid	Alanine and Aspartate Metabolism
creatine	Amino Acid	Creatine Metabolism
creatinine	Amino Acid	Creatine Metabolism
guanidinoacetate	Amino Acid	Creatine Metabolism
4-hydroxyglutamate	Amino Acid	Glutamate Metabolism
beta-citrylglutamate	Amino Acid	Glutamate Metabolism
carboxyethyl-GABA	Amino Acid	Glutamate Metabolism
gamma-carboxyglutamate	Amino Acid	Glutamate Metabolism
glutamate	Amino Acid	Glutamate Metabolism
glutamine	Amino Acid	Glutamate Metabolism
N-acetyl-aspartyl-glutamate (NAAG)	Amino Acid	Glutamate Metabolism
N-acetylglutamate	Amino Acid	Glutamate Metabolism
N-acetylglutamine	Amino Acid	Glutamate Metabolism
pyroglutamine	Amino Acid	Glutamate Metabolism
S-1-pyrroline-5-carboxylate	Amino Acid	Glutamate Metabolism
5-oxoproline	Amino Acid	Glutathione Metabolism
cys-gly, oxidized	Amino Acid	Glutathione Metabolism
cysteine-glutathione disulfide	Amino Acid	Glutathione Metabolism
cysteinylglycine	Amino Acid	Glutathione Metabolism
glutathione, oxidized (GSSG)	Amino Acid	Glutathione Metabolism
allo-threonine	Amino Acid	Glycine, Serine and Threonine Metabolism
betaine	Amino Acid	Glycine, Serine and Threonine Metabolism

dimethylglycine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
glycine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
N-acetylglycine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
N-acetylserine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
N-acetylthreonine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
O-acetylhomoserine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
sarcosine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
serine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
threonine	**Amino Acid**	**Glycine, Serine and Threonine Metabolism**
1-methylguanidine	**Amino Acid**	**Guanidino and Acetamido Metabolism**
4-guanidinobutanoate	**Amino Acid**	**Guanidino and Acetamido Metabolism**
guanidinosuccinate	**Amino Acid**	**Guanidino and Acetamido Metabolism**
1-methylhistidine	**Amino Acid**	**Histidine Metabolism**
1-methylimidazoleacetate	**Amino Acid**	**Histidine Metabolism**
3-methylhistidine	**Amino Acid**	**Histidine Metabolism**
formiminoglutamate	**Amino Acid**	**Histidine Metabolism**
histidine	**Amino Acid**	**Histidine Metabolism**
hydantoin-5-propionic acid	**Amino Acid**	**Histidine Metabolism**
imidazole lactate	**Amino Acid**	**Histidine Metabolism**
imidazole propionate	**Amino Acid**	**Histidine Metabolism**
N-acetyl-1-methylhistidine	**Amino Acid**	**Histidine Metabolism**
N-acetyl-3-methylhistidine	**Amino Acid**	**Histidine Metabolism**
N-acetylhistidine	**Amino Acid**	**Histidine Metabolism**
trans-urocanate	**Amino Acid**	**Histidine Metabolism**
2-hydroxy-3-methylvalerate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
2-methylbutyrylcarnitine (C5)	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
3-hydroxy-2-ethylpropionate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
3-hydroxyisobutyrate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
3-methyl-2-oxobutyrate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
3-methyl-2-oxovalerate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
3-methylglutaconate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
4-methyl-2-oxopentanoate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**

alpha-hydroxyisocaproate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
alpha-hydroxyisovalerate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
beta-hydroxyisovalerate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
ethylmalonate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
isobutyrylcarnitine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
isoleucine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
isovalerate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
isovalerylcarnitine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
isovalerylglycine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
leucine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
methylsuccinate	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
N-acetylisoleucine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
N-acetylleucine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
N-acetylvaline	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
tiglylcarnitine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
valine	**Amino Acid**	**Leucine, Isoleucine and Valine Metabolism**
2-aminoadipate	**Amino Acid**	**Lysine Metabolism**
3-methylglutarylcarnitine	**Amino Acid**	**Lysine Metabolism**
5-hydroxylysine	**Amino Acid**	**Lysine Metabolism**
6-oxopiperidine-2-carboxylate	**Amino Acid**	**Lysine Metabolism**
glutarate (pentanedioate)	**Amino Acid**	**Lysine Metabolism**
glutarylcarnitine (C5)	**Amino Acid**	**Lysine Metabolism**
lysine	**Amino Acid**	**Lysine Metabolism**
N2-acetyllysine	**Amino Acid**	**Lysine Metabolism**
N6,N6,N6-trimethyllysine	**Amino Acid**	**Lysine Metabolism**
N6-acetyllysine	**Amino Acid**	**Lysine Metabolism**
N-acetyl-cadaverine	**Amino Acid**	**Lysine Metabolism**
pipecolate	**Amino Acid**	**Lysine Metabolism**
2-aminobutyrate	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
2-hydroxybutyrate/2-hydroxyisobutyrate	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**

alpha-ketobutyrate	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
cystathionine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
cysteine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
cysteine s-sulfate	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
cysteine sulfinic acid	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
cystine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
hypotaurine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
methionine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
methionine sulfone	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
methionine sulfoxide	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
N-acetylmethionine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
N-acetyltaurine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
N-formylmethionine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
N-methyltaurine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
S-adenosylhomocysteine (SAH)	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
S-methylcysteine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
S-methylmethionine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
taurine	**Amino Acid**	**Methionine, Cysteine, SAM and Taurine Metabolism**
2-hydroxyphenylacetate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
3-(3-hydroxyphenyl)propionate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**

3-(3-hydroxyphenyl)propionate sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
3-(4-hydroxyphenyl)lactate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
3-hydroxyphenylacetate sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
3-methoxytyrosine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
3-phenylpropionate (hydrocinnamate)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
4-hydroxyphenylacetate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
4-hydroxyphenylacetatoylcarnitine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
4-hydroxyphenylpyruvate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
5-bromotryptophan	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
catechol glucuronide	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
dopamine sulfate (1)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
dopamine sulfate (2)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
gentisate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
homovanillate (HVA)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
N-acetylphenylalanine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
N-acetyltyrosine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
N-formylphenylalanine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
o-cresol sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
p-cresol sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
p-cresol-glucuronide	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenol sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenylacetate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenylalanine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenyllactate (PLA)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenylpropionylglycine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
phenylpyruvate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
thyroxine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
tyramine O-sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
tyrosine	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
vanillactate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
vanillic alcohol sulfate	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**
vanillylmandelate (VMA)	**Amino Acid**	**Phenylalanine and Tyrosine Metabolism**

4-acetamidobutanoate	**Amino Acid**	**Polyamine Metabolism**
5-methylthioadenosine (MTA)	**Amino Acid**	**Polyamine Metabolism**
acisoga	**Amino Acid**	**Polyamine Metabolism**
N-acetylputrescine	**Amino Acid**	**Polyamine Metabolism**
spermidine	**Amino Acid**	**Polyamine Metabolism**
3-hydroxykynurenine	**Amino Acid**	**Tryptophan Metabolism**
3-indoxyl sulfate	**Amino Acid**	**Tryptophan Metabolism**
5-hydroxyindole sulfate	**Amino Acid**	**Tryptophan Metabolism**
5-hydroxyindoleacetate	**Amino Acid**	**Tryptophan Metabolism**
C-glycosyltryptophan	**Amino Acid**	**Tryptophan Metabolism**
indole-3-carboxylic acid	**Amino Acid**	**Tryptophan Metabolism**
indoleacetate	**Amino Acid**	**Tryptophan Metabolism**
indoleacetylglutamine	**Amino Acid**	**Tryptophan Metabolism**
indolelactate	**Amino Acid**	**Tryptophan Metabolism**
indolepropionate	**Amino Acid**	**Tryptophan Metabolism**
kynurenate	**Amino Acid**	**Tryptophan Metabolism**
kynurenine	**Amino Acid**	**Tryptophan Metabolism**
N-acetylkynurenine (2)	**Amino Acid**	**Tryptophan Metabolism**
N-acetyltryptophan	**Amino Acid**	**Tryptophan Metabolism**
picolinate	**Amino Acid**	**Tryptophan Metabolism**
serotonin	**Amino Acid**	**Tryptophan Metabolism**
thioproline	**Amino Acid**	**Tryptophan Metabolism**
tryptophan	**Amino Acid**	**Tryptophan Metabolism**
tryptophan betaine	**Amino Acid**	**Tryptophan Metabolism**
xanthurenate	**Amino Acid**	**Tryptophan Metabolism**
2-oxoarginine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
arginine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
argininosuccinate	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
citrulline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
dimethylarginine (SDMA + ADMA)	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
homoarginine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
homocitrulline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**

N2,N5-diacetylornithine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N-acetylarginine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N-acetylcitrulline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N-alpha-acetylornithine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N-delta-acetylornithine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N-methylproline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
ornithine	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
pro-hydroxy-pro	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
proline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
trans-4-hydroxyproline	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
urea	**Amino Acid**	**Urea cycle; Arginine and Proline Metabolism**
N6-carboxymethyllysine	**Carbohydrate**	**Advanced Glycation End-product**
6-sialyl-N-acetyllactosamine	**Carbohydrate**	**Aminosugar Metabolism**
erythronate	**Carbohydrate**	**Aminosugar Metabolism**
glucuronate	**Carbohydrate**	**Aminosugar Metabolism**
N-acetylglucosamine/N-acetylgalactosamine	**Carbohydrate**	**Aminosugar Metabolism**
N-acetylglucosaminylasparagine	**Carbohydrate**	**Aminosugar Metabolism**
N-acetylneuraminate	**Carbohydrate**	**Aminosugar Metabolism**
sucrose	**Carbohydrate**	**Disaccharides and Oligosaccharides**
fructose	**Carbohydrate**	**Fructose, Mannose and Galactose Metabolism**
galactonate	**Carbohydrate**	**Fructose, Mannose and Galactose Metabolism**
mannitol/sorbitol	**Carbohydrate**	**Fructose, Mannose and Galactose Metabolism**
mannose	**Carbohydrate**	**Fructose, Mannose and Galactose Metabolism**
maltose	**Carbohydrate**	**Glycogen Metabolism**
maltotriose	**Carbohydrate**	**Glycogen Metabolism**
1,5-anhydroglucitol (1,5-AG)	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**
3-phosphoglycerate	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**
glucose	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**
glycerate	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**

lactate	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**
pyruvate	**Carbohydrate**	**Glycolysis, Gluconeogenesis, and Pyruvate Metabolism**
arabinose	**Carbohydrate**	**Pentose Metabolism**
arabitol/xylitol	**Carbohydrate**	**Pentose Metabolism**
arabonate/xylonate	**Carbohydrate**	**Pentose Metabolism**
ribitol	**Carbohydrate**	**Pentose Metabolism**
ribonate	**Carbohydrate**	**Pentose Metabolism**
xylose	**Carbohydrate**	**Pentose Metabolism**
ascorbate (Vitamin C)	**Cofactors and Vitamins**	**Ascorbate and Aldarate Metabolism**
gulonate	**Cofactors and Vitamins**	**Ascorbate and Aldarate Metabolism**
oxalate (ethanedioate)	**Cofactors and Vitamins**	**Ascorbate and Aldarate Metabolism**
threonate	**Cofactors and Vitamins**	**Ascorbate and Aldarate Metabolism**
bilirubin (E,E)	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
bilirubin (E,Z or Z,E)	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
bilirubin (Z,Z)	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
biliverdin	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
heme	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
I-urobilinogen	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
L-urobilin	**Cofactors and Vitamins**	**Hemoglobin and Porphyrin Metabolism**
1-methylnicotinamide	**Cofactors and Vitamins**	**Nicotinate and Nicotinamide Metabolism**
N1-Methyl-2-pyridone-5-carboxamide	**Cofactors and Vitamins**	**Nicotinate and Nicotinamide Metabolism**

nicotinamide	**Cofactors and Vitamins**	**Nicotinate and Nicotinamide Metabolism**
quinolinate	**Cofactors and Vitamins**	**Nicotinate and Nicotinamide Metabolism**
trigonelline (N'-methylnicotinate)	**Cofactors and Vitamins**	**Nicotinate and Nicotinamide Metabolism**
pantothenate	**Cofactors and Vitamins**	**Pantothenate and CoA Metabolism**
alpha-CEHC glucuronide	**Cofactors and Vitamins**	**Tocopherol Metabolism**
alpha-CEHC sulfate	**Cofactors and Vitamins**	**Tocopherol Metabolism**
alpha-tocopherol	**Cofactors and Vitamins**	**Tocopherol Metabolism**
gamma-CEHC	**Cofactors and Vitamins**	**Tocopherol Metabolism**
gamma-CEHC glucuronide	**Cofactors and Vitamins**	**Tocopherol Metabolism**
gamma-tocopherol/beta-tocopherol	**Cofactors and Vitamins**	**Tocopherol Metabolism**
4-oxo-retinoic acid	**Cofactors and Vitamins**	**Vitamin A Metabolism**
retinol (Vitamin A)	**Cofactors and Vitamins**	**Vitamin A Metabolism**
pyridoxate	**Cofactors and Vitamins**	**Vitamin B6 Metabolism**
phosphate	**Energy**	**Oxidative Phosphorylation**
2-methylcitrate/homocitrate	**Energy**	**TCA Cycle**
aconitate [cis or trans]	**Energy**	**TCA Cycle**
alpha-ketoglutarate	**Energy**	**TCA Cycle**
citraconate/glutaconate	**Energy**	**TCA Cycle**
citrate	**Energy**	**TCA Cycle**
fumarate	**Energy**	**TCA Cycle**
malate	**Energy**	**TCA Cycle**
succinate	**Energy**	**TCA Cycle**
succinylcarnitine	**Energy**	**TCA Cycle**

carnitine	**Lipid**	**Carnitine Metabolism**
deoxycarnitine	**Lipid**	**Carnitine Metabolism**
diacylglycerol (14:0/18:1, 16:0/16:1) [1]	**Lipid**	**Diacylglycerol**
diacylglycerol (14:0/18:1, 16:0/16:1) [2]	**Lipid**	**Diacylglycerol**
diacylglycerol (16:1/18:2 [2], 16:0/18:3 [1])	**Lipid**	**Diacylglycerol**
linoleoyl-arachidonoyl-glycerol (18:2/20:4) [1]	**Lipid**	**Diacylglycerol**
linoleoyl-arachidonoyl-glycerol (18:2/20:4) [2]	**Lipid**	**Diacylglycerol**
linoleoyl-docosahexaenoyl-glycerol (18:2/22:6)	**Lipid**	**Diacylglycerol**
linoleoyl-linolenoyl-glycerol (18:2/18:3)	**Lipid**	**Diacylglycerol**
linoleoyl-linoleoyl-glycerol (18:2/18:2)	**Lipid**	**Diacylglycerol**
oleoyl-arachidonoyl-glycerol (18:1/20:4)	**Lipid**	**Diacylglycerol**
oleoyl-linolenoyl-glycerol (18:1/18:3)	**Lipid**	**Diacylglycerol**
oleoyl-linoleoyl-glycerol (18:1/18:2) [1]	**Lipid**	**Diacylglycerol**
oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	**Lipid**	**Diacylglycerol**
oleoyl-oleoyl-glycerol (18:1/18:1) [1]	**Lipid**	**Diacylglycerol**
oleoyl-oleoyl-glycerol (18:1/18:1) [2]	**Lipid**	**Diacylglycerol**
palmitoleoyl-linoleoyl-glycerol (16:1/18:2)	**Lipid**	**Diacylglycerol**
palmitoyl-arachidonoyl-glycerol (16:0/20:4) [1]	**Lipid**	**Diacylglycerol**
palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]	**Lipid**	**Diacylglycerol**
palmitoyl-docosahexaenoyl-glycerol	**Lipid**	**Diacylglycerol**
palmitoyl-linoleoyl-glycerol (16:0/18:2) [1]	**Lipid**	**Diacylglycerol**
palmitoyl-linoleoyl-glycerol (16:0/18:2) [2]	**Lipid**	**Diacylglycerol**
palmitoyl-oleoyl-glycerol (16:0/18:1) [1]	**Lipid**	**Diacylglycerol**
palmitoyl-oleoyl-glycerol (16:0/18:1) [2]	**Lipid**	**Diacylglycerol**
12-HETE	**Lipid**	**Eicosanoid**
linoleoyl ethanolamide	**Lipid**	**Endocannabinoid**
N-oleoyltaurine	**Lipid**	**Endocannabinoid**
oleoyl ethanolamide	**Lipid**	**Endocannabinoid**
palmitoyl ethanolamide	**Lipid**	**Endocannabinoid**
stearoyl ethanolamide	**Lipid**	**Endocannabinoid**
oleoylcholine	**Lipid**	**Fatty Acid Metabolism (Acyl Choline)**
palmitoylcholine	**Lipid**	**Fatty Acid Metabolism (Acyl Choline)**

hexanoylglutamine	**Lipid**	**Fatty Acid Metabolism (Acyl Glutamine)**
N-octanoylglutamine	**Lipid**	**Fatty Acid Metabolism (Acyl Glutamine)**
butyrylcarnitine	**Lipid**	**Fatty Acid Metabolism (also BCAA Metabolism)**
methylmalonate (MMA)	**Lipid**	**Fatty Acid Metabolism (also BCAA Metabolism)**
propionylcarnitine	**Lipid**	**Fatty Acid Metabolism (also BCAA Metabolism)**
propionylglycine	**Lipid**	**Fatty Acid Metabolism (also BCAA Metabolism)**
3-hydroxybutyrylcarnitine (1)	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
3-hydroxybutyrylcarnitine (2)	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
acetylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
adipoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
cis-4-decenoyl carnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
decanoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
hexanoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
laurylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
linoleoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
myristoleoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
myristoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
octanoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
oleoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
palmitoleoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
palmitoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
pimeloylcarnitine/3-methyladipoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
stearoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
suberoylcarnitine	**Lipid**	**Fatty Acid Metabolism(Acyl Carnitine)**
3,4-methylene heptanoylglycine	**Lipid**	**Fatty Acid Metabolism(Acyl Glycine)**
hexanoylglycine	**Lipid**	**Fatty Acid Metabolism(Acyl Glycine)**
N-palmitoylglycine	**Lipid**	**Fatty Acid Metabolism(Acyl Glycine)**
malonate	**Lipid**	**Fatty Acid Synthesis**
malonylcarnitine	**Lipid**	**Fatty Acid Synthesis**
oleamide	**Lipid**	**Fatty Acid, Amide**
palmitic amide	**Lipid**	**Fatty Acid, Amide**
2-aminoheptanoate	**Lipid**	**Fatty Acid, Amino**

2-aminooctanoate	**Lipid**	**Fatty Acid, Amino**
15-methylpalmitate	**Lipid**	**Fatty Acid, Branched**
17-methylstearate	**Lipid**	**Fatty Acid, Branched**
pristanate	**Lipid**	**Fatty Acid, Branched**
2-hydroxyadipate	**Lipid**	**Fatty Acid, Dicarboxylate**
2-hydroxyglutarate	**Lipid**	**Fatty Acid, Dicarboxylate**
3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	**Lipid**	**Fatty Acid, Dicarboxylate**
3-methyladipate	**Lipid**	**Fatty Acid, Dicarboxylate**
4-hydroxy-2-oxoglutaric acid	**Lipid**	**Fatty Acid, Dicarboxylate**
adipate	**Lipid**	**Fatty Acid, Dicarboxylate**
azelate (nonanedioate)	**Lipid**	**Fatty Acid, Dicarboxylate**
docosadioate	**Lipid**	**Fatty Acid, Dicarboxylate**
dodecanedioate	**Lipid**	**Fatty Acid, Dicarboxylate**
eicosanodioate	**Lipid**	**Fatty Acid, Dicarboxylate**
hexadecanedioate	**Lipid**	**Fatty Acid, Dicarboxylate**
maleate	**Lipid**	**Fatty Acid, Dicarboxylate**
octadecanedioate	**Lipid**	**Fatty Acid, Dicarboxylate**
pimelate (heptanedioate)	**Lipid**	**Fatty Acid, Dicarboxylate**
sebacate (decanedioate)	**Lipid**	**Fatty Acid, Dicarboxylate**
suberate (octanedioate)	**Lipid**	**Fatty Acid, Dicarboxylate**
tetradecanedioate	**Lipid**	**Fatty Acid, Dicarboxylate**
undecanedioate	**Lipid**	**Fatty Acid, Dicarboxylate**
12,13-DiHOME	**Lipid**	**Fatty Acid, Dihydroxy**
9,10-DiHOME	**Lipid**	**Fatty Acid, Dihydroxy**
13-HODE + 9-HODE	**Lipid**	**Fatty Acid, Monohydroxy**
16-hydroxypalmitate	**Lipid**	**Fatty Acid, Monohydroxy**
2-hydroxydecanoate	**Lipid**	**Fatty Acid, Monohydroxy**
2-hydroxylaurate	**Lipid**	**Fatty Acid, Monohydroxy**
2-hydroxyoctanoate	**Lipid**	**Fatty Acid, Monohydroxy**
2-hydroxypalmitate	**Lipid**	**Fatty Acid, Monohydroxy**
2-hydroxystearate	**Lipid**	**Fatty Acid, Monohydroxy**
3-hydroxydecanoate	**Lipid**	**Fatty Acid, Monohydroxy**

3-hydroxyhexanoate	**Lipid**	**Fatty Acid, Monohydroxy**
3-hydroxylaurate	**Lipid**	**Fatty Acid, Monohydroxy**
3-hydroxyoctanoate	**Lipid**	**Fatty Acid, Monohydroxy**
3-hydroxysebacate	**Lipid**	**Fatty Acid, Monohydroxy**
5-hydroxyhexanoate	**Lipid**	**Fatty Acid, Monohydroxy**
9-hydroxystearate	**Lipid**	**Fatty Acid, Monohydroxy**
glycerol	**Lipid**	**Glycerolipid Metabolism**
glycerol 3-phosphate	**Lipid**	**Glycerolipid Metabolism**
glycerophosphoglycerol	**Lipid**	**Glycerolipid Metabolism**
chiro-inositol	**Lipid**	**Inositol Metabolism**
myo-inositol	**Lipid**	**Inositol Metabolism**
3-hydroxybutyrate (BHBA)	**Lipid**	**Ketone Bodies**
acetoacetate	**Lipid**	**Ketone Bodies**
10-heptadecenoate (17:1n7)	**Lipid**	**Long Chain Fatty Acid**
10-nonadecenoate (19:1n9)	**Lipid**	**Long Chain Fatty Acid**
arachidate (20:0)	**Lipid**	**Long Chain Fatty Acid**
eicosenoate (20:1)	**Lipid**	**Long Chain Fatty Acid**
erucate (22:1n9)	**Lipid**	**Long Chain Fatty Acid**
margarate (17:0)	**Lipid**	**Long Chain Fatty Acid**
myristate (14:0)	**Lipid**	**Long Chain Fatty Acid**
myristoleate (14:1n5)	**Lipid**	**Long Chain Fatty Acid**
nonadecanoate (19:0)	**Lipid**	**Long Chain Fatty Acid**
oleate/vaccenate (18:1)	**Lipid**	**Long Chain Fatty Acid**
palmitate (16:0)	**Lipid**	**Long Chain Fatty Acid**
palmitoleate (16:1n7)	**Lipid**	**Long Chain Fatty Acid**
pentadecanoate (15:0)	**Lipid**	**Long Chain Fatty Acid**
stearate (18:0)	**Lipid**	**Long Chain Fatty Acid**
1-adrenoyl-GPC (22:4)	**Lipid**	**Lysolipid**
1-arachidonoyl-GPA (20:4)	**Lipid**	**Lysolipid**
1-arachidonoyl-GPC (20:4n6)	**Lipid**	**Lysolipid**
1-arachidonoyl-GPE (20:4n6)	**Lipid**	**Lysolipid**
1-arachidonoyl-GPI (20:4)	**Lipid**	**Lysolipid**

1-arachidoyl-GPC (20:0)	**Lipid**	**Lysolipid**
1-behenoyl-GPC (22:0)	**Lipid**	**Lysolipid**
1-dihomo-linolenoyl-GPC (20:3n3 or 6)	**Lipid**	**Lysolipid**
1-dihomo-linolenoyl-GPE (20:3n3 or 6)	**Lipid**	**Lysolipid**
1-dihomo-linoleoyl-GPC (20:2)	**Lipid**	**Lysolipid**
1-docosahexaenoyl-GPC (22:6)	**Lipid**	**Lysolipid**
1-docosahexaenoyl-GPE (22:6)	**Lipid**	**Lysolipid**
1-docosapentaenoyl-GPC (22:5n3)	**Lipid**	**Lysolipid**
1-docosapentaenoyl-GPC (22:5n6)	**Lipid**	**Lysolipid**
1-eicosapentaenoyl-GPC (20:5)	**Lipid**	**Lysolipid**
1-eicosapentaenoyl-GPE (20:5)	**Lipid**	**Lysolipid**
1-eicosenoyl-GPC (20:1)	**Lipid**	**Lysolipid**
1-erucoyl-GPC (22:1)	**Lipid**	**Lysolipid**
1-lignoceroyl-GPC (24:0)	**Lipid**	**Lysolipid**
1-linolenoyl-GPC (18:3)	**Lipid**	**Lysolipid**
1-linoleoyl-GPA (18:2)	**Lipid**	**Lysolipid**
1-linoleoyl-GPC (18:2)	**Lipid**	**Lysolipid**
1-linoleoyl-GPE (18:2)	**Lipid**	**Lysolipid**
1-linoleoyl-GPG (18:2)	**Lipid**	**Lysolipid**
1-linoleoyl-GPI (18:2)	**Lipid**	**Lysolipid**
1-margaroyl-GPC (17:0)	**Lipid**	**Lysolipid**
1-margaroyl-GPE (17:0)	**Lipid**	**Lysolipid**
1-meadoyl-GPC (20:3n9)	**Lipid**	**Lysolipid**
1-myristoyl-GPC (14:0)	**Lipid**	**Lysolipid**
1-nonadecanoyl-GPC (19:0)	**Lipid**	**Lysolipid**
1-oleoyl-GPC (18:1)	**Lipid**	**Lysolipid**
1-oleoyl-GPE (18:1)	**Lipid**	**Lysolipid**
1-oleoyl-GPG (18:1)	**Lipid**	**Lysolipid**
1-oleoyl-GPI (18:1)	**Lipid**	**Lysolipid**
1-oleoyl-GPS (18:1)	**Lipid**	**Lysolipid**
1-palmitoleoyl-GPC (16:1)	**Lipid**	**Lysolipid**
1-palmitoleoyl-GPE (16:1)	**Lipid**	**Lysolipid**

1-palmitoleoyl-GPI (16:1)	**Lipid**	**Lysolipid**
1-palmitoyl-GPC (16:0)	**Lipid**	**Lysolipid**
1-palmitoyl-GPE (16:0)	**Lipid**	**Lysolipid**
1-palmitoyl-GPG (16:0)	**Lipid**	**Lysolipid**
1-palmitoyl-GPI (16:0)	**Lipid**	**Lysolipid**
1-pentadecanoyl-GPC (15:0)	**Lipid**	**Lysolipid**
1-stearoyl-GPC (18:0)	**Lipid**	**Lysolipid**
1-stearoyl-GPE (18:0)	**Lipid**	**Lysolipid**
1-stearoyl-GPI (18:0)	**Lipid**	**Lysolipid**
2-arachidonoyl-GPC (20:4)	**Lipid**	**Lysolipid**
2-arachidonoyl-GPE (20:4)	**Lipid**	**Lysolipid**
2-docosahexaenoyl-GPC (22:6)	**Lipid**	**Lysolipid**
2-docosahexaenoyl-GPE (22:6)	**Lipid**	**Lysolipid**
2-linoleoyl-GPC (18:2)	**Lipid**	**Lysolipid**
2-linoleoyl-GPE (18:2)	**Lipid**	**Lysolipid**
2-myristoyl-GPC (14:0)	**Lipid**	**Lysolipid**
2-oleoyl-GPC (18:1)	**Lipid**	**Lysolipid**
2-oleoyl-GPE (18:1)	**Lipid**	**Lysolipid**
2-palmitoleoyl-GPC (16:1)	**Lipid**	**Lysolipid**
2-palmitoyl-GPC (16:0)	**Lipid**	**Lysolipid**
2-palmitoyl-GPE (16:0)	**Lipid**	**Lysolipid**
2-stearoyl-GPE (18:0)	**Lipid**	**Lysolipid**
2-stearoyl-GPI (18:0)	**Lipid**	**Lysolipid**
1-palmityl-GPC (O-16:0)	**Lipid**	**Lyso-phospho-ether**
1-palmityl-GPE (O-16:0)	**Lipid**	**Lyso-phospho-ether**
1-stearyl-GPC (O-18:0)	**Lipid**	**Lyso-phospho-ether**
1-(1-enyl-oleoyl)-GPC (P-18:1)	**Lipid**	**Lysoplasmalogen**
1-(1-enyl-oleoyl)-GPE (P-18:1)	**Lipid**	**Lysoplasmalogen**
1-(1-enyl-palmitoyl)-GPC (P-16:0)	**Lipid**	**Lysoplasmalogen**
1-(1-enyl-palmitoyl)-GPE (P-16:0)	**Lipid**	**Lysoplasmalogen**
1-(1-enyl-stearoyl)-GPC (P-18:0)	**Lipid**	**Lysoplasmalogen**
1-(1-enyl-stearoyl)-GPE (P-18:0)	**Lipid**	**Lysoplasmalogen**

10-undecenoate (11:1n1)	**Lipid**	**Medium Chain Fatty Acid**
5-dodecenoate (12:1n7)	**Lipid**	**Medium Chain Fatty Acid**
caprate (10:0)	**Lipid**	**Medium Chain Fatty Acid**
caproate (6:0)	**Lipid**	**Medium Chain Fatty Acid**
caprylate (8:0)	**Lipid**	**Medium Chain Fatty Acid**
heptanoate (7:0)	**Lipid**	**Medium Chain Fatty Acid**
laurate (12:0)	**Lipid**	**Medium Chain Fatty Acid**
3-hydroxy-3-methylglutarate	**Lipid**	**Mevalonate Metabolism**
1-arachidonylglycerol (20:4)	**Lipid**	**Monoacylglycerol**
1-dihomo-linolenylglycerol (20:3)	**Lipid**	**Monoacylglycerol**
1-linolenoylglycerol (18:3)	**Lipid**	**Monoacylglycerol**
1-linoleoylglycerol (18:2)	**Lipid**	**Monoacylglycerol**
1-myristoylglycerol (14:0)	**Lipid**	**Monoacylglycerol**
1-oleoylglycerol (18:1)	**Lipid**	**Monoacylglycerol**
1-palmitoleoylglycerol (16:1)	**Lipid**	**Monoacylglycerol**
1-palmitoylglycerol (16:0)	**Lipid**	**Monoacylglycerol**
2-linoleoylglycerol (18:2)	**Lipid**	**Monoacylglycerol**
1-stearoyl-2-arachidonoyl-GPS (18:0/20:4)	**Lipid**	**Phosphatidylserine (PS)**
1-stearoyl-2-oleoyl-GPS (18:0/18:1)	**Lipid**	**Phosphatidylserine (PS)**
1,2-dilinoleoyl-GPC (18:2/18:2)	**Lipid**	**Phospholipid Metabolism**
1,2-dilinoleoyl-GPE (18:2/18:2)	**Lipid**	**Phospholipid Metabolism**
1,2-dipalmitoyl-GPC (16:0/16:0)	**Lipid**	**Phospholipid Metabolism**
1,2-dipalmitoyl-GPE (16:0/16:0)	**Lipid**	**Phospholipid Metabolism**
1-arachidoyl-2-arachidonoyl-GPC (20:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-arachidonoyl-GPC (18:2/20:4n6)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-arachidonoyl-GPE (18:2/20:4)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-docosahexaenoyl-GPC (18:2/22:6)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-docosapentaenyol-GPC (18:2/22:5n3)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-eicosapentaenoyl-GPC (18:2/20:5)	**Lipid**	**Phospholipid Metabolism**
1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	**Lipid**	**Phospholipid Metabolism**
1-margaroyl-2-arachidonoyl-GPC (17:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-margaroyl-2-docosahexaenoyl-GPC (17:0/22:6)	**Lipid**	**Phospholipid Metabolism**

1-margaroyl-2-linoleoyl-GPC (17:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-margaroyl-2-oleoyl-GPC (17:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-myristoyl-2-docosahexaenoyl-GPC (14:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-myristoyl-2-linoleoyl-GPC (14:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-myristoyl-2-palmitoyl-GPC (14:0/16:0)	**Lipid**	**Phospholipid Metabolism**
1-oleoyl-2-arachidonoyl-GPI (18:1/20:4)	**Lipid**	**Phospholipid Metabolism**
1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)	**Lipid**	**Phospholipid Metabolism**
1-oleoyl-2-docosahexaenoyl-GPE (18:1/22:6)	**Lipid**	**Phospholipid Metabolism**
1-oleoyl-2-linoleoyl-GPC (18:1/18:2)	**Lipid**	**Phospholipid Metabolism**
1-oleoyl-2-linoleoyl-GPE (18:1/18:2)	**Lipid**	**Phospholipid Metabolism**
1-palmitoleoyl-2-docosahexaenoyl-GPC (16:1/22:6)	**Lipid**	**Phospholipid Metabolism**
1-palmitoleoyl-2-linolenoyl-GPC (16:1/18:3)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-adrenoyl-GPC (16:0/22:4)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-docosahexaenoyl-GPI (16:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-eicosapentaenoyl-GPC (16:0/20:5)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-eicosapentaenoyl-GPE (16:0/20:5)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-oleoyl-GPI (16:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-palmitoleoyl-GPE (16:0/16:1)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	**Lipid**	**Phospholipid Metabolism**
1-palmitoyl-2-stearoyl-GPE (16:0/18:0)	**Lipid**	**Phospholipid Metabolism**

1-palmityl-2-arachidonoyl-GPC (O-16:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-palmityl-2-linoleoyl-GPC (O-16:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-palmityl-2-oleoyl-GPC (O-16:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-palmityl-2-palmitoyl-GPC (O-16:0/16:0)	**Lipid**	**Phospholipid Metabolism**
1-palmityl-2-stearoyl-GPC (O-16:0/18:0)	**Lipid**	**Phospholipid Metabolism**
1-pentadecanoyl-2-arachidonoyl-GPC (15:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-pentadecanoyl-2-docosahexaenoyl-GPC (15:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-pentadecanoyl-2-linoleoyl-GPC (15:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-adrenoyl-GPC (18:0/22:4)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-adrenoyl-GPE (18:0/22:4)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-dihomo-linolenoyl-GPC (18:0/20:3n3 or 6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-dihomo-linolenoyl-GPE (18:0/20:3n3 or 6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-dihomo-linolenoyl-GPI (18:0/20:3n3 or 6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosahexaenoyl-GPE (18:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosahexaenoyl-GPI (18:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosahexaenoyl-GPS (18:0/22:6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosapentaenoyl-GPC (18:0/22:5n3)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosapentaenoyl-GPC (18:0/22:5n6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosapentaenoyl-GPE (18:0/22:5n3)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-docosapentaenoyl-GPE (18:0/22:5n6)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-linoleoyl-GPE (18:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-meadoyl-GPC (18:0/20:3n9)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-stearoyl-2-oleoyl-GPI (18:0/18:1)	**Lipid**	**Phospholipid Metabolism**
1-stearyl-2-arachidonoyl-GPC (O-18:0/20:4)	**Lipid**	**Phospholipid Metabolism**

1-stearyl-2-docosapentaenoyl-GPC (O-18:0/22:5n3)	**Lipid**	**Phospholipid Metabolism**
arachidonoylcholine	**Lipid**	**Phospholipid Metabolism**
choline	**Lipid**	**Phospholipid Metabolism**
choline phosphate	**Lipid**	**Phospholipid Metabolism**
cytidine 5'-diphosphocholine	**Lipid**	**Phospholipid Metabolism**
dihomo-linolenoyl-choline	**Lipid**	**Phospholipid Metabolism**
docosahexaenoylcholine	**Lipid**	**Phospholipid Metabolism**
glycerophosphoethanolamine	**Lipid**	**Phospholipid Metabolism**
glycerophosphoinositol	**Lipid**	**Phospholipid Metabolism**
glycerophosphorylcholine (GPC)	**Lipid**	**Phospholipid Metabolism**
phosphatidylcholine (14:0/14:0, 16:0/12:0)	**Lipid**	**Phospholipid Metabolism**
phosphatidylcholine (15:0/18:1, 17:0/16:1)	**Lipid**	**Phospholipid Metabolism**
phosphatidylcholine (16:0/22:5n3, 18:1/20:4)	**Lipid**	**Phospholipid Metabolism**
phosphatidylcholine (18:0/20:2, 20:0/18:2)	**Lipid**	**Phospholipid Metabolism**
phosphatidylcholine (18:0/20:5, 16:0/22:5n6)	**Lipid**	**Phospholipid Metabolism**
phosphoethanolamine	**Lipid**	**Phospholipid Metabolism**
trimethylamine N-oxide	**Lipid**	**Phospholipid Metabolism**
1-(1-enyl-oleoyl)-2-docosahexaenoyl-GPE (P-18:1/22:6)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-docosahexaenoyl-GPC (P-16:0/22:6)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-docosahexaenoyl-GPE (P-16:0/22:6)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-eicosapentaenoyl-GPE (P-16:0/20:5)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-myristoyl-GPC (P-16:0/14:0)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)	**Lipid**	**Plasmalogen**
1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)	**Lipid**	**Plasmalogen**

1-(1-enyl-stearoyl)-2-arachidonoyl-GPC (P-18:0/20:4)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-dihomo-linolenoyl-GPE (P-18:0/20:3)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-docosahexaenoyl-GPC (P-18:0/22:6)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-docosahexaenoyl-GPE (P-18:0/22:6)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-docosapentaenoyl-GPE (P-18:0/22:5n3)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-linoleoyl-GPC (P-18:0/18:2)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-oleoyl-GPC (P-18:0/18:1)	Lipid	Plasmalogen
1-(1-enyl-stearoyl)-2-oleoyl-GPE (P-18:0/18:1)	Lipid	Plasmalogen
adrenate (22:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
arachidonate (20:4n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
dihomo-linoleate (20:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
dihomo-linolenate (20:3n3 or n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
docosadienoate (22:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
docosahexaenoate (DHA; 22:6n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
docosapentaenoate (n3 DPA; 22:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
docosapentaenoate (n6 DPA; 22:5n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
eicosapentaenoate (EPA; 20:5n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
linoleate (18:2n6)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
linolenate [alpha or gamma; (18:3n3 or 6)]	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
stearidonate (18:4n3)	Lipid	Polyunsaturated Fatty Acid (n3 and n6)
chenodeoxycholate	Lipid	Primary Bile Acid Metabolism
cholate	Lipid	Primary Bile Acid Metabolism
glycochenodeoxycholate	Lipid	Primary Bile Acid Metabolism
glycochenodeoxycholate glucuronide (1)	Lipid	Primary Bile Acid Metabolism
glycochenodeoxycholate sulfate	Lipid	Primary Bile Acid Metabolism
glycocholate	Lipid	Primary Bile Acid Metabolism
tauro-beta-muricholate	Lipid	Primary Bile Acid Metabolism
taurochenodeoxycholate	Lipid	Primary Bile Acid Metabolism
taurocholate	Lipid	Primary Bile Acid Metabolism

3b-hydroxy-5-cholenoic acid	**Lipid**	**Secondary Bile Acid Metabolism**
7-ketodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
deoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
glycocholenate sulfate	**Lipid**	**Secondary Bile Acid Metabolism**
glycodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
glycodeoxycholate sulfate	**Lipid**	**Secondary Bile Acid Metabolism**
glycohyocholate	**Lipid**	**Secondary Bile Acid Metabolism**
glycolithocholate	**Lipid**	**Secondary Bile Acid Metabolism**
glycolithocholate sulfate	**Lipid**	**Secondary Bile Acid Metabolism**
glycoursodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
hyocholate	**Lipid**	**Secondary Bile Acid Metabolism**
lithocholate	**Lipid**	**Secondary Bile Acid Metabolism**
taurocholenate sulfate	**Lipid**	**Secondary Bile Acid Metabolism**
taurodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
taurolithocholate	**Lipid**	**Secondary Bile Acid Metabolism**
taurolithocholate 3-sulfate	**Lipid**	**Secondary Bile Acid Metabolism**
tauroursodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
ursodeoxycholate	**Lipid**	**Secondary Bile Acid Metabolism**
behenoyl dihydrosphingomyelin (d18:0/22:0)	**Lipid**	**Sphingolipid Metabolism**
behenoyl sphingomyelin (d18:1/22:0)	**Lipid**	**Sphingolipid Metabolism**
glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	**Lipid**	**Sphingolipid Metabolism**
glycosyl-N-stearoyl-sphingosine (d18:1/18:0)	**Lipid**	**Sphingolipid Metabolism**
lactosyl-N-nervonoyl-sphingosine (d18:1/24:1)	**Lipid**	**Sphingolipid Metabolism**
lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)	**Lipid**	**Sphingolipid Metabolism**
lignoceroyl sphingomyelin (d18:1/24:0)	**Lipid**	**Sphingolipid Metabolism**
myristoyl dihydrosphingomyelin (d18:0/14:0)	**Lipid**	**Sphingolipid Metabolism**
N-behenoyl-sphingadienine (d18:2/22:0)	**Lipid**	**Sphingolipid Metabolism**
N-palmitoyl-sphinganine (d18:0/16:0)	**Lipid**	**Sphingolipid Metabolism**
N-palmitoyl-sphingosine (d18:1/16:0)	**Lipid**	**Sphingolipid Metabolism**
N-stearoyl-sphingosine (d18:1/18:0)	**Lipid**	**Sphingolipid Metabolism**
palmitoyl dihydrosphingomyelin (d18:0/16:0)	**Lipid**	**Sphingolipid Metabolism**
palmitoyl sphingomyelin (d18:1/16:0)	**Lipid**	**Sphingolipid Metabolism**

sphinganine	**Lipid**	**Sphingolipid Metabolism**
sphinganine-1-phosphate	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/14:0, d16:1/16:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/15:0, d16:1/17:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/18:1, d18:2/18:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/20:0, d16:1/22:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/20:1, d18:2/20:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:1/24:1, d18:2/24:0)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:2/14:0, d18:1/14:1)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:2/16:0, d18:1/16:1)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)	**Lipid**	**Sphingolipid Metabolism**
sphingomyelin (d18:2/24:1, d18:1/24:2)	**Lipid**	**Sphingolipid Metabolism**
sphingosine	**Lipid**	**Sphingolipid Metabolism**
sphingosine 1-phosphate	**Lipid**	**Sphingolipid Metabolism**
stearoyl sphingomyelin (d18:1/18:0)	**Lipid**	**Sphingolipid Metabolism**
tricosanoyl sphingomyelin (d18:1/23:0)	**Lipid**	**Sphingolipid Metabolism**
16a-hydroxy DHEA 3-sulfate	**Lipid**	**Steroid**
17alpha-hydroxypregnanolone glucuronide	**Lipid**	**Steroid**
17alpha-hydroxypregnenolone sulfate	**Lipid**	**Steroid**
21-hydroxypregnenolone disulfate	**Lipid**	**Steroid**
4-androsten-3alpha,17alpha-diol monosulfate (2)	**Lipid**	**Steroid**
4-androsten-3alpha,17alpha-diol monosulfate (3)	**Lipid**	**Steroid**
4-androsten-3beta,17beta-diol disulfate (1)	**Lipid**	**Steroid**
4-androsten-3beta,17beta-diol disulfate (2)	**Lipid**	**Steroid**
4-androsten-3beta,17beta-diol monosulfate (1)	**Lipid**	**Steroid**
4-androsten-3beta,17beta-diol monosulfate (2)	**Lipid**	**Steroid**
5alpha-androstan-3alpha,17beta-diol disulfate	**Lipid**	**Steroid**
5alpha-androstan-3alpha,17beta-diol monosulfate (1)	**Lipid**	**Steroid**
5alpha-androstan-3alpha,17beta-diol monosulfate (2)	**Lipid**	**Steroid**

5alpha-androstan-3beta,17alpha-diol disulfate	Lipid	Steroid
5alpha-androstan-3beta,17beta-diol disulfate	Lipid	Steroid
5alpha-androstan-3beta,17beta-diol monosulfate (2)	Lipid	Steroid
5alpha-pregnan-3(alpha or beta),20beta-diol disulfate	Lipid	Steroid
5alpha-pregnan-3beta,20alpha-diol disulfate	Lipid	Steroid
5alpha-pregnan-3beta,20alpha-diol monosulfate (2)	Lipid	Steroid
5alpha-pregnan-3beta,20beta-diol monosulfate (1)	Lipid	Steroid
andro steroid monosulfate (1)	Lipid	Steroid
androsterone sulfate	Lipid	Steroid
cortisol	Lipid	Steroid
cortisone	Lipid	Steroid
dehydroisoandrosterone sulfate (DHEA-S)	Lipid	Steroid
epiandrosterone sulfate	Lipid	Steroid
etiocholanolone glucuronide	Lipid	Steroid
pregn steroid monosulfate	Lipid	Steroid
pregnanediol-3-glucuronide	Lipid	Steroid
pregnanolone/allopregnanolone sulfate	Lipid	Steroid
pregnen-diol disulfate	Lipid	Steroid
pregnenolone sulfate	Lipid	Steroid
3beta,7alpha-dihydroxy-5-cholestenoate	Lipid	Sterol
3-hydroxy-5-cholestenoic acid	Lipid	Sterol
4-cholesten-3-one	Lipid	Sterol
7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	Lipid	Sterol
beta-sitosterol	Lipid	Sterol
campesterol	Lipid	Sterol
cholesterol	Lipid	Sterol
allantoin	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing
hypoxanthine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing
inosine	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing
inosine 5'-monophosphate (IMP)	Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing

N1-methylinosine	**Nucleotide**	**Purine Metabolism, (Hypo)Xanthine/Inosine containing**
urate	**Nucleotide**	**Purine Metabolism, (Hypo)Xanthine/Inosine containing**
xanthine	**Nucleotide**	**Purine Metabolism, (Hypo)Xanthine/Inosine containing**
xanthosine	**Nucleotide**	**Purine Metabolism, (Hypo)Xanthine/Inosine containing**
adenine	**Nucleotide**	**Purine Metabolism, Adenine containing**
adenosine	**Nucleotide**	**Purine Metabolism, Adenine containing**
adenosine 5'-diphosphate (ADP)	**Nucleotide**	**Purine Metabolism, Adenine containing**
adenosine 5'-monophosphate (AMP)	**Nucleotide**	**Purine Metabolism, Adenine containing**
N1-methyladenosine	**Nucleotide**	**Purine Metabolism, Adenine containing**
N6-carbamoylthreonyladenosine	**Nucleotide**	**Purine Metabolism, Adenine containing**
N6-methyladenosine	**Nucleotide**	**Purine Metabolism, Adenine containing**
N6-succinyladenosine	**Nucleotide**	**Purine Metabolism, Adenine containing**
7-methylguanine	**Nucleotide**	**Purine Metabolism, Guanine containing**
guanosine	**Nucleotide**	**Purine Metabolism, Guanine containing**
N2,N2-dimethylguanosine	**Nucleotide**	**Purine Metabolism, Guanine containing**
3-methylcytidine	**Nucleotide**	**Pyrimidine Metabolism, Cytidine containing**
cytidine	**Nucleotide**	**Pyrimidine Metabolism, Cytidine containing**
cytidine 5'-monophosphate (5'-CMP)	**Nucleotide**	**Pyrimidine Metabolism, Cytidine containing**
cytosine	**Nucleotide**	**Pyrimidine Metabolism, Cytidine containing**
N4-acetylcytidine	**Nucleotide**	**Pyrimidine Metabolism, Cytidine containing**
dihydroorotate	**Nucleotide**	**Pyrimidine Metabolism, Orotate containing**
orotate	**Nucleotide**	**Pyrimidine Metabolism, Orotate containing**
orotidine	**Nucleotide**	**Pyrimidine Metabolism, Orotate containing**
3-aminoisobutyrate	**Nucleotide**	**Pyrimidine Metabolism, Thymine containing**
5,6-dihydrothymine	**Nucleotide**	**Pyrimidine Metabolism, Thymine containing**
3-ureidopropionate	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
5-methyluridine (ribothymidine)	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
beta-alanine	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
N-acetyl-beta-alanine	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
pseudouridine	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**

uracil	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
uridine	**Nucleotide**	**Pyrimidine Metabolism, Uracil containing**
4-hydroxyphenylacetylglutamine	**Peptide**	**Acetylated Peptides**
phenylacetylcarnitine	**Peptide**	**Acetylated Peptides**
phenylacetylglutamate	**Peptide**	**Acetylated Peptides**
phenylacetylglutamine	**Peptide**	**Acetylated Peptides**
phenylacetylglycine	**Peptide**	**Acetylated Peptides**
alpha-glutamylalanine	**Peptide**	**Dipeptide**
alpha-glutamylglycine	**Peptide**	**Dipeptide**
aspartylaspartate	**Peptide**	**Dipeptide**
aspartylisoleucine	**Peptide**	**Dipeptide**
aspartylleucine	**Peptide**	**Dipeptide**
cyclo(ala-pro)	**Peptide**	**Dipeptide**
cyclo(met-pro)	**Peptide**	**Dipeptide**
cyclo(pro-val)	**Peptide**	**Dipeptide**
glycylglycine	**Peptide**	**Dipeptide**
histidylglycine	**Peptide**	**Dipeptide**
histidylleucine	**Peptide**	**Dipeptide**
histidylphenylalanine	**Peptide**	**Dipeptide**
histidyltryptophan	**Peptide**	**Dipeptide**
isoleucylalanine	**Peptide**	**Dipeptide**
isoleucylglutamate	**Peptide**	**Dipeptide**
isoleucylglycine	**Peptide**	**Dipeptide**
isoleucylleucine/leucylisoleucine	**Peptide**	**Dipeptide**
isoleucylthreonine	**Peptide**	**Dipeptide**
leucylglutamine	**Peptide**	**Dipeptide**
leucylglycine	**Peptide**	**Dipeptide**
leucylleucine	**Peptide**	**Dipeptide**
methionylalanine	**Peptide**	**Dipeptide**
phenylalanylarginine	**Peptide**	**Dipeptide**
phenylalanylglycine	**Peptide**	**Dipeptide**
phenylalanylisoleucine	**Peptide**	**Dipeptide**

phenylalanylleucine	**Peptide**	**Dipeptide**
phenylalanylphenylalanine	**Peptide**	**Dipeptide**
phenylalanyltryptophan	**Peptide**	**Dipeptide**
prolylalanine	**Peptide**	**Dipeptide**
prolylglycine	**Peptide**	**Dipeptide**
prolylphenylalanine	**Peptide**	**Dipeptide**
prolylproline	**Peptide**	**Dipeptide**
pyroglutamylvaline	**Peptide**	**Dipeptide**
serylalanine	**Peptide**	**Dipeptide**
threonylalanine	**Peptide**	**Dipeptide**
tryptophylleucine	**Peptide**	**Dipeptide**
valylarginine	**Peptide**	**Dipeptide**
valylglutamine	**Peptide**	**Dipeptide**
valylglycine	**Peptide**	**Dipeptide**
valylleucine	**Peptide**	**Dipeptide**
valylphenylalanine	**Peptide**	**Dipeptide**
N-acetylcarnosine	**Peptide**	**Dipeptide Derivative**
ADSGEGDFXAEGGGVR	**Peptide**	**Fibrinogen Cleavage Peptide**
DSGEGDFXAEGGGVR	**Peptide**	**Fibrinogen Cleavage Peptide**
gamma-glutamylalanine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamyl-alpha-lysine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamyl-epsilon-lysine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylglutamate	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylglutamine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylglycine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylhistidine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylisoleucine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylleucine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylmethionine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylphenylalanine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylthreonine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamyltryptophan	**Peptide**	**Gamma-glutamyl Amino Acid**

gamma-glutamyltyrosine	**Peptide**	**Gamma-glutamyl Amino Acid**
gamma-glutamylvaline	**Peptide**	**Gamma-glutamyl Amino Acid**
bradykinin	**Peptide**	**Polypeptide**
bradykinin, des-arg(9)	**Peptide**	**Polypeptide**
bradykinin, hydroxy-pro(3)	**Peptide**	**Polypeptide**
tartronate (hydroxymalonate)	**Xenobiotics**	**Bacterial/Fungal**
2-ethylphenylsulfate	**Xenobiotics**	**Benzoate Metabolism**
2-hydroxyhippurate (salicylurate)	**Xenobiotics**	**Benzoate Metabolism**
3-hydroxyhippurate	**Xenobiotics**	**Benzoate Metabolism**
3-methoxycatechol sulfate (1)	**Xenobiotics**	**Benzoate Metabolism**
3-methoxycatechol sulfate (2)	**Xenobiotics**	**Benzoate Metabolism**
3-methyl catechol sulfate (1)	**Xenobiotics**	**Benzoate Metabolism**
3-methyl catechol sulfate (2)	**Xenobiotics**	**Benzoate Metabolism**
4-ethylphenylsulfate	**Xenobiotics**	**Benzoate Metabolism**
4-hydroxyhippurate	**Xenobiotics**	**Benzoate Metabolism**
4-methylcatechol sulfate	**Xenobiotics**	**Benzoate Metabolism**
4-vinylphenol sulfate	**Xenobiotics**	**Benzoate Metabolism**
benzoate	**Xenobiotics**	**Benzoate Metabolism**
catechol sulfate	**Xenobiotics**	**Benzoate Metabolism**
hippurate	**Xenobiotics**	**Benzoate Metabolism**
methyl-4-hydroxybenzoate sulfate	**Xenobiotics**	**Benzoate Metabolism**
O-methylcatechol sulfate	**Xenobiotics**	**Benzoate Metabolism**
propyl 4-hydroxybenzoate sulfate	**Xenobiotics**	**Benzoate Metabolism**
1,2,3-benzenetriol sulfate (1)	**Xenobiotics**	**Chemical**
1,2,3-benzenetriol sulfate (2)	**Xenobiotics**	**Chemical**
1,3-propanediol	**Xenobiotics**	**Chemical**
2-aminophenol sulfate	**Xenobiotics**	**Chemical**
2-methoxyresorcinol sulfate	**Xenobiotics**	**Chemical**
3-acetylphenol sulfate	**Xenobiotics**	**Chemical**
3-hydroxypyridine sulfate	**Xenobiotics**	**Chemical**
4-hydroxychlorothalonil	**Xenobiotics**	**Chemical**
6-hydroxyindole sulfate	**Xenobiotics**	**Chemical**

benzoylcarnitine	**Xenobiotics**	**Chemical**
dimethyl sulfone	**Xenobiotics**	**Chemical**
ectoine	**Xenobiotics**	**Chemical**
EDTA	**Xenobiotics**	**Chemical**
ethyl glucuronide	**Xenobiotics**	**Chemical**
iminodiacetate (IDA)	**Xenobiotics**	**Chemical**
lanthionine	**Xenobiotics**	**Chemical**
N-methylpipecolate	**Xenobiotics**	**Chemical**
O-sulfo-L-tyrosine	**Xenobiotics**	**Chemical**
rhodamine B	**Xenobiotics**	**Chemical**
succinimide	**Xenobiotics**	**Chemical**
sulfate	**Xenobiotics**	**Chemical**
trizma acetate	**Xenobiotics**	**Chemical**
1-hydroxy-2-naphthalenecarboxylate	**Xenobiotics**	**Drug**
2-acetamidophenol sulfate	**Xenobiotics**	**Drug**
2-hydroxyacetaminophen sulfate	**Xenobiotics**	**Drug**
2-hydroxyibuprofen	**Xenobiotics**	**Drug**
2-methoxyacetaminophen glucuronide	**Xenobiotics**	**Drug**
2-methoxyacetaminophen sulfate	**Xenobiotics**	**Drug**
3-(cystein-S-yl)acetaminophen	**Xenobiotics**	**Drug**
3-(N-acetyl-L-cystein-S-yl) acetaminophen	**Xenobiotics**	**Drug**
3-hydroxyquinine	**Xenobiotics**	**Drug**
4-acetamidophenol	**Xenobiotics**	**Drug**
4-acetamidophenylglucuronide	**Xenobiotics**	**Drug**
4-acetaminophen sulfate	**Xenobiotics**	**Drug**
4-acetylphenol sulfate	**Xenobiotics**	**Drug**
4-aminophenol sulfate (2)	**Xenobiotics**	**Drug**
4-hydroxycoumarin	**Xenobiotics**	**Drug**
allopurinol	**Xenobiotics**	**Drug**
allopurinol riboside	**Xenobiotics**	**Drug**
alpha-hydroxymetoprolol	**Xenobiotics**	**Drug**
amoxicillin	**Xenobiotics**	**Drug**

aripiprazole	**Xenobiotics**	**Drug**
atenolol	**Xenobiotics**	**Drug**
atorvastatin (lipitor)	**Xenobiotics**	**Drug**
candesartan	**Xenobiotics**	**Drug**
carbamazepine	**Xenobiotics**	**Drug**
carbamazepine 10,11-epoxide	**Xenobiotics**	**Drug**
carbamazepine glucuronide	**Xenobiotics**	**Drug**
carboxyibuprofen	**Xenobiotics**	**Drug**
Cetirizine	**Xenobiotics**	**Drug**
chlorthalidone	**Xenobiotics**	**Drug**
deferasirox (DFX)	**Xenobiotics**	**Drug**
deferoxamine (DFO)	**Xenobiotics**	**Drug**
desmethylnaproxen	**Xenobiotics**	**Drug**
desmethylnaproxen sulfate	**Xenobiotics**	**Drug**
diltiazem	**Xenobiotics**	**Drug**
diphenhydramine	**Xenobiotics**	**Drug**
doxycycline	**Xenobiotics**	**Drug**
enalapril	**Xenobiotics**	**Drug**
escitalopram	**Xenobiotics**	**Drug**
fexofenadine	**Xenobiotics**	**Drug**
fluoxetine	**Xenobiotics**	**Drug**
furosemide	**Xenobiotics**	**Drug**
gabapentin	**Xenobiotics**	**Drug**
homoveratric acid	**Xenobiotics**	**Drug**
hydrochlorothiazide	**Xenobiotics**	**Drug**
hydroquinone sulfate	**Xenobiotics**	**Drug**
hydroxypioglitazone (M-IV)	**Xenobiotics**	**Drug**
ibuprofen	**Xenobiotics**	**Drug**
ibuprofen acyl glucuronide	**Xenobiotics**	**Drug**
ketopioglitazone	**Xenobiotics**	**Drug**
lamotrigine	**Xenobiotics**	**Drug**
lidocaine	**Xenobiotics**	**Drug**

lisinopril	**Xenobiotics**	**Drug**
metformin	**Xenobiotics**	**Drug**
metoprolol	**Xenobiotics**	**Drug**
metoprolol acid metabolite	**Xenobiotics**	**Drug**
mycophenolic acid	**Xenobiotics**	**Drug**
mycophenolic acid glucuronide	**Xenobiotics**	**Drug**
naproxen	**Xenobiotics**	**Drug**
N-desmethyl tramadol	**Xenobiotics**	**Drug**
N-ethylglycinexylidide	**Xenobiotics**	**Drug**
O-desmethyltramadol	**Xenobiotics**	**Drug**
O-desmethyltramadol glucuronide	**Xenobiotics**	**Drug**
O-desmethylvenlafaxine	**Xenobiotics**	**Drug**
o-hydroxyatorvastatin	**Xenobiotics**	**Drug**
o-hydroxyatorvastatin lactone	**Xenobiotics**	**Drug**
olmesartan	**Xenobiotics**	**Drug**
omeprazole	**Xenobiotics**	**Drug**
oxycodone	**Xenobiotics**	**Drug**
oxypurinol	**Xenobiotics**	**Drug**
pantoprazole	**Xenobiotics**	**Drug**
p-hydroxyatorvastatin	**Xenobiotics**	**Drug**
p-hydroxyatorvastatin lactone	**Xenobiotics**	**Drug**
pioglitazone	**Xenobiotics**	**Drug**
pivaloylcarnitine	**Xenobiotics**	**Drug**
prednisolone	**Xenobiotics**	**Drug**
prednisone	**Xenobiotics**	**Drug**
pregabalin	**Xenobiotics**	**Drug**
quetiapine	**Xenobiotics**	**Drug**
quinine	**Xenobiotics**	**Drug**
ranitidine	**Xenobiotics**	**Drug**
rivaroxaban	**Xenobiotics**	**Drug**
salicylate	**Xenobiotics**	**Drug**
salicyluric glucuronide	**Xenobiotics**	**Drug**

S-carboxymethyl-L-cysteine	**Xenobiotics**	**Drug**
sertraline	**Xenobiotics**	**Drug**
sildenafil	**Xenobiotics**	**Drug**
sitagliptin	**Xenobiotics**	**Drug**
solifenacin	**Xenobiotics**	**Drug**
sulfamethoxazole	**Xenobiotics**	**Drug**
tadalafil	**Xenobiotics**	**Drug**
topiramate	**Xenobiotics**	**Drug**
Tramadol	**Xenobiotics**	**Drug**
valsartan	**Xenobiotics**	**Drug**
venlafaxine	**Xenobiotics**	**Drug**
verapamil	**Xenobiotics**	**Drug**
warfarin	**Xenobiotics**	**Drug**
2,3-dihydroxyisovalerate	**Xenobiotics**	**Food Component/Plant**
2-keto-3-deoxy-gluconate	**Xenobiotics**	**Food Component/Plant**
2-piperidinone	**Xenobiotics**	**Food Component/Plant**
4-allylphenol sulfate	**Xenobiotics**	**Food Component/Plant**
4-vinylguaiacol sulfate	**Xenobiotics**	**Food Component/Plant**
acesulfame	**Xenobiotics**	**Food Component/Plant**
cinnamoylglycine	**Xenobiotics**	**Food Component/Plant**
dihydroferulic acid	**Xenobiotics**	**Food Component/Plant**
ergothioneine	**Xenobiotics**	**Food Component/Plant**
erythritol	**Xenobiotics**	**Food Component/Plant**
eugenol sulfate	**Xenobiotics**	**Food Component/Plant**
ferulic acid 4-sulfate	**Xenobiotics**	**Food Component/Plant**
ferulylglycine (1)	**Xenobiotics**	**Food Component/Plant**
gluconate	**Xenobiotics**	**Food Component/Plant**
homostachydrine	**Xenobiotics**	**Food Component/Plant**
indolin-2-one	**Xenobiotics**	**Food Component/Plant**
isoeugenol sulfate	**Xenobiotics**	**Food Component/Plant**
linamarin	**Xenobiotics**	**Food Component/Plant**
methyl glucopyranoside (alpha + beta)	**Xenobiotics**	**Food Component/Plant**

methyl indole-3-acetate	**Xenobiotics**	**Food Component/Plant**
N-(2-furoyl)glycine	**Xenobiotics**	**Food Component/Plant**
N-acetylalliin	**Xenobiotics**	**Food Component/Plant**
naringenin 7-glucuronide	**Xenobiotics**	**Food Component/Plant**
phytanate	**Xenobiotics**	**Food Component/Plant**
piperine	**Xenobiotics**	**Food Component/Plant**
pyrraline	**Xenobiotics**	**Food Component/Plant**
quinate	**Xenobiotics**	**Food Component/Plant**
retinal	**Xenobiotics**	**Food Component/Plant**
saccharin	**Xenobiotics**	**Food Component/Plant**
S-allylcysteine	**Xenobiotics**	**Food Component/Plant**
solanidine	**Xenobiotics**	**Food Component/Plant**
stachydrine	**Xenobiotics**	**Food Component/Plant**
syringol sulfate	**Xenobiotics**	**Food Component/Plant**
tartarate	**Xenobiotics**	**Food Component/Plant**
theanine	**Xenobiotics**	**Food Component/Plant**
thymol sulfate	**Xenobiotics**	**Food Component/Plant**
umbelliferone sulfate	**Xenobiotics**	**Food Component/Plant**
3-hydroxycotinine glucuronide	**Xenobiotics**	**Tobacco Metabolite**
cotinine	**Xenobiotics**	**Tobacco Metabolite**
cotinine N-oxide	**Xenobiotics**	**Tobacco Metabolite**
hydroxycotinine	**Xenobiotics**	**Tobacco Metabolite**
1,3,7-trimethylurate	**Xenobiotics**	**Xanthine Metabolism**
1,3-dimethylurate	**Xenobiotics**	**Xanthine Metabolism**
1,7-dimethylurate	**Xenobiotics**	**Xanthine Metabolism**
1-methylurate	**Xenobiotics**	**Xanthine Metabolism**
1-methylxanthine	**Xenobiotics**	**Xanthine Metabolism**
3,7-dimethylurate	**Xenobiotics**	**Xanthine Metabolism**
3-methylxanthine	**Xenobiotics**	**Xanthine Metabolism**
5-acetylamino-6-amino-3-methyluracil	**Xenobiotics**	**Xanthine Metabolism**
5-acetylamino-6-formylamino-3-methyluracil	**Xenobiotics**	**Xanthine Metabolism**
7-methylurate	**Xenobiotics**	**Xanthine Metabolism**

7-methylxanthine	**Xenobiotics**	**Xanthine Metabolism**
caffeic acid sulfate	**Xenobiotics**	**Xanthine Metabolism**
caffeine	**Xenobiotics**	**Xanthine Metabolism**
paraxanthine	**Xenobiotics**	**Xanthine Metabolism**
theobromine	**Xenobiotics**	**Xanthine Metabolism**
theophylline	**Xenobiotics**	**Xanthine Metabolism**
X - 01911	**Unknown**	**Unknown**
X - 02249	**Unknown**	**Unknown**
X - 02269	**Unknown**	**Unknown**
X - 07765	**Unknown**	**Unknown**
X - 09789	**Unknown**	**Unknown**
X - 11261	**Unknown**	**Unknown**
X - 11299	**Unknown**	**Unknown**
X - 11305	**Unknown**	**Unknown**
X - 11308	**Unknown**	**Unknown**
X - 11315	**Unknown**	**Unknown**
X - 11334	**Unknown**	**Unknown**
X - 11357	**Unknown**	**Unknown**
X - 11372	**Unknown**	**Unknown**
X - 11378	**Unknown**	**Unknown**
X - 11381	**Unknown**	**Unknown**
X - 11407	**Unknown**	**Unknown**
X - 11429	**Unknown**	**Unknown**
X - 11438	**Unknown**	**Unknown**
X - 11440	**Unknown**	**Unknown**
X - 11441	**Unknown**	**Unknown**
X - 11442	**Unknown**	**Unknown**
X - 11444	**Unknown**	**Unknown**
X - 11452	**Unknown**	**Unknown**
X - 11470	**Unknown**	**Unknown**
X - 11478	**Unknown**	**Unknown**
X - 11483	**Unknown**	**Unknown**

X - 11485	**Unknown**	**Unknown**
X - 11491	**Unknown**	**Unknown**
X - 11522	**Unknown**	**Unknown**
X - 11530	**Unknown**	**Unknown**
X - 11537	**Unknown**	**Unknown**
X - 11538	**Unknown**	**Unknown**
X - 11540	**Unknown**	**Unknown**
X - 11549	**Unknown**	**Unknown**
X - 11564	**Unknown**	**Unknown**
X - 11593	**Unknown**	**Unknown**
X - 11787	**Unknown**	**Unknown**
X - 11795	**Unknown**	**Unknown**
X - 11805	**Unknown**	**Unknown**
X - 11838	**Unknown**	**Unknown**
X - 11843	**Unknown**	**Unknown**
X - 11847	**Unknown**	**Unknown**
X - 11849	**Unknown**	**Unknown**
X - 11850	**Unknown**	**Unknown**
X - 11852	**Unknown**	**Unknown**
X - 11858	**Unknown**	**Unknown**
X - 11871	**Unknown**	**Unknown**
X - 11880	**Unknown**	**Unknown**
X - 11905	**Unknown**	**Unknown**
X - 12007	**Unknown**	**Unknown**
X - 12013	**Unknown**	**Unknown**
X - 12026	**Unknown**	**Unknown**
X - 12063	**Unknown**	**Unknown**
X - 12100	**Unknown**	**Unknown**
X - 12101	**Unknown**	**Unknown**
X - 12104	**Unknown**	**Unknown**
X - 12117	**Unknown**	**Unknown**
X - 12126	**Unknown**	**Unknown**

X - 12127	**Unknown**	**Unknown**
X - 12170	**Unknown**	**Unknown**
X - 12199	**Unknown**	**Unknown**
X - 12206	**Unknown**	**Unknown**
X - 12212	**Unknown**	**Unknown**
X - 12216	**Unknown**	**Unknown**
X - 12221	**Unknown**	**Unknown**
X - 12230	**Unknown**	**Unknown**
X - 12231	**Unknown**	**Unknown**
X - 12257	**Unknown**	**Unknown**
X - 12261	**Unknown**	**Unknown**
X - 12283	**Unknown**	**Unknown**
X - 12329	**Unknown**	**Unknown**
X - 12407	**Unknown**	**Unknown**
X - 12410	**Unknown**	**Unknown**
X - 12411	**Unknown**	**Unknown**
X - 12442	**Unknown**	**Unknown**
X - 12450	**Unknown**	**Unknown**
X - 12459	**Unknown**	**Unknown**
X - 12462	**Unknown**	**Unknown**
X - 12472	**Unknown**	**Unknown**
X - 12511	**Unknown**	**Unknown**
X - 12524	**Unknown**	**Unknown**
X - 12543	**Unknown**	**Unknown**
X - 12544	**Unknown**	**Unknown**
X - 12565	**Unknown**	**Unknown**
X - 12636	**Unknown**	**Unknown**
X - 12680	**Unknown**	**Unknown**
X - 12681	**Unknown**	**Unknown**
X - 12686	**Unknown**	**Unknown**
X - 12688	**Unknown**	**Unknown**
X - 12701	**Unknown**	**Unknown**

X - 12707	**Unknown**	**Unknown**
X - 12714	**Unknown**	**Unknown**
X - 12718	**Unknown**	**Unknown**
X - 12726	**Unknown**	**Unknown**
X - 12729	**Unknown**	**Unknown**
X - 12730	**Unknown**	**Unknown**
X - 12731	**Unknown**	**Unknown**
X - 12738	**Unknown**	**Unknown**
X - 12739	**Unknown**	**Unknown**
X - 12740	**Unknown**	**Unknown**
X - 12753	**Unknown**	**Unknown**
X - 12798	**Unknown**	**Unknown**
X - 12812	**Unknown**	**Unknown**
X - 12814	**Unknown**	**Unknown**
X - 12816	**Unknown**	**Unknown**
X - 12818	**Unknown**	**Unknown**
X - 12819	**Unknown**	**Unknown**
X - 12822	**Unknown**	**Unknown**
X - 12830	**Unknown**	**Unknown**
X - 12831	**Unknown**	**Unknown**
X - 12844	**Unknown**	**Unknown**
X - 12846	**Unknown**	**Unknown**
X - 12847	**Unknown**	**Unknown**
X - 12849	**Unknown**	**Unknown**
X - 12851	**Unknown**	**Unknown**
X - 12906	**Unknown**	**Unknown**
X - 13431	**Unknown**	**Unknown**
X - 13435	**Unknown**	**Unknown**
X - 13529	**Unknown**	**Unknown**
X - 13553	**Unknown**	**Unknown**
X - 13658	**Unknown**	**Unknown**
X - 13684	**Unknown**	**Unknown**

X - 13723	**Unknown**	**Unknown**
X - 13729	**Unknown**	**Unknown**
X - 13737	**Unknown**	**Unknown**
X - 13835	**Unknown**	**Unknown**
X - 13844	**Unknown**	**Unknown**
X - 13846	**Unknown**	**Unknown**
X - 13866	**Unknown**	**Unknown**
X - 13891	**Unknown**	**Unknown**
X - 14056	**Unknown**	**Unknown**
X - 14314	**Unknown**	**Unknown**
X - 14568	**Unknown**	**Unknown**
X - 14626	**Unknown**	**Unknown**
X - 14658	**Unknown**	**Unknown**
X - 14662	**Unknown**	**Unknown**
X - 14838	**Unknown**	**Unknown**
X - 14939	**Unknown**	**Unknown**
X - 15245	**Unknown**	**Unknown**
X - 15461	**Unknown**	**Unknown**
X - 15469	**Unknown**	**Unknown**
X - 15486	**Unknown**	**Unknown**
X - 15492	**Unknown**	**Unknown**
X - 15497	**Unknown**	**Unknown**
X - 15503	**Unknown**	**Unknown**
X - 15666	**Unknown**	**Unknown**
X - 15728	**Unknown**	**Unknown**
X - 16071	**Unknown**	**Unknown**
X - 16083	**Unknown**	**Unknown**
X - 16087	**Unknown**	**Unknown**
X - 16124	**Unknown**	**Unknown**
X - 16132	**Unknown**	**Unknown**
X - 16135	**Unknown**	**Unknown**
X - 16397	**Unknown**	**Unknown**

X - 16570	**Unknown**	**Unknown**
X - 16576	**Unknown**	**Unknown**
X - 16580	**Unknown**	**Unknown**
X - 16649	**Unknown**	**Unknown**
X - 16654	**Unknown**	**Unknown**
X - 16935	**Unknown**	**Unknown**
X - 16938	**Unknown**	**Unknown**
X - 16944	**Unknown**	**Unknown**
X - 16946	**Unknown**	**Unknown**
X - 16947	**Unknown**	**Unknown**
X - 16964	**Unknown**	**Unknown**
X - 17010	**Unknown**	**Unknown**
X - 17145	**Unknown**	**Unknown**
X - 17146	**Unknown**	**Unknown**
X - 17162	**Unknown**	**Unknown**
X - 17185	**Unknown**	**Unknown**
X - 17189	**Unknown**	**Unknown**
X - 17299	**Unknown**	**Unknown**
X - 17306	**Unknown**	**Unknown**
X - 17325	**Unknown**	**Unknown**
X - 17327	**Unknown**	**Unknown**
X - 17328	**Unknown**	**Unknown**
X - 17335	**Unknown**	**Unknown**
X - 17337	**Unknown**	**Unknown**
X - 17340	**Unknown**	**Unknown**
X - 17343	**Unknown**	**Unknown**
X - 17348	**Unknown**	**Unknown**
X - 17350	**Unknown**	**Unknown**
X - 17351	**Unknown**	**Unknown**
X - 17353	**Unknown**	**Unknown**
X - 17354	**Unknown**	**Unknown**
X - 17355	**Unknown**	**Unknown**

X - 17357	**Unknown**	**Unknown**
X - 17359	**Unknown**	**Unknown**
X - 17361	**Unknown**	**Unknown**
X - 17367	**Unknown**	**Unknown**
X - 17398	**Unknown**	**Unknown**
X - 17438	**Unknown**	**Unknown**
X - 17469	**Unknown**	**Unknown**
X - 17653	**Unknown**	**Unknown**
X - 17654	**Unknown**	**Unknown**
X - 17655	**Unknown**	**Unknown**
X - 17673	**Unknown**	**Unknown**
X - 17676	**Unknown**	**Unknown**
X - 17677	**Unknown**	**Unknown**
X - 17685	**Unknown**	**Unknown**
X - 17690	**Unknown**	**Unknown**
X - 18249	**Unknown**	**Unknown**
X - 18345	**Unknown**	**Unknown**
X - 18603	**Unknown**	**Unknown**
X - 18779	**Unknown**	**Unknown**
X - 18886	**Unknown**	**Unknown**
X - 18887	**Unknown**	**Unknown**
X - 18889	**Unknown**	**Unknown**
X - 18899	**Unknown**	**Unknown**
X - 18901	**Unknown**	**Unknown**
X - 18913	**Unknown**	**Unknown**
X - 18914	**Unknown**	**Unknown**
X - 18921	**Unknown**	**Unknown**
X - 18922	**Unknown**	**Unknown**
X - 18938	**Unknown**	**Unknown**
X - 19141	**Unknown**	**Unknown**
X - 19183	**Unknown**	**Unknown**
X - 19438	**Unknown**	**Unknown**

X - 19561	**Unknown**	**Unknown**
X - 21258	**Unknown**	**Unknown**
X - 21285	**Unknown**	**Unknown**
X - 21286	**Unknown**	**Unknown**
X - 21295	**Unknown**	**Unknown**
X - 21310	**Unknown**	**Unknown**
X - 21312	**Unknown**	**Unknown**
X - 21318	**Unknown**	**Unknown**
X - 21319	**Unknown**	**Unknown**
X - 21339	**Unknown**	**Unknown**
X - 21343	**Unknown**	**Unknown**
X - 21353	**Unknown**	**Unknown**
X - 21358	**Unknown**	**Unknown**
X - 21364	**Unknown**	**Unknown**
X - 21365	**Unknown**	**Unknown**
X - 21383	**Unknown**	**Unknown**
X - 21410	**Unknown**	**Unknown**
X - 21411	**Unknown**	**Unknown**
X - 21441	**Unknown**	**Unknown**
X - 21442	**Unknown**	**Unknown**
X - 21444	**Unknown**	**Unknown**
X - 21448	**Unknown**	**Unknown**
X - 21467	**Unknown**	**Unknown**
X - 21470	**Unknown**	**Unknown**
X - 21471	**Unknown**	**Unknown**
X - 21474	**Unknown**	**Unknown**
X - 21607	**Unknown**	**Unknown**
X - 21626	**Unknown**	**Unknown**
X - 21657	**Unknown**	**Unknown**
X - 21658	**Unknown**	**Unknown**
X - 21659	**Unknown**	**Unknown**
X - 21662	**Unknown**	**Unknown**

X - 21666	**Unknown**	**Unknown**
X - 21668	**Unknown**	**Unknown**
X - 21729	**Unknown**	**Unknown**
X - 21735	**Unknown**	**Unknown**
X - 21736	**Unknown**	**Unknown**
X - 21737	**Unknown**	**Unknown**
X - 21752	**Unknown**	**Unknown**
X - 21792	**Unknown**	**Unknown**
X - 21795	**Unknown**	**Unknown**
X - 21796	**Unknown**	**Unknown**
X - 21803	**Unknown**	**Unknown**
X - 21807	**Unknown**	**Unknown**
X - 21815	**Unknown**	**Unknown**
X - 21821	**Unknown**	**Unknown**
X - 21829	**Unknown**	**Unknown**
X - 21834	**Unknown**	**Unknown**
X - 21840	**Unknown**	**Unknown**
X - 21845	**Unknown**	**Unknown**
X - 21849	**Unknown**	**Unknown**
X - 22035	**Unknown**	**Unknown**
X - 22102	**Unknown**	**Unknown**
X - 22145	**Unknown**	**Unknown**
X - 22147	**Unknown**	**Unknown**
X - 22162	**Unknown**	**Unknown**
X - 22379	**Unknown**	**Unknown**
X - 22412	**Unknown**	**Unknown**
X - 22475	**Unknown**	**Unknown**
X - 22509	**Unknown**	**Unknown**
X - 22515	**Unknown**	**Unknown**
X - 22520	**Unknown**	**Unknown**
X - 22716	**Unknown**	**Unknown**
X - 22764	**Unknown**	**Unknown**

X - 22771	**Unknown**	**Unknown**
X - 22775	**Unknown**	**Unknown**
X - 22834	**Unknown**	**Unknown**
X - 22836	**Unknown**	**Unknown**
X - 23160	**Unknown**	**Unknown**
X - 23196	**Unknown**	**Unknown**
X - 23283	**Unknown**	**Unknown**
X - 23291	**Unknown**	**Unknown**
X - 23293	**Unknown**	**Unknown**
X - 23294	**Unknown**	**Unknown**
X - 23295	**Unknown**	**Unknown**
X - 23297	**Unknown**	**Unknown**
X - 23314	**Unknown**	**Unknown**
X - 23369	**Unknown**	**Unknown**
X - 23585	**Unknown**	**Unknown**
X - 23587	**Unknown**	**Unknown**
X - 23588	**Unknown**	**Unknown**
X - 23590	**Unknown**	**Unknown**
X - 23593	**Unknown**	**Unknown**
X - 23636	**Unknown**	**Unknown**
X - 23637	**Unknown**	**Unknown**
X - 23639	**Unknown**	**Unknown**
X - 23641	**Unknown**	**Unknown**
X - 23644	**Unknown**	**Unknown**
X - 23648	**Unknown**	**Unknown**
X - 23649	**Unknown**	**Unknown**
X - 23652	**Unknown**	**Unknown**
X - 23662	**Unknown**	**Unknown**
X - 23665	**Unknown**	**Unknown**
X - 23666	**Unknown**	**Unknown**
X - 23669	**Unknown**	**Unknown**
X - 23671	**Unknown**	**Unknown**

X - 23680	**Unknown**	**Unknown**
X - 23739	**Unknown**	**Unknown**
X - 23749	**Unknown**	**Unknown**
X - 23756	**Unknown**	**Unknown**
X - 23765	**Unknown**	**Unknown**
X - 23780	**Unknown**	**Unknown**
X - 23782	**Unknown**	**Unknown**
X - 23997	**Unknown**	**Unknown**
X - 24020	**Unknown**	**Unknown**
X - 24027	**Unknown**	**Unknown**
X - 24106	**Unknown**	**Unknown**
X - 24129	**Unknown**	**Unknown**
X - 24243	**Unknown**	**Unknown**
X - 24272	**Unknown**	**Unknown**
X - 24293	**Unknown**	**Unknown**
X - 24295	**Unknown**	**Unknown**
X - 24306	**Unknown**	**Unknown**
X - 24307	**Unknown**	**Unknown**
X - 24309	**Unknown**	**Unknown**
X - 24328	**Unknown**	**Unknown**
X - 24329	**Unknown**	**Unknown**
X - 24337	**Unknown**	**Unknown**
X - 24411	**Unknown**	**Unknown**
X - 24422	**Unknown**	**Unknown**
X - 24431	**Unknown**	**Unknown**
X - 24432	**Unknown**	**Unknown**
X - 24435	**Unknown**	**Unknown**
X - 24449	**Unknown**	**Unknown**
X - 24452	**Unknown**	**Unknown**
X - 24455	**Unknown**	**Unknown**
X - 24456	**Unknown**	**Unknown**
X - 24462	**Unknown**	**Unknown**

X - 24475	**Unknown**	**Unknown**
X - 24490	**Unknown**	**Unknown**
X - 24512	**Unknown**	**Unknown**
X - 24513	**Unknown**	**Unknown**
X - 24527	**Unknown**	**Unknown**
X - 24529	**Unknown**	**Unknown**
X - 24531	**Unknown**	**Unknown**
X - 24542	**Unknown**	**Unknown**
X - 24544	**Unknown**	**Unknown**
X - 24546	**Unknown**	**Unknown**
X - 24549	**Unknown**	**Unknown**
X - 24550	**Unknown**	**Unknown**
X - 24551	**Unknown**	**Unknown**
X - 24552	**Unknown**	**Unknown**
X - 24554	**Unknown**	**Unknown**
X - 24555	**Unknown**	**Unknown**
X - 24557	**Unknown**	**Unknown**
X - 24558	**Unknown**	**Unknown**
X - 24559	**Unknown**	**Unknown**
X - 24561	**Unknown**	**Unknown**
X - 24568	**Unknown**	**Unknown**
X - 24571	**Unknown**	**Unknown**
X - 24576	**Unknown**	**Unknown**
X - 24580	**Unknown**	**Unknown**
X - 24585	**Unknown**	**Unknown**
X - 24588	**Unknown**	**Unknown**
X - 24637	**Unknown**	**Unknown**
X - 24682	**Unknown**	**Unknown**
X - 24693	**Unknown**	**Unknown**
X - 24699	**Unknown**	**Unknown**
X - 24736	**Unknown**	**Unknown**
X - 24738	**Unknown**	**Unknown**

X - 24747	**Unknown**	**Unknown**
X - 24748	**Unknown**	**Unknown**
X - 24765	**Unknown**	**Unknown**
X - 24766	**Unknown**	**Unknown**
X - 24803	**Unknown**	**Unknown**
X - 24804	**Unknown**	**Unknown**
X - 24806	**Unknown**	**Unknown**
X - 24809	**Unknown**	**Unknown**
X - 24812	**Unknown**	**Unknown**
X - 24813	**Unknown**	**Unknown**
X - 24831	**Unknown**	**Unknown**
X - 24832	**Unknown**	**Unknown**
X - 24845	**Unknown**	**Unknown**
X - 24849	**Unknown**	**Unknown**
X - 24870	**Unknown**	**Unknown**

References

Abe, K., Takahashi, A., Imaizumi, H., Hayashi, M., Okai, K., Kanno, Y., Watanabe, H. and Ohira, H. (2016) 'Interleukin-21 plays a critical role in the pathogenesis and severity of type I autoimmune hepatitis', *Springerplus,* 5(1), pp. 777.

Al-Naamani, N., Sagliani, K. D., Dolnikowski, G. G., Warburton, R. R., Toksoz, D., Kayyali, U., Hill, N. S., Fanburg, B. L., Roberts, K. E. and Preston, I. R. (2016) 'Plasma 12- and 15-hydroxyeicosanoids are predictors of survival in pulmonary arterial hypertension', *Pulm Circ,* 6(2), pp. 224-33.

Al-Shabrawey, M., Mussell, R., Kahook, K., Tawfik, A., Eladl, M., Sarthy, V., Nussbaum, J., El-Marakby, A., Park, S. Y., Gurel, Z., Sheibani, N. and Maddipati, K. R. (2011) 'Increased expression and activity of 12-lipoxygenase in oxygen-induced ischemic retinopathy and proliferative diabetic retinopathy: implications in retinal neovascularization', *Diabetes,* 60(2), pp. 614-24.

Alhadad, A., Miniati, M., Alhadad, H., Gottsäter, A. and Bajc, M. (2012) 'The value of tomographic ventilation/perfusion scintigraphy (V/PSPECT) for follow-up and prediction of recurrence in pulmonary embolism', *Thromb Res,* 130(6), pp. 877-81.

Alias, S., Redwan, B., Panzenboeck, A., Winter, M. P., Schubert, U., Voswinckel, R., Frey, M. K., Jakowitsch, J., Alimohammadi, A., Hobohm, L., Mangold, A., Bergmeister, H., Sibilia, M., Wagner, E. F., Mayer, E., Klepetko, W., Hoelzenbein, T. J., Preissner, K. T. and Lang, I. M. (2014) 'Defective angiogenesis delays thrombus resolution: a potential pathogenetic mechanism underlying chronic thromboembolic pulmonary hypertension', *Arterioscler Thromb Vasc Biol,* 34(4), pp. 810-819.

Ang, J. E., Revell, V., Mann, A., Mäntele, S., Otway, D. T., Johnston, J. D., Thumser, A. E., Skene, D. J. and Raynaud, F. (2012) 'Identification of human plasma metabolites exhibiting time-of-day variation using an untargeted liquid chromatography-mass spectrometry metabolomic approach', *Chronobiol Int,* 29(7), pp. 868-81.

Angelin, A., Gil-de-Gómez, L., Dahiya, S., Jiao, J., Guo, L., Levine, M. H., Wang, Z., Quinn, W. J., Kopinski, P. K., Wang, L., Akimova, T., Liu, Y., Bhatti, T. R., Han, R., Laskin, B. L., Baur, J. A., Blair, I. A., Wallace, D. C., Hancock, W. W. and Beier, U. H. (2017) 'Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments', *Cell Metab,* 25(6), pp. 1282-1293.e7.

Annibali, V., Ristori, G., Angelini, D. F., Serafini, B., Mechelli, R., Cannoni, S., Romano, S., Paolillo, A., Abderrahim, H., Diamantini, A., Borsellino, G., Aloisi, F., Battistini, L. and Salvetti, M. (2011) 'CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis', *Brain,* 134(Pt 2), pp. 542-54.

Annunziato, F., Romagnani, C. and Romagnani, S. (2015) 'The 3 major types of innate and adaptive cell-mediated effector immunity', *J Allergy Clin Immunol,* 135(3), pp. 626-35.

Apoil, P. A., Puissant-Lubrano, B., Congy-Jolivet, N., Peres, M., Tkaczuk, J., Roubinet, F. and Blancher, A. (2017) 'Reference values for T, B and NK human lymphocyte subpopulations in adults', *Data Brief,* 12, pp. 400-404.

Archer, S. L., Gomberg-Maitland, M., Maitland, M. L., Rich, S., Garcia, J. G. and Weir, E. K. (2008) 'Mitochondrial metabolism, redox signaling, and fusion: a mitochondria-ROS-HIF-1alpha-Kv1.5 O2-sensing pathway at the intersection of pulmonary hypertension and cancer', *Am J Physiol Heart Circ Physiol,* 294(2), pp. H570-8.

Arroyo-Villa, I., Bautista-Caro, M. B., Balsa, A., Aguado-Acín, P., Bonilla-Hernán, M. G., Plasencia, C., Villalba, A., Nuño, L., Puig-Kröger, A., Martín-Mola, E. and Miranda-Carús, M. E. (2014) 'Constitutively altered frequencies of circulating follicullar helper T cell counterparts and their subsets in rheumatoid arthritis', *Arthritis Res Ther,* 16(6), pp. 500.

Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D. and Isner, J. M. (1998) 'Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization', *Circ Res,* 83(3), pp. 233-40.

Asano, K., Ikegami, H., Fujisawa, T., Nishino, M., Nojima, K., Kawabata, Y., Noso, S., Hiromine, Y., Fukai, A. and Ogihara, T. (2007) 'Molecular scanning of interleukin-21 gene and genetic susceptibility to type 1 diabetes', *Hum Immunol,* 68(5), pp. 384-91.

Asherson, R. A., Harris, E. N., Bernstein, R. M., Mackworth-Young, C. G. and Hughes, G. R. (1984) 'Immunological studies in 'primary' idiopathic pulmonary hypertension', *Eur J Rheumatol Inflamm,* 7(3), pp. 75-9.

Ataga, K. I., Cappellini, M. D. and Rachmilewitz, E. A. (2007) 'Beta-thalassaemia and sickle cell anaemia as paradigms of hypercoagulability', *Br J Haematol,* 139(1), pp. 3-13.

Atkinson, J. E., Olson, J. W., Altiere, R. J. and Gillespie, M. N. (1987) 'Evidence that hypoxic pulmonary vascular remodeling in rats is polyamine dependent', *J Appl Physiol (1985),* 62(4), pp. 1562-8.

Attridge, K., Wang, C. J., Wardzinski, L., Kenefeck, R., Chamberlain, J. L., Manzotti, C., Kopf, M. and Walker, L. S. (2012) 'IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis', *Blood,* 119(20), pp. 4656-64.

Aubert, G., Martin, O. J., Horton, J. L., Lai, L., Vega, R. B., Leone, T. C., Koves, T., Gardell, S. J., Krüger, M., Hoppel, C. L., Lewandowski, E. D., Crawford, P. A., Muoio, D. M. and Kelly, D. P. (2016) 'The Failing Heart Relies on Ketone Bodies as a Fuel', *Circulation,* 133(8), pp. 698-705.

Austin, E. D. and Loyd, J. E. (2014) 'The genetics of pulmonary arterial hypertension', *Circ Res,* 115(1), pp. 189-202.

Austin, E. D., Ma, L., LeDuc, C., Berman Rosenzweig, E., Borczuk, A., Phillips, J. A., Palomero, T., Sumazin, P., Kim, H. R., Talati, M. H., West, J., Loyd, J. E. and Chung, W. K. (2012) 'Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension', *Circ Cardiovasc Genet,* 5(3), pp. 336-43.

Austin, E. D., Rock, M. T., Mosse, C. A., Vnencak-Jones, C. L., Yoder, S. M., Robbins, I. M., Loyd, J. E. and Meyrick, B. O. (2010a) 'T lymphocyte subset abnormalities in the blood and lung in pulmonary arterial hypertension', *Respir Med,* 104(3), pp. 454-62.

Austin, E. D., Rock, M. T., Mosse, C. A., Vnencak-Jones, C. L., Yoder, S. M., Robbins, I. M., Loyd, J. E. and Meyrick, B. O. (2010b) 'T lymphocyte subset abnormalities in the blood and lung in pulmonary arterial hypertension', *Respiratory Medicine,* 104(3), pp. 454-462.

Azizi, M. H., Nayernouri, T. and Azizi, F. (2008) 'A brief history of the discovery of the circulation of blood in the human body', *Arch Iran Med,* 11(3), pp. 345-50.

Baccala, R. and Theofilopoulos, A. N. (2005) 'The new paradigm of T-cell homeostatic proliferation-induced autoimmunity', *Trends Immunol,* 26(1), pp. 5-8.

Badesch, D. B., Raskob, G. E., Elliott, C. G., Krichman, A. M., Farber, H. W., Frost, A. E., Barst, R. J., Benza, R. L., Liou, T. G., Turner, M., Giles, S., Feldkircher, K., Miller, D. P. and McGoon, M. D. (2010) 'Pulmonary arterial hypertension: baseline characteristics from the REVEAL Registry', *Chest,* 137(2), pp. 376-87.

Bakhle, Y. and Ferreira, S. (2011) 'Lung Metabolism of Eicosanoids: Prostaglandins, Prostacyclin, Thromboxane, and Leukotrienes', *Comprehensive Physiology*: John Wiley & Sons, Inc., pp. 365–386.

Balabanian, K., Foussat, A., Dorfmüller, P., Durand-Gasselin, I., Capel, F., Bouchet-Delbos, L., Portier, A., Marfaing-Koka, A., Krzysiek, R., Rimaniol, A. C., Simonneau, G., Emilie, D. and Humbert, M. (2002) 'CX(3)C chemokine fractalkine in pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 165(10), pp. 1419-25.

Balion, C. M., Santaguida, P., McKelvie, R., Hill, S. A., McQueen, M. J., Worster, A. and Raina, P. S. (2008) 'Physiological, pathological, pharmacological, biochemical and hematological factors affecting BNP and NT-proBNP', *Clin Biochem,* 41(4-5), pp. 231-9.

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J. and Ahmed, R. (2006) 'Restoring function in exhausted CD8 T cells during chronic viral infection', *Nature,* 439(7077), pp. 682-7.

Barnes, J. W., Tian, L., Heresi, G. A., Farver, C. F., Asosingh, K., Comhair, S. A., Aulak, K. S. and Dweik, R. A. (2015) 'O-linked β-N-acetylglucosamine transferase directs cell proliferation in idiopathic pulmonary arterial hypertension', *Circulation,* 131(14), pp. 1260-8.

Barrett, S. P., Toh, B. H., Alderuccio, F., van Driel, I. R. and Gleeson, P. A. (1995) 'Organ-specific autoimmunity induced by adult thymectomy and cyclophosphamide-induced lymphopenia', *Eur J Immunol,* 25(1), pp. 238-44.

Bartke, N. and Hannun, Y. A. (2009) 'Bioactive sphingolipids: metabolism and function', *J Lipid Res,* 50 Suppl, pp. S91-6.

Bartuś, M., Łomnicka, M., Kostogrys, R. B., Kaźmierczak, P., Watała, C., Słominska, E. M., Smoleński, R. T., Pisulewski, P. M., Adamus, J., Gebicki, J. and Chlopicki, S. (2008) '1-Methylnicotinamide (MNA) prevents endothelial dysfunction in hypertriglyceridemic and diabetic rats', *Pharmacol Rep,* 60(1), pp. 127-38.

Bassit, R. A., Sawada, L. A., Bacurau, R. F., Navarro, F., Martins, E., Santos, R. V., Caperuto, E. C., Rogeri, P. and Costa Rosa, L. F. (2002) 'Branched-chain amino acid supplementation and the immune response of long-distance athletes', *Nutrition,* 18(5), pp. 376-9.

Batt, J., Ahmed, S. S., Correa, J., Bain, A. and Granton, J. (2014) 'Skeletal muscle dysfunction in idiopathic pulmonary arterial hypertension', *Am J Respir Cell Mol Biol,* 50(1), pp. 74-86.

Bedi, K. C., Snyder, N. W., Brandimarto, J., Aziz, M., Mesaros, C., Worth, A. J., Wang, L. L., Javaheri, A., Blair, I. A., Margulies, K. B. and Rame, J. E. (2016) 'Evidence for Intramyocardial Disruption of Lipid Metabolism and Increased Myocardial Ketone Utilization in Advanced Human Heart Failure', *Circulation,* 133(8), pp. 706-16.

Benza, R. L., Miller, D. P., Gomberg-Maitland, M., Frantz, R. P., Foreman, A. J., Coffey, C. S., Frost, A., Barst, R. J., Badesch, D. B., Elliott, C. G., Liou, T. G. and McGoon, M. D. (2010) 'Predicting survival in pulmonary arterial hypertension: insights from the Registry to Evaluate Early and Long-Term Pulmonary Arterial Hypertension Disease Management (REVEAL)', *Circulation,* 122(2), pp. 164-72.

Bertero, T., Oldham, W. M., Cottrill, K. A., Pisano, S., Vanderpool, R. R., Yu, Q., Zhao, J., Tai, Y., Tang, Y., Zhang, Y. Y., Rehman, S., Sugahara, M., Qi, Z., Gorcsan, J., Vargas, S. O., Saggar, R., Wallace, W. D., Ross, D. J., Haley, K. J., Waxman, A. B., Parikh, V. N., De Marco, T., Hsue, P. Y., Morris, A., Simon, M. A., Norris, K. A., Gaggioli, C., Loscalzo, J., Fessel, J. and Chan, S. Y. (2016) 'Vascular stiffness mechanoactivates YAP/TAZ-dependent glutaminolysis to drive pulmonary hypertension', *J Clin Invest,* 126(9), pp. 3313-35.

Berton, R., Conceição, M. S., Libardi, C. A., Canevarolo, R. R., Gáspari, A. F., Chacon-Mikahil, M. P., Zeri, A. C. and Cavaglieri, C. R. (2017) 'Metabolic time-course response after resistance exercise: A metabolomics approach', *J Sports Sci,* 35(12), pp. 1211-1218.

Bettencourt, I. A. and Powell, J. D. (2017) 'Targeting Metabolism as a Novel Therapeutic Approach to Autoimmunity, Inflammation, and Transplantation', *J Immunol,* 198(3), pp. 999-1005.

Bhargava, P., Li, C., Stanya, K. J., Jacobi, D., Dai, L., Liu, S., Gangl, M. R., Harn, D. A. and Lee, C. H. (2012) 'Immunomodulatory glycan LNFPIII alleviates hepatosteatosis and insulin resistance through direct and indirect control of metabolic pathways', *Nat Med,* 18(11), pp. 1665-72.

Bhatt, S., Matthews, J., Parvin, S., Sarosiek, K. A., Zhao, D., Jiang, X., Isik, E., Letai, A. and Lossos, I. S. (2015) 'Direct and immune-mediated cytotoxicity of interleukin-21 contributes to antitumor effects in mantle cell lymphoma', *Blood,* 126(13), pp. 1555-64.

Bhattacharya, J., Nanjo, S. and Staub, N. C. (1982) 'Factors affecting lung microvascular pressure', *Ann N Y Acad Sci,* 384, pp. 107-14.

Bhattacharya, J. and Staub, N. C. (1980) 'Direct measurement of microvascular pressures in the isolated perfused dog lung', *Science,* 210(4467), pp. 327-8.

Biedroń, R., Ciszek, M., Tokarczyk, M., Bobek, M., Kurnyta, M., Słominska, E. M., Smoleński, R. T. and Marcinkiewicz, J. (2008) '1-Methylnicotinamide and nicotinamide: two related anti-inflammatory agents that differentially affect the functions of activated macrophages', *Arch Immunol Ther Exp (Warsz),* 56(2), pp. 127-34.

Boer, F. (2003) 'Drug handling by the lungs', *Br J Anaesth,* 91(1), pp. 50-60.

Bohnhorst, J., Bjørgan, M. B., Thoen, J. E., Natvig, J. B. and Thompson, K. M. (2001a) 'Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjögren's syndrome', *J Immunol,* 167(7), pp. 3610-8.

Bohnhorst, J. O., Thoen, J. E., Natvig, J. B. and Thompson, K. M. (2001b) 'Significantly depressed percentage of CD27+ (memory) B cells among peripheral blood B cells in patients with primary Sjögren's syndrome', *Scand J Immunol,* 54(4), pp. 421-7.

Bonafé, L., Troxler, H., Kuster, T., Heizmann, C. W., Chamoles, N. A., Burlina, A. B. and Blau, N. (2000) 'Evaluation of urinary acylglycines by electrospray tandem mass spectrometry in mitochondrial energy metabolism defects and organic acidurias', *Mol Genet Metab,* 69(4), pp. 302-11.

Bonderman, D., Wilkens, H., Wakounig, S., Schäfers, H. J., Jansa, P., Lindner, J., Simkova, I., Martischnig, A. M., Dudczak, J., Sadushi, R., Skoro-Sajer, N., Klepetko, W. and Lang, I. M. (2009) 'Risk factors for chronic thromboembolic pulmonary hypertension', *Eur Respir J,* 33(2), pp. 325-31.

Bonelli, M., Savitskaya, A., von Dalwigk, K., Steiner, C. W., Aletaha, D., Smolen, J. S. and Scheinecker, C. (2008) 'Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE)', *Int Immunol,* 20(7), pp. 861-8.

Bonnet, D., Martin, D., Pascale De Lonlay, Villain, E., Jouvet, P., Rabier, D., Brivet, M. and Saudubray, J. M. (1999) 'Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children', *Circulation,* 100(22), pp. 2248-53.

Bonnet, S., Dumas-de-La-Roque, E., Bégueret, H., Marthan, R., Fayon, M., Dos Santos, P., Savineau, J. P. and Baulieu, E. E. (2003) 'Dehydroepiandrosterone (DHEA) prevents and reverses chronic hypoxic pulmonary hypertension', *Proc Natl Acad Sci U S A,* 100(16), pp. 9488-93.

Boudonck, K. J., Mitchell, M. W., Német, L., Keresztes, L., Nyska, A., Shinar, D. and Rosenstock, M. (2009) 'Discovery of metabolomics biomarkers for early detection of nephrotoxicity', *Toxicol Pathol,* 37(3), pp. 280-92.

Braverman, N. E. and Moser, A. B. (2012) 'Functions of plasmalogen lipids in health and disease', *Biochim Biophys Acta,* 1822(9), pp. 1442-52.

Brenner, O. (1935) 'Pathology of the vessels of the pulmonary circulation part I', *Arch Intern Med (Chic). ,* 56(2), pp. 211-237.

Brittain, E. L. and Chan, S. Y. (2016) 'Integration of complex data sources to provide biologic insight into pulmonary vascular disease (2015 Grover Conference Series)', *Pulm Circ,* 6(3), pp. 251-60.

Brittain, E. L., Talati, M., Fessel, J. P., Zhu, H., Penner, N., Calcutt, M. W., West, J. D., Funke, M., Lewis, G. D., Gerszten, R. E., Hamid, R., Pugh, M. E., Austin, E. D., Newman, J. H. and Hemnes, A. R. (2016) 'Fatty Acid Metabolic Defects and Right Ventricular Lipotoxicity in Human Pulmonary Arterial Hypertension', *Circulation,* 133(20), pp. 1936-44.

Broshtilova, V., Lozanov, V. and Miteva, L. (2013) 'Polyamine metabolism changes in psoriasis', *Indian J Dermatol,* 58(4), pp. 306-9.

Brown, C. C., Esterhazy, D., Sarde, A., London, M., Pullabhatla, V., Osma-Garcia, I., Al-Bader, R., Ortiz, C., Elgueta, R., Arno, M., de Rinaldis, E., Mucida, D., Lord, G. M. and Noelle, R. J. (2015) 'Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program', *Immunity,* 42(3), pp. 499-511.

Bucerius, J. (2016) 'Monitoring Vasculitis with 18F-FDG PET', *Q J Nucl Med Mol Imaging,* 60(3), pp. 219-35.

Bujak, R., Mateo, J., Blanco, I., Izquierdo-García, J. L., Dudzik, D., Markuszewski, M. J., Peinado, V. I., Laclaustra, M., Barberá, J. A., Barbas, C. and Ruiz-Cabello, J. (2016) 'New Biochemical Insights into the Mechanisms of Pulmonary Arterial Hypertension in Humans', *PLoS One,* 11(8), pp. e0160505.

Bulati, M., Buffa, S., Candore, G., Caruso, C., Dunn-Walters, D. K., Pellicanò, M., Wu, Y. C. and Colonna Romano, G. (2011) 'B cells and immunosenescence: a focus on IgG+IgD-CD27- (DN) B cells in aged humans', *Ageing Res Rev,* 10(2), pp. 274-84.

Bulati, M., Buffa, S., Martorana, A., Gervasi, F., Camarda, C., Azzarello, D. M., Monastero, R., Caruso, C. and Colonna-Romano, G. (2015) 'Double negative (IgG+IgD-CD27-) B cells are increased in a cohort of moderate-severe Alzheimer's disease patients and show a pro-inflammatory trafficking receptor phenotype', *J Alzheimers Dis,* 44(4), pp. 1241-51.

Bull, T. M., Cool, C. D., Serls, A. E., Rai, P. R., Parr, J., Neid, J. M., Geraci, M. W., Campbell, T. B., Voelkel, N. F. and Badesch, D. B. (2003) 'Primary pulmonary hypertension, Castleman's disease and human herpesvirus-8', *Eur Respir J,* 22(3), pp. 403-7.

Byersdorfer, C. A., Tkachev, V., Opipari, A. W., Goodell, S., Swanson, J., Sandquist, S., Glick, G. D. and Ferrara, J. L. (2013) 'Effector T cells require fatty acid metabolism during murine graft-versus-host disease', *Blood,* 122(18), pp. 3230-7.

Calder, P. C. (2006) 'Branched-chain amino acids and immunity', *J Nutr,* 136(1 Suppl), pp. 288S-93S.

Cappellini, M. D. (2007) 'Coagulation in the pathophysiology of hemolytic anemias', *Hematology Am Soc Hematol Educ Program*, pp. 74-8.

Caruso, R., Costanzo, A. and Monteleone, G. (2009) 'Pathogenic role of interleukin-21 in psoriasis', *Cell Cycle,* 8(22), pp. 3629-30.

Casero, R. A. and Marton, L. J. (2007) 'Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases', *Nat Rev Drug Discov,* 6(5), pp. 373-90.

Cha, Y. J., Kim, D. H., Jung, W. H. and Koo, J. S. (2014) 'Expression of sarcosine metabolism-related proteins according to metastatic site in breast cancer', *Int J Clin Exp Pathol,* 7(11), pp. 7824-33.

Chaisson, N. F. and Hassoun, P. M. (2013) 'Systemic sclerosis-associated pulmonary arterial hypertension', *Chest,* 144(4), pp. 1346-56.

Cham, C. M., Driessens, G., O'Keefe, J. P. and Gajewski, T. F. (2008) 'Glucose deprivation inhibits multiple key gene expression events and effector functions in CD8+ T cells', *Eur J Immunol,* 38(9), pp. 2438-50.

Champion, H. C., Michelakis, E. D. and Hassoun, P. M. (2009) 'Comprehensive invasive and noninvasive approach to the right ventricle-pulmonary circulation unit: state of the art and clinical and research implications', *Circulation,* 120(11), pp. 992-1007.

Chan, S. Y. and Loscalzo, J. (2012) 'The emerging paradigm of network medicine in the study of human disease', *Circ Res,* 111(3), pp. 359-74.

Chao, P. Z., Hsieh, M. S., Lee, F. P., Chen, S. Y., Cheng, C. W., Chang, H. W., Lin, Y. T., Ting, L. L., Lin, Y. F. and Chen, C. H. (2015) 'Serum level of interleukin-21 is elevated in chronic rhinosinusitis', *Am J Rhinol Allergy,* 29(1), pp. e1-6.

Che, Y., Qiu, J., Jin, T., Yin, F., Li, M. and Jiang, Y. (2016) 'Circulating memory T follicular helper subsets, Tfh2 and Tfh17, participate in the pathogenesis of Guillain-Barré syndrome', *Sci Rep,* 6, pp. 20963.

Chida, A., Shintani, M., Matsushita, Y., Sato, H., Eitoku, T., Nakayama, T., Furutani, Y., Hayama, E., Kawamura, Y., Inai, K., Ohtsuki, S., Saji, T., Nonoyama, S. and Nakanishi, T. (2014) 'Mutations of NOTCH3 in childhood pulmonary arterial hypertension', *Mol Genet Genomic Med,* 2(3), pp. 229-39.

Chida, A., Shintani, M., Nakayama, T., Furutani, Y., Hayama, E., Inai, K., Saji, T., Nonoyama, S. and Nakanishi, T. (2012) 'Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension', *Circ J,* 76(6), pp. 1501-8.

Chimenti, M. S., Triggianese, P., Conigliaro, P., Candi, E., Melino, G. and Perricone, R. (2015) 'The interplay between inflammation and metabolism in rheumatoid arthritis', *Cell Death Dis,* 6, pp. e1887.

Chua, J., Zhou, W., Ho, J. K., Patel, N. A., Mackensen, G. B. and Mahajan, A. (2013) 'Acute right ventricular pressure overload compromises left ventricular function by altering septal strain and rotation', *J Appl Physiol (1985),* 115(2), pp. 186-93.

Cohen, S., Danzaki, K. and MacIver, N. J. (2017) 'Nutritional effects on T-cell immunometabolism', *Eur J Immunol,* 47(2), pp. 225-235.

Cohn, L., Elias, J. A. and Chupp, G. L. (2004) 'Asthma: mechanisms of disease persistence and progression', *Annu Rev Immunol,* 22, pp. 789-815.

Colonna-Romano, G., Bulati, M., Aquino, A., Pellicanò, M., Vitello, S., Lio, D., Candore, G. and Caruso, C. (2009) 'A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people', *Mech Ageing Dev,* 130(10), pp. 681-90.

Comroe, J. H. (1966) 'The main functions of the pulmonary circulation', *Circulation,* 33(1), pp. 146-58.

Cool, C. D., Voelkel, N. F. and Bull, T. (2011) 'Viral infection and pulmonary hypertension: is there an association?', *Expert Rev Respir Med,* 5(2), pp. 207-16.

Cosmi, B., Nijkeuter, M., Valentino, M., Huisman, M. V., Barozzi, L. and Palareti, G. (2011) 'Residual emboli on lung perfusion scan or multidetector computed tomography after a first episode of acute pulmonary embolism', *Intern Emerg Med,* 6(6), pp. 521-8.

Cotter, D. G., Schugar, R. C. and Crawford, P. A. (2013) 'Ketone body metabolism and cardiovascular disease', *Am J Physiol Heart Circ Physiol,* 304(8), pp. H1060-76.

Cournand, A., Lauson, H. and Bloomfield, R. (1944) 'Recording of right heart pressures in man', *Proc Soc Exp Biol Med. ,* (55), pp. 34-36.

Cournand, A. and Ranges, H. (1941) Catheterisation of the right auricle in man.

Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. D., Richards, D. W., Lester, M. S. and Jones, M. (1945) ' Measurement of cardiac output in man using the technique of catheterization of the right auricle or ventricle', *J Clin Invest, 24(1)*, pp. 106-16

Crivellato, E. (2011) 'The role of angiogenic growth factors in organogenesis', *Int J Dev Biol,* 55(4-5), pp. 365-75.

Cugno, M., Ingegnoli, F., Gualtierotti, R. and Fantini, F. (2010) 'Potential effect of anti-tumour necrosis factor-alpha treatment on reducing the cardiovascular risk related to rheumatoid arthritis', *Curr Vasc Pharmacol,* 8(2), pp. 285-92.

D'Alonzo, G. E., Barst, R. J., Ayres, S. M., Bergofsky, E. H., Brundage, B. H., Detre, K. M., Fishman, A. P., Goldring, R. M., Groves, B. M. and Kernis, J. T. (1991) 'Survival in patients with primary pulmonary hypertension. Results from a national prospective registry', *Ann Intern Med,* 115(5), pp. 343-9.

Dang, C. V. (2010) 'Glutaminolysis: supplying carbon or nitrogen or both for cancer cells?', *Cell Cycle,* 9(19), pp. 3884-6.

Dassanayaka, S. and Jones, S. P. (2014) 'O-GlcNAc and the cardiovascular system', *Pharmacol Ther,* 142(1), pp. 62-71.

Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., Mackey, E. W., Miller, J. D., Leslie, A. J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E. J., Coovadia, H. M., Goulder, P. J., Klenerman, P., Ahmed, R., Freeman, G. J. and Walker, B. D. (2006) 'PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression', *Nature,* 443(7109), pp. 350-4.

De Milito, A., Mörch, C., Sönnerborg, A. and Chiodi, F. (2001) 'Loss of memory (CD27) B lymphocytes in HIV-1 infection', *AIDS,* 15(8), pp. 957-64.

Delcroix, M., Lang, I., Pepke-Zaba, J., Jansa, P., D'Armini, A. M., Snijder, R., Bresser, P., Torbicki, A., Mellemkjaer, S., Lewczuk, J., Simkova, I., Barberà, J. A., de Perrot, M., Hoeper, M. M., Gaine, S., Speich, R., Gomez-Sanchez, M. A., Kovacs, G., Jaïs, X., Ambroz, D., Treacy, C., Morsolini, M., Jenkins, D., Lindner, J., Dartevelle, P., Mayer, E. and Simonneau, G. (2016) 'Long-Term Outcome of Patients With Chronic Thromboembolic Pulmonary Hypertension: Results From an International Prospective Registry', *Circulation,* 133(9), pp. 859-71.

deMello, D. E. and Reid, L. M. (2000) 'Embryonic and early fetal development of human lung vasculature and its functional implications', *Pediatr Dev Pathol,* 3(5), pp. 439-49.

Deng, Z., Morse, J. H., Slager, S. L., Cuervo, N., Moore, K. J., Venetos, G., Kalachikov, S., Cayanis, E., Fischer, S. G., Barst, R. J., Hodge, S. E. and Knowles, J. A. (2000) 'Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene', *Am J Hum Genet,* 67(3), pp. 737-44.

Dorfmüller, P. (2013) 'Pulmonary hypertension: pathology', *Handb Exp Pharmacol,* 218, pp. 59-75.

Dorfmüller, P., Humbert, M., Perros, F., Sanchez, O., Simonneau, G., Müller, K. M. and Capron, F. (2007) 'Fibrous remodeling of the pulmonary venous system in pulmonary arterial hypertension associated with connective tissue diseases', *Hum Pathol,* 38(6), pp. 893-902.

Dorfmüller, P., Perros, F., Balabanian, K. and Humbert, M. (2003) 'Inflammation in pulmonary arterial hypertension', *Eur Respir J,* 22(2), pp. 358-63.

Dorfmüller, P., Zarka, V., Durand-Gasselin, I., Monti, G., Balabanian, K., Garcia, G., Capron, F., Coulomb-Lherminé, A., Marfaing-Koka, A., Simonneau, G., Emilie, D. and Humbert, M. (2002) 'Chemokine RANTES in severe pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 165(4), pp. 534-9.

Doughty, C. A., Bleiman, B. F., Wagner, D. J., Dufort, F. J., Mataraza, J. M., Roberts, M. F. and Chiles, T. C. (2006) 'Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth', *Blood,* 107(11), pp. 4458-65.

Dromparis, P., Sutendra, G. and Michelakis, E. D. (2010) 'The role of mitochondria in pulmonary vascular remodeling', *J Mol Med (Berl),* 88(10), pp. 1003-10.

Dumas de la Roque, E., Quignard, J. F., Ducret, T., Dahan, D., Courtois, A., Begueret, H., Marthan, R. and Savineau, J. P. (2013) 'Beneficial effect of dehydroepiandrosterone on pulmonary hypertension in a rodent model of pulmonary hypertension in infants', *Pediatr Res,* 74(2), pp. 163-9.

Dupuis, J., Cernacek, P., Tardif, J. C., Stewart, D. J., Gosselin, G., Dyrda, I., Bonan, R. and Crépeau, J. (1998) 'Reduced pulmonary clearance of endothelin-1 in pulmonary hypertension', *Am Heart J,* 135(4), pp. 614-20.

Duquenne, C., Cornec, D., Marhadour, T., Jousse-Joulin, S., Cantagrel, A., Pavy, S., Devauchelle-Pensec, V. and Saraux, A. (2015) 'Lymphopenia in early arthritis: Impact on diagnosis and 3-year outcomes (ESPOIR cohort)', *Joint Bone Spine,* 82(6), pp. 417-22.

Duraiswamy, J., Ibegbu, C. C., Masopust, D., Miller, J. D., Araki, K., Doho, G. H., Tata, P., Gupta, S., Zilliox, M. J., Nakaya, H. I., Pulendran, B., Haining, W. N., Freeman, G. J. and Ahmed, R. (2011) 'Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults', *J Immunol,* 186(7), pp. 4200-12.

Dweik, R. A., Rounds, S., Erzurum, S. C., Archer, S., Fagan, K., Hassoun, P. M., Hill, N. S., Humbert, M., Kawut, S. M., Krowka, M., Michelakis, E., Morrell, N. W., Stenmark, K., Tuder, R. M., Newman, J. and Phenotypes, A. C. o. P. H. (2014) 'An official American Thoracic Society Statement: pulmonary hypertension phenotypes', *Am J Respir Crit Care Med,* 189(3), pp. 345-55.

Edwards, A. L., Gunningham, S. P., Clare, G. C., Hayman, M. W., Smith, M., Frampton, C. M., Robinson, B. A., Troughton, R. W. and Beckert, L. E. (2013) 'Professional killer cell deficiencies and decreased survival in pulmonary arterial hypertension', *Respirology,* 18(8), pp. 1271-7.

Eisenberg, T., Abdellatif, M., Schroeder, S., Primessnig, U., Stekovic, S., Pendl, T., Harger, A., Schipke, J., Zimmermann, A., Schmidt, A., Tong, M., Ruckenstuhl, C., Dammbrueck, C., Gross, A. S., Herbst, V., Magnes, C., Trausinger, G., Narath, S., Meinitzer, A., Hu, Z., Kirsch, A., Eller, K., Carmona-Gutierrez, D., Büttner, S., Pietrocola, F., Knittelfelder, O., Schrepfer, E., Rockenfeller, P., Simonini, C., Rahn, A., Horsch, M., Moreth, K., Beckers, J., Fuchs, H., Gailus-Durner, V., Neff, F., Janik, D., Rathkolb, B., Rozman, J., de Angelis, M. H., Moustafa, T., Haemmerle, G., Mayr, M., Willeit, P., von Frieling-Salewsky, M., Pieske, B., Scorrano, L., Pieber, T., Pechlaner, R., Willeit, J., Sigrist, S. J., Linke, W. A., Mühlfeld, C., Sadoshima, J.,

Dengjel, J., Kiechl, S., Kroemer, G., Sedej, S. and Madeo, F. (2016) 'Cardioprotection and lifespan extension by the natural polyamine spermidine', *Nat Med,* 22(12), pp. 1428-1438.

El Alwani, M., Wu, B. X., Obeid, L. M. and Hannun, Y. A. (2006) 'Bioactive sphingolipids in the modulation of the inflammatory response', *Pharmacol Ther,* 112(1), pp. 171-83.

Engelmann, B. (2004) 'Plasmalogens: targets for oxidants and major lipophilic antioxidants', *Biochem Soc Trans,* 32(Pt 1), pp. 147-50.

Fartoukh, M., Emilie, D., Le Gall, C., Monti, G., Simonneau, G. and Humbert, M. (1998) 'Chemokine macrophage inflammatory protein-1alpha mRNA expression in lung biopsy specimens of primary pulmonary hypertension', *Chest,* 114(1 Suppl), pp. 50S-51S.

Faul, J. L., Nishimura, T., Berry, G. J., Benson, G. V., Pearl, R. G. and Kao, P. N. (2000) 'Triptolide attenuates pulmonary arterial hypertension and neointimal formation in rats', *Am J Respir Crit Care Med,* 162(6), pp. 2252-8.

Fedorowicz, A., Mateuszuk, Ł., Kopec, G., Skórka, T., Kutryb-Zając, B., Zakrzewska, A., Walczak, M., Jakubowski, A., Łomnicka, M., Słomińska, E. and Chlopicki, S. (2016) 'Activation of the nicotinamide N-methyltransferase (NNMT)-1-methylnicotinamide (MNA) pathway in pulmonary hypertension', *Respir Res,* 17(1), pp. 108.

Fessel, J. P., Hamid, R., Wittmann, B. M., Robinson, L. J., Blackwell, T., Tada, Y., Tanabe, N., Tatsumi, K., Hemnes, A. R. and West, J. D. (2012) 'Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming', *Pulm Circ,* 2(2), pp. 201-13.

Fessel, J. P., Loyd, J. E. and Austin, E. D. (2011) 'The genetics of pulmonary arterial hypertension in the post-BMPR2 era', *Pulm Circ,* 1(3), pp. 305-19.

Fisher, A. B. (1984) 'Intermediary metabolism of the lung', *Environ Health Perspect,* 55, pp. 149-58.

Forcade, E., Kim, H. T., Cutler, C., Wang, K., Alho, A. C., Nikiforow, S., Ho, V. T., Koreth, J., Armand, P., Alyea, E. P., Blazar, B. R., Soiffer, R. J., Antin, J. H. and Ritz, J. (2016) 'Circulating T follicular helper cells with increased function during chronic graft-versus-host disease', *Blood,* 127(20), pp. 2489-97.

Forfia, P. R., Fisher, M. R., Mathai, S. C., Housten-Harris, T., Hemnes, A. R., Borlaug, B. A., Chamera, E., Corretti, M. C., Champion, H. C., Abraham, T. P., Girgis, R. E. and Hassoun, P. M. (2006) 'Tricuspid annular displacement predicts survival in pulmonary hypertension', *Am J Respir Crit Care Med,* 174(9), pp. 1034-41.

Franceschelli, S., Ferrone, A., Pesce, M., Riccioni, G. and Speranza, L. (2013) 'Biological functional relevance of asymmetric dimethylarginine (ADMA) in cardiovascular disease', *Int J Mol Sci,* 14(12), pp. 24412-21.

Frank L. Powell, P. D. W. a. J. B. W., 4 - , In , edited by V. Courtney Broaddus, Robert J. Mason, Joel D. Ernst, Talmadge E. King, Stephen C. Lazarus, John F. Murray, Jay A. Nadel, Arthur S. Slutsky and Michael B. Gotway, W.B. Saunders, Philadelphia, 2016, Pages 44-75. '4- Ventilation, Blood Flow, and Gas Exchange'.

Frauwirth, K. A. and Thompson, C. B. (2004) 'Regulation of T lymphocyte metabolism', *J Immunol,* 172(8), pp. 4661-5.

Frost, A. E., Badesch, D. B., Barst, R. J., Benza, R. L., Elliott, C. G., Farber, H. W., Krichman, A., Liou, T. G., Raskob, G. E., Wason, P., Feldkircher, K., Turner, M. and McGoon, M. D. (2011) 'The changing picture of patients with pulmonary arterial hypertension in the United States: how REVEAL differs from historic and non-US Contemporary Registries', *Chest,* 139(1), pp. 128-37.

Gaddum, J., Hebb, C., Silver, A. and Swan, A. (1953) '5-Hydroxytryptamine; pharmacological action and destruction in perfused lungs', *Q J Exp Physiol Cogn Med Sci,* 38(4), pp. 255-62.

Galiè, N., Humbert, M., Vachiery, J. L., Gibbs, S., Lang, I., Torbicki, A., Simonneau, G., Peacock, A., Vonk Noordegraaf, A., Beghetti, M., Ghofrani, A., Gomez Sanchez, M. A., Hansmann, G., Klepetko, W., Lancellotti, P., Matucci, M., McDonagh, T., Pierard, L. A., Trindade, P. T., Zompatori, M. and Hoeper, M. (2015) '2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension: The Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS): Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC), International Society for Heart and Lung Transplantation (ISHLT)', *Eur Respir J,* 46(4), pp. 903-75.

Gavin, M. A., Torgerson, T. R., Houston, E., DeRoos, P., Ho, W. Y., Stray-Pedersen, A., Ocheltree, E. L., Greenberg, P. D., Ochs, H. D. and Rudensky, A. Y. (2006) 'Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development', *Proc Natl Acad Sci U S A,* 103(17), pp. 6659-64.

Germain, M., Eyries, M., Montani, D., Poirier, O., Girerd, B., Dorfmüller, P., Coulet, F., Nadaud, S., Maugenre, S., Guignabert, C., Carpentier, W., Vonk-Noordegraaf, A., Lévy, M., Chaouat, A., Lambert, J. C., Bertrand, M., Dupuy, A. M., Letenneur, L., Lathrop, M., Amouyel, P., de Ravel, T. J., Delcroix, M., Austin, E. D., Robbins, I. M., Hemnes, A. R., Loyd, J. E., Berman-Rosenzweig, E., Barst, R. J., Chung, W. K., Simonneau, G., Trégouët, D. A., Humbert, M. and Soubrier, F. (2013) 'Genome-wide association analysis identifies a susceptibility locus for pulmonary arterial hypertension', *Nat Genet,* 45(5), pp. 518-21.

Gerner, E. W. and Meyskens, F. L. (2004) 'Polyamines and cancer: old molecules, new understanding', *Nat Rev Cancer,* 4(10), pp. 781-92.

Gerriets, V. A. and MacIver, N. J. (2014) 'Role of T cells in malnutrition and obesity', *Front Immunol,* 5, pp. 379.

Gharibi, T., Majidi, J., Kazemi, T., Dehghanzadeh, R., Motallebnezhad, M. and Babaloo, Z. (2016) 'Biological effects of IL-21 on different immune cells and its role in autoimmune diseases', *Immunobiology,* 221(2), pp. 357-67.

Ghio, S., Pazzano, A. S., Klersy, C., Scelsi, L., Raineri, C., Camporotondo, R., D'Armini, A. and Visconti, L. O. (2011) 'Clinical and prognostic relevance of echocardiographic evaluation of right ventricular geometry in patients with idiopathic pulmonary arterial hypertension', *Am J Cardiol,* 107(4), pp. 628-32.

Giordano, E., Hillary, R. A., Vary, T. C., Pegg, A. E., Sumner, A. D., Caldarera, C. M., Zhang, X. Q., Song, J., Wang, J., Cheung, J. Y. and Shantz, L. M. (2012) 'Overexpression of ornithine decarboxylase decreases ventricular systolic function during induction of cardiac hypertrophy', *Amino Acids,* 42(2-3), pp. 507-518.

Goerke, J. (1998) 'Pulmonary surfactant: functions and molecular composition', *Biochim Biophys Acta,* 1408(2-3), pp. 79-89.

Golias, C., Charalabopoulos, A., Stagikas, D., Charalabopoulos, K. and Batistatou, A. (2007) 'The kinin system--bradykinin: biological effects and clinical implications. Multiple role of the kinin system--bradykinin', *Hippokratia,* 11(3), pp. 124-8.

Gomez-Arroyo, J., Mizuno, S., Szczepanek, K., Van Tassell, B., Natarajan, R., dos Remedios, C. G., Drake, J. I., Farkas, L., Kraskauskas, D., Wijesinghe, D. S., Chalfant, C. E., Bigbee, J., Abbate, A., Lesnefsky, E. J., Bogaard, H. J. and Voelkel, N. F. (2013) 'Metabolic gene remodeling and

mitochondrial dysfunction in failing right ventricular hypertrophy secondary to pulmonary arterial hypertension', *Circ Heart Fail,* 6(1), pp. 136-44.

Grinnan, D., Farr, G., Fox, A. and Sweeney, L. (2016) 'The Role of Hyperglycemia and Insulin Resistance in the Development and Progression of Pulmonary Arterial Hypertension', *J Diabetes Res,* 2016, pp. 2481659.

Guglani, L. and Khader, S. A. (2010) 'Th17 cytokines in mucosal immunity and inflammation', *Curr Opin HIV AIDS,* 5(2), pp. 120-7.

Guo, Y. and Nolle, R. J. (2013) 'Retinoic acid on stage in antitumor immunity', *Oncoimmunology,* 2(2), pp. e22985.

Gupta, I., Ganguly, S., Rozanas, C. R., Stuehr, D. J. and Panda, K. (2016) 'Ascorbate attenuates pulmonary emphysema by inhibiting tobacco smoke and Rtp801-triggered lung protein modification and proteolysis', *Proc Natl Acad Sci U S A,* 113(29), pp. E4208-17.

Güder, G., Frantz, S., Bauersachs, J., Allolio, B., Ertl, G., Angermann, C. E. and Störk, S. (2010) 'Low circulating androgens and mortality risk in heart failure', *Heart,* 96(7), pp. 504-9.

Haas, C. (2004) '[Pulmonary hypertension associated with systemic lupus erythematosus]', *Bull Acad Natl Med,* 188(6), pp. 985-97; discussion 997.

Hachulla, E., Gressin, V., Guillevin, L., Carpentier, P., Diot, E., Sibilia, J., Kahan, A., Cabane, J., Francès, C., Launay, D., Mouthon, L., Allanore, Y., Tiev, K. P., Clerson, P., de Groote, P. and Humbert, M. (2005) 'Early detection of pulmonary arterial hypertension in systemic sclerosis: a French nationwide prospective multicenter study', *Arthritis Rheum,* 52(12), pp. 3792-800.

Hagan, G., Southwood, M., Treacy, C., Ross, R. M., Soon, E., Coulson, J., Sheares, K., Screaton, N., Pepke-Zaba, J., Morrell, N. W. and Rudd, J. H. (2011) '(18)FDG PET imaging can quantify increased cellular metabolism in pulmonary arterial hypertension: A proof-of-principle study', *Pulm Circ,* 1(4), pp. 448-55.

Hall, J. A., Cannons, J. L., Grainger, J. R., Dos Santos, L. M., Hand, T. W., Naik, S., Wohlfert, E. A., Chou, D. B., Oldenhove, G., Robinson, M., Grigg, M. E., Kastenmayer, R., Schwartzberg, P. L. and Belkaid, Y. (2011) 'Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha', *Immunity,* 34(3), pp. 435-47.

Hall, S., Brogan, P., Haworth, S. G. and Klein, N. (2009) 'Contribution of inflammation to the pathology of idiopathic pulmonary arterial hypertension in children', *Thorax,* 64(9), pp. 778-83.

Halliday, S. J. and Hemnes, A. R. (2017) 'Identifying "super responders" in pulmonary arterial hypertension', *Pulm Circ,* 7(2), pp. 300-311.

Hampl, V., Bíbová, J., Povýsilová, V. and Herget, J. (2003) 'Dehydroepiandrosterone sulphate reduces chronic hypoxic pulmonary hypertension in rats', *Eur Respir J,* 21(5), pp. 862-5.

Han, M., Pendem, S., Teh, S. L., Sukumaran, D. K., Wu, F. and Wilson, J. X. (2010) 'Ascorbate protects endothelial barrier function during septic insult: Role of protein phosphatase type 2A', *Free Radic Biol Med,* 48(1), pp. 128-35.

Hannun, Y. A. and Obeid, L. M. (2008) 'Principles of bioactive lipid signalling: lessons from sphingolipids', *Nat Rev Mol Cell Biol,* 9(2), pp. 139-50.

Harrison, R. E., Flanagan, J. A., Sankelo, M., Abdalla, S. A., Rowell, J., Machado, R. D., Elliott, C. G., Robbins, I. M., Olschewski, H., McLaughlin, V., Gruenig, E., Kermeen, F., Halme, M., Räisänen-Sokolowski, A., Laitinen, T., Morrell, N. W. and Trembath, R. C. (2003) 'Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary

hypertension related to hereditary haemorrhagic telangiectasia', *J Med Genet,* 40(12), pp. 865-71.

Hashimoto-Kataoka, T., Hosen, N., Sonobe, T., Arita, Y., Yasui, T., Masaki, T., Minami, M., Inagaki, T., Miyagawa, S., Sawa, Y., Murakami, M., Kumanogoh, A., Yamauchi-Takihara, K., Okumura, M., Kishimoto, T., Komuro, I., Shirai, M., Sakata, Y. and Nakaoka, Y. (2015) 'Interleukin-6/interleukin-21 signaling axis is critical in the pathogenesis of pulmonary arterial hypertension', *Proc Natl Acad Sci U S A,* 112(20), pp. E2677-86.

Hato, T., Kimura, Y., Morisada, T., Koh, G. Y., Miyata, K., Tabata, M., Kadomatsu, T., Endo, M., Urano, T., Arai, F., Araki, K., Suda, T., Kobayashi, K. and Oike, Y. (2009) 'Angiopoietins contribute to lung development by regulating pulmonary vascular network formation', *Biochem Biophys Res Commun,* 381(2), pp. 218-23.

Hayashi, K., Takahashi, M., Nishida, W., Yoshida, K., Ohkawa, Y., Kitabatake, A., Aoki, J., Arai, H. and Sobue, K. (2001) 'Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids', *Circ Res,* 89(3), pp. 251-8.

He, Z., Jin, L., Liu, Z. F., Hu, L., Dang, E. L., Feng, Z. Z., Li, Q. J. and Wang, G. (2012) 'Elevated serum levels of interleukin 21 are associated with disease severity in patients with psoriasis', *Br J Dermatol,* 167(1), pp. 191-3.

Heger, Z., Merlos Rodrigo, M. A., Michalek, P., Polanska, H., Masarik, M., Vit, V., Plevova, M., Pacik, D., Eckschlager, T., Stiborova, M. and Adam, V. (2016) 'Sarcosine Up-Regulates Expression of Genes Involved in Cell Cycle Progression of Metastatic Models of Prostate Cancer', *PLoS One,* 11(11), pp. e0165830.

Hemkens, L. G. and Bucher, H. C. (2014) 'HIV infection and cardiovascular disease', *Eur Heart J,* 35(21), pp. 1373-81.

Hemnes, A. R., Brittain, E. L., Trammell, A. W., Fessel, J. P., Austin, E. D., Penner, N., Maynard, K. B., Gleaves, L., Talati, M., Absi, T., Disalvo, T. and West, J. (2014) 'Evidence for right ventricular lipotoxicity in heritable pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 189(3), pp. 325-34.

Heresi, G. A., Malin, S. K., Barnes, J. W., Tian, L., Kirwan, J. P. and Dweik, R. A. (2017) 'Abnormal Glucose Metabolism and High-Energy Expenditure in Idiopathic Pulmonary Arterial Hypertension', *Ann Am Thorac Soc,* 14(2), pp. 190-199.

Hillary, R. A. and Pegg, A. E. (2003) 'Decarboxylases involved in polyamine biosynthesis and their inactivation by nitric oxide', *Biochim Biophys Acta,* 1647(1-2), pp. 161-6.

Hislop, A. (2005) 'Developmental biology of the pulmonary circulation', *Paediatr Respir Rev,* 6(1), pp. 35-43.

Hislop, A. A. and Pierce, C. M. (2000) 'Growth of the vascular tree', *Paediatr Respir Rev,* 1(4), pp. 321-7.

Hochberg, M. C. (1997) 'Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus', *Arthritis Rheum,* 40(9), pp. 1725.

Hoeper, M. and Gibbs, S. (2014) 'The changing landscape of pulmonary arterial hypertension and implications for patient care', *Eur Respir Rev,* 23(134), pp. 450-7.

Hoeper, M. M., Barst, R. J., Bourge, R. C., Feldman, J., Frost, A. E., Galié, N., Gómez-Sánchez, M. A., Grimminger, F., Grünig, E., Hassoun, P. M., Morrell, N. W., Peacock, A. J., Satoh, T., Simonneau, G., Tapson, V. F., Torres, F., Lawrence, D., Quinn, D. A. and Ghofrani, H. A. (2013a) 'Imatinib mesylate as add-on therapy for pulmonary arterial hypertension: results of the randomized IMPRES study', *Circulation,* 127(10), pp. 1128-38.

Hoeper, M. M., Bogaard, H. J., Condliffe, R., Frantz, R., Khanna, D., Kurzyna, M., Langleben, D., Manes, A., Satoh, T., Torres, F., Wilkins, M. R. and Badesch, D. B. (2013b) 'Definitions and diagnosis of pulmonary hypertension', *J Am Coll Cardiol,* 62(25 Suppl), pp. D42-50.

Hoet, P. H. and Nemery, B. (2000) 'Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions', *Am J Physiol Lung Cell Mol Physiol,* 278(3), pp. L417-33.

Holt, P., Clague, R. B., Hay, F., Nineham, L. and Wade, E. (1980) 'Is idiopathic pulmonary hypertension a connective tissue disease? (abstract)', *Arthritis and Rheumatism,* 23(6), pp. 690.

Hong, J. J., Amancha, P. K., Rogers, K., Ansari, A. A. and Villinger, F. (2013) 'Re-evaluation of PD-1 expression by T cells as a marker for immune exhaustion during SIV infection', *PLoS One,* 8(3), pp. e60186.

Houssaini, A., Abid, S., Mouraret, N., Wan, F., Rideau, D., Saker, M., Marcos, E., Tissot, C. M., Dubois-Randé, J. L., Amsellem, V. and Adnot, S. (2013) 'Rapamycin reverses pulmonary artery smooth muscle cell proliferation in pulmonary hypertension', *Am J Respir Cell Mol Biol,* 48(5), pp. 568-77.

Howie, D., Cobbold, S. P., Adams, E., Ten Bokum, A., Necula, A. S., Zhang, W., Huang, H., Roberts, D. J., Thomas, B., Hester, S. S., Vaux, D. J., Betz, A. G. and Waldmann, H. (2017) 'Foxp3 drives oxidative phosphorylation and protection from lipotoxicity', *JCI Insight,* 2(3), pp. e89160.

Huang, W., Eickhoff, J. C., Mehraein-Ghomi, F., Church, D. R., Wilding, G. and Basu, H. S. (2015) 'Expression of spermidine/spermine N(1) -acetyl transferase (SSAT) in human prostate tissues is related to prostate cancer progression and metastasis', *Prostate,* 75(11), pp. 1150-9.

Huertas, A., Phan, C., Bordenave, J., Tu, L., Thuillet, R., Le Hiress, M., Avouac, J., Tamura, Y., Allanore, Y., Jovan, R., Sitbon, O., Guignabert, C. and Humbert, M. (2016) 'Regulatory T Cell Dysfunction in Idiopathic, Heritable and Connective Tissue-Associated Pulmonary Arterial Hypertension', *Chest,* 149(6), pp. 1482-93.

Huertas, A., Tu, L., Gambaryan, N., Girerd, B., Perros, F., Montani, D., Fabre, D., Fadel, E., Eddahibi, S., Cohen-Kaminsky, S., Guignabert, C. and Humbert, M. (2012) 'Leptin and regulatory T-lymphocytes in idiopathic pulmonary arterial hypertension', *Eur Respir J,* 40(4), pp. 895-904.

Hughes, J. and Morrell, N. (2001) 'Vascular structure and function', *Pulmonary Circulation*: Imperial College Press, pp. 14-43.

Hughes, J. and Morrell, N. (2011) 'Endothelium', *Pulmonary Circulation*: Imperial College Press, London, pp. 124-158.

Hughes, J., Morrell, N. and Imperial College Press (Cardiopulmonary Medicine Series), P. 2001. Pulmonary Circulation: from Basic Mechanisms to Clinical Practice.: Imperial College Press (Cardiopulmonary Medicine Series).

Hughes, T., Kim-Howard, X., Kelly, J. A., Kaufman, K. M., Langefeld, C. D., Ziegler, J., Sanchez, E., Kimberly, R. P., Edberg, J. C., Ramsey-Goldman, R., Petri, M., Reveille, J. D., Martín, J., Brown, E. E., Vilá, L. M., Alarcón, G. S., James, J. A., Gilkeson, G. S., Moser, K. L., Gaffney, P. M., Merrill, J. T., Vyse, T. J., Alarcón-Riquelme, M. E., Nath, S. K., Harley, J. B., Sawalha, A. H. and Network, B. (2011) 'Fine-mapping and transethnic genotyping establish IL2/IL21 genetic association with lupus and localize this genetic effect to IL21', *Arthritis Rheum,* 63(6), pp. 1689-97.

Humbert, M., Monti, G., Brenot, F., Sitbon, O., Portier, A., Grangeot-Keros, L., Duroux, P., Galanaud, P., Simonneau, G. and Emilie, D. (1995) 'Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension', *Am J Respir Crit Care Med,* 151(5), pp. 1628-31.

Humbert, M., Sitbon, O., Chaouat, A., Bertocchi, M., Habib, G., Gressin, V., Yaici, A., Weitzenblum, E., Cordier, J. F., Chabot, F., Dromer, C., Pison, C., Reynaud-Gaubert, M., Haloun, A., Laurent, M., Hachulla, E. and Simonneau, G. (2006) 'Pulmonary arterial hypertension in France: results from a national registry', *Am J Respir Crit Care Med,* 173(9), pp. 1023-30.

Humbert, M., Sitbon, O., Chaouat, A., Bertocchi, M., Habib, G., Gressin, V., Yaïci, A., Weitzenblum, E., Cordier, J. F., Chabot, F., Dromer, C., Pison, C., Reynaud-Gaubert, M., Haloun, A., Laurent, M., Hachulla, E., Cottin, V., Degano, B., Jaïs, X., Montani, D., Souza, R. and Simonneau, G. (2010) 'Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era', *Circulation,* 122(2), pp. 156-63.

Hussain, H., Shornick, L. P., Shannon, V. R., Wilson, J. D., Funk, C. D., Pentland, A. P. and Holtzman, M. J. (1994) 'Epidermis contains platelet-type 12-lipoxygenase that is overexpressed in germinal layer keratinocytes in psoriasis', *Am J Physiol,* 266(1 Pt 1), pp. C243-53.

Idrees, M., Alnajashi, K., Abdulhameed, J., Khan, A., Batubara, E., Alotay, A., Fayed, A., Aldammas, S., Alseif, M., Alawwad, H., Abusabaa, Y., Almobrad, M., Kashour, T. and SAPH, R. T. (2015) 'Saudi experience in the management of pulmonary arterial hypertension; the outcome of PAH therapy with the exclusion of chronic parenteral prostacyclin', *Ann Thorac Med,* 10(3), pp. 204-11.

Imrich, R. (2002) 'The role of neuroendocrine system in the pathogenesis of rheumatic diseases (minireview)', *Endocr Regul,* 36(2), pp. 95-106.

Issan, Y., Hochhauser, E., Guo, A., Gotlinger, K. H., Kornowski, R., Leshem-Lev, D., Lev, E., Porat, E., Snir, E., Thompson, C. I., Abraham, N. G. and Laniado-Schwartzman, M. (2013) 'Elevated level of pro-inflammatory eicosanoids and EPC dysfunction in diabetic patients with cardiac ischemia', *Prostaglandins Other Lipid Mediat,* 100-101, pp. 15-21.

Izzedine, H., Cacoub, P., Launay-Vacher, V., Bagnis, C. and Deray, G. (2002) 'Lymphopenia in Wegener's granulomatosis. A new clinical activity index?', *Nephron,* 92(2), pp. 466-71.

Jais, X., Launay, D., Yaici, A., Le Pavec, J., Tchérakian, C., Sitbon, O., Simonneau, G. and Humbert, M. (2008) 'Immunosuppressive therapy in lupus- and mixed connective tissue disease-associated pulmonary arterial hypertension: a retrospective analysis of twenty-three cases', *Arthritis Rheum,* 58(2), pp. 521-31.

Jamieson, S. W., Kapelanski, D. P., Sakakibara, N., Manecke, G. R., Thistlethwaite, P. A., Kerr, K. M., Channick, R. N., Fedullo, P. F. and Auger, W. R. (2003) 'Pulmonary endarterectomy: experience and lessons learned in 1,500 cases', *Ann Thorac Surg,* 76(5), pp. 1457-62; discussion 1462-4.

Jankowska, E. A., Biel, B., Majda, J., Szklarska, A., Lopuszanska, M., Medras, M., Anker, S. D., Banasiak, W., Poole-Wilson, P. A. and Ponikowski, P. (2006) 'Anabolic deficiency in men with chronic heart failure: prevalence and detrimental impact on survival', *Circulation,* 114(17), pp. 1829-37.

Jenkins, D. (2015) 'Pulmonary endarterectomy: the potentially curative treatment for patients with chronic thromboembolic pulmonary hypertension', *Eur Respir Rev,* 24(136), pp. 263-71.

Jernigan, N., Walker, B. and Resta, T. (2009) 'Pulmonary Endothelium and Vasomotor Control', in Voelkel, NF & Rounds, S. (eds.) *The Pulmonary Endothelium: Function in health and disease*. Chichester, UK.: John Wiley & Sons, Ltd,, pp. 185-202.

Jiang, Y., Li, Y. and Zhu, B. (2015) 'T-cell exhaustion in the tumor microenvironment', *Cell Death Dis,* 6, pp. e1792.

Jorens, P. G., Van Marck, E., Snoeckx, A. and Parizel, P. M. (2009) 'Nonthrombotic pulmonary embolism', *Eur Respir J,* 34(2), pp. 452-74.

Kayyali, U. and Fanburg, B. (2009) 'Pulmonary Endothelial Cell Surface Metabolic Functions', in Voelkel, N. & Rounds, S. (eds.) *The Pulmonary Endothelium: Function in health and disease*. Chichester, UK: John Wiley & Sons Ltd. , pp. 105-112.

Keane, K. N., Calton, E. K., Carlessi, R., Hart, P. H. and Newsholme, P. (2017) 'The bioenergetics of inflammation: insights into obesity and type 2 diabetes', *Eur J Clin Nutr,* 71(7), pp. 904-912.

Kepp, O., Galluzzi, L. and Kroemer, G. (2011) 'Mitochondrial control of the NLRP3 inflammasome', *Nat Immunol,* 12(3), pp. 199-200.

Kerekes, G., Nurmohamed, M. T., González-Gay, M. A., Seres, I., Paragh, G., Kardos, Z., Baráth, Z., Tamási, L., Soltész, P. and Szekanecz, Z. (2014) 'Rheumatoid arthritis and metabolic syndrome', *Nat Rev Rheumatol,* 10(11), pp. 691-6.

Kim, N. H. and Lang, I. M. (2012) 'Risk factors for chronic thromboembolic pulmonary hypertension', *Eur Respir Rev,* 21(123), pp. 27-31.

Kimura, M. and Yamaguchi, S. (1999) 'Screening for fatty acid beta oxidation disorders. Acylglycine analysis by electron impact ionization gas chromatography-mass spectrometry', *J Chromatogr B Biomed Sci Appl,* 731(1), pp. 105-10.

Kirsch, S., Thijssen, S., Alarcon Salvador, S., Heine, G. H., van Bentum, K., Fliser, D., Sester, M. and Sester, U. (2012) 'T-cell numbers and antigen-specific T-cell function follow different circadian rhythms', *J Clin Immunol,* 32(6), pp. 1381-9.

Kirtava, Z., Blomberg, J., Bredberg, A., Henriksson, G., Jacobsson, L. and Manthorpe, R. (1995) 'CD4+ T-lymphocytopenia without HIV infection: increased prevalence among patients with primary Sjögren's syndrome', *Clin Exp Rheumatol,* 13(5), pp. 609-16.

Kovacs, G., Berghold, A., Scheidl, S. and Olschewski, H. (2009) 'Pulmonary arterial pressure during rest and exercise in healthy subjects: a systematic review', *Eur Respir J,* 34(4), pp. 888-94.

Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D. and Muoio, D. M. (2008) 'Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance', *Cell Metab,* 7(1), pp. 45-56.

Kummer, W. (2011) 'Pulmonary vascular innervation and its role in responses to hypoxia: size matters!', *Proc Am Thorac Soc,* 8(6), pp. 471-6.

Laczy, B., Hill, B. G., Wang, K., Paterson, A. J., White, C. R., Xing, D., Chen, Y. F., Darley-Usmar, V., Oparil, S. and Chatham, J. C. (2009) 'Protein O-GlcNAcylation: a new signaling paradigm for the cardiovascular system', *Am J Physiol Heart Circ Physiol,* 296(1), pp. H13-28.

Lahm, T., Crisostomo, P. R., Markel, T. A., Wang, M., Lillemoe, K. D. and Meldrum, D. R. (2007) 'The critical role of vascular endothelial growth factor in pulmonary vascular remodeling after lung injury', *Shock,* 28(1), pp. 4-14.

Lam, C. S., Borlaug, B. A., Kane, G. C., Enders, F. T., Rodeheffer, R. J. and Redfield, M. M. (2009) 'Age-associated increases in pulmonary artery systolic pressure in the general population', *Circulation,* 119(20), pp. 2663-70.

Lammers, S., Scott, D., Hunter, K., Tan, W., Shandas, R. and Stenmark, K. R. (2012) 'Mechanics and function of the pulmonary vasculature: implications for pulmonary vascular disease and right ventricular function', *Compr Physiol,* 2(1), pp. 295-319.

Lane, K. B., Machado, R. D., Pauciulo, M. W., Thomson, J. R., Phillips, J. A., Loyd, J. E., Nichols, W. C., Trembath, R. C. and Consortium, I. P. (2000) 'Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension', *Nat Genet,* 26(1), pp. 81-4.

Lang, I. M., Dorfmüller, P. and Vonk Noordegraaf, A. (2016) 'The Pathobiology of Chronic Thromboembolic Pulmonary Hypertension', *Ann Am Thorac Soc,* 13 Suppl 3, pp. S215-21.

Lang, I. M., Gaine, S.P. (2015) 'Recent advances in targeting the prostacyclin pathway in pulmonary arterial hypertension', *Eur Respir Rev*, 24(138), pp. 630-641.

Lang, I. M., Pesavento, R., Bonderman, D. and Yuan, J. X. (2013) 'Risk factors and basic mechanisms of chronic thromboembolic pulmonary hypertension: a current understanding', *Eur Respir J,* 41(2), pp. 462-8.

Langleben, D., Barst, R. J., Badesch, D., Groves, B. M., Tapson, V. F., Murali, S., Bourge, R. C., Ettinger, N., Shalit, E., Clayton, L. M., Jöbsis, M. M., Blackburn, S. D., Crow, J. W., Stewart, D. J. and Long, W. (1999) 'Continuous infusion of epoprostenol improves the net balance between pulmonary endothelin-1 clearance and release in primary pulmonary hypertension', *Circulation,* 99(25), pp. 3266-71.

Langleben, D., Dupuis, J., Langleben, I., Hirsch, A. M., Baron, M., Senécal, J. L. and Giovinazzo, M. (2006) 'Etiology-specific endothelin-1 clearance in human precapillary pulmonary hypertension', *Chest,* 129(3), pp. 689-95.

Larsen, C. M., Faulenbach, M., Vaag, A., Vølund, A., Ehses, J. A., Seifert, B., Mandrup-Poulsen, T. and Donath, M. Y. (2007) 'Interleukin-1-receptor antagonist in type 2 diabetes mellitus', *N Engl J Med,* 356(15), pp. 1517-26.

Last, R. L., Jones, A. D. and Shachar-Hill, Y. (2007) 'Towards the plant metabolome and beyond', *Nat Rev Mol Cell Biol,* 8(2), pp. 167-74.

Lawson, C. A., Brown, A. K., Bejarano, V., Douglas, S. H., Burgoyne, C. H., Greenstein, A. S., Boylston, A. W., Emery, P., Ponchel, F. and Isaacs, J. D. (2006) 'Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood', *Rheumatology (Oxford),* 45(10), pp. 1210-7.

Le Coz, C., Joublin, A., Pasquali, J. L., Korganow, A. S., Dumortier, H. and Monneaux, F. (2013) 'Circulating TFH subset distribution is strongly affected in lupus patients with an active disease', *PLoS One,* 8(9), pp. e75319.

Lee, C. F., Lo, Y. C., Cheng, C. H., Furtmüller, G. J., Oh, B., Andrade-Oliveira, V., Thomas, A. G., Bowman, C. E., Slusher, B. S., Wolfgang, M. J., Brandacher, G. and Powell, J. D. (2015) 'Preventing Allograft Rejection by Targeting Immune Metabolism', *Cell Rep,* 13(4), pp. 760-70.

Lee, C. F. and Tian, R. (2015) 'Mitochondrion as a Target for Heart Failure Therapy- Role of Protein Lysine Acetylation', *Circ J,* 79(9), pp. 1863-70.

Lessig, J. and Fuchs, B. (2009) 'Plasmalogens in biological systems: their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis', *Curr Med Chem,* 16(16), pp. 2021-41.

Leuchte, H. H., Holzapfel, M., Baumgartner, R. A., Ding, I., Neurohr, C., Vogeser, M., Kolbe, T., Schwaiblmair, M. and Behr, J. (2004) 'Clinical significance of brain natriuretic peptide in primary pulmonary hypertension', *J Am Coll Cardiol,* 43(5), pp. 764-70.

Lewis, G. D., Farrell, L., Wood, M. J., Martinovic, M., Arany, Z., Rowe, G. C., Souza, A., Cheng, S., McCabe, E. L., Yang, E., Shi, X., Deo, R., Roth, F. P., Asnani, A., Rhee, E. P., Systrom, D. M., Semigran, M. J., Vasan, R. S., Carr, S. A., Wang, T. J., Sabatine, M. S., Clish, C. B. and Gerszten, R. E. (2010) 'Metabolic signatures of exercise in human plasma', *Sci Transl Med,* 2(33), pp. 33ra37.

Li, J., Tian, Z., Zheng, H. Y., Zhang, W., Duan, M. H., Liu, Y. T., Cao, X. X. and Zhou, D. B. (2013) 'Pulmonary hypertension in POEMS syndrome', *Haematologica,* 98(3), pp. 393-8.

Ling, Y., Johnson, M. K., Kiely, D. G., Condliffe, R., Elliot, C. A., Gibbs, J. S., Howard, L. S., Pepke-Zaba, J., Sheares, K. K., Corris, P. A., Fisher, A. J., Lordan, J. L., Gaine, S., Coghlan, J. G., Wort, S. J., Gatzoulis, M. A. and Peacock, A. J. (2012) 'Changing demographics, epidemiology, and survival of incident pulmonary arterial hypertension: results from the pulmonary hypertension registry of the United Kingdom and Ireland', *Am J Respir Crit Care Med,* 186(8), pp. 790-6.

Liu, Y., Gong, Y., Qu, C., Zhang, Y., You, R., Yu, N., Lu, G., Huang, Y., Zhang, H., Gao, Y. and Guo, X. (2017) 'CD32b expression is down-regulated on double-negative memory B cells in patients with Hashimoto's thyroiditis', *Mol Cell Endocrinol,* 440, pp. 1-7.

Lomas-Neira, J., Venet, F., Chung, C. S., Thakkar, R., Heffernan, D. and Ayala, A. (2014) 'Neutrophil-endothelial interactions mediate angiopoietin-2-associated pulmonary endothelial cell dysfunction in indirect acute lung injury in mice', *Am J Respir Cell Mol Biol,* 50(1), pp. 193-200.

Lucarelli, G., Ditonno, P., Bettocchi, C., Spilotros, M., Rutigliano, M., Vavallo, A., Galleggiante, V., Fanelli, M., Larocca, A. M., Germinario, C. A., Maiorano, E., Selvaggi, F. P. and Battaglia, M. (2013) 'Serum sarcosine is a risk factor for progression and survival in patients with metastatic castration-resistant prostate cancer', *Future Oncol,* 9(6), pp. 899-907.

Lundbaek, J. A. and Andersen, O. S. (1994) 'Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers', *J Gen Physiol,* 104(4), pp. 645-73.

Lévi, F. A., Canon, C., Touitou, Y., Reinberg, A. and Mathé, G. (1988) 'Seasonal modulation of the circadian time structure of circulating T and natural killer lymphocyte subsets from healthy subjects', *J Clin Invest,* 81(2), pp. 407-13.

Ma, L., Roman-Campos, D., Austin, E. D., Eyries, M., Sampson, K. S., Soubrier, F., Germain, M., Trégouët, D. A., Borczuk, A., Rosenzweig, E. B., Girerd, B., Montani, D., Humbert, M., Loyd, J. E., Kass, R. S. and Chung, W. K. (2013) 'A novel channelopathy in pulmonary arterial hypertension', *N Engl J Med,* 369(4), pp. 351-361.

Maceyka, M. and Spiegel, S. (2014) 'Sphingolipid metabolites in inflammatory disease', *Nature,* 510(7503), pp. 58-67.

Maecker, H. T., McCoy, J. P. and Nussenblatt, R. (2012) 'Standardizing immunophenotyping for the Human Immunology Project', *Nat Rev Immunol,* 12(3), pp. 191-200.

Mainguy, V., Maltais, F., Saey, D., Gagnon, P., Martel, S., Simon, M. and Provencher, S. (2010) 'Peripheral muscle dysfunction in idiopathic pulmonary arterial hypertension', *Thorax,* 65(2), pp. 113-7.

Mansoor, J. K., Schelegle, E. S., Davis, C. E., Walby, W. F., Zhao, W., Aksenov, A. A., Pasamontes, A., Figueroa, J. and Allen, R. (2014) 'Analysis of volatile compounds in exhaled breath

condensate in patients with severe pulmonary arterial hypertension', *PLoS One,* 9(4), pp. e95331.

Marsboom, G., Wietholt, C., Haney, C. R., Toth, P. T., Ryan, J. J., Morrow, E., Thenappan, T., Bache-Wiig, P., Piao, L., Paul, J., Chen, C. T. and Archer, S. L. (2012) 'Lung ^{18}F-fluorodeoxyglucose positron emission tomography for diagnosis and monitoring of pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 185(6), pp. 670-9.

Martin, D. A., Towne, J. E., Kricorian, G., Klekotka, P., Gudjonsson, J. E., Krueger, J. G. and Russell, C. B. (2013) 'The emerging role of IL-17 in the pathogenesis of psoriasis: preclinical and clinical findings', *J Invest Dermatol,* 133(1), pp. 17-26.

Mateuszuk, Ł., Khomich, T. I., Słomińska, E., Gajda, M., Wójcik, L., Łomnicka, M., Gwóźdź, P. and Chłopicki, S. (2009) 'Activation of nicotinamide N-methyltrasferase and increased formation of 1-methylnicotinamide (MNA) in atherosclerosis', *Pharmacol Rep,* 61(1), pp. 76-85.

Mauad, T., Pozzan, G., Lanças, T., Overbeek, M. J., Souza, R., Jardim, C., Dolhnikoff, M., Mello, G., Pires-Neto, R. C., Bernardi, F. e. C. and Grünberg, K. (2014) 'Immunopathological aspects of schistosomiasis-associated pulmonary arterial hypertension', *J Infect,* 68(1), pp. 90-8.

Mazzoccoli, G., Sothern, R. B., De Cata, A., Giuliani, F., Fontana, A., Copetti, M., Pellegrini, F. and Tarquini, R. (2011) 'A timetable of 24-hour patterns for human lymphocyte subpopulations', *J Biol Regul Homeost Agents,* 25(3), pp. 387-95.

McCabe, C., Deboeck, G., Harvey, I., Ross, R. M., Gopalan, D., Screaton, N. and Pepke-Zaba, J. (2013) 'Inefficient exercise gas exchange identifies pulmonary hypertension in chronic thromboembolic obstruction following pulmonary embolism', *Thromb Res,* 132(6), pp. 659-65.

McCabe, C., White, P. A., Hoole, S. P., Axell, R. G., Priest, A. N., Gopalan, D., Taboada, D., MacKenzie Ross, R., Morrell, N. W., Shapiro, L. M. and Pepke-Zaba, J. (2014) 'Right ventricular dysfunction in chronic thromboembolic obstruction of the pulmonary artery: a pressure-volume study using the conductance catheter', *J Appl Physiol (1985),* 116(4), pp. 355-63.

McGoon, M. D., Benza, R. L., Escribano-Subias, P., Jiang, X., Miller, D. P., Peacock, A. J., Pepke-Zaba, J., Pulido, T., Rich, S., Rosenkranz, S., Suissa, S. and Humbert, M. (2013) 'Pulmonary arterial hypertension: epidemiology and registries', *J Am Coll Cardiol,* 62(25 Suppl), pp. D51-9.

McKinney, E. F., Lee, J. C., Jayne, D. R., Lyons, P. A. and Smith, K. G. (2015) 'T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection', *Nature,* 523(7562), pp. 612-6.

Meana, C., Rubín, J. M., Bordallo, C., Suárez, L., Bordallo, J. and Sánchez, M. (2016) 'Correlation between endogenous polyamines in human cardiac tissues and clinical parameters in patients with heart failure', *J Cell Mol Med,* 20(2), pp. 302-12.

Medford, H. M. and Marsh, S. A. (2014) 'The role of O-GlcNAc transferase in regulating the gene transcription of developing and failing hearts', *Future Cardiol,* 10(6), pp. 801-12.

Mellins, R. B. (1982) 'Fleischner Lecture. Metabolic functions of the lung and their clinical relevance', *AJR Am J Roentgenol,* 138(6), pp. 999-1009.

Mendes, J. F., Siqueira, E. M., de Brito E Silva, J. G. and Arruda, S. F. (2016) 'Vitamin A deficiency modulates iron metabolism independent of hemojuvelin (Hfe2) and bone morphogenetic protein 6 (Bmp6) transcript levels', *Genes Nutr,* 11, pp. 1.

Meyer, J. A. (1990) 'Werner Forssmann and catheterization of the heart, 1929', *Ann Thorac Surg,* 49(3), pp. 497-9.

Michalek, R. D., Gerriets, V. A., Jacobs, S. R., Macintyre, A. N., MacIver, N. J., Mason, E. F., Sullivan, S. A., Nichols, A. G. and Rathmell, J. C. (2011) 'Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets', *J Immunol,* 186(6), pp. 3299-303.

Michelakis, E. D., McMurtry, M. S., Wu, X. C., Dyck, J. R., Moudgil, R., Hopkins, T. A., Lopaschuk, G. D., Puttagunta, L., Waite, R. and Archer, S. L. (2002) 'Dichloroacetate, a metabolic modulator, prevents and reverses chronic hypoxic pulmonary hypertension in rats: role of increased expression and activity of voltage-gated potassium channels', *Circulation,* 105(2), pp. 244-50.

Mikirova, N., Casciari, J., Rogers, A. and Taylor, P. (2012) 'Effect of high-dose intravenous vitamin C on inflammation in cancer patients', *J Transl Med,* 10, pp. 189.

Milburn, M. V. and Lawton, K. A. (2013) 'Application of metabolomics to diagnosis of insulin resistance', *Annu Rev Med,* 64, pp. 291-305.

Millar, F. R., Summers, C., Griffiths, M. J., Toshner, M. R. and Proudfoot, A. G. (2016) 'The pulmonary endothelium in acute respiratory distress syndrome: insights and therapeutic opportunities', *Thorax,* 71(5), pp. 462-73.

Milledge, J. S. (1984) 'Angiotensin converting enzyme and hypoxia', *Bull Eur Physiopathol Respir,* 20(6), pp. 481-5.

Milovic, V. and Turchanowa, L. (2003) 'Polyamines and colon cancer', *Biochem Soc Trans,* 31(2), pp. 381-3.

Min, B., Yamane, H., Hu-Li, J. and Paul, W. E. (2005) 'Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms', *J Immunol,* 174(10), pp. 6039-44.

Minneci, P. C., Deans, K. J., Zhi, H., Yuen, P. S., Star, R. A., Banks, S. M., Schechter, A. N., Natanson, C., Gladwin, M. T. and Solomon, S. B. (2005) 'Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin', *J Clin Invest,* 115(12), pp. 3409-17.

Miravitlles, M., Soler-Cataluña, J. J., Calle, M. and Soriano, J. B. (2013) 'Treatment of COPD by clinical phenotypes: putting old evidence into clinical practice', *Eur Respir J,* 41(6), pp. 1252-6.

Montecino-Rodriguez, E., Berent-Maoz, B. and Dorshkind, K. (2013) 'Causes, consequences, and reversal of immune system aging', *J Clin Invest,* 123(3), pp. 958-65.

Morita, R., Schmitt, N., Bentebibel, S. E., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S., Sabzghabaei, N., Lavecchio, E. M., Punaro, M., Pascual, V., Banchereau, J. and Ueno, H. (2011) 'Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion', *Immunity,* 34(1), pp. 108-21.

Morris, A. J., Panchatcharam, M., Cheng, H. Y., Federico, L., Fulkerson, Z., Selim, S., Miriyala, S., Escalante-Alcalde, D. and Smyth, S. S. (2009) 'Regulation of blood and vascular cell function by bioactive lysophospholipids', *J Thromb Haemost,* 7 Suppl 1, pp. 38-43.

Morrison, R. F. and Seidel, E. R. (1995) 'Vascular endothelial cell proliferation: regulation of cellular polyamines', *Cardiovasc Res,* 29(6), pp. 841-7.

Mura, M., Han, B., Andrade, C. F., Seth, R., Hwang, D., Waddell, T. K., Keshavjee, S. and Liu, M. (2006) 'The early responses of VEGF and its receptors during acute lung injury: implication of VEGF in alveolar epithelial cell survival', *Crit Care,* 10(5), pp. R130.

Márquez, A., Orozco, G., Martínez, A., Palomino-Morales, R., Fernández-Arquero, M., Mendoza, J. L., Taxonera, C., Díaz-Rubio, M., Gómez-García, M., Nieto, A., López-Nevot, M. A., de la Concha,

E. G., Martín, J. and Urcelay, E. (2009) 'Novel association of the interleukin 2-interleukin 21 region with inflammatory bowel disease', *Am J Gastroenterol,* 104(8), pp. 1968-75.

Naeije, R. and Chesler, N. (2012) 'Pulmonary circulation at exercise', *Compr Physiol,* 2(1), pp. 711-41.

Naeije, R. and Manes, A. (2014) 'The right ventricle in pulmonary arterial hypertension', *Eur Respir Rev,* 23(134), pp. 476-87.

Nagan, N. and Zoeller, R. A. (2001) 'Plasmalogens: biosynthesis and functions', *Prog Lipid Res,* 40(3), pp. 199-229.

Nagaya, N., Nishikimi, T., Uematsu, M., Satoh, T., Kyotani, S., Sakamaki, F., Kakishita, M., Fukushima, K., Okano, Y., Nakanishi, N., Miyatake, K. and Kangawa, K. (2000) 'Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension', *Circulation,* 102(8), pp. 865-70.

Nguyen, C. H., Stadler, S., Brenner, S., Huttary, N., Krieger, S., Jäger, W., Dolznig, H. and Krupitza, G. (2016) 'Cancer cell-derived 12(S)-HETE signals via 12-HETE receptor, RHO, ROCK and MLC2 to induce lymph endothelial barrier breaching', *Br J Cancer,* 115(3), pp. 364-70.

Nguyen, K. A., Peer, N., de Villiers, A., Mukasa, B., Matsha, T. E., Mills, E. J. and Kengne, A. P. (2017) 'Metabolic Syndrome in People Living with Human Immunodeficiency Virus: An Assessment of the Prevalence and the Agreement between Diagnostic Criteria', *Int J Endocrinol,* 2017, pp. 1613657.

Nijkeuter, M., Hovens, M. M., Davidson, B. L. and Huisman, M. V. (2006) 'Resolution of thromboemboli in patients with acute pulmonary embolism: a systematic review', *Chest,* 129(1), pp. 192-7.

Núñez, J., Miñana, G., Bodí, V., Núñez, E., Sanchis, J., Husser, O. and Llàcer, A. (2011) 'Low lymphocyte count and cardiovascular diseases', *Curr Med Chem,* 18(21), pp. 3226-33.

Ogawara, H., Handa, H., Morita, K., Hayakawa, M., Kojima, J., Amagai, H., Tsumita, Y., Kaneko, Y., Tsukamoto, N., Nojima, Y. and Murakami, H. (2003) 'High Th1/Th2 ratio in patients with chronic idiopathic thrombocytopenic purpura', *Eur J Haematol,* 71(4), pp. 283-8.

Oka, M., Karoor, V., Homma, N., Nagaoka, T., Sakao, E., Golembeski, S. M., Limbird, J., Imamura, M., Gebb, S. A., Fagan, K. A. and McMurtry, I. F. (2007) 'Dehydroepiandrosterone upregulates soluble guanylate cyclase and inhibits hypoxic pulmonary hypertension', *Cardiovasc Res,* 74(3), pp. 377-87.

Olson, J. W., Hacker, A. D., Altiere, R. J. and Gillespie, M. N. (1984) 'Polyamines and the development of monocrotaline-induced pulmonary hypertension', *Am J Physiol,* 247(4 Pt 2), pp. H682-5.

Olson, J. W., Hacker, A. D., Atkinson, J. E., Altiere, R. J. and Gillespie, M. N. (1986) 'Polyamine content in rat lung during development of hypoxia-induced pulmonary hypertension', *Biochem Pharmacol,* 35(4), pp. 714-6.

Orfanos, S. E., Armaganidis, A., Glynos, C., Psevdi, E., Kaltsas, P., Sarafidou, P., Catravas, J. D., Dafni, U. G., Langleben, D. and Roussos, C. (2000) 'Pulmonary capillary endothelium-bound angiotensin-converting enzyme activity in acute lung injury', *Circulation,* 102(16), pp. 2011-8.

Orfanos, S. E., Langleben, D., Khoury, J., Schlesinger, R. D., Dragatakis, L., Roussos, C., Ryan, J. W. and Catravas, J. D. (1999) 'Pulmonary capillary endothelium-bound angiotensin-converting enzyme activity in humans', *Circulation,* 99(12), pp. 1593-9.

Orfanos, S. E., Mavrommati, I., Korovesi, I. and Roussos, C. (2004) 'Pulmonary endothelium in acute lung injury: from basic science to the critically ill', *Intensive Care Med,* 30(9), pp. 1702-14.

Orlinska, U., Olson, J. W. and Gillespie, M. N. (1988) 'Polyamine content in pulmonary arteries from rats with monocrotaline-induced pulmonary hypertension', *Res Commun Chem Pathol Pharmacol,* 62(2), pp. 187-94.

Ormiston, M. L., Chang, C., Long, L. L., Soon, E., Jones, D., Machado, R., Treacy, C., Toshner, M. R., Campbell, K., Riding, A., Southwood, M., Pepke-Zaba, J., Exley, A., Trembath, R. C., Colucci, F., Wills, M., Trowsdale, J. and Morrell, N. W. (2012) 'Impaired natural killer cell phenotype and function in idiopathic and heritable pulmonary arterial hypertension', *Circulation,* 126(9), pp. 1099-109.

Otsubo, C., Bharathi, S., Uppala, R., Ilkayeva, O. R., Wang, D., McHugh, K., Zou, Y., Wang, J., Alcorn, J. F., Zuo, Y. Y., Hirschey, M. D. and Goetzman, E. S. (2015) 'Long-chain Acylcarnitines Reduce Lung Function by Inhibiting Pulmonary Surfactant', *J Biol Chem,* 290(39), pp. 23897-904.

Paglieroni, T. G. and Holland, P. V. (1994) 'Circannual variation in lymphocyte subsets, revisited', *Transfusion,* 34(6), pp. 512-6.

Paley, M. A., Kroy, D. C., Odorizzi, P. M., Johnnidis, J. B., Dolfi, D. V., Barnett, B. E., Bikoff, E. K., Robertson, E. J., Lauer, G. M., Reiner, S. L. and Wherry, E. J. (2012) 'Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection', *Science,* 338(6111), pp. 1220-5.

Park, S. H., Chen, W. C., Hoffman, C., Marsh, L. M., West, J. and Grunig, G. (2013) 'Modification of hemodynamic and immune responses to exposure with a weak antigen by the expression of a hypomorphic BMPR2 gene', *PLoS One,* 8(1), pp. e55180.

Patel, C. H. and Powell, J. D. (2017) 'Targeting T cell metabolism to regulate T cell activation, differentiation and function in disease', *Curr Opin Immunol,* 46, pp. 82-88.

Patel, D., Kandhi, S., Kelly, M., Neo, B. H. and Wolin, M. S. (2014) 'Dehydroepiandrosterone promotes pulmonary artery relaxation by NADPH oxidation-elicited subunit dimerization of protein kinase G 1α', *Am J Physiol Lung Cell Mol Physiol,* 306(4), pp. L383-91.

Peacock, A. J., Murphy, N. F., McMurray, J. J., Caballero, L. and Stewart, S. (2007) 'An epidemiological study of pulmonary arterial hypertension', *Eur Respir J,* 30(1), pp. 104-9.

Pearce, E. L., Poffenberger, M. C., Chang, C. H. and Jones, R. G. (2013) 'Fueling immunity: insights into metabolism and lymphocyte function', *Science,* 342(6155), pp. 1242454.

Pearce, E. L., Walsh, M. C., Cejas, P. J., Harms, G. M., Shen, H., Wang, L. S., Jones, R. G. and Choi, Y. (2009) 'Enhancing CD8 T-cell memory by modulating fatty acid metabolism', *Nature,* 460(7251), pp. 103-7.

Pegg, A. E. (2009) 'Mammalian polyamine metabolism and function', *IUBMB Life,* 61(9), pp. 880-94.

Pengo, V., Lensing, A. W., Prins, M. H., Marchiori, A., Davidson, B. L., Tiozzo, F., Albanese, P., Biasiolo, A., Pegoraro, C., Iliceto, S., Prandoni, P. and Group, T. P. H. S. (2004) 'Incidence of chronic thromboembolic pulmonary hypertension after pulmonary embolism', *N Engl J Med,* 350(22), pp. 2257-64.

Pepke-Zaba, J., Delcroix, M., Lang, I., Mayer, E., Jansa, P., Ambroz, D., Treacy, C., D'Armini, A. M., Morsolini, M., Snijder, R., Bresser, P., Torbicki, A., Kristensen, B., Lewczuk, J., Simkova, I., Barberà, J. A., de Perrot, M., Hoeper, M. M., Gaine, S., Speich, R., Gomez-Sanchez, M. A., Kovacs, G., Hamid, A. M., Jaïs, X. and Simonneau, G. (2011) 'Chronic thromboembolic pulmonary hypertension (CTEPH): results from an international prospective registry', *Circulation,* 124(18), pp. 1973-81.

Pernice, F., Floccari, F., Nostro, L., Caccamo, C., Belghity, N., Mantuano, S., Romeo, A., Barilla', A., Aloisi, C., Ruello, A., Frisina, N. and Buemi, M. (2006) 'Oxidative stress, sister chromatid

exchanges and apoptosis in the pathogenesis of lymphocytopenia in ESRD patients', *J Nephrol,* 19(5), pp. 613-20.

Perreault, M., Białek, A., Trottier, J., Verreault, M., Caron, P., Milkiewicz, P. and Barbier, O. (2013) 'Role of glucuronidation for hepatic detoxification and urinary elimination of toxic bile acids during biliary obstruction', *PLoS One,* 8(11), pp. e80994.

Perros, F., Dorfmüller, P., Montani, D., Hammad, H., Waelput, W., Girerd, B., Raymond, N., Mercier, O., Mussot, S., Cohen-Kaminsky, S., Humbert, M. and Lambrecht, B. N. (2012) 'Pulmonary lymphoid neogenesis in idiopathic pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 185(3), pp. 311-21.

Perros, F., Dorfmüller, P., Souza, R., Durand-Gasselin, I., Mussot, S., Mazmanian, M., Hervé, P., Emilie, D., Simonneau, G. and Humbert, M. (2007) 'Dendritic cell recruitment in lesions of human and experimental pulmonary hypertension', *Eur Respir J,* 29(3), pp. 462-8.

Piao, L., Fang, Y. H., Parikh, K., Ryan, J. J., Toth, P. T. and Archer, S. L. (2013) 'Cardiac glutaminolysis: a maladaptive cancer metabolism pathway in the right ventricle in pulmonary hypertension', *J Mol Med (Berl),* 91(10), pp. 1185-97.

Piao, L., Marsboom, G. and Archer, S. L. (2010) 'Mitochondrial metabolic adaptation in right ventricular hypertrophy and failure', *J Mol Med (Berl),* 88(10), pp. 1011-20.

Piazza, G. and Goldhaber, S. Z. (2011) 'Chronic thromboembolic pulmonary hypertension', *N Engl J Med,* 364(4), pp. 351-60.

Pickup, J. C. (2004) 'Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes', *Diabetes Care,* 27(3), pp. 813-23.

Pietra, G. G. (1994) 'Histopathology of primary pulmonary hypertension', *Chest,* 105(2 Suppl), pp. 2S-6S.

Pinsky, M. R. (2016) 'The right ventricle: interaction with the pulmonary circulation', *Crit Care,* 20, pp. 266.

Pitzalis, C., Jones, G. W., Bombardieri, M. and Jones, S. A. (2014) 'Ectopic lymphoid-like structures in infection, cancer and autoimmunity', *Nat Rev Immunol,* 14(7), pp. 447-62.

Poli, A., Michel, T., Thérésine, M., Andrès, E., Hentges, F. and Zimmer, J. (2009) 'CD56bright natural killer (NK) cells: an important NK cell subset', *Immunology,* 126(4), pp. 458-65.

Pousada, G., Baloira, A., Fontán, D., Núñez, M. and Valverde, D. (2016) 'Mutational and clinical analysis of the ENG gene in patients with pulmonary arterial hypertension', *BMC Genet,* 17(1), pp. 72.

Powell, F., Wagner, P. and West, J. (2016) 'Ventilation, Blood Flow, and Gas Exchange', in Courtney Broaddus, V., Mason, R., Ernst, J., King, T., Lazarus, S., Murray, J., Nadel, J., Slutsky, A. & Gotway, M. (eds.) *Murray and Nadel's Textbook of Respiratory Medicine (Sixth Edition)*. Philadelphia: W.B. Saunders, pp. 44-75.

Preston, I. R., Tang, G., Tilan, J. U., Hill, N. S. and Suzuki, Y. J. (2005) 'Retinoids and pulmonary hypertension', *Circulation,* 111(6), pp. 782-90.

Price, L. C., Montani, D., Tcherakian, C., Dorfmüller, P., Souza, R., Gambaryan, N., Chaumais, M. C., Shao, D. M., Simonneau, G., Howard, L. S., Adcock, I. M., Wort, S. J., Humbert, M. and Perros, F. (2011) 'Dexamethasone reverses monocrotaline-induced pulmonary arterial hypertension in rats', *Eur Respir J,* 37(4), pp. 813-22.

Pugh, M. E., Robbins, I. M., Rice, T. W., West, J., Newman, J. H. and Hemnes, A. R. (2011) 'Unrecognized glucose intolerance is common in pulmonary arterial hypertension', *J Heart Lung Transplant,* 30(8), pp. 904-11.

Puwanant, S., Park, M., Popović, Z. B., Tang, W. H., Farha, S., George, D., Sharp, J., Puntawangkoon, J., Loyd, J. E., Erzurum, S. C. and Thomas, J. D. (2010) 'Ventricular geometry, strain, and rotational mechanics in pulmonary hypertension', *Circulation,* 121(2), pp. 259-66.

Qin, Y., Zhou, A., Ben, X., Shen, J., Liang, Y. and Li, F. (2001) 'All-trans retinoic acid in pulmonary vascular structural remodeling in rats with pulmonary hypertension induced by monocrotaline', *Chin Med J (Engl),* 114(5), pp. 462-5.

Quarck, R., Nawrot, T., Meyns, B. and Delcroix, M. (2009) 'C-reactive protein: a new predictor of adverse outcome in pulmonary arterial hypertension', *J Am Coll Cardiol,* 53(14), pp. 1211-8.

Rafikova, O., Meadows, M. L., Kinchen, J. M., Mohney, R. P., Maltepe, E., Desai, A. A., Yuan, J. X., Garcia, J. G., Fineman, J. R., Rafikov, R. and Black, S. M. (2016) 'Metabolic Changes Precede the Development of Pulmonary Hypertension in the Monocrotaline Exposed Rat Lung', *PLoS One,* 11(3), pp. e0150480.

Rasmussen, T. K., Andersen, T., Hvid, M., Hetland, M. L., Hørslev-Petersen, K., Stengaard-Pedersen, K., Holm, C. K. and Deleuran, B. (2010) 'Increased interleukin 21 (IL-21) and IL-23 are associated with increased disease activity and with radiographic status in patients with early rheumatoid arthritis', *J Rheumatol,* 37(10), pp. 2014-20.

Raverdeau, M. and Mills, K. H. (2014) 'Modulation of T cell and innate immune responses by retinoic Acid', *J Immunol,* 192(7), pp. 2953-8.

Raychaudhuri, B., Bonfield, T. L., Malur, A., Hague, K., Kavuru, M. S., Arroliga, A. C. and Thomassen, M. J. (2002a) 'Circulating monocytes from patients with primary pulmonary hypertension are hyporesponsive', *Clin Immunol,* 104(2), pp. 191-8.

Raychaudhuri, B., Malur, A., Bonfield, T. L., Abraham, S., Schilz, R. J., Farver, C. F., Kavuru, M. S., Arroliga, A. C. and Thomassen, M. J. (2002b) 'The prostacyclin analogue treprostinil blocks NFkappaB nuclear translocation in human alveolar macrophages', *J Biol Chem,* 277(36), pp. 33344-8.

Raymond, R. J., Hinderliter, A. L., Willis, P. W., Ralph, D., Caldwell, E. J., Williams, W., Ettinger, N. A., Hill, N. S., Summer, W. R., de Boisblanc, B., Schwartz, T., Koch, G., Clayton, L. M., Jöbsis, M. M., Crow, J. W. and Long, W. (2002) 'Echocardiographic predictors of adverse outcomes in primary pulmonary hypertension', *J Am Coll Cardiol,* 39(7), pp. 1214-9.

Reiter, C. D., Wang, X., Tanus-Santos, J. E., Hogg, N., Cannon, R. O., Schechter, A. N. and Gladwin, M. T. (2002) 'Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease', *Nat Med,* 8(12), pp. 1383-9.

Rhoads, J. P., Major, A. S. and Rathmell, J. C. (2017) 'Fine tuning of immunometabolism for the treatment of rheumatic diseases', *Nat Rev Rheumatol,* 13(5), pp. 313-320.

Rhodes, C. J., Ghataorhe, P., Wharton, J., Rue-Albrecht, K. C., Hadinnapola, C., Watson, G., Bleda, M., Haimel, M., Coghlan, G., Corris, P. A., Howard, L. S., Kiely, D. G., Peacock, A. J., Pepke-Zaba, J., Toshner, M. R., Wort, S. J., Gibbs, J. S., Lawrie, A., Gräf, S., Morrell, N. W. and Wilkins, M. R. (2017) 'Plasma Metabolomics Implicates Modified Transfer RNAs and Altered Bioenergetics in the Outcomes of Pulmonary Arterial Hypertension', *Circulation,* 135(5), pp. 460-475.

Rich, S., Kieras, K., Hart, K., Groves, B. M., Stobo, J. D. and Brundage, B. H. (1986) 'Antinuclear antibodies in primary pulmonary hypertension', *J Am Coll Cardiol,* 8(6), pp. 1307-11.

Rocha, B., Dautigny, N. and Pereira, P. (1989) 'Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo', *Eur J Immunol,* 19(5), pp. 905-11.

Romme Christensen, J., Börnsen, L., Ratzer, R., Piehl, F., Khademi, M., Olsson, T., Sørensen, P. S. and Sellebjerg, F. (2013) 'Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression', *PLoS One,* 8(3), pp. e57820.

Rosenzweig, E. B., Morse, J. H., Knowles, J. A., Chada, K. K., Khan, A. M., Roberts, K. E., McElroy, J. J., Juskiw, N. K., Mallory, N. C., Rich, S., Diamond, B. and Barst, R. J. (2008) 'Clinical implications of determining BMPR2 mutation status in a large cohort of children and adults with pulmonary arterial hypertension', *J Heart Lung Transplant,* 27(6), pp. 668-74.

Ross, D. J., Hough, G., Hama, S., Aboulhosn, J., Belperio, J. A., Saggar, R., Van Lenten, B. J., Ardehali, A., Eghbali, M., Reddy, S., Fogelman, A. M. and Navab, M. (2015) 'Proinflammatory high-density lipoprotein results from oxidized lipid mediators in the pathogenesis of both idiopathic and associated types of pulmonary arterial hypertension', *Pulm Circ,* 5(4), pp. 640-8.

Rother, R. P., Bell, L., Hillmen, P. and Gladwin, M. T. (2005) 'The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease', *JAMA,* 293(13), pp. 1653-62.

Rudd, J. H., Myers, K. S., Bansilal, S., Machac, J., Rafique, A., Farkouh, M., Fuster, V. and Fayad, Z. A. (2007) '(18)Fluorodeoxyglucose positron emission tomography imaging of atherosclerotic plaque inflammation is highly reproducible: implications for atherosclerosis therapy trials', *J Am Coll Cardiol,* 50(9), pp. 892-6.

Rudd, J. H., Warburton, E. A., Fryer, T. D., Jones, H. A., Clark, J. C., Antoun, N., Johnström, P., Davenport, A. P., Kirkpatrick, P. J., Arch, B. N., Pickard, J. D. and Weissberg, P. L. (2002) 'Imaging atherosclerotic plaque inflammation with [18F]-fluorodeoxyglucose positron emission tomography', *Circulation,* 105(23), pp. 2708-11.

Russo, G. L. (2009) 'Dietary n-6 and n-3 polyunsaturated fatty acids: from biochemistry to clinical implications in cardiovascular prevention', *Biochem Pharmacol,* 77(6), pp. 937-46.

Ryan, J. W., Roblero, J., Stewart, J. M. and Leary, W. P. (1971) 'Metabolism of vasoactive polypeptides in the pulmonary circulation', *Chest,* 59, pp. Suppl:8S-9S.

Sada, Y., Dohi, Y., Uga, S., Higashi, A., Kinoshita, H. and Kihara, Y. (2016) 'Non-suppressive regulatory T cell subset expansion in pulmonary arterial hypertension', *Heart Vessels,* 31(8), pp. 1319-26.

Said, S. I. (1982) 'Metabolic functions of the pulmonary circulation', *Circ Res,* 50(3), pp. 325-33.

Sakaguchi, S. and Sakaguchi, N. (2005) 'Regulatory T cells in immunologic self-tolerance and autoimmune disease', *Int Rev Immunol,* 24(3-4), pp. 211-26.

Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M. (2008) 'Regulatory T cells and immune tolerance', *Cell,* 133(5), pp. 775-87.

Salvagno, G. L., Danese, E. and Lippi, G. (2017) 'Preanalytical variables for liquid chromatography-mass spectrometry (LC-MS) analysis of human blood specimens', *Clin Biochem,* 50(10-11), pp. 582-586.

Sanchez, O., Marcos, E., Perros, F., Fadel, E., Tu, L., Humbert, M., Dartevelle, P., Simonneau, G., Adnot, S. and Eddahibi, S. (2007) 'Role of endothelium-derived CC chemokine ligand 2 in idiopathic pulmonary arterial hypertension', *Am J Respir Crit Care Med,* 176(10), pp. 1041-7.

Sanders, R. J., Ofman, R., Duran, M., Kemp, S. and Wanders, R. J. (2006) 'Omega-oxidation of very long-chain fatty acids in human liver microsomes. Implications for X-linked adrenoleukodystrophy', *J Biol Chem,* 281(19), pp. 13180-7.

Sansbury, B. E., DeMartino, A. M., Xie, Z., Brooks, A. C., Brainard, R. E., Watson, L. J., DeFilippis, A. P., Cummins, T. D., Harbeson, M. A., Brittian, K. R., Prabhu, S. D., Bhatnagar, A., Jones, S. P. and Hill, B. G. (2014) 'Metabolomic analysis of pressure-overloaded and infarcted mouse hearts', *Circ Heart Fail,* 7(4), pp. 634-42.

Santagostino, A., Garbaccio, G., Pistorio, A., Bolis, V., Camisasca, G., Pagliaro, P. and Girotto, M. (1999) 'An Italian national multicenter study for the definition of reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults', *Haematologica,* 84(6), pp. 499-504.

Sato, H., Ota, H., Sugimura, K., Aoki, T., Tatebe, S., Miura, M., Yamamoto, S., Yaoita, N., Suzuki, H., Satoh, K., Takase, K. and Shimokawa, H. (2016) 'Balloon Pulmonary Angioplasty Improves Biventricular Functions and Pulmonary Flow in Chronic Thromboembolic Pulmonary Hypertension', *Circ J,* 80(6), pp. 1470-7.

Schermuly, R. T., Dony, E., Ghofrani, H. A., Pullamsetti, S., Savai, R., Roth, M., Sydykov, A., Lai, Y. J., Weissmann, N., Seeger, W. and Grimminger, F. (2005) 'Reversal of experimental pulmonary hypertension by PDGF inhibition', *J Clin Invest,* 115(10), pp. 2811-21.

Scherrer, L. A. and Gross, R. W. (1989) 'Subcellular distribution, molecular dynamics and catabolism of plasmalogens in myocardium', *Mol Cell Biochem,* 88(1-2), pp. 97-105.

Schipper, R. G., Romijn, J. C., Cuijpers, V. M. and Verhofstad, A. A. (2003) 'Polyamines and prostatic cancer', *Biochem Soc Trans,* 31(2), pp. 375-80.

Schlosser, K., Taha, M., Deng, Y., McIntyre, L. A., Mei, S. H. J. and Stewart, D. J. (2017) 'High circulating angiopoietin-2 levels exacerbate pulmonary inflammation but not vascular leak or mortality in endotoxin-induced lung injury in mice', *Thorax*.

Schwarze-Zander, C., Pabst, S., Hammerstingl, C., Ohlig, J., Wasmuth, J. C., Boesecke, C., Stoffel-Wagner, B., Carstensen, A., Nickenig, G., Strassburg, C. P., Rockstroh, J. K., Skowasch, D. and Schueler, R. (2015) 'Pulmonary hypertension in HIV infection: a prospective echocardiographic study', *HIV Med,* 16(9), pp. 578-82.

Seeger, T., Haffez, F., Fischer, A., Koehl, U., Leistner, D. M., Seeger, F. H., Boon, R. A., Zeiher, A. M. and Dimmeler, S. (2013) 'Immunosenescence-associated microRNAs in age and heart failure', *Eur J Heart Fail,* 15(4), pp. 385-93.

Shriver, L. P. and Manchester, M. (2011) 'Inhibition of fatty acid metabolism ameliorates disease activity in an animal model of multiple sclerosis', *Sci Rep,* 1, pp. 79.

Simon, D., Balogh, P., Bognár, A., Kellermayer, Z., Engelmann, P., Németh, P., Farkas, N., Minier, T., Lóránd, V., Czirják, L. and Berki, T. (2016) 'Reduced non-switched memory B cell subsets cause imbalance in B cell repertoire in systemic sclerosis', *Clin Exp Rheumatol*.

Simonneau, G., Torbicki, A., Dorfmüller, P. and Kim, N. (2017) 'The pathophysiology of chronic thromboembolic pulmonary hypertension', *Eur Respir Rev,* 26(143).

Sitbon, O., Humbert, M., Jaïs, X., Ioos, V., Hamid, A. M., Provencher, S., Garcia, G., Parent, F., Hervé, P. and Simonneau, G. (2005) 'Long-term response to calcium channel blockers in idiopathic pulmonary arterial hypertension', *Circulation,* 111(23), pp. 3105-11.

Sitbon, O., Humbert, M., Nunes, H., Parent, F., Garcia, G., Hervé, P., Rainisio, M. and Simonneau, G. (2002) 'Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival', *J Am Coll Cardiol,* 40(4), pp. 780-8.

Sitbon, O., Lascoux-Combe, C., Delfraissy, J. F., Yeni, P. G., Raffi, F., De Zuttere, D., Gressin, V., Clerson, P., Sereni, D. and Simonneau, G. (2008) 'Prevalence of HIV-related pulmonary arterial hypertension in the current antiretroviral therapy era', *Am J Respir Crit Care Med,* 177(1), pp. 108-13.

Sole, M. J., Drobac, M., Schwartz, L., Hussain, M. N. and Vaughan-Neil, E. F. (1979) 'The extraction of circulating catecholamines by the lungs in normal man and in patients with pulmonary hypertension', *Circulation,* 60(1), pp. 160-3.

Soon, E., Holmes, A. M., Treacy, C. M., Doughty, N. J., Southgate, L., Machado, R. D., Trembath, R. C., Jennings, S., Barker, L., Nicklin, P., Walker, C., Budd, D. C., Pepke-Zaba, J. and Morrell, N. W. (2010) 'Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension', *Circulation,* 122(9), pp. 920-7.

Stan, R. (2009) 'Pulmonary Endothelial Cell Surface Metabolic Functions', in Voelkel, N. & Rounds, S. (eds.) *Anatomy of the Pulmonary Endothelium, in The Pulmonary Endothelium: Function in health and disease*. Chichester, UK: John Wiley & Sons, pp. 105-112.

Starling, E. and Verney, E. (1925) 'The Secretion of Urine as Studied on the Isolated Kidney', *Proc. R. Soc. Lond. B,* (97), pp. 321-363.

Stervbo, U., Bozzetti, C., Baron, U., Jürchott, K., Meier, S., Mälzer, J. N., Nienen, M., Olek, S., Rachwalik, D., Schulz, A. R., Neumann, A., Babel, N., Grützkau, A. and Thiel, A. (2015) 'Effects of aging on human leukocytes (part II): immunophenotyping of adaptive immune B and T cell subsets', *Age (Dordr),* 37(5), pp. 93.

Strange, G., Gabbay, E., Kermeen, F., Williams, T., Carrington, M., Stewart, S. and Keogh, A. (2013) 'Time from symptoms to definitive diagnosis of idiopathic pulmonary arterial hypertension: The delay study', *Pulm Circ,* 3(1), pp. 89-94.

Strange, G., Lau, E. M., Giannoulatou, E., Corrigan, C., Kotlyar, E., Kermeen, F., Williams, T., Celermajer, D. S., Dwyer, N., Whitford, H., Wrobel, J. P., Feenstra, J., Lavender, M., Whyte, K., Collins, N., Steele, P., Proudman, S., Thakkar, V., Keating, D., Keogh, A. and Registry, P. (2017) 'Survival of Idiopathic Pulmonary Arterial Hypertension Patients in the Modern Era in Australia and New Zealand', *Heart Lung Circ*.

Straub, R. H., Schölmerich, J. and Zietz, B. (2000) 'Replacement therapy with DHEA plus corticosteroids in patients with chronic inflammatory diseases--substitutes of adrenal and sex hormones', *Z Rheumatol,* 59 Suppl 2, pp. II/108-18.

Sudhakaran, P. R., Binu, S. and Soumya, S. J. (2014) 'Effect of sarcosine on endothelial function relevant to angiogenesis', *J Cancer Res Ther,* 10(3), pp. 603-10.

Sukumar, M., Liu, J., Ji, Y., Subramanian, M., Crompton, J. G., Yu, Z., Roychoudhuri, R., Palmer, D. C., Muranski, P., Karoly, E. D., Mohney, R. P., Klebanoff, C. A., Lal, A., Finkel, T., Restifo, N. P. and Gattinoni, L. (2013) 'Inhibiting glycolytic metabolism enhances CD8+ T cell memory and antitumor function', *J Clin Invest,* 123(10), pp. 4479-88.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N. and Yancopoulos, G. D. (1996) 'Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis', *Cell,* 87(7), pp. 1171-80.

Sutendra, G., Bonnet, S., Rochefort, G., Haromy, A., Folmes, K. D., Lopaschuk, G. D., Dyck, J. R. and Michelakis, E. D. (2010) 'Fatty acid oxidation and malonyl-CoA decarboxylase in the vascular remodeling of pulmonary hypertension', *Sci Transl Med,* 2(44), pp. 44ra58.

Sutendra, G., Dromparis, P., Bonnet, S., Haromy, A., McMurtry, M. S., Bleackley, R. C. and Michelakis, E. D. (2011) 'Pyruvate dehydrogenase inhibition by the inflammatory cytokine TNFα

contributes to the pathogenesis of pulmonary arterial hypertension', *J Mol Med (Berl),* 89(8), pp. 771-83.

Sutendra, G. and Michelakis, E. D. (2014) 'The metabolic basis of pulmonary arterial hypertension', *Cell Metab,* 19(4), pp. 558-73.

Sutter, I., Klingenberg, R., Othman, A., Rohrer, L., Landmesser, U., Heg, D., Rodondi, N., Mach, F., Windecker, S., Matter, C. M., Lüscher, T. F., von Eckardstein, A. and Hornemann, T. (2016) 'Decreased phosphatidylcholine plasmalogens--A putative novel lipid signature in patients with stable coronary artery disease and acute myocardial infarction', *Atherosclerosis,* 246, pp. 130-40.

Sutter, I., Velagapudi, S., Othman, A., Riwanto, M., Manz, J., Rohrer, L., Rentsch, K., Hornemann, T., Landmesser, U. and von Eckardstein, A. (2015) 'Plasmalogens of high-density lipoproteins (HDL) are associated with coronary artery disease and anti-apoptotic activity of HDL', *Atherosclerosis,* 241(2), pp. 539-46.

Suzuki, C., Takahashi, M., Morimoto, H., Izawa, A., Ise, H., Hongo, M., Hoshikawa, Y., Ito, T., Miyashita, H., Kobayashi, E., Shimada, K. and Ikeda, U. (2006) 'Mycophenolate mofetil attenuates pulmonary arterial hypertension in rats', *Biochem Biophys Res Commun,* 349(2), pp. 781-8.

Szabó, K., Papp, G., Szántó, A., Tarr, T. and Zeher, M. (2016) 'A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjögren's syndrome and systemic lupus erythematosus', *Clin Exp Immunol,* 183(1), pp. 76-89.

Szczeklik, W., Sanak, M., Mastalerz, L., Sokołowska, B. M., Gielicz, A., Soja, J., Kumik, J., Musiał, J. and Szczeklik, A. (2012) '12-hydroxy-eicosatetraenoic acid (12-HETE): a biomarker of Churg-Strauss syndrome', *Clin Exp Allergy,* 42(4), pp. 513-22.

Sztrymf, B., Coulet, F., Girerd, B., Yaici, A., Jais, X., Sitbon, O., Montani, D., Souza, R., Simonneau, G., Soubrier, F. and Humbert, M. (2008) 'Clinical outcomes of pulmonary arterial hypertension in carriers of BMPR2 mutation', *Am J Respir Crit Care Med,* 177(12), pp. 1377-83.

Taboada, D., Pepke-Zaba, J., Jenkins, D. P., Berman, M., Treacy, C. M., Cannon, J. E., Toshner, M., Dunning, J. J., Ng, C., Tsui, S. S. and Sheares, K. K. (2014) 'Outcome of pulmonary endarterectomy in symptomatic chronic thromboembolic disease', *Eur Respir J,* 44(6), pp. 1635-45.

Tadic, M., Ivanovic, B. and Grozdic, I. (2011) 'Metabolic syndrome impacts the right ventricle: true or false?', *Echocardiography,* 28(5), pp. 530-8.

Takahashi, Y., Sakaguchi, K., Horio, H., Hiramatsu, K., Moriya, S., Takahashi, K. and Kawakita, M. (2015) 'Urinary N1, N12-diacetylspermine is a non-invasive marker for the diagnosis and prognosis of non-small-cell lung cancer', *Br J Cancer,* 113(10), pp. 1493-501.

Talati, M. H., Brittain, E. L., Fessel, J. P., Penner, N., Atkinson, J., Funke, M., Grueter, C., Jerome, W. G., Freeman, M., Newman, J. H., West, J. and Hemnes, A. R. (2016) 'Mechanisms of Lipid Accumulation in the Bone Morphogenetic Protein Receptor Type 2 Mutant Right Ventricle', *Am J Respir Crit Care Med,* 194(6), pp. 719-28.

Tamby, M. C., Chanseaud, Y., Humbert, M., Fermanian, J., Guilpain, P., Garcia-de-la-Peña-Lefebvre, P., Brunet, S., Servettaz, A., Weill, B., Simonneau, G., Guillevin, L., Boissier, M. C. and Mouthon, L. (2005) 'Anti-endothelial cell antibodies in idiopathic and systemic sclerosis associated pulmonary arterial hypertension', *Thorax,* 60(9), pp. 765-72.

Tamby, M. C., Humbert, M., Guilpain, P., Servettaz, A., Dupin, N., Christner, J. J., Simonneau, G., Fermanian, J., Weill, B., Guillevin, L. and Mouthon, L. (2006) 'Antibodies to fibroblasts in

idiopathic and scleroderma-associated pulmonary hypertension', *Eur Respir J,* 28(4), pp. 799-807.

Tamosiuniene, R., Tian, W., Dhillon, G., Wang, L., Sung, Y. K., Gera, L., Patterson, A. J., Agrawal, R., Rabinovitch, M., Ambler, K., Long, C. S., Voelkel, N. F. and Nicolls, M. R. (2011) 'Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension', *Circ Res,* 109(8), pp. 867-79.

Tanaka, Y., Kume, S., Araki, H., Nakazawa, J., Chin-Kanasaki, M., Araki, S., Nakagawa, F., Koya, D., Haneda, M., Maegawa, H. and Uzu, T. (2015) '1-Methylnicotinamide ameliorates lipotoxicity-induced oxidative stress and cell death in kidney proximal tubular cells', *Free Radic Biol Med,* 89, pp. 831-41.

Tangye, S. G. (2015) 'Advances in IL-21 biology - enhancing our understanding of human disease', *Curr Opin Immunol,* 34, pp. 107-15.

Taniguchi, K., Shimazaki, C., Fujimoto, Y., Shimura, K., Uchiyama, H., Matsumoto, Y., Kuroda, J., Horiike, S. and Taniwaki, M. (2009) 'Tocilizumab is effective for pulmonary hypertension associated with multicentric Castleman's disease', *Int J Hematol,* 90(1), pp. 99-102.

Taraseviciene-Stewart, L., Gera, L., Hirth, P., Voelkel, N. F., Tuder, R. M. and Stewart, J. M. (2002) 'A bradykinin antagonist and a caspase inhibitor prevent severe pulmonary hypertension in a rat model', *Can J Physiol Pharmacol,* 80(4), pp. 269-74.

Taraseviciene-Stewart, L., Nicolls, M. R., Kraskauskas, D., Scerbavicius, R., Burns, N., Cool, C., Wood, K., Parr, J. E., Boackle, S. A. and Voelkel, N. F. (2007) 'Absence of T cells confers increased pulmonary arterial hypertension and vascular remodeling', *Am J Respir Crit Care Med,* 175(12), pp. 1280-9.

Taraseviciene-Stewart, L., Scerbavicius, R., Stewart, J. M., Gera, L., Demura, Y., Cool, C., Kasper, M. and Voelkel, N. F. (2005) 'Treatment of severe pulmonary hypertension: a bradykinin receptor 2 agonist B9972 causes reduction of pulmonary artery pressure and right ventricular hypertrophy', *Peptides,* 26(8), pp. 1292-300.

Teichert-Kuliszewska, K., Kutryk, M. J., Kuliszewski, M. A., Karoubi, G., Courtman, D. W., Zucco, L., Granton, J. and Stewart, D. J. (2006) 'Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension', *Circ Res,* 98(2), pp. 209-17.

Tierney, D. F., Marton, L. J., Hacker, A. D. and Lowe, N. (1985) 'Polyamines in clinical disorders', *West J Med,* 142(1), pp. 63-73.

Tonelli, A. R., Haserodt, S., Aytekin, M. and Dweik, R. A. (2013) 'Nitric oxide deficiency in pulmonary hypertension: Pathobiology and implications for therapy', *Pulm Circ,* 3(1), pp. 20-30.

Townsley, M. I. (2012) 'Structure and composition of pulmonary arteries, capillaries, and veins', *Compr Physiol,* 2(1), pp. 675-709.

Tsien, C., Davuluri, G., Singh, D., Allawy, A., Ten Have, G., Thapaliya, S., Schulze, J., Barnes, D., McCullough, A., Engelen, M., Deutz, N. and Dasarathy, S. (2015) 'Metabolic and molecular responses to leucine-enriched branched chain amino acid supplementation in the skeletal muscle of alcoholic cirrhosis', *Hepatology,* 61(6), pp. 2018-29.

Tuder, R. M., Chacon, M., Alger, L., Wang, J., Taraseviciene-Stewart, L., Kasahara, Y., Cool, C. D., Bishop, A. E., Geraci, M., Semenza, G. L., Yacoub, M., Polak, J. M. and Voelkel, N. F. (2001) 'Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension: evidence for a process of disordered angiogenesis', *J Pathol,* 195(3), pp. 367-74.

Tuder, R. M., Groves, B., Badesch, D. B. and Voelkel, N. F. (1994) 'Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension', *Am J Pathol,* 144(2), pp. 275-85.

Uehara, M., Enomoto, N., Mikamo, M., Oyama, Y., Kono, M., Fujisawa, T., Inui, N., Nakamura, Y. and Suda, T. (2016) 'Impact of angiopoietin-1 and -2 on clinical course of idiopathic pulmonary fibrosis', *Respir Med,* 114, pp. 18-26.

Ulrich, S., Nicolls, M., Voelkel, N. and Speich, R. 'A high prevalence of lymphocytopenia in various forms of pulmonary hypertension, a possible link to underlying autoimmunity?', *Assemblee annuelle commune des Societes Suisses de Cardiologie, de Pneumologie, de Chirurgie Thoracique et de Medecine Intensive*, Lausanne

Ulrich, S., Nicolls, M. R., Taraseviciene, L., Speich, R. and Voelkel, N. (2008a) 'Increased regulatory and decreased CD8+ cytotoxic T cells in the blood of patients with idiopathic pulmonary arterial hypertension', *Respiration,* 75(3), pp. 272-80.

Ulrich, S., Taraseviciene-Stewart, L., Huber, L. C., Speich, R. and Voelkel, N. (2008b) 'Peripheral blood B lymphocytes derived from patients with idiopathic pulmonary arterial hypertension express a different RNA pattern compared with healthy controls: a cross sectional study', *Respir Res,* 9, pp. 20.

Utzschneider, D. T., Legat, A., Fuertes Marraco, S. A., Carrié, L., Luescher, I., Speiser, D. E. and Zehn, D. (2013) 'T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion', *Nat Immunol,* 14(6), pp. 603-10.

Valencia, X., Yarboro, C., Illei, G. and Lipsky, P. E. (2007) 'Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus', *J Immunol,* 178(4), pp. 2579-88.

van den Hoogen, F., Khanna, D., Fransen, J., Johnson, S. R., Baron, M., Tyndall, A., Matucci-Cerinic, M., Naden, R. P., Medsger, T. A., Carreira, P. E., Riemekasten, G., Clements, P. J., Denton, C. P., Distler, O., Allanore, Y., Furst, D. E., Gabrielli, A., Mayes, M. D., van Laar, J. M., Seibold, J. R., Czirjak, L., Steen, V. D., Inanc, M., Kowal-Bielecka, O., Müller-Ladner, U., Valentini, G., Veale, D. J., Vonk, M. C., Walker, U. A., Chung, L., Collier, D. H., Ellen Csuka, M., Fessler, B. J., Guiducci, S., Herrick, A., Hsu, V. M., Jimenez, S., Kahaleh, B., Merkel, P. A., Sierakowski, S., Silver, R. M., Simms, R. W., Varga, J. and Pope, J. E. (2013) '2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative', *Ann Rheum Dis,* 72(11), pp. 1747-55.

van der Windt, G. J. and Pearce, E. L. (2012) 'Metabolic switching and fuel choice during T-cell differentiation and memory development', *Immunol Rev,* 249(1), pp. 27-42.

Ventetuolo, C. E., Baird, G. L., Barr, R. G., Bluemke, D. A., Fritz, J. S., Hill, N. S., Klinger, J. R., Lima, J. A., Ouyang, P., Palevsky, H. I., Palmisciano, A. J., Krishnan, I., Pinder, D., Preston, I. R., Roberts, K. E. and Kawut, S. M. (2016) 'Higher Estradiol and Lower Dehydroepiandrosterone-Sulfate Levels Are Associated with Pulmonary Arterial Hypertension in Men', *Am J Respir Crit Care Med,* 193(10), pp. 1168-75.

Viglietta, V., Baecher-Allan, C., Weiner, H. L. and Hafler, D. A. (2004) 'Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis', *J Exp Med,* 199(7), pp. 971-9.

Vignali, D. A., Collison, L. W. and Workman, C. J. (2008) 'How regulatory T cells work', *Nat Rev Immunol,* 8(7), pp. 523-32.

Vilá, L. M., Alarcón, G. S., McGwin, G., Bastian, H. M., Fessler, B. J., Reveille, J. D. and Group, L. S. (2006) 'Systemic lupus erythematosus in a multiethnic US cohort, XXXVII: association of

lymphopenia with clinical manifestations, serologic abnormalities, disease activity, and damage accrual', *Arthritis Rheum,* 55(5), pp. 799-806.

Voelkel, N. F. and Gomez-Arroyo, J. (2014) 'The role of vascular endothelial growth factor in pulmonary arterial hypertension. The angiogenesis paradox', *Am J Respir Cell Mol Biol,* 51(4), pp. 474-84.

Voelkel, N. F., Vandivier, R. W. and Tuder, R. M. (2006) 'Vascular endothelial growth factor in the lung', *Am J Physiol Lung Cell Mol Physiol,* 290(2), pp. L209-21.

Vonk Noordegraaf, A. and Galiè, N. (2011) 'The role of the right ventricle in pulmonary arterial hypertension', *Eur Respir Rev,* 20(122), pp. 243-53.

Vyas, V. K. and Ghate, M. (2011) 'Recent developments in the medicinal chemistry and therapeutic potential of dihydroorotate dehydrogenase (DHODH) inhibitors', *Mini Rev Med Chem,* 11(12), pp. 1039-55.

Wada, T., Jesmin, S., Gando, S., Yanagida, Y., Mizugaki, A., Sultana, S. N., Zaedi, S. and Yokota, H. (2013) 'The role of angiogenic factors and their soluble receptors in acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS) associated with critical illness', *J Inflamm (Lond),* 10(1), pp. 6.

Wagenvoort, C. A. (1995) 'Pathology of pulmonary thromboembolism', *Chest,* 107(1 Suppl), pp. 10S-17S.

Wanders, R. J., Komen, J. and Kemp, S. (2011) 'Fatty acid omega-oxidation as a rescue pathway for fatty acid oxidation disorders in humans', *FEBS J,* 278(2), pp. 182-94.

Wang, C., Huang, X. F., Cai, Q. Q., Cao, X. X., Duan, M. H., Cai, H., Zhou, D. B. and Li, J. (2017) 'Prognostic study for overall survival in patients with newly diagnosed POEMS syndrome', *Leukemia,* 31(1), pp. 100-106.

Wang, J., Ioan-Facsinay, A., van der Voort, E. I., Huizinga, T. W. and Toes, R. E. (2007) 'Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells', *Eur J Immunol,* 37(1), pp. 129-38.

Wang, J., Shan, Y., Jiang, Z., Feng, J., Li, C., Ma, L. and Jiang, Y. (2013) 'High frequencies of activated B cells and T follicular helper cells are correlated with disease activity in patients with new-onset rheumatoid arthritis', *Clin Exp Immunol,* 174(2), pp. 212-20.

Wang, L., Li, W., Yang, Y., Wu, W., Cai, Q., Ma, X., Xiong, C., He, J. and Fang, W. (2016a) 'Quantitative assessment of right ventricular glucose metabolism in idiopathic pulmonary arterial hypertension patients: a longitudinal study', *Eur Heart J Cardiovasc Imaging,* 17(10), pp. 1161-8.

Wang, L., Zhao, P., Ma, L., Shan, Y., Jiang, Z., Wang, J. and Jiang, Y. (2014) 'Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus', *J Rheumatol,* 41(9), pp. 1781-92.

Wang, X., Zhu, Y., Zhang, M., Wang, H., Jiang, Y. and Gao, P. (2016b) 'Ulcerative Colitis Is Characterized by a Decrease in Regulatory B Cells', *J Crohns Colitis,* 10(10), pp. 1212-23.

Warburg, O. (1956) 'On the origin of cancer cells', *Science,* 123(3191), pp. 309-14.

Wartski, M. and Collignon, M. A. (2000) 'Incomplete recovery of lung perfusion after 3 months in patients with acute pulmonary embolism treated with antithrombotic agents. THESEE Study Group. Tinzaparin ou Heparin Standard: Evaluation dans l'Embolie Pulmonaire Study', *J Nucl Med,* 41(6), pp. 1043-8.

Wawrzyńska, L., Kurzyna, M., Kuca, P., Fijałkowska, A., Kober, J., Florczyk, M. and Torbicki, A. (2004) '[Autoimmune thyroid diseases in patients with primary pulmonary hypertension]', *Pneumonol Alergol Pol,* 72(1-2), pp. 19-22.

Wei, C., Anolik, J., Cappione, A., Zheng, B., Pugh-Bernard, A., Brooks, J., Lee, E. H., Milner, E. C. and Sanz, I. (2007) 'A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus', *J Immunol,* 178(10), pp. 6624-33.

Wende, A. R. (2016) 'Post-translational modifications of the cardiac proteome in diabetes and heart failure', *Proteomics Clin Appl,* 10(1), pp. 25-38.

Weng, T. P., Fu, T. C., Wang, C. H., Hsu, C. C. and Wang, J. S. (2014) 'Activation of lymphocyte autophagy/apoptosis reflects haemodynamic inefficiency and functional aerobic impairment in patients with heart failure', *Clin Sci (Lond),* 127(10), pp. 589-602.

Wenzel, S. E. (2012) 'Asthma phenotypes: the evolution from clinical to molecular approaches', *Nat Med,* 18(5), pp. 716-25.

West, J., Niswender, K. D., Johnson, J. A., Pugh, M. E., Gleaves, L., Fessel, J. P. and Hemnes, A. R. (2013) 'A potential role for insulin resistance in experimental pulmonary hypertension', *Eur Respir J,* 41(4), pp. 861-71.

West, J. B. (2008) 'Ibn al-Nafis, the pulmonary circulation, and the Islamic Golden Age', *J Appl Physiol (1985),* 105(6), pp. 1877-80.

West, J. B. (2011) 'Comparative physiology of the pulmonary circulation', *Compr Physiol,* 1(3), pp. 1525-39.

Wherry, E. J., Blattman, J. N., Murali-Krishna, K., van der Most, R. and Ahmed, R. (2003) 'Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment', *J Virol,* 77(8), pp. 4911-27.

Wilkens, H., Bauer, M., Forestier, N., König, J., Eichler, A., Schneider, S., Schäfers, H. J. and Sybrecht, G. W. (2003) 'Influence of inhaled iloprost on transpulmonary gradient of big endothelin in patients with pulmonary hypertension', *Circulation,* 107(11), pp. 1509-13.

Winnike, J. H., Busby, M. G., Watkins, P. B. and O'Connell, T. M. (2009) 'Effects of a prolonged standardized diet on normalizing the human metabolome', *Am J Clin Nutr,* 90(6), pp. 1496-501.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. and Turner, N. D. (2004) 'Glutathione metabolism and its implications for health', *J Nutr,* 134(3), pp. 489-92.

Xiao, L., Wei, Y., Zhang, Y. N., Luo, X., Yang, B. Y., Yu, S. F., Wu, X. M., Wu, C. Y. and Li, H. B. (2015) 'Increased IL-21 expression in chronic rhinosinusitis with nasalpolyps', *Clin Exp Allergy,* 45(2), pp. 404-13.

Xin, Y., Lv, J. Q., Wang, Y. Z., Zhang, J. and Zhang, X. (2015) 'Effect of all-trans retinoic acids (ATRA) on the expression of α-smooth muscle actin (α-SMA) in the lung tissues of rats with pulmonary arterial hypertension (PAH)', *Genet Mol Res,* 14(4), pp. 14308-13.

Xu, H., Liu, R., He, B., Bi, C. W., Bi, K. and Li, Q. (2016) 'Polyamine Metabolites Profiling for Characterization of Lung and Liver Cancer Using an LC-Tandem MS Method with Multiple Statistical Data Mining Strategies: Discovering Potential Cancer Biomarkers in Human Plasma and Urine', *Molecules,* 21(8).

Xu, W., Koeck, T., Lara, A. R., Neumann, D., DiFilippo, F. P., Koo, M., Janocha, A. J., Masri, F. A., Arroliga, A. C., Jennings, C., Dweik, R. A., Tuder, R. M., Stuehr, D. J. and Erzurum, S. C. (2007)

'Alterations of cellular bioenergetics in pulmonary artery endothelial cells', *Proc Natl Acad Sci U S A,* 104(4), pp. 1342-7.

Yan, B., A, J., Wang, G., Lu, H., Huang, X., Liu, Y., Zha, W., Hao, H., Zhang, Y., Liu, L., Gu, S., Huang, Q., Zheng, Y. and Sun, J. (2009) 'Metabolomic investigation into variation of endogenous metabolites in professional athletes subject to strength-endurance training', *J Appl Physiol (1985),* 106(2), pp. 531-8.

Yang, L., Venneti, S. and Nagrath, D. (2017) 'Glutaminolysis: A Hallmark of Cancer Metabolism', *Annu Rev Biomed Eng,* 19, pp. 163-194.

Yi, J. S., Cox, M. A. and Zajac, A. J. (2010) 'T-cell exhaustion: characteristics, causes and conversion', *Immunology,* 129(4), pp. 474-81.

Yin, P., Lehmann, R. and Xu, G. (2015a) 'Effects of pre-analytical processes on blood samples used in metabolomics studies', *Anal Bioanal Chem,* 407(17), pp. 4879-92.

Yin, Y., Choi, S. C., Xu, Z., Perry, D. J., Seay, H., Croker, B. P., Sobel, E. S., Brusko, T. M. and Morel, L. (2015b) 'Normalization of CD4+ T cell metabolism reverses lupus', *Sci Transl Med,* 7(274), pp. 274ra18.

Yoon, J. K., Kim, D. H. and Koo, J. S. (2014) 'Implications of differences in expression of sarcosine metabolism-related proteins according to the molecular subtype of breast cancer', *J Transl Med,* 12, pp. 149.

Yoshikawa, N., Shimizu, N., Uehara, M., Oda, A., Matsumiya, R., Matsubara, E., Kobayashi, H., Hosono, O., Kuribara-Souta, A., Baba, H., Nagamura, F., Kiryu, S. and Tanaka, H. (2017) 'The effects of bolus supplementation of branched-chain amino acids on skeletal muscle mass, strength, and function in patients with rheumatic disorders during glucocorticoid treatment', *Mod Rheumatol,* 27(3), pp. 508-517.

Zachara, N. E., O'Donnell, N., Cheung, W. D., Mercer, J. J., Marth, J. D. and Hart, G. W. (2004) 'Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells', *J Biol Chem,* 279(29), pp. 30133-42.

Zamanian, R., Hemnes, A., Praestgaard, A., Tracy, R., Lima, J., Bluemke, D. and Kawut, S. (2012) 'Insulin Resistance And Right Ventricular Structure And Function: The MESA-Right Ventricle Study (abstract)', *American Journal of Respiratory and Critical Care Medicine,* (185), pp. A3453.

Zamanian, R. T., Hansmann, G., Snook, S., Lilienfeld, D., Rappaport, K. M., Reaven, G. M., Rabinovitch, M. and Doyle, R. L. (2009) 'Insulin resistance in pulmonary arterial hypertension', *Eur Respir J,* 33(2), pp. 318-24.

Zervou, S., Whittington, H. J., Russell, A. J. and Lygate, C. A. (2016) 'Augmentation of Creatine in the Heart', *Mini Rev Med Chem,* 16(1), pp. 19-28.

Zhang, S., Zeng, X., Ren, M., Mao, X. and Qiao, S. (2017) 'Novel metabolic and physiological functions of branched chain amino acids: a review', *J Anim Sci Biotechnol,* 8, pp. 10.

Zhao, L., Ashek, A., Wang, L., Fang, W., Dabral, S., Dubois, O., Cupitt, J., Pullamsetti, S. S., Cotroneo, E., Jones, H., Tomasi, G., Nguyen, Q. D., Aboagye, E. O., El-Bahrawy, M. A., Barnes, G., Howard, L. S., Gibbs, J. S., Gsell, W., He, J. G. and Wilkins, M. R. (2013) 'Heterogeneity in lung (18)FDG uptake in pulmonary arterial hypertension: potential of dynamic (18)FDG positron emission tomography with kinetic analysis as a bridging biomarker for pulmonary vascular remodeling targeted treatments', *Circulation,* 128(11), pp. 1214-24.

Zhao, Y., Peng, J., Lu, C., Hsin, M., Mura, M., Wu, L., Chu, L., Zamel, R., Machuca, T., Waddell, T., Liu, M., Keshavjee, S., Granton, J. and de Perrot, M. (2014a) 'Metabolomic heterogeneity of pulmonary arterial hypertension', *PLoS One,* 9(2), pp. e88727.

Zhao, Y. D., Yun, H. Z., Peng, J., Yin, L., Chu, L., Wu, L., Michalek, R., Liu, M., Keshavjee, S., Waddell, T., Granton, J. and de Perrot, M. (2014b) 'De novo synthesize of bile acids in pulmonary arterial hypertension lung', *Metabolomics,* 10(6), pp. 1169-1175.

Zhenyukh, O., Civantos, E., Ruiz-Ortega, M., Sánchez, M. S., Vázquez, C., Peiró, C., Egido, J. and Mas, S. (2017) 'High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation', *Free Radic Biol Med,* 104, pp. 165-177.

Zoeller, R. A., Grazia, T. J., LaCamera, P., Park, J., Gaposchkin, D. P. and Farber, H. W. (2002) 'Increasing plasmalogen levels protects human endothelial cells during hypoxia', *Am J Physiol Heart Circ Physiol,* 283(2), pp. H671-9.