Novel Biomarkers in Vascular Remodelling and Inflammation in Pulmonary Arterial Hypertension

By

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For Mom and Dad,

you guys are the best.

Declaration

I, Rijan Gurung, hereby declare that this thesis is my own work. Where other sources of information have been used, they have been acknowledged.

I confirm that the practical procedures resulting in experimental data presented in this thesis were performed by me except for the following:

- Collection of blood from patients with pulmonary arterial hypertensive patients was conducted by the medical team under Dr. Carmine Dario Vizza at the Pulmonary Hypertension Center, Policlinico Umberto I, Rome, Italy.
- Collection of blood from patients with coronary artery disease was conducted by Dr. Sudheer Koganti at the Royal Free Hospital, London.
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Signed:

Abstract

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease driven by vascular remodelling and inflammation. Presenting symptoms of PAH are nonspecific, making diagnosis often late when the disease is irreversible. Endothelial damage occurs early in the disease progress and medial thickening due to proliferating smooth muscle cells in the distal arteries is the earliest known pathology. Circulating microparticles (MPs) are vesicles released by various cells and used as markers of cell activation during inflammation and vascular damage in various vasculopathies. Thus, the aim was to identify circulating MPs, with a special interest to smooth muscle MPs, to be used as biomarkers in PAH.

Initially, I characterised smooth muscle MPs derived from growing smooth muscle cells in culture. Smooth muscle MPs were positive for platelet derived growth factor receptor-β (PDGFR-β), endoglin, intracellular cell adhesion molecule (ICAM-1) and neural glial antigen 2 (NG2) but negative for platelet endtholial cell adhesion molecule-1 (PECAM-1). High levels of endoglin+/ICAM-1+ and low levels of PDGFRβ+/NG2+ MPs were derived from human umbilical cord vein endothelial cells. PDGF, tumour necrosis factor-α, transforming growth factorβ, and endothelin-1 were growth factors and cytokines that could stimulate the release of MPs from growing smooth muscle cells.

Having characterised smooth muscle MPs (SMMPs), I investigated their levels in plasma from pulmonary arterial hypertension patients and compared them with other vascular inflammatory diseases. Circulating levels of total, smooth muscle,

endothelial, leukocyte, and platelet MPs were elevated in PAH patients compared to age-matched healthy controls and in patients with myocardial ischemia and HIV. PAH drugs, particularly prostacyclin mimetics were effective in decreasing MP numbers in cell culture and in patients after long-term therapy.

The function of MPs and mechanism of their release inhibition by the prostacyclin analogue treprostinil was investigated. MPs in plasma and cultured smooth muscle cells were procoagulant, as measured using a thrombin generation assay, and induced smooth muscle proliferation. Treprostinil inhibited SMMP release via the prostacyclin receptor and the prostaglandin E2 receptor, and also inhibited cell proliferation. Furthermore, the mimetic inhibited calcineurin/nuclear factor of activated T-cells (NFAT) signalling, which was partially reversed by blockade of peroxisome proliferator activated receptor. As calcineurin/NFAT is a driver of smooth muscle proliferation and remodelling, it may be a novel target through which prostacyclin may be signalling.

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Abbreviations

5-HT	Serotonin
6MWD	6-minute walking distance
ACT	Activated clotting time
ACVRL1	Activin A Receptor Type II-like 1
ADM	Adrenomedullin
ADMA	Assymetric dimethyl arginine
ADP	Adenosine diphosphate
Akt	Also known as protein kinase B
ALK	Activin receptor-like kinase
ANG	Angiopoietin
ANP	Atrial natriuretic peptide
AP-1	Activator protein-1
APAH	Associated pulmonary arterial hypertension
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7
ARIES	Ambrisentan for the Treatment of Pulmonary Arterial Hypertension, Randomized, Double-blind, Placebo-controlled, Multicentre, Efficacy study 1 and 2
ATP	Adenosine triphosphate
AUC	Area under the curve
Bcl-2	B-cell lymphoma 2
BK_{Ca}	Large conductance calcium activated potassium channel
BMPR2	Bone morphogenetic protein receptor type 2
BNP	Brain natriuretic peptide
BREATHE	Bosentan Randomised trial of Endothelin Antagonist Therapy
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAV1	Caveolin 1
CCL	Chemokine ligand
CHD	coronary heart disease
CI	Confidence interval

Cl-	Chloride
CnA	Calcineurin A
CPM	Carboxypeptidase M
CRP	C-reactive protein
CsA	Cyclosporine A
CTD	Connective tissue disease
CTEPH	Chronic thromboembolic pulmonary arterial hypertension
cTnI	Cardiac tropronin I
cTnT	Cardiac troponin T
DMEM	Dulbecco's Modified Eagle's Medium
DP	Prostaglandin D receptor
EARLY	Endothelin Antagonist Trial in Mildly Symptomatic Pulmonary Arterial Hypertensive Patients
EBM	Endothelial growth basal medium
EC	Endothelial cell
ECG	Echocardiogram
EDTA	Ethylenediaminetetraacetic acid
EIF2AK4	Eukaryotic translation initiation factor-2 alpha kinase 4
ELISA	Enzyme-linked immunosorbent assay
EMP	Endothelial microparticle
eNOS	Endothelial nitric oxide synthase
EP	Prostaglandin E receptor
ERA	Endothelin receptor antagonist
ERK	Extracellular-signal-regulated kinase
ET-1	Endothelin-1
$ET_{A/B}$	Endothelin A/B
ETRA	Endothelin receptor antagonist
FBS	Foetal bovine serum
FCS	Feotal calf serum
FITC	Fluorescein isothiocyanate
FPAH	Familial pulmonary arterial hypertension
GF15	Growth factor 15
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α	Hypoxia-inducible factor α
HIV	Human immunodeficiency virus
hsCRP	Highly specific CRP
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule
IFNγ	Interferon gamma
IL-1/6	Interleukin-1
iNOS	Inducible nitric oxide synthase
IP3	Inositol-1, 4, 5-triphosphate
IPAH	Idiopathic pulmonary arterial hypertension
ITIM	Immunoreceptor tyrosine inhibitory motif
K+	Potassium
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	Calcium activated potassium channel
KCNK3	Potassium channel subfamily K member 3
Kv1.5	Voltage gated potassium channel 1.5
LMP	Leukocyte microparticlee
LRP1	Low density lipoprotein receptor-related protein 1
MAPK	Mitogen activated protein kinase
MCAM	melanoma cell adhesion molecule
MCP-1	Monocyte chemoattractant protein-1
mmHg	Millimetre of mercury
MP	Microparticle
mPAP	Mean pulmonary artery pressure
mRAP	Mean right atrial pressure
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa B
NG2	Neural glial 2
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
Non-STEMI	Non-ST-elevated myocardial infarction

OPN	Osteopontin
PACES	Pulmonary Arterial Hypertension Combination Study of Epoprostenol and Sildenafil
PAEC	Pulmonary artery endothelial cell
PAH	Pulmonary arterial hypertension
PASM	Pulmonary arterial smooth muscle
PASMC	Pulmonary arterial smooth muscle cell
PCH	Pulmonary capillary hemangiomatosis
PCWP	Pulmonary capillary wedge pressure
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PE	Phycoerythrin
PEC	Pulmonary endothelial cell
PECAM-1	Platelet endothelial cell adhesion molecule-1
PGI_2	Prostacyclin
PGI_2S	Prostacyclin synthase
PH	Pulmonary hypertension
PHIRST	Pulmonary Arterial Hypertension and Response Trial
PI	Propidium iodide
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PMP	Platelet microparticle
PMS	phenazine methosulfate
PPAR	Peroxisome proliferator-activated receptor
PPP	Platelet poor plasma
PPRE	Peroxisome proliferator response element
PS	Phosphatidylserine
PVOD	Pulmonary veno-occlusive disease
PVR	Pulmonary vascular resistance
RANTES	Regulated on activation, normal T cell expressed and secreted
REVEAL	Registry to Evaluate Early Long-term pulmonary arterial hypertension disease management

RHC	Right heart catheter
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
RV	Right ventricle
RXR	Retinoid X receptor
Sch	Schistosomiasis
SM22α	Smooth muscle 22α
SMC	Smooth muscle cell
SMMP	Smooth muscle microparticle
SOCE	Sore operated calcium entry
STAT	Signal transducers and activators of transcription
STEMI	ST-elevated myocardial infarction
STEP-1	Safety and Pilot Efficacy Trial in Combination with Bosentan for the Evaluation in Pulmonary Arterial Hypertension
TASK	TWIK-related acid sensitive potassium channel
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TGF-β	Transforming growth factor beta
THBS1	Thrombospondin 1
TnC	Troponin C
TNF-α	Tumour necrosis factor α
TP	Thromboxane receptor
TRIUMPH	Treprostinil Sodium Inhalation used in the Management of Pulmonary Arterial Hypertension
TRPC	Transient receptor potential channel
VCAM	vascular cell adhesion molecule
VDCC	Voltage dependent calcium channel
VE-cadherin	Vascular endothelial cadherin
VEGF-1/2	Vascular endothelial growth factor-1/2
VLA_4	Very late antigen 4
VO_2	Peak oxygen uptake
vWF	Van Willebrand Factor

WHO	World Health Organisation
WU	Wood units
α-SMA	α-smooth muscle actin

Introduction

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare and progressive vascular remodelling disease which is ultimately fatal unless patients undergo lung transplant. Patients with PAH belong to a group having pulmonary hypertension (PH) which is defined as an increase in mean pulmonary artery pressure (mPAP) >25 mmHg at rest as assessed by right heart catheterisation (RHC). This compares to a normal mPAP at rest of 14±3 mmHg with an upper limit of approximately 20 mmHg. Patients with PAH are characterised as having pre-capillary PH defined by a pulmonary capillary wedge pressure (PCWP) <15 and a pulmonary vascular resistance (PVR) >3 Wood units (WU) without the presence of other precapillary causes of PH brought on by other lung diseases (Hoeper et al., 2013).

PAH affects people of all age groups. Without treatment, the median survival after diagnosis for patients is only 3 years for adults (D'Alonzo et al., 1991) and less than 10 months in children (Takatsuki and Ivy, 2013). In neonates and infants, the development of PAH most probably arises from the failure of the neonatal vasculature to dilate at birth (Rabinovitch, 2012). The remarkably reduced number of alveolar ducts and abnormally muscularised pulmonary arteries at the alveolar duct and wall levels are characteristic of the disease. In older infants, children and adults, PAH is also characterised by intimal hyperplasia, which leads to pulmonary artery occlusion, a rise in vascular pressure and the formation of plexiform lesions (Rabinovitch, 2012). The severity of PAH can be tested using clinical parameters that measure pulmonary haemodynamics, exercise capacity

and World Health Organisation (WHO) functional class, a reliable predictor of survival at time of diagnosis as well as during follow-up (Galie et al., 2015b).

1.1 Pulmonary hypertension Classification

Pulmonary arterial hypertension is, as mentioned before, a group of patients with PH. There are 5 categories or "groups" in PH under the WHO Classification of Pulmonary Hypertension most recently updated at the 5th World Conference in Nice 2013 (Simonneau et al., 2013). The first group is termed PAH and includes a wide variety of causes of PH that share similar vascular remodelling characteristics. Under PAH are 5 main classes: Idiopathic (IPAH), heritable or familial (FPAH), drug and toxin induced, associated pulmonary arterial hypertension (APAH), and persistent PH of the newborn (Table 1). All forms of PAH leads to reduced survival and pathological sequelae such as plexiform lesions (McLaughlin and McGoon, 2006).

All other groups signify pre-capillary PH in presence of other causes. Group 2 represents PH due to left heart disease. Group 3 represents PH associated with parenchymal lung disease and/or hypoxia. Group 4 represents chronic theromboemoblic pulmonary hypertension (CTEPH), which is the obstruction of the pulmonary vasculature resulting from unresolved embolus masses that undergo fibrosis. Group 5 represents PH with unclear and/or multifactorial mechanisms.

Classification of Pulmonary Hypertension

Group 1: Pulmonary arterial hypertension (PAH)

1.1 Idiopathic (IPAH)
1.2 Heritable (HPAH)
- 1.2.1 BMPR2 mutation
- 1.2.2 Other mutations

1.3 Drugs or toxins induced
1.4 Associated with:
- 1.4.1 Connective tissue disease
- 1.4.2 Human immunodifficiency virus (HIV) infection
- 1.4.3 Portal hypertension
- 1.4.4 Congenital heart disease
- 1.4.5 Schistosomiasis

1'. Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH)

1'.1 Idiopathic

1'.2 Heritable

- 1'.2.1 EIF2AK4 mutation
- 1'.2.2 Other mutations

1'.3 Drugs, toxins and radiation induced

1'.4 Associated with:

- 1'.4.1 Connective Tissue Disease
- 1'.4.2 HIV Infection

1'' Persistent pulmonary hypertension of the newborn

Group 2: Pulmonary hypertension due to left heart disease

2.1 Left ventricular systolic dysfunction

2.2 Left ventricular diastolic dysfunction

2.3 Valvular disease

2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

2.5 Congenital/acquired pulmonary veins stenosis

Group 3: Pulmonary hypertension due to lung disease and/or hypoxia

3.1 Chronic obstructive pulmonary disease (COPD)

3.2 Interstitial lung disease

3.3 Other pulmonary disease 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
Group 4: Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions 4.1 Chronic thromboembolic pulnmonary hypertension (CTEPH) 4.2 Other pulmonary artery obstructions 4.2.1 Angiosarcoma 4.2.2 Other intravascular tumors 4.2.3 Arteritis 4.2.4 Congential pulmonary arteries stenoses 4.2.5 Parasites (hydatidosis)
Group 5: Pulmonary hypertension with unclear and/or multifactorial mechanisms 5.1 Haematological disorders: haemolytic anaemia, myeloproliferative disorders, splenectomy 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomatosis neurofibromatosis 5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders 5.4 Others: pulmonary tumoral thrombohic microangiopathy, fibrosing mediastinitis, chronic renal failure (with/without dialysis), segmental pulmonary hypertension

Table 1 – Clinical classification of pulmonary hypertension. Adapted from (Adapted from Galie et al., 2015).

Functional classification of pulmonary hypertension

Class I	Patients with pulmonary hypertension without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnoea or fatigue, chest pain or near syncope
Class II	Patients with pulmonary hypertension resulting in slight limitation of physical activity but comfortable at rest. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class III	Patients with pulmonary hypertension resulting in marked limitation of physical activity but comfortable at rest. Less than ordinary activity causes undue dyspnoea or fatigue, chest pain or near syncope.
Class IV	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. Patients manifest signs of right heart failure. Dyspnoea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity

Table 2 - Current World Health Organisation / New York Heart Association Classification of functional status in patients with pulmonary hypertension. The severity of PAH in patients can be classified using the WHO functional class system. Patients with the mildest form of disease in early-stage PAH are placed in class I and patients with the most severe are placed in class IV. The WHO functional class system is helpful in aiding decision making for PAH therapy and serves as an accurate predictor of patient mortality. (Adapted from Galie et al., 2015).

1.1.1 Idiopathic pulmonary arterial hypertension

Idiopathic pulmonary arterial hypertension (IPAH) is a form of PAH where the cause of the disease is undetermined, thus no family history and risk factor is identified to be present. It is characterised histopathologically by muscularisation of the precapillary arterioles, medial thickening due to vascular smooth muscle cell proliferation, and angioproliferative plexiform lesions, which are complex glomeruloid-like vascular structures of endothelial cells originating from pulmonary arteries (McLaughlin and McGoon, 2006; Jonigk et al., 2011). There is also cell proliferation in the intima though their origin is not clear as they may be smooth muscle-like cells that could originate as stem cells, fibrocytes or transform from endothelial cells. IPAH is a rare disease, with incidences of 2-5 million per year and a male:female ratio of 1:2.7 (Pugh and Hemnes, 2010). According to the first US National Institutes of Health registry created in 1981, the mean age of patients with IPAH was 36 years. Now that PAH is diagnosed more frequently, the mean age of diagnosis has increased to 50-65 years in many registries. Moreover, survival has also improved among patients, while the female predominance has been variable and may not even exist in the elderly (Galie et al., 2015b). A 2013 study recruited 32 study participants and revealed that the overall mean age of IPAH patients was 56±16 years at the time of symptom onset and 59±17 years at the time of diagnosis by right heart catheter. Males were older than females at symptom onset as mean age was 58versus 53 years, respectively (Strange et al., 2013). Symptoms of the disease include dyspnea and fatigue, due to decreased gas exchange not only as a result of the vascular remodelling but also a loss of peripheral blood vessels coined “vascular pruning”. This is often

followed by chest pain or angina (McLaughlin and McGoon, 2006). PAH will lead to right ventricular hypertrophy as the heart compensates to generate enough force to pump blood through the pulmonary circulation against a higher pressure. Unremitted hypertrophy will lead to right ventricular failure and ultimately death.

1.1.2 Heritable pulmonary arterial hypertension

PAH with familial cases of identified gene mutations is known as heritable. Germline mutations in the gene coding for the bone morphogenetic protein receptor type II (BMPR2), which is a member of the transforming growth factor (TGF)-β signalling family, are present in up to 80% of the familial cases of PAH. BMPR2 mutations have also been shown to be present in up to 26% of IPAH (Thomson et al., 2000; Eyries et al., 2013; Austin and Loyd, 2014). Bi-allelic mutations in eukaryotic translation initiation factor 2 alpha kinase 4 (EIF2AK4), which encodes a serine threonine kinase expressed in response to amino acid deprivation, have been reported in all familial pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis and in 25% of histologically confirmed PVOD and PCH (Eyries et al., 2013). Mutations in other genes of the TGF-β family have also been detected in particular PAH presentations such as the ACVRL1 gene encoding for the TGF-β co-receptor, endoglin, a gene that is also mutated in hereditary hemorrhagic telangiectasia (Harrison et al., 2003). Other mutations that have also been shown to be involved in PAH are the activin receptor-like kinase -1 (ALK-1), SMAD9, Caveolin 1 (CAV1), and the potassium channel subfamily K gene KCNK3 (Galie et al., 2015b).

1.1.3 Drug- and toxin-induced PAH

The exposure to various prominent drugs and toxins has been found to be associated with PAH. In the 1960s and later in 1990s, the weight loss stimulant drugs aminorex and fenfluramine derivatives were closely linked with PAH outbreaks (Souza et al., 2008; McLaughlin et al., 2015). In 2012, benfluorex, a drug sharing similar structural and pharmacological characteristics as fenfluramine was withdrawn in the European Union due to the risk of right-valve disease, was also revealed to be associated with increased incidence of PAH (Savale et al., 2012). The anticancer drug and broad-spectrum tyrosine kinase inhibitor, dasatinib, was linked with a series of cases of drug-induced PAH in patients with chronic myelogenous leukemia, and in most cases patients did not fully recover hemodynamically (Montani et al., 2012). Additionally, interferon therapy has also been associated to PAH development (McLaughlin et al., 2015).

1.1.4 Associated pulmonary arterial hypertension

PAH has been shown to be associated with various other diseases such as connective tissue disease (CTD), human immunodeficiency virus (HIV), portal hypertension, congenital heart disease (CHD), and schistosomiasis. Approximately 15-25% of total PAH cases are accounted to CTD-associated PAH in worldwide registries (Badesch et al., 2010). The leading causes are systemic sclerosis (SSc) and systemic lupus erythematosus (SLE). Moreover, the leading cause of SSc is PAH (Humbert et al., 2011). 30% of patients with CTD-associated PAH have 1 year mortality, as opposed to 15% of patients with IPAH.

Remarkably, cases of reversible PAH have been reported in patients with mixed CTD and SLE (McLaughlin et al., 2015). Patients with HIV have increased risk in developing PAH, with prevalence of 0.5% (Benza et al., 2012). The clinical and haemodynamic presentations are similar to IPAH. The USA REVEAL (Registry to Evaluate Early Long-term PAH Disease Management) registry showed that survival was at 93% at 1 year and 75% at 3 years. Both PAH drugs and highly active anti-retroviral drugs used in HIV have been reported to reverse PAH (Degano and Sitbon, 2009). Approximately 6% of patients with portal hypertension develop PAH independent of liver disease severity, though the long-term prognosis is associated with both liver and pulmonary vascular disease. PAH associated with portal hypertension, or portopulmonary hypertension, has been associated with increased mortality during and after liver transplantation, particularly if the mPAP is higher than 35 mm Hg. With a 3 year survival of 40%, prognosis in portopulmonary hypertension is worse than in IPAH/HPAH with 64% survival (Krowka et al., 2012). As disease management is improving, more and more children with PAH associated with congenital heart disease (CHD) are surviving to adulthood. Approximately 10% of adults with CHD develop PAH (Engelfriet et al., 2007). PH is one of the most prominent complications seen in schistosomiasis (Sch), an infectious disease caused by parasitic trematode worms. A prevalence of 4.6% of PAH among patients diagnosed with hepatosplenic schistosomiasis mansoni (Lapa et al., 2009), and approximately 20% of newly diagnosed PAH cases in endemic countries may be due to Sch-PAH. The 3-year mortality of patients with Sch-PAH is 15% (Dos Santos Fernandes et al., 2010) and it has been suggested that these patients respond well with improvements in functional class, 6-minute walking distance, cardiac index and pulmonary vascular

resistance (PVR) to PAH therapies including phosphodiesterase-5 inhibitors and endothelin antagonists (Fernandes et al., 2012).

1.1.5 Pulmonary veno-occlusive disease, pulmonary capillary hemangiomatosis, and persistent PH of the newborn

As pulmonary veno-occlusive disease (PVOD) and pulmonary capillary hemangiomatosis (PCH) share closer similarities to group 1 PAH than any other group such as the ability to carry the BMPR2 mutation and being diseases of vascular remodelling with the presence of lesions, they are classified as 1' (Montani et al., 2009a). Significant differences in PVOD and PCH presentation from group 1 PAH include chest computed tomography findings showing pulmonary opacities, lymph node enlargement and signs of oedema, as well as causal homozygous EIF2AK4 mutations in heritable cases (Montani et al., 2009b; Eyries et al., 2013). Likewise, due to the difference and similarities, persistent PH of the newborn is classified as 1" (McLaughlin et al., 2015).

1.2 Remodelling in PAH

Historically, endothelial dysfunction was assumed to be responsible for the onset of vascular remodelling. More recently, numerous studies have suggested that other vessel wall cells including smooth muscle cells, fibroblasts and non-resident vascular cells, such as bone marrow-derived stem cells could play a major role in disease initiation as well (Davie et al., 2009). Five vascular abnormalities have been established in the remodelling process in PAH: 1) abnormal muscularisation

of distal precapillary arteries, 2) loss of precapillary arteries, 3) thickening of large pulmonary arteries, 4) neointimal formation in smaller vessels less than 100-500 μm in diameter and 5) formation of plexiform lesions within the affected vasculature (Figure 1; Rabinovitch, 2012). Pathological features of PAH also include the formation of thrombotic lesions resulting from endothelial dysfunction-induced local thrombosis (Humbert et al., 2004a). Dysregulated endothelial proliferation may also lead to the formation of aberrant channels in the obliterated lumen and adventitia of vessels, perhaps as a result of apoptotic-resistant endothelial cell expansion or circulating endothelial progenitor cell accumulation at areas of vascular injury (Masri et al., 2007; Rabinovitch, 2012).

Indeed, a key factor in the remodelling process is enhanced pulmonary arterial smooth muscle (PASM) proliferation accompanied by depressed apoptosis of these cells (Sakao et al., 2010). Many factors are involved in driving their proliferation such as the BMPR-2 mutations, increased expression and activity of the platelet derived growth factor (PDGF) receptor and the serotonin (5-HT) transporter, and de novo expression of the anti-apoptotic protein survivin. In addition, the levels of various growth factors such as PDGF, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are also elevated, which all increase cell proliferation and promote remodelling (Humbert et al., 2004b; Hassoun et al., 2009). Reduction in the expression and function of the voltage-gated potassium channel, Kv1.5 has been associated with vasoconstriction seen in PAH as well as increase of the anti-apoptotic protein, Bcl-2 (Bonnet et al., 2007). Upregulation of remodelling-associated genes such as hypoxia-inducible

factor-1 α (HIF-1α), VEGF-α, VEGF-1/-2, angiopoietin-1 (ANG-1), Tie-2, thrombospondin (THBS1)-1, the stem cell growth factor receptor c-kit, and the vascular sprouting-associated markers NOTCH4 and matrix metalloproteinase 9 are also present in PAH (Jonigk et al., 2011).

Classically in PAH, there are three major pathways that have been proposed to cause or contribute to the remodelling: the prostacyclin (PGI_2), endothelin (ET), and nitric oxide (NO) pathways (McLaughlin and McGoon, 2006). There is impaired production of vasodilator such as NO and PGI_2 and prolonged overexpression of vasoconstrictor agents such as endothelin-1 (ET-1) and thromboxane A_2, which results in elevated pulmonary artery pressure, cell proliferation and thrombosis in the lung. These pathways will be further discussed in later sections of this chapter.

1.3 Pathways in pulmonary arterial hypertension

1.3.1 Nitric oxide pathway

Nitric oxide (NO) is a potent vasodilator that is synthesised by NO synthase (NOS) via the conversion of L-arginine to NO and L-citrulline in a process requiring NADPH and O_2 as cosubstrates and (6R)-tetrahydrobiopterin (BH_4), FAD, FMN and iron protoporphyrin IX (haem) as cofactors (Korhonen et al., 2005). 3 isoforms of NOS have been identified: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). eNOS is expressed mainly in endothelial cells while nNOS is expressed in the brain and peripheral nervous system. Both eNOS and nNOS are constitutively expressed. eNOS and nNOs are activated by increases in intracellular calcium levels which stabilises binding of calmodulin to constitutive NOS, thus leading to the production of NO. When intracellular calcium levels decrease, the production discontinues, thus NO production is transient and short-lasting. In contrast, iNOS expression is found in most resting cells and its gene expression in various inflammatory and tissue cells is induced by microbial products such as lipopolysaccharide (LPS) and double stranded RNA, or proinflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor α (TNFα) and interferon γ (IFNγ). iNOS can produce high levels of NO for prolonged periods due to its tight binding to calmodulin even at low intracellular calcium levels.

NO is an important regulator of endothelial proliferation and survival, smooth muscle proliferation and platelet function. High levels of the NOS inhibitor

asymmetric dimethylarginine (ADMA) have been shown in PAH (Pullamsetti et al., 2011), while low levels of cofactors such as BH_4 have been shown to cause PH (Khoo, 2005). NO can activate cytoplasmic (soluble) guanylyl cyclase (cGC) which leads to the generation of cyclic guanosine monophosphate (cGMP). Cyclic GMP in turn stimulates protein kinase G, which through its downstream phosphorylation of channels, receptors, kinases and phosphatases is able to reduce vascular tone and cardiac hypertrophy seen in PAH (Takimoto et al., 2005; Kass et al., 2007). NO is also able to modulate the vasculature independent of cGMP through protein S-nitrosylation of thiol groups contained in proteins or nitration of aromatic amino acids (Kass et al., 2007).

1.3.2 Endothelin pathway

Endothelin 1 (ET-1) is a 21-amino acid peptide that is produced in vascular endothelial cells. It is derived from big ET-1 via the action of phosphorimidon-sensitive metalloproteinase, endothelin converting enzyme (ECE), which is present in several isoforms in both vascular endothelial and smooth muscle cells. It is a potent vasoconstrictor and mitogen for vascular smooth muscle and thus is implicated in the pathology of PAH and vascular remodelling. ET-1 is able to mediate its effect on the pulmonary vasculature by binding to its two G-protein-coupled receptors, ET_A and ET_B, which exhibit species, developmental and regional differences (Davie et al., 2002). Both receptors are involved in ET-1-mediated contraction in the distal pulmonary arteries, whereas ET_A is largely responsible for the contraction of proximal arteries (Davie et al., 2002). ET_B is involved in the clearance of ET-1, possibly explaining why treatment with dual

ET-1 receptor antagonists (ERAs) actually increases ET-1 levels (Williamson et al., 2000). Furthermore, ET_B receptors are also expressed in endothelial cells, which upon activation, can release vasodilator and anti-proliferative substances such as PGI_2 and NO, which may counter the vasoconstricting effects of ET-1. Levels of ET-1 are raised in lungs of patients with PAH (Cacoub et al., 1997) and shown to be a poor predictor of survival. In rats, higher circulating ET-1 has been associated with increased susceptibility to hypoxia-induced pulmonary vascular remodelling (Aguirre et al., 2011). In cell culture, ET-1 is a strong driver of growth of PASM cells (PASMCs) from fawn hooded rats (Zamora et al., 1996; Wort et al., 2001). ET-1 secretion from endothelial cells has been shown to be inhibited by prostacyclin in a dose dependent manner (Prins et al., 1994). In patients, epoprostenol was reported to have a beneficial effect in the homeostasis of ET1 by promoting its clearance (Langleben et al., 1999).

1.3.3 Prostacyclin pathway

Prostacyclin (PGI_2) is a 20 carbon prostanoid derivative that was discovered by John Vane and colleagues in the late 1970s as a naturally occurring vasoactive regulator that works as a powerful vasodilator and inhibitor of platelet aggregation and cell proliferation (Takubowski et al., 1994; Gryglewski, 2008). In contrast, thromboxane A_2, which is also derived from COX, is a potent vasoconstrictor, mitogen and platelet activator, and thus opposes PGI_2 in regulating the cellular functions in the vasculature (Anderson and Nawarskas, 2010). PGI_2 is synthesised within vascular endothelial and smooth muscle cells in response to the oxidation

of arachidonic acid by cyclooxygenase (COX)- 1 and -2 enzymes (Flavahan, 2007). A 1982 study conducted by Lewis Rubin showed that intravenous PGI_2 in primary PH patients (synonymous with IPAH) showed responsive pulmonary vasodilation that was dose dependent (Rubin et al., 1982; Nemenoff et al., 2008). An increase in cardiac output without a large fall in systemic blood pressure was also seen, which led to future promising studies in the field.

PGI_2 is very unstable and has a half-life at physiological pH and temperature of approximately three minutes either *in vitro* or in *vivo* (Dusting et al., 1978; Clapp and Gurung, 2015). Due to this, a series of compounds have been made which are chemically based around PGI_2 that are not susceptible to hydrolysis in solution and hav a longer biological half-life *in vivo*. These prostacyclin analogues are further explained in section 1.6.4.

1.4 Biomarkers in PAH

The diagnostic approach for PAH is based on the patient's history and physical examination by echocardiogram with confirmation by right heart catheterisation, the gold standard for haemodynamic evaluation despite being highly invasive (Bazan and Fares, 2015). Although echocardiography is less invasive, it has limited accuracy for estimating hemodynamic measures such as PAP (Fisher et al., 2009). When considering treatment decisions for PAH, echocardiography alone is not sufficient and RHC is required (Galie et al., 2015b). Despite advances in drug therapeutics, late diagnosis and a lack of indices have continued to make it difficult to improve the efficacy of treatments and patient survival in PAH. Many of the clinical and hemodynamic parameters currently used for disease confirmation and/or progression lack standardisation, reproducibility and are invasive. In an effort to bypass these limitations, a number of circulating biomarkers have been investigated in PAH as potential objective and non-invasive tools for diagnosis, prognosis, and response to therapy (Pezzuto et al., 2015).

A biomarker is defined by The National Institutes of Health Biomarker Definition Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Recently, biomarkers have also been described as disease-associated changes in body tissue and fluids (Poste, 2011). Ideally, a biomarker should be a surrogate for clinical end points that is observer independent, widely available,

non-invasive, disease-specific, a sign of disease activity, a target for treatment, and statistically significant (Pezzuto et al., 2015).

1.4.1 Microparticles

MPs are procoagulant vesicles that are released in the circulation by various cells within the vasculature upon cell activation and/or apoptosis (Simak and Gelderman, 2006; Lacroix and Dignat-George, 2013). They are also able transport inflammatory components from their cells of origin as well as miRNA and bind and fuse to their target cells through receptor-ligand interactions and thus mediate inflammation and coagulation (Diehl et al., 2012). They are elevated in the circulation in PAH and appear to correlate with severity of disease, though their origin has only been characterised for endothelial cells and leukocytes (Amabile et al., 2008). A marker that indicates both inflammatory status as well as vascular remodelling and damage could be extremely useful as a biomarker in PAH. Indeed, MPs are pro-inflammatory, pro-thrombotic submicron phospholipid vesicles (mostly 100nm to 1μm in diameter) derived from eukaryotic cells that bleb off from the plasma membranes of various cells such as platelets, leukocytes and endothelial cells in response to different types of stimulation such as inflammatory insult by cytokines (Simak and Gelderman, 2006; Dignat-George and Boulanger, 2011).

The most extensively studied are platelet-derived MPs (PMPs), which were thought to hold pathophysiological importance as they expose procoagulant anionic phospholipids such as phosphotidylserine on their outer surfaces. In 1946,

Chargaff and West demonstrated that high speed centrifugation could prolong the clotting of platelet poor plasma (PPP), suggesting that subcellular particles that were procoagulant could be present in plasma and removed by sedimentation (Chargaff and West, 1946). Later in 1967, Wolf was able to show that activated platelets shed membrane fragments which he termed "platelet dust". These particles were also associated with phospholipid-related procoagulant activity in plasma, specifically platelet factor 3 (Wolf, 1967).

By definition, MPs are procoagulant phospholipid microvesicles that contain certain membrane receptors as well as other proteins associated with the parent cells from which they were derived. Through flow cytometry, targeting cell-specific antigens or combinations of these antigens allows the identification of their cellular origin. MPs may vary in size, the smallest ones being comparable to the size of exosomes (40-80nm), which are intracellular multivesicular bodies fused with the plasma membrane. A crucial distinction between the subcellular fragments is their source and mechanism of derivation (Orozco and Lewis, 2010). Unlike exosomes, MPs richly expose phosphatidylserine (PS) on their membranes, and the calcium-dependent phospholipid, annexin V, which has a high affinity for PS, can be utilised to detect MPs (Simak and Gelderman, 2006). The upper size limit of MPs is considered to be 1.5μm in diameter but typically in assays 1μm is used as larger MPs can be the same size or even larger than platelets, making the distinction of MPs from platelets or platelet-MP aggregates difficult (Simak and Gelderman, 2006). Endothelial and platelet microparticles have been shown to serve as transport vesicles for microRNAs which can

influence cardiovascular diseases such as coronary artery disease, where different mRNA profiles have been observed between stable and unstable coronary artery disease (Diehl et al., 2012).

MP release from membranes is a highly controlled process, unlike the degradation of plasma membranes associated with necrotic cells which is random (Simak and Gelderman, 2006). The release could be cell specific and/or agonist/stimulant specific, thereby resulting in MP release from different cell types. Cell stimulation leads to increased intracellular calcium levels, which is essential for MP release from cells (Figure 1). This activates the Mg^{2+} ATP-independent Ca^{2+}-dependent enzyme scramblase, which allows phospholipids to move randomly between both leaflets of the bilayer, thereby disrupting and collapsing the lipid symmetry regulated by the enzymes flippase and floppase (Simak and Gelderman, 2006). Flippase is a Mg^{2+} adenosine triphosphate (ATP)-dependent aminopholipid translocase that is responsible for the transport of phospholipids from the outer leaflet to the inner, while its counterpart floppase is responsible for the transport from the inner leaflet to the outer, thus ensuring a transmembrane enzymatic balance. Elevated intracellular calcium levels inactivates flippase while activating floppase and scramblase, thereby disrupting the membrane equilibrium and initiating the budding off process of the microparticle within minutes, a process known as “blebbing” (Zwaal and Schroit, 1997). During this stage, phosphatidylserine (PS) is externalised, allowing the detection of MPs using the annexin V protein which potently binds to PS. The increased cytosolic calcium also activates enzymes such as calpain which cleaves cytoskeleton filaments,

which leads to MP blebbing (Chironi et al., 2009; Lacroix and Dignat-George, 2013). Caspase 3-induced Rho kinase I activation has also been implicated in the actin-myosin contraction and cytoskeletal restructuring seen in the blebbing process (Boulanger et al., 2006). Tramontano and colleagues reported that the Rho kinase inhibitor Y-27632 was able to inhibit TNFα-induced microparticle release from cultured human coronary artery endothelial cells, implicating the involvement of the Rho kinase pathway in the blebbing process (Tramontano et al., 2004).

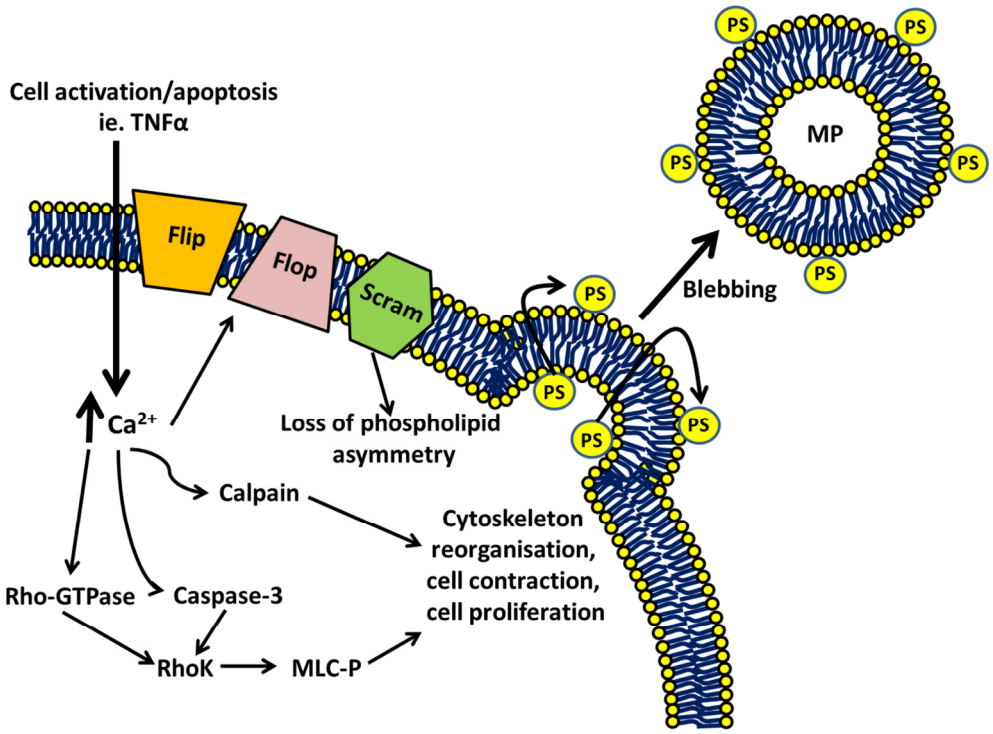

Figure 1: Schematic of the microparticle formation process

Cells undergoing activation (ie. via TNFα stimulation) or apoptosis have raised intracellular calcium, which can inactivate the Mg2+ ATP-dependent aminophospholipid flippase and activate floppase and scramblase. This leads to the loss of phospholipid asymmetry, which contributes to the formation of microparticles, a process known as blebbing. Increased calcium also activates calpain as well as caspase-3 and the Rho-kinase pathway, which lead to cytoskeleton reorganisation, cell contraction, and proliferation, and also contribute to microparticle blebbing.

Abbreviations: Flip=flippase; Flop=floppase; Ca^{2+}=calcium; TNFα=tumour necrosis factor α; Rho K=Rho kinase; PS=phophatidyl serine; MLC-P=myosin light chain phosphorylation

Most platelet-activating agonists such as adenosine diphosphate (ADP), adrenaline, thrombin, collagen, and calcium ionophores (e.g. A23187 and ionomycin) are able to induce PMP release with varying potency: adrenaline < ADP < thrombin < collagen < thrombin + collagen < A23187 (Horstman and Ahn, 1999). Other stimuli that cause MP release from platelets, endothelial cells, and various other cell types include TNF-α and other inflammatory cytokines, bacterial lipopolysaccharides, ROS, CRP, uremic toxins, the complement membrane attack complex C5b9, anti-platelet antibodies, and mechanical stimuli such as sheer stress (Hamilton et al., 1990; Simak and Gelderman, 2006; Dignat-George and Boulanger, 2011).

MPs can communicate, initiate signalling, cell contact or transfer of receptors (Simak and Gelderman, 2006). Additionally, they may be involved in organ defense systems, specifically in inflammation, tissue regeneration and stress response (Tushuizen et al., 2011). It has been proposed that as PS serves as a marker for injured cells and helps identify these cells as being ready for removal by phagocytosis of the cell. By releasing PS+MPs, cells may be able to receive a window of opportunity for reparation in a faster manner that conserves metabolic energy compared to internalising the PS into their inner plasma membrane leaflet (Simak and Gelderman, 2006).

1.4.1.1 Microparticles in PAH

Past studies have shown that microparticles correlate with PAH severity and may hold the potential to be a valuable tool for disease diagnosis. Bakouboula and colleagues reported that levels of procoagulant endoglin (CD105)+ and tissue factor+ MPs were elevated in the plasma of PAH patients compared to healthy controls (Bakouboula et al., 2008). The raised MP levels correlated with elevated levels of proinflammatory markers such as highly specific CRP (hsCRP) and MCP-1. Endothelium derived endoglin+ MPs were further increased in blood taken from the pulmonary artery compared to blood taken from the jugular vein, which may suggest an increase in production of MPs at the vicinity of the artery, a possible trapping in cell aggregates as seen in coronary diseases, or sequestration from the pulmonary vasculature (Héloire et al., 2003). Interestingly, values of the MP gradient across the pulmonary precapillary circulation between the occluded pulmonary artery and jugular vein were significantly correlated with mPAP. Amabile and colleagues also reported that platelet endothelial cell adhesion molecule (PECAM/CD31)+ and vascular endothelial (VE)-cadherin (CD144)+ MPs were also elevated and correlated with haemodynamic severity measured by mPAP in PAH patients (Amabile et al., 2008). Platelet MPs double positive for PECAM and the platelet surface marker CD41 were also shown to be elevated in PAH.

1.4.2 Right ventricular dysfunction/neurohormonal activation

1.4.2.1 Natriuretic peptides

Natriuretic peptides are a family of hormones that share a similar molecular structure involved in the regulation of blood volume and pressure through their action as a diuretic, natriuretic, vasodilator and inhibitor of the renin angiotensin aldosterone system (Yoshimura et al., 1991). Atrial and brain natriuretic peptides (ANP and BNP, respectively) are the major peptides involved in the natriuretic peptide system and are both elevated in PH associated with right ventricular (RV) wall stress. BNP is considered to be more sensitive to ventricular dysfunction as it is released by ventricular tissue whereas ANP is mainly produced by atrial myocytes (Maeda et al., 1998). BNP and its cleavage product pro-BNP are to date the only serologic markers indicated in PAH treatment guidelines for use as prognostic indicators (Pezzuto et al., 2015).

BNP elevation has been observed in various types of PH including IPAH, PAH associated with CTD and congenital systemic-to-pulmonary shunts, CTEPH, PH associated with lung fibrosis, as well as in acute pulmonary embolism (Nagaya et al., 2000, 2002; Kucher, 2003; Leuchte et al., 2004; Wilkins et al., 2005).

Both BNP and ANP correlate with RV overload (Nagaya et al., 1998). Elevated BNP resulting from RV overload correlated positively with mean PAP, PVR,

mean right atrial pressure, RV end-diastolic pressure, and RV myocardial mass index, and negatively with CO and RV ejection fraction in pulmonary hypertensive patients. Findings showing negative correlation with 6-minute walking distance (6MWD), peak oxygen uptake (VO_2), and positive correlation with the WHO class in PAH were later observed (Leuchte et al., 2004). PH patients with a supramedian level of baseline BNP (>150 pg/ml) had a lower survival rate than those with an inframedian (50-100 pg/ml). Plasma BNP lowered significantly in survivors on treatment during the follow-up but increased (>180 pg/ml) in non-survivors (Nagaya et al., 2000).

The N-terminal part of the prohormone of BNP, NT-pro-BNP, has also been studied in a heterogenous group of PH patients. Levels of plasma NT-pro-BNP levels were elevated in a cohort of 61 patients with various forms of pre-capillary PH, which correlated positively with hemodynamic parameters such as right atrial pressure, mPAP, PVR, and HR, as well as lung function VO_2 (Andreassen et al., 2006). Leuchte and colleagues also observed that sixty-six of 118 (55.9%) PH patients had NT-pro-BNP levels that were 2.5 fold higher than in normal patients. (Leuchte, 2007). Interestingly, a longitudinal study revealed that a >15% /year reduction in circulating NT-pro-BNP was associated with better survival in patients with PAH (Mauritz et al., 2011).

As biomarkers, BNP and NT-pro-BNP have limitations regarding being influenced by demographic characteristics such as obesity, sex and age (Pezzuto et al., 2015). Additionally, as NT-pro-BNP is cleared by the kidneys, renal

insufficiency may result in high levels of the pro-hormone independently from a decline in pulmonary haemodynamics or RV overload (Leuchte, 2007). Indeed, NT-pro-BNP levels have been reported higher in scleroderma-related PAH than in IPAH, despite a severe haemodynamic impairment in the latter patient cohort.

1.4.2.2 Other Biomarkers of myocardial injury

Cardiac troponins are components of the thin actin filaments of cardiac muscle and are integral in the contraction of the heart. They consist of 3 regulatory proteins: troponin C (TnC), TnI (cTnI), and TnT (cTnT). High troponin levels in plasma are associated with myocyte damage. Cardiac troponins play a major role in the diagnosis and prognosis of acute coronary syndromes as well as detecting myocardial damage and right ventricular dysfunction in pulmonary embolism (Meyer et al., 2000; Antman, 2002). Unfortunately, cardiac troponins do not represent a sensitive early biomarker of early disease. Increases in cTnT levels were detected in only 14% of a heterogeneous group of 56 patients with PH (51 with PAH and 5 with CTEPH), though these levels of cTnT were shown to be a strong independent marker for PVR, 6MWD and death (Torbicki et al., 2003). In another study, cTnT elevation was also observed but in only 27.3% of a hetergoenous group of PH patients (Filusch et al., 2010).

1.4.3 Inflammation/oxidative stress

Inflammation has been shown to play an active role in disease progression in PAH. The pulmonary vessel wall with plexiform lesions contain inflammatory

cell infiltrates and are surrounded by immune cells such as T- and B-lymphocytes, macrophages, and mast cells in PAH (Tuder et al., 1994; Voelkel et al., 2014).

Numerous plasma cytokines are increased in PAH such as interleukin (IL)-1α, -1β, -2, -4, -6, -8, -10, and -12p70, tumour necrosis factor (TNF)-β, monocyte chemoattractant protein-1 (MCP-1), and osteopontin (OPN) (Soon et al., 2010; Rabinovitch et al., 2014). Transgenic mice experiments have showed that overexpression of IL-6 is associated with vascular remodelling, development of PAH, and an exaggerated response to hypoxia, while IL-6-defiency is protective from hypoxia-induced PAH (Savale et al., 2009). IL-2, -6 and -8, -10, and -12p70 appear to be predictors of survival in a study involving a cohort of 60 IPAH and heritable PAH patients. Serum cytokines correlated better with survival than right heart function and 6MWD, though correlation with haemodynamic parameters was not seen (Soon et al., 2010). Elevated levels of epidermal growth factor and IL-6 has also been observed in paediatric PAH, of which the latter was significantly associated with adverse outcome (Duncan et al., 2012). Katsushi and colleagues were able to show that epoprostenol therapy led to a significant decrease in elevated circulating levels of MCP-1 in PAH patients (Katsushi et al., 2004).

Increased oxidative stress has been observed in PAH patients as well as in animal models (Cracowski et al., 2001; DeMarco et al., 2008). Interestingly, PGI_2 infusion has shown to have an anti-inflammatory effect on the lung tissue,

reducing oxidative stress, as measured via a decrease in the levels of arachidonic acid-derived metabolites 5-oxo-eicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid, and leukotriene B_4 in the lungs of PH patients (Bowers et al., 2004). The increased production of reactive oxygen species (ROS) was shown more recently to be a common consequence of BMPR2 mutations in heritable PAH in both human and mice cultured smooth muscle cells (Lane et al., 2011). As increased peroxide and superoxide production preceded RV systolic pressure elevation, it suggested a potential role for ROS in the pathogenesis of PAH.

Isoprostanes are also markers of oxidative stress as they are products of membrane lipid peroxidation and have been found to regulate the bronchoconstriction and vasoconstriction and inflammation in the pulmonary vasculature (Montuschi et al., 2004; Janssen, 2008). Thromboxane receptor (TP) activation and signalling via the RhoA/ROCK pathway as well as prostaglandin E_2 receptor (EP_2) activation have been implicated in the smooth muscle contraction caused by E-ring and F-ring compounds of isoprostanes (Janssen and Tazzeo, 2002; Tazzeo et al., 2003). Isoprostanes may also stimulate smooth muscle and endothelial cells to induce proinflammatory cytokine release (Janssen, 2008). The elevated urinary isoprostane $F_{2\alpha}$ levels in PAH patients that inversely correlate with pulmonary vasoreactivity have been noted (Cracowski et al., 2001). Moreover, baseline levels of F2-isoprostane in the urine were associated with mortality in PAH patients (Cracowski, 2012). The role of isoprostanes as markers for oxidative stress has been implicated in various other cardiovascular diseases (eg. atherosclerosis, coronary artery disease) as well as lung diseases (eg. Asthma and chronic

obstructive pulmonary disease), neurological diseases (eg. Alzheimer's disease, Huntington's disease), renal diseases (eg. Hemodialysis), and liver diseases (eg. acute and chronic alcoholic liver disease) (Montuschi et al., 2004).

Increased circulating levels of C-reactive protein (CRP), a marker of inflammation and tissue damage, in PAH and CTEPH were observed in patients compared to controls (Quarck et al., 2009). In PAH patients, levels of CRP were associated closely with the New York Heart Association functional class, right atrial pressure, and 6MWD, and were higher in non-survivors that survivors. PAH patients with normalised CRP levels after treatment continued to have a higher survival rate. In CTEPH patients who had undergone enderarterectomy to surgically remove obstructive arterial deposits, CRP levels were significantly decreased after 12 months post-surgery. CRP has been widely been considered a non-specific bystander inflammatory marker for many diseases (eg. acute myocardial ischemia, atherosclerosis, and Chrohn's disease) (Lagrand et al., 1999; Vermeire et al., 2005; Quarck et al., 2009). However, it also has the capability of activating endothelial cells to induce the expression of adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1), and cytokines such as IL-1, -6, and TNFα and chemokines such monocyte chemoattractant protein -1 (MCP-1) and mediate inflammatory processes during atherosclerosis (Labarrere and Zaloga, 2004).

1.4.4.1 Vascular remodelling and damage

Angiopoentins are angiogenic factors important in vascular development and maturation and are produced by VSMCs and precursor pericytes (Brindle, 2006). Ang-1 downregulates excessive proliferation of endothelial cells and stabilizes new blood vessel formation by binding to the extracellular domain of the tyrosine kinase receptor Tie2 (Chu et al., 2004). Ang-2 is the primary antagonist of Ang-1 and has been recognised as a ligand that may be involved in the pathogenesis of IPAH vascular hyperplasia (Augustin et al., 2009). Kumpers and colleagues conducted a study measuring plasma Ang-1, Ang-2, soluble Tie2 and VEGF in a retrospective cohort of 81 IPAH patients and a prospective cohort of 25 IPAH patients, which revealed elevated plasma levels of all aniogenic factors in disease compared with controls (Kümpers et al., 2010). Elevated Ang-2 was associated with elevated PVR, cardiac index, and mixed venous oxygen saturation and was considered an independent risk factor for mortality. After 3 months of PAH-directed therapy, Ang-2 levels were positively correlated with increased right atrial pressure and PVR and inversely correlated with mixed VO_2 saturation. Additionally, Ang-2 mRNA and protein were also found in histological samples of IPAH lung tissue (Kümpers et al., 2010).

Osteopontin (OPN) is a pleiotropic cytokine broadly expressed in various cell types including fibroblasts and cardiomyocytes and is upregulated in inflammation, other conditions such as cancer (Rangaswami et al., 2004, 2006). Its expression can be induced by proinflammatory cytokines such as IL-1β and TNF as well as TGFβ, angiotensin II, and hyperglycaemic and hypoxic conditions.

It is capable of recruiting monocytes and macrophages, and T-lymphocytes to areas of inflammation (Hullinger et al., 2001; Lorenzen et al., 2011). Osteopontin induces proliferation of smooth muscle cells and is involved in monocyte recruitment during pulmonary arterial remodelling in sustained hypoxia (Gadeau et al., 1993; Burke et al., 2009). OPN gene expression has also been shown to be elevated in hypoxia-induced pulmonary hypertensive rats and IPAH patients (Hoshikawa et al., 2003, 2006) Though its origination is unclear, they may behave similarly like soluble cytokines to promote communication between the extracellular matrix and cardiomyocytes or as an immobilized constituent of the extracellular matrix (Rosenberg et al., 2012). Elevated levels of OPN have been observed in IPAH patients and been shown to be an independent marker for survival, correlating with disease severity (Lorenzen et al., 2014). Both *in vitro* and *in vivo* studies have revealed that OPN expression is increased in pulmonary artery adventitial fibroblasts during PH-associated vascular remodelling (Anwar et al., 2012). OPN was also an independent predictor of right ventricular (RV) dilatation and dysfunction, which was also confirmed in animal models of cardiac hypertrophy and failure and also patients with heart failure (Singh et al., 1999; Rosenberg et al., 2008).

Since NO is too unstable to be measured in gaseous form in the blood, exhaled NO levels in bronchoalveolar lavage fluid are measured instead and shown to be decreased in IPAH patients compared to controls (Girgis et al., 2005). Exhaled NO levels were shown to be elevated following either intravenous or inhaled epoprostenol as well as bosentan therapy (Forrest et al., 1999; Ozkan et al., 2001;

Girgis et al., 2005). As biomarkers, exhaled NO presents various confounding variables such as sex, age, infection, atopy, and food and drug intake (American Thoracic Society and European Respiratory Society, 2005).

A reduction in the bioavailability of NO is likely to partly result in the increased synthesis of the potent competitive inhibitor of NOS, assymetric dimethyl arginine (ADMA). Levels are elevated in IPAH patients and are positively correlated with right atrial pressure and negatively with mixed VO_2 saturation, stroke volume, cardiac index and survival (Kielstein, 2005). Garenflo and colleagues reported that levels of ADMA are also increased in PAH associated with CHD and CTEPH patients, which were decreased for CTEPH patients who underwent pulmonary endarterectomy (Gorenflo et al., 2001; Skoro-Sajer et al., 2007).

Serotonin (5-HT) is a monoamine neurotransmitter that is thought to mediate pulmonary vascular remodelling in PAH by inducing cell proliferation of pulmonary arterial smooth muscle cells and fibroblasts and causing vasoconstriction. Animal model experiments revealed that exogenous 5-HT could promote the development of PAH in rats, while the inhibition of the serotonin receptors 5-HT_{1b} and 5-HT_{2a} inhibited this development (Eddahibi et al., 2001; Keegan et al., 2001; Hironaka et al., 2003). IPAH patients, 5-HT levels are increased and positively correlates with PVR (Hervé et al., 1995). More recently, Callebert and colleagues conducted a study involving 16 PAH patients, which showed that serotonin was neither a predictive marker for disease severity nor a predictor of haemodynamic improvement after epoprostenol therapy, though a

larger study would be necessary to firmly confirm this finding (Callebert et al., 2015).

The large multimeric glycoprotein plasma van Willebrand factor (vWF) is produced by endothelial cells and involved in platelet aggregation and adhesion at sites of vascular injury. Plasma vWF is elevated in severe PAH and shown to decrease following prostacyclin treatment. A study by Kawut and colleagues involving a cohort of 66 PAH patients demonstrated that elevated vWF at baseline and follow-up were negatively correlated with survival (Kawut, 2005). Interestingly, vWF levels decreased with long-term prostacyclin infusion (Friedman et al., 1997).

D-dimer is a marker for cross-linked fibrin and thus may be a used to detect microvascular thrombosis. Elevated D-dimer is observed in IPAH and correlated with the New York Heart Association class, resting oxygen saturation, 6MWT and PAP (Shitrit, 2002; Shitrit et al., 2002). Levels negatively correlated with survival at 1 year. However, correlations between haemodynamic parameters and D-dimer plasma levels were not seen in PAH associated with systemic sclerosis (Kiatchoosakun et al., 2007). As D-dimer is elevated in many other diseases as well, its use as a marker for PAH is probably limited.

1.4.4.2 Endothelin-1

PH patients have been shown to have elevated ET-1 levels in lung tissue and plasma as well as elevated mRNA in the endothelial cells of lung specimens (Giaid et al., 1993; Rubens et al., 2001). The increase in ET-1 or big ET-1 plasma levels were shown to be related to pulmonary haemodynamics in congestive heart failure and IPAH. ET-3, which is produced by various cell types including endothelial cells in numerous organs, has a high affinity for ETB receptors but not ETA (Galie, 2004). As the activation of endothelial ETB has been shown to lead to beneficial effects such as the release of vasodilator and anti-proliferative agents such as prostacyclin and nitric oxide as well as the prevention of endothelial apoptosis, the study of ET-3, in particular the ET-1/ET-3 ratio, was studied by Montani and colleagues as a prognostic factor of PAH (Hirata et al., 1993; Shichiri et al., 1997; Montani et al., 2007). Notably, the ET-1/ET-3 ratio was associated with a decline in clinical status, haemodynamics and prognosis in PAH patients during vasoactive drug treatments (Montani et al., 2007).

Langleban and colleagues reported that epoprostenol may have a beneficial effect on endothelin-1 clearance and release in patients with primary (idiopathic)PH. 82% of the group treated with epoprostenol and conventional therapy had an arterial/venous ET-1 level ratio <1 compared to only 29% of the control group on conventional therapy alone (Langleben et al., 1999). A later study showed that inhaled iloprost was able to decrease the transpulmonary big ET-1 ratio likely through increasing pulmonary clearance as levels of big ET-1 were increased in

the pulmonary artery and decreased in the radial artery (Wilkens, 2003). Elevated ET-1 was considered an independent predictor of clinical worsening in the long term (Vizza et al., 2008, 2013). To add, demographic features such as African ethnicity, male gender, and older age are associated with higher ET-1 plasma levels, whilst patients on angiotensin-converting enzyme inhibitors, β-blockers, statins, and vasodilators are associated with lower levels (Shah, 2007). Thus, various potential confounding factors could affect the use of ET-1 as a biomarker.

1.4.5 Markers for end-organ failure

As renal dysfunction is associated with haemodynamic impairment in PAH as well as an independent predictor of mortality, renal function measurements such as serum creatinine levels or creatinine clearance represent important prognostic biomarkers (Shah et al., 2008; Benza et al., 2010). This is important particularly when evaluating biomarkers such as NT-proBNP undergoing renal clearance.

Hyponatremia has been shown to be strongly related to RV dysfunction, WHO functional class, and poor survival in PAH (Forfia et al., 2008). Though the mechanism behind hyponatremia has not yet been elucidated, increased neurohormonal activation induced by advanced RV dysfunction has been hypothesised.

Elevated uric acid, which is the final oxidation product of purine metabolism and an endogenous free radical scavenger, has been observed in PAH and correlated

with disease severity (Bendayan et al., 2003). Its production in the disease is likely to be by ischemic lung or RV tissue (Voelkel et al., 2000). Increases in plasma uric acid was independently associated with mortality in IPAH, and were decreased following successful prostacyclin therapy (Hoeper et al., 1999). Unfortunately, plasma levels of uric acid is influenced by a multitude of factors including age, sex, renal failure, hypoxemia, allopurinol, and intake of diuretics, thereby limiting its potential as a biomarker.

RV dysfunction seen in PAH can lead to liver congestion and low perfusion to the liver, and can lead to liver dysfunction. Thus, liver dysfunction has also been implicated as a potential marker for PAH (Richman et al., 1961). To add, the prognosis of heart failure was shown to be associated with elevated levels bilirubin and aspartate aminotransferase, which is an indicator of liver dysfunction (Batin et al., 1995). A 2010 study involving a cohort of 37 PAH patients confirmed this finding by showing that hyperbilirubinemia was associated with advanced right heart failure and reduced survival (Takeda et al., 2010).

1.4.6 New markers of transcriptional regulation

miRNAs are small non-coding RNA molecules that are 21-23 nucleotides and involved in transcriptional and post-transcriptional regulation of gene expression. They have been shown regulate cell proliferation and apoptosis in physiological processes such as cardiac fibrosis, hypertrophy, angiogenesis and heart failure (Catalucci et al., 2009). miRNA can be detected in fluid samples, suggesting their biological functions may occur outside cells through paracrine signaling (Gupta et

al., 2010). The miR-204 gene has been implicated in PAH as it is pro-proliferative and apoptotic role in pulmonary arterial smooth muscles. Its expression was downregulated in PASMCs from pulmonary hypertension patients which may have been attributed to elevated signal transducer and activator of transcription 3 (STAT3) by circulating factors such as ET-1, PDGF, and angiotensin II (Courboulin et al., 2011). miR-204 suppression and STAT3 activation also led to increased expression of nuclear factor of activated T-cells (NFAT), which further drove the proliferative and anti-apoptotic processes potentially responsible for vascular remodelling in PAH. miR-204 expression is decreased in PAH patient lungs and the lungs of rodents exposed to chronic hypoxia or monocrotaline (Courboulin et al., 2011). Levels correlated with PVR in humans and mPAP in rodents as measures for disease severity. Interestingly, nebulization with a miR-204 mimic reversed the pathology of monocrotaline-induced PAH in murine. In another study, miR-21 expression was increased in human PASMCs under hypoxic conditions but decreased in mouse monocrotaline models (Sarkar et al., 2010) and in human lung samples from IPAH patients. miR-21 has been implicated as an "oncomir" because it is consistently upregulated in multiple cancers such as chronic lymphocyte leukemia and breast cancer and promotes cell proliferation, apoptosis, and metastasis (Ou et al., 2014). Another study revealed that TGFβ and BMP4 stimulation may rapidly induce miR-21 expression, thereby suggesting that impaired BMPRII receptor signalling may be associated with reduced miR-21 expression and thus increased vascular remodelling. In contrast the miR21 oncomir, miR-145, is reduced in a number of cancers including lung, bladder, gastric, and nasopharyngeal cancer (Xu et al., 2012). It is the most abundant miRNA in normal vascular walls and vascular smooth muscle cells and

may play a role in the regulation of smooth muscle cell proliferation and plasticity (Cheng et al., 2009; Cordes et al., 2009). Its expression has been shown to be further increased in IPAH and HPAH patient lung tissue compared to controls (Caruso et al., 2012). Its expression was also elevated in cultured PASMCs from patients with BMPR2 mutations and in wild type mice exposed to hypoxia, whilst miR-145 deficiency was protective from PAH development. The proto-oncogene encoding a serine/threonine protein kinase, PIM-1, has been recognised as a potential marker for PAH as it shows high specificity for vascular lesions in disease (Padma and Nagarajan, 1991; Paulin et al., 2011). It is expressed very low in healthy human tissues but plasma levels were increased in PAH and correlated with disease severity as measured by WHO functional class, cardia index, 6MWD, and NT-proBNP. PIM-1 is involved in the NFAT/STAT3 signalling pathway, suggesting that it may be involved in the pro-proliferative and anti-apoptotic phenotype characteristic of smooth muscle cells in disease (Paulin et al., 2011). Interestingly, after blood sample collection, PIM-1 expression in the buffy coat was increased in PAH unlike other inflammatory diseases such as scleroderma, which may indicate specificity for PAH.

1.5 Therapies in PAH

Clinically, background treatments for PAH include warfarin, digoxin, diuretics, and oxygen. The cardiac glycoside digoxin and diuretics provide symptomatic relief but fail to provide long term clinical benefit (Rich et al., 1998; Rhodes et al., 2009). The anticoagulant warfarin, a vitamin K sparing antagonist, has been

shown to slightly improve survival in patients though its effect is difficult to estimate without a randomised clinical trial (Johnson et al., 2006). The L-type calcium channel blocker, nifedipine, has shown to provide some benefit and its treatment has shown to increase cardiac output and decrease pulmonary resistance in ~10-15% of IPAH patients (Tonelli et al., 2010). Unfortunately, these effects have not been consistent and many patients did not respond to vasodilator therapy (Rubin, 1985).

There are three main classes of drugs for the treatment of PAH: prostacyclin (or stable analogues), phosphodiesterase type 5 (PDE5) inhibitors and endothelin-1 (ET-1) antagonists. Though all of these drug classes have shown to provide significant improvements in symptoms clinically such as in exercise capacity, only prostacyclin has shown to increase survival (Barst et al., 2011; Ivy et al., 2013). Though the disease progression can be delayed through current therapy, there is no cure for PAH to date.

1.5.1 PDE-5 inhibitors in PAH

The inhibition of cGMP-degrading enzyme phosphodiesterase-5 (PDE-5) leads to vasodilatation at sites expressing the enzyme (Anderson and Nawarskas, 2010). Due to the abundant expression of PDE-5 in the pulmonary vasculature, PDE-5 inhibitors were investigated for use as therapy in PAH. Sildenafil, tadalafil, and verdanifil are PDE-5 inhibitors that are approved for erectile dysfunction and are also capable of causing significant vasodilatation in the pulmonary vasculature

with maximum effects observed after 60, 75-90, and 40-45 minutes, respectively (Ghofrani et al., 2004).

Sildenafil is an orally active, potent and selective PDE-5 inhibitor. The randomised controlled trial Sildenafil Use in Pulmonary Arterial Hypertension (SUPER-1) trial performed in 278 PAH patients treated with sildenafil showed improved haemodynamics, exercise capacity and symptoms (Galie et al., 2005). Another randomised controlled trial led conducted by Barst and colleagues revealed beneficial effects of oral sildenafil citrate in 235 treatment naïve paediatric PAH patients (Barst et al., 2012).

While sildenafil has a half-life of 4 hours *in vivo* and is dispensed thrice a day, the selective PDE-5 inhibitor tadalafil has a 17.5 hour half-life *in vivo* and is only dispensed once a day (Galie et al., 2009; Falk et al., 2010). The PHIRST (Pulmonary Arterial Hypertension and Response Trial) study on 406 PAH patients treated with tadalafil showed improvements in symptoms, haemodynamics, exercise capacity and time to clinical worsening at 40 mg once daily dosage (Galie et al., 2009).

1.5.2 Endothelin antagonists in PAH

Bosentan is an orally active dual ET_A and ET_B receptor antagonist and the first ERA to be synthesised. Rat experiments have shown that bosentan can reduce pulmonary vascular hypertrophy without inducing systemic vasodilatation (Chen

et al., 1995). Bosentan has been studied in PAH patients in five randomised control trials: Pilot, BREATHE (Bosentan Randomised trial of Endothelin Antagnoist THErapy)-1, BREATHE-2, BREATHE-5 and EARLY (Endothelin Antagonist TRial in mildly symptomatic PAH patients), which showed effectiveness in decreasing mPAP and PVR and increasing cardiac output while improving functional class in patients (III to II), time to clinical worsening, exercise capacity, and echocardiographic and Doppler variables (Channick et al., 2001; Rubin et al., 2002; Humbert, 2004; Galie, 2006; Galiè et al., 2008b; Strange et al., 2011). Bosentan has been approved for treatment of PAH patients in WHO functional class II and patients with PAH associated with congenital systemic-to-pulmonary shunts and Eisenmenger's syndrome (Galie, 2006).

Ambrisentan is a non-sulfonamide, propanoic acid-class of ERA that has a relatively selectivity for ET_A receptors (Davie et al., 2009). A pilot study and two large randomised controlled trials, ARIES 1 and 2, have revealed its efficacy on improving exercise capacity, symptoms, haemodynamics and time to clinical worsening in IPAH and PAH associated with connective tissue disease and HIV infection (Galiè et al., 2008a). Ambrisentan has been approved for WHO functional class II and III patients.

Sitaxentan is another ET_A receptor antagonist with a greater selectivity towards ET_A receptors than ambrisentan (ref). The orally active drug has been evaluated in 2 randomised controlled trials, STRIDE (Sitaxentan To Relieve Impared Exercise) 1 and 2 in patients with IPAH or PAH associated with CTD or coronary

heart disease (CHD) in WHO functional class II and III (Barst et al., 2004, 2006b). The trials revealed improvement in exercise capacity measured by predicted peak oxygen consumption and haemodynamics measured by PAP and PVR. Sitaxentan was initially authorised by the European Medicines Agency (EMA) as Thelin (Pfizer, New York, USA) in 2006 but was later withdrawn by the manufacturer in 2010 from the worldwide market after 9 cases of severe hepatitis-like drug reactions were reported (Hoeper et al., 2011). The EMA withdrew marketing authorisation soon after (European Medicines Agency, 2010).

1.5.3 Prostacyclin pathway

The synthetic PGI_2 Epoprostenol has a short half-life of <6 minutes and administration via peripheral veins also causes painful vein irritation (Steiropoulos et al., 2008). Thus, the need for continuous administration by infusion pump and a permanent tunnelled catheter is needed. Epoprostenol has been shown to improve symptoms in PAH, exercise capacity measured by the 6 minute walking distance (6MWD) and haemodynamics assessed by mean PAP, cardiac index and PVR, and is the only treatment known to improve survival in IPAH in a randomised study (Barst et al., 1996). Other prostanoids are chemically stable in solution and have considerably longer plasma half-lives: iloprost with 20-30 minutes, beraprost with 40-60 minutes, and treprostinil with 180-270 minutes (Demolis et al., 1993; Olschewski et al., 2004; Wade et al., 2004). Prostacyclin analogues can be administered via various routes of administration such as intravenous (iloprost, treprostinil), oral (beraprost, treprostinil), inhaled (iloprost, treprostinil), and

subcutaneous (treprostinil). The order of in vivo stability of the prostacyclin analogues is iloprost<beraprost<cicaprost<<treprostinil (Clapp and Patel, 2010).

Iloprost is typically administered by inhalation with the theoretical benefit of being selective for the pulmonary circulation, though it is also available for i.v., and has a half-life of 20 to 25 minutes (Higenbottam et al., 1998; Steiropoulos et al., 2008; Galie et al., 2015b). The Aerosolized iloprost Randomized (AIR) study group trial, which was a randomized controlled trial evaluating inhaled iloprost, showed that daily inhalations (6-9 times, 2.5-5μg per inhalation, median 30ug daily) in patients with PAH and CTEPH increased exercise capacity and improved symptoms, PVR, and clinical events compared to placebo inhalation (Olschewski et al., 2002). Another randomised control trial known as the STEP (Safety and pilot efficacy Trial in combination with bosentan for Evaluation in Pulmonary arterial hypertension) trial revealed that inhaled iloprost helped increase exercise capacity in patients already treated with bosentan (McLaughlin et al., 2006). Frequent side-effects of inhaled iloprost include flushing and jaw pain, though is known to be well tolerated by most patients.

Beraprost was the first chemically stable and orally active prostacyclin analogue (Galie et al., 2015b). It was revealed in a randomised controlled trial performed by the ALPHABET (Arterial Pulmonary Hypertension and Beraprost European) study group in Europe and another group in the USA of its ability to improve exercise capacity though only up to 3-6 months (Galiè et al., 2002; Barst et al.,

2003). However, no significant haemodynamic benefits were observed. Common adverse effects include headache, flushing, jaw pain and diarrhoea.

Treprostinil is a tricyclic benzidene analogue of PGI_2 that is chemically very stable and can be administered at ambient temperature. Thus, it can be administered intravenously (half-life = 4.4 hours) as well as subcutaneously (half-life = 4.6 hours) by a micro-infusion pump and a small subcutaneous catheter (Laliberte et al., 2004). Though patients require doses 2-3 times higher does than epoprostenol the superior stability of treprostinil allows greater convenience for patients as they can be supplied in premixed and prefilled syringes (Benedict et al., 2007). In 2002, Simonneau and colleagues studied the effects of treprostinil in a large randomised controlled trial performed in this condition and showed marked improvements in haemodynamics such as mean right atrial pressure, mPAP, cardiac index, PVR, and mixed venous oxygen saturation, as well as exercise capacity, and symptoms in PAH patients (Simonneau et al., 2002). Side effects included infusion-site pain and erythema, cough, headache, throat irritation, nausea, and flushing. In IPAH patients, subcutaneous treprostinil has been shown to improve survival over the course of 4 years compared to predicted survival using the National Institute of Health formula (Barst et al., 2006a). Recent advances have led to the development of aerosolised treatment delivered through an ultrasonic pulse-delivery nebuliser system, which has shown decreases in PVR and mPAP (Sandifer et al., 2005; Voswinckel et al., 2006; Nadler and Edelman, 2010). The peak effect and plasma level of treprostinil was observed at 10-15 minutes following inhalation. The phase II randomised controlled trial

known as the TRIUMP (Treprostinil sodium Inhalation Used in the Management of Pulmonary arterial Hypertension) trial revealed that inhaled treprostinil in patients on bosentan or sildenafil showed mild to moderate improvements in exercise capacity as measured by 6MWD (Benza et al., 2011).

1.6 Cellular targets for prostacyclin

In 1994, the main target for PGI_2 was discovered to be the seven-transmembane prostaglandin I (IP) receptor, which is expressed abundantly in blood vessels, leukocytes, and thrombocytes (Narumiya et al., 1999). The receptor is coupled to the stimulatory G protein, Gs, which activates adenylyl cyclase and leads to cyclic adenosine monophosphate (cAMP) generation (Figure 2). This second messenger is responsible for multiple biological effects of PGI_2 through primarily activating protein kinase A (PKA). Cyclic AMP is broken down by specific PDEs, chiefly by PDE 1, 3, 4, which regulate basal levels and analogue-induced elevation in the lung (Phillips et al., 2005; Murray et al., 2007; Schermuly et al., 2007).

Prostacyclin and its analogues can act on other prostaglandin receptors that also contribute to their therapeutic action. The activation of prostaglandin receptors EP_2, EP_4, and DP_1 leads to elevation in cAMP as they are coupled to Gs, and leads to vasorelaxation. In contrast, the activation of the EP_1, EP_3, FP and TP receptors lead to a contractile effect as they are coupled to the G proteins, Gi and Gq, which are involved in either reducing cAMP levels and/or elevating intracellular calcium

levels (Woodward et al., 2011). PGI_2 has been shown to have low selectivity for prostanoid receptors, and is able to activate EP_1, EP_3, and TP receptors albeit at higher concentrations compared to natural ligands (15-45 fold for EP_1 and EP_3 and <100-fold for TP). (Bennett and Sanger, 1982; Kennedy et al., 1982; Lawrence et al., 1992). At clinical doses, however, PGI_2 is likely to activate EP_3 receptors. PGI_2 analogues in general potently bind to the IP receptors, though iloprost has high affinity for EP_1 receptors (Ki=1nM) (Abramovitz et al., 2000). This is consistent with EP_1 receptor blockade enhancing iloprost-induced vasorelaxation in isolated rabbit perfused lungs and in guinea pig aorta (Clapp et al., 1998; Schermuly et al., 2007). Treprostinil on the other hand has low affinity for EP_1 recptor (ki=212nM) but high affinity for DP_1 (ki=4.4nM) and EP_2 (ki=3.6nM) receptors (Whittle et al., 2012; Clapp and Gurung, 2015). Thus the pharmacology of these two prostacyclins is distinct (Clapp and Gurung, 2015).

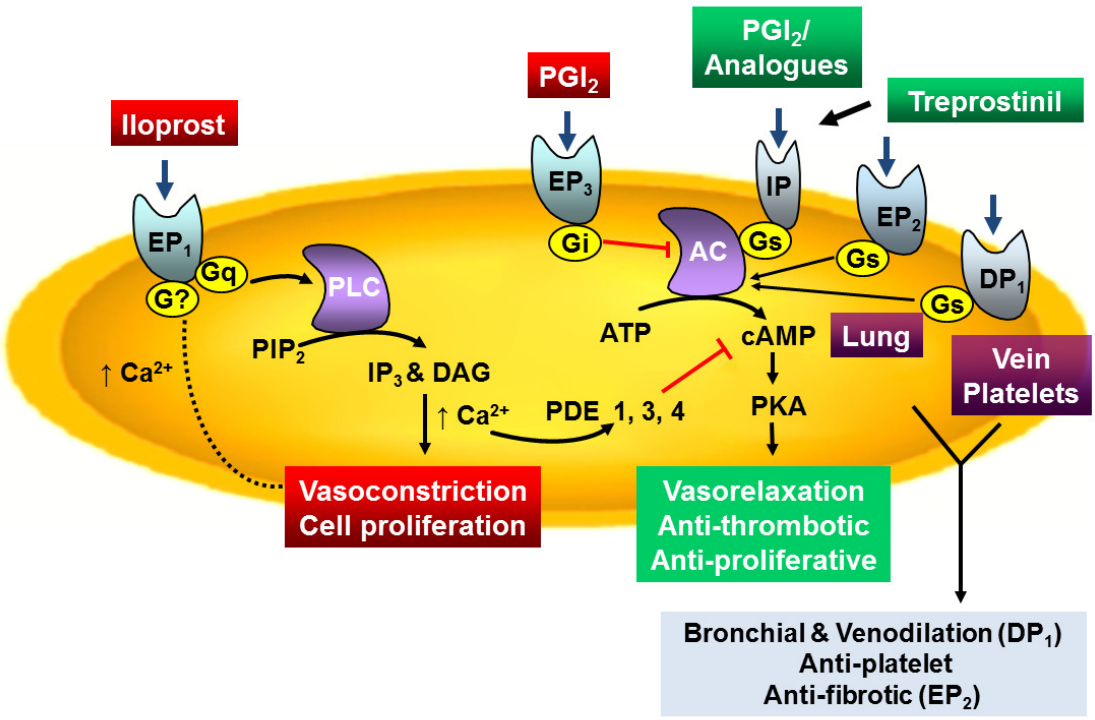

Figure 2. Schematic of prostacyclin signalling in smooth muscle cells

Prostacyclin and its analogues are able to act on a variety of receptors, which include the prostacyclin (IP) receptor, and the prostaglandin receptors EP1, EP_2, and EP3, and DP_1, which are each coupled to a G-protein that enables downstream signalling. The activation of the IP, EP_2, and DP_1 receptors lead to vasodilatory and antiproliferative effects while the activation of the EP1 and EP_3 receptors lead to vasoconstriction and cell proliferative effects.

Abbreviations: PLC=phospholipase C; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3=Inositol triphosphate; DAG=diacylglycerol; Ca^{2+}=calcium; PDE=phosphodiesterase-5; ATP=adenosine triphosphate; AC=adenylyl cyclase; cAMP; cyclic adenosine monophosphate; PKA=protein kinase A

1.5.4 Combination therapy in PAH

The simultaneous use of more than one PAH-targeted drug coined as "combination therapy" has become a standard care in many PAH centres. Drug combinations have been shown to be safe and effective in several cases (Ghofrani et al., 2003; Hoeper et al., 2004). A study in 2005 showed that combination treatment with either bosentan, sildenafil and iloprost significantly improved the survival, lung transplantation, and need for intravenous iloprost treatment in PAH patients compared to a historical control group (Hoeper et al., 2005). The STEP-1 (SafeTy and pilot Efficacy trial in combination with bosentan for the evaluation in Pulmonary arterial hypertension) study that studied the safety and efficacy of inhaled iloprost and bosentan over 12 weeks of treatment showed only a slight improvement in the post-inhalation 6-minute walking distance (6MWD) and time to clinical worsening, though no improvement in haemodynamics was observed. A similar randomised controlled trial called the COMBI (COMbination therapy of Bosentan and aerosolised Iloprost in IPAH) trial however failed to show an effect on 6MWD or time to clinical worsening when investigating effects of inhaled iloprost with bosentan (Hoeper, 2006). The TRIUMPH (TReprostinil sodium Inhalation Used in the Management of Pulmonary arterial hypertension) trial studied the addition of inhaled treprostinil to either bosentan or sildenafil therapy in patients with PAH, and demonstrated an improvement in 6MWD, though improvement in functional class and time to clinical worsening did not occur (Benza et al., 2012). Similarly, the PACES (Pulmonary Arterial hypertension Combination study of Epoprostenol and Sildenafil) trial showed that the combination of sildenafil to epoprostenol in PAH brought improvements in

6MWD as well as time to clinical worsening after 12 weeks (Simonneau et al., 2008). Most recently, the AMBITION (Ambrisentan and Tadalafil in patients with pulmonary arterial hypertension) trial studied the efficacy of the combination of the selective ETA receptor antagonist ambrisentan with the PDE-5 inhibitor tadalafil compared to monotherapy (ambrisentan or tadalafil). The combination of the two drugs provided greater reduction in pro-BNP levels, higher percentage of patients with satisfactory clinical response and improved the 6MWD compared to the pooled monotherapy group, though no significant differences in haemodynamics were observed (Galie et al., 2015a). A sequential approach to combination therapy is the most widely utilised strategy for PAH, though initial (upfront) combination therapy based on the known mortality of PAH is also recommended in the 2015 ESC/ERS guidelines for PAH (Galie et al., 2015b).

1.6.1 Peroxisome proliferator-activated receptors in prostacyclin signalling

As prostacyclin synthase (PGI_2S) is expressed highly in vascular smooth muscles not only in the plasma membrane but also in the perinuclear region, signalling of PGI_2 via a family of transcription factors known as peroxisome proliferator-activated receptors (PPARs) is now recognised (Smith et al., 1983). There are three isoforms of the PPARs: PPARα, PPARβ/δ, and PPARγ, which are encoded by the genes PPARA, PPARD and PPARG, respectively. Classically, PPARs can form a heterodimer with the retinoid X receptor (RXR), which then associates

with coactivators and bind with peroxisome proliferator response elements (PPREs) to regulate the expression of target genes. As the name suggests, PPARs are involved in the function of peroxisomes, which are small organelles involved in fatty acid metabolism (Reddy et al., 1973) . but they are also involved in regulating various other processes such as insulin sensitivity, glucose homeostasis, fatty acid oxidation, inflammation, cell proliferation, apoptosis, cytokine production and vasculoprotection (Hamblin et al., 2009). PPARs are most commonly activated by ligand binding and contain a central DNA-binding domain that recognises response elements in the promoter regions of their target genes. Endogenous ligands include prostaglandins (*e.g.* 15-Deoxy-delta 12, 14- PGJ_2), fatty acids, lipoxygenase metabolites (e.g. 8-HETE) and a variety of synthetic agents (Forman et al., 1995). PGI_2 analogues iloprost and carbacyclin can act as PPAR ligands by directly binding and inducing gene transcription of PPAR-α and PPAR-δ (synonymous with PPAR-β) in vitro, activating them as efficiently as endogenous and synthetic ligands. (Forman et al., 1997). The structural basis for the binding of iloprost to PPAR-α and -β was confirmed through crystal structures of the ligand binding domain (Jin et al., 2011). Treprostinil and carbacyclin, have also been shown to activate PPARγ in an IP receptor-dependent manner (Falcetti et al., 2007).

All three isoforms of PPARs are highly expressed in endothelial cells and to varying degrees in other cell types, with PPAR-β having the most widespread expression (Hamblin et al., 2009). Message levels for the three isoforms are reported in normal pulmonary smooth muscle, while reduced staining of PPAR-γ

was reported in IPAH lungs (Li et al., 2012). Reduced PPAR-γ expression was shown in the lungs of rodents with hypoxia-induced PH and in vascular lesions in a rat model of severe PAH caused by hypoxia in the presence of the VEGF blocker, sugen (Ameshima et al., 2003). PPARγ expression in endothelial cells and proliferating cells within the intima and plexiform lesions was non-existent (Falcetti et al., 2007) PPARγ knockdown in endothelial cells *in vitro* resulted in an abnormal, proliferating, apoptosis-resistant phenotype, while *in vivo* experiments in mice led to the development of PAH and muscularisation of distal pulmonary arteries (Ameshima et al., 2003; Guignabert et al., 2009). The loss of function mutation in BMPR-2, which normally suppresses cellular growth in the vasculature, is prominent in heritable PAH and has shown to decrease endogenous PPARγ activity whilst enhancing pathways such as the PDGF/mitogen activated protein kinase (MAPK)-mediated extracellular-signal-regulated kinase (ERK) pathways associated with vascular remodelling (Hansmann and Zamanian, 2009). This would suggest that the lack or loss of PPARγ, the IP receptor and PGI_2S may be responsible for the hyperproliferative cellular phenotype displayed in PAH. Interestingly, though targeted deletion of PPARγ in smooth muscle was shown to cause PAH, its expression was shown to be distinctly increased in the medial layer of distal pulmonary arteries from PAH patients on various treatments (Falcetti et al, 2010). Additionally, in this study, its expression was not dependent on drug treatment, unlike the IP receptor. Upregulation of PPAR in smooth muscle cells may serve as a compensatory mechanism, through which progression in remodelling in PAH might be limited.

1.6.2 NFAT and calcineurin and their cellular functions

PPARs can also act to negatively regulate gene expression through recruitment of corepressors and transrepression of various pro-inflammatory and pro-proliferative transcription factors including nuclear factor kappa B (NFκB), Smad-3, activator protein-1 (AP1), signal transducers and activators of transcription (STAT) proteins, and nuclear factor of activated T cells (NFAT) (Macian, 2005; Ricote and Glass, 2007). NFAT is a key player in the mechanism of the proliferation of smooth muscle that leads to the remodelling in PAH (de Frutos et al., 2007a, 2010). The NFAT family consists of 5 members: NFAT1 (NFATc2), NFAT (NFATc, NFATc1), NFAT3 (NFATc4), NFAT (NFATc3) and NFAT5 (Musson et al., 2012). They all share an NFAT homology region in the N-terminal of the protein that mediates regulatory functions such as the binding to the Ca^{2+}-dependent phosphatase protein, calcineurin which dephosphorylates NFAT and promotes its translocation into the nucleus (Nilsson et al., 2007). The homology region also consists of nuclear localisation and export sequences, and phosphorylation sites containing localised serine-rich region and 3 serine-proline repeats for various serine/threonine kinases to activate nuclear export (Hill-Eubanks et al., 2003). Multiple reports have shown that PKA can also phosphorylate NFAT and engage in the nuclear export of NFAT (Chow and Davis, 2000; Sheridan et al., 2002). The C-terminal region of NFAT contains a DNA binding domain that is moderately homologous to binding domains of the Rel-family proteins such as the transcription factor NFκB (Hill-Eubanks et al., 2003). NFAT consists of a 59kDa catalytic A subunit, which contains a

calmodulin binding domain and an autoinhibitory region, and a 19kDa calcium binding regulatory B subunit. Calcineurin is ubiquitously expressed, though it is present at approximately 10-fold higher concentrations in brain and muscle than other tissues types (Olson and Williams, 2000). Separate mammalian calcineurin A (CnA) catalytic genes give rise to three isoforms, CnAα (PPP3CA), CnAβ (PPP3CB), CnAγ (PPP3CC). Two separate B subunit regulatory genes B1 and B2 have been identified in vertebrates. CnAα, CnAβ, and B1 gene products are ubiquitously expressed throughout the body, while calcineurin Aγ and B2 are expressed more locally in specific tissues such as the brain and testes (Molkentin, 2004). CnAα has been shown to regulate vascular ATP-sensitive potassium channels by inhibiting PKA-dependent phosphorylation of the channel as well as the catalytic subunit (RII) of PKA itself, thereby opposing the vasodilatory action of the potassium channel (Orie et al., 2009). PDGF, a key driver of smooth muscle cell proliferation in PAH, has been shown to activate CnAβ and induce the nuclear translocation of NFATc3 from the cytosol and contribute to smooth muscle proliferation in rat aorta (Jabr et al., 2007).

Hypothesis:

Circulating microparticles (MPs) derived from smooth muscle cells are elevated in PAH and can serve as biomarkers of vascular remodelling and inflammation. Measurement of MP levels in plasma may serve to indicate disease severity as well as allow the assessment of the impact of front-line therapy in patients.

Furthermore, these MPs are procoagulant vesicles that can promote inflammation and play a part in vascular damage and remodelling.

Aims and Objectives:

- Pulmonary arterial smooth muscle cells and their MPs from patients with pulmonary arterial hypertension and healthy donors will be grown in culture and characterised. In parallel, human umbilical cord endothelial cells and their MPs will also be characterised. This will help elucidate cell surface markers that are smooth muscle specific and aid in the identification of smooth muscle MPs in plasma.
- Smooth muscle, endothelial, and leukocyte MP levels will be measured in plasma collected from PAH patients before and after long-term therapy. These MP levels will be compared with plasma from age and sex matched controls as well as from patients with coronary artery disease and HIV.
- The procoagulant function of MP from PAH patients and cultured smooth muscle cells samples will be investigated. Additionally, the mechanism of prostacyclin therapy on inhibiting MP release will be studied.

Methods

2. Methods

2.1 PAH patient characteristics

Human pulmonary arterial smooth muscle cells (PASMCs) were isolated from the lungs of children and adults with IPAH who had undergone transplantation after failed treatment or who had died and control adults. Human lung tissue was taken after obtaining patient/relative consent with ethical approval from Great Ormond Street Hospital (ICH & GOSH REC 05/Q0508/45), Papworth Hospital (REC H00/531/T), the Assistance Public - Hôpitaux de Paris (Institutional Review Board IRB00006477, agreement No. 11-045) or Brompton & Harefield Trust (NHLI REC 01-210) through Dr. John Wharton at Imperial College London. Child patients had received epoprostenol and bosentan therapy for 1.34 years and adult patients had received varying prostacyclin therapy for a minimum of 1.2 years with either bosentan or sildenafil or a combination.

Control PASMCs were isolated from donor lungs unsuitable for transplantation but were otherwise histologically normal or were from lung parenchymal strips cut from the macroscopically normal regions of the diseased lungs taken as far as possible away from the tumour as possible.

HUVECs were isolated from healthy babies after delivery with ethical approval from West Middlesex Hospital Maternity following written parental consent.

2.2 Cell Isolation

2.2.1 Isolation of PASMCs

PASMCs were enzymatically isolated from distal pulmonary arteries using a modified method (Falcetti et al., 2010). Distal pulmonary arteries were dissected at room temperature using a phase contrast microscope (Olympus, CK2) from the lungs of PAH patients and normal donors and kept in cold (4^{o}C) normal physiological saline solution. The saline solution contained 112mM NaCl, 5mM KCl, 1mM $MgCl_2$, 25mM $NaHCO_3$, 0.5mM KH_2PO_4, 0.5 mM Na_2HPO_4, 10mM glucose, and 1.8mM $CaCl_2$ (all chemicals from Sigma-Aldridge, Dorset, Poole, UK). The segments of arteries were left to sterilise in 3% penicillin/streptomycin (Invitrogen, Paisley, UK) and 1.5µg/ml gentamycin solution (Sigma-Aldridge, Poole, Dorset, UK) for 30 minutes at 4^{o}C. The arteries were then dissected into ~3 mm wide pieces in a flow hood and placed in a 50 ml falcon tube with 1ml aliquot of dissociation cocktail and incubated at 37^{o}C with continuous shaking for 30 minutes. Aliquots of the dissociation cocktail were prepared by adding 0.125mg/ml elastase (Lorne Laboratories, Reading, UK), 10 mg/ml collagenase (Sigma-Aldridge, Poole, Dorset, UK), 0.06mg/ml trypsin inhibitor (Sigma-Aldridge, Pool, Dorset, UK), 3.75 mg/ml bovine serum albumin (Sigma-Aldridge, Poole, Dorset, UK), 100µl MEM vitamins (GIBCO, Invitrogen, Paisley, UK) and 3 ml DMEM/Ham's F12 media (Life Technologies, Paisley, UK) to 10ml DMEM/F12 HEPES (GIBCO, Invitrogen, Paisley, UK) Following the incubation, the tissue was then passed through a 40µm cell strainer into a 50ml falcon containing 10 ml of growth medium with the following composition: DMEM/F12 containing with 10% foetal bovine serum (FBS) (GIBCO, South American Breed,

Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (GIBCO, Invitrogen, Paisley, UK). The cell suspension was centrifuged at 180g for 5 minutes at room temperature to pellet cells, which was then resuspended in 10 ml of fresh growth medium by gentle agitation to disperse cells into a homogeneous suspension. Cells were transferred into a T-25 flask and cultured in a humidified atmosphere at 37^{o}C and 5% CO_2. Growth media was changed every 3 days until cells reached 80% confluence, after which they were frozen down to maximise the viability of these cells and cryo-preserved in liquid nitrogen at -170^{o}C until used for experiments.

2.2.2 Isolation of human umbilical vein endothelial cells (HUVECs)

Umbilical cords were collected from healthy babies at delivery and human umbilical vein endothelial cells (HUVECs) were isolated within 7 days using a modified method (Baudin et al., 2007). Blood was removed from the fresh umbilical cords, which were then submerged in sterile RPMI 1640 medium (Life Technologies, Paisley, UK) to wash any excess blood. The ends of the cords were cut off to remove bacteria and then closed using clamps. RPMI 1640 medium (Life Technologies, Paisley, UK) was syringed into the cord vein until taut (with 10-20ml of media) and left in a sterile hood for 5 minutes. The spiralled end of the cords was unclamped to allow the removal of RPMI and the edge of the umbilical cord cut off, again to avoid infection then re-clamped. 10-30 ml of 20mg/ml of collagenase I (Life Technologies, Paisley, UK) reconstituted in RPMI was syringed into the cord veins until taut and the umbilical cords incubated at 37^{o}C and 5% CO_2 for 15 minutes. The cords were massaged so as to manually dislodge the HUVECs from the basal lamina. To inactivate the collagenase, 10-30 ml of

RPMI containing 20% foetal bovine serum (FBS) (GIBCO, Invitrogen, Paisley, UK) was then syringed into the cord veins until taut. The end was unclamped and the collagenase solution containing endothelial cells was collected in a 50 ml falcon tube and then centrifuged (type, manufacturer) at 5,000g for 5 minutes. The supernatant was decanted and 5 ml of MCDB 131 medium (Life Technologies, Paisley, UK) was added to the pellet and pipetted up and down to obtain a homogenous cell mixture. This was then transferred into a T-25 flask and cells incubated at 37^{o}C under 5% $CO_{2.}$ The media was replaced after cell attachment to endothelial basal medium (LONZA, Walkersville, Maryland, USA) with 10% FBS, which was changed with fresh media every 3 days. Upon reaching 80% confluence (Figure 3), the cells were split (as explained in the next section) to be used for experiments as well as frozen down and cryo-preserved at -170^{o}C for long term storage.

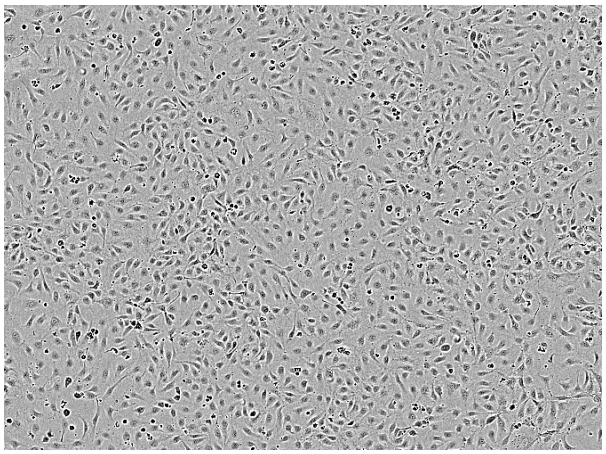

Figure 3. Cultured human umiblican vein endothelial cells (HUVECs)

Image shown are cultured endothelial cells isolated from the umbilical cord of a healthy baby after delivery examined under a phase contrast microscope.

10x magnification

2. Subculture of human cells

Frozen cryo-vials containing either PASMCs, PAECs or HUVECs were rapidly thawed at 37^{o}C in a water bath to minimise ice crystal formation which may damage cell viability. These cells were seeded in a T-75 flask containing 10ml of their respective growth media. The growth media for smooth muscle cells consisted of DMEM/Ham's F-12 containing 10% FBS and 1% penicillin/streptomycin, while the growth media for endothelial cells consisted of EBM containing 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37^{o}C and 5% CO_2 in a humidified atmosphere for 24 hours to allow attachment to the flask botton, after which the media was replaced by fresh growth media. The growth media was changed every 3 days and cells grown until reaching 80% confluence Once the cells reached this level of confluency, they were then ready for subculture. This was done by first washing cells with 5ml of warm PBS (Invitrogen, Paisley, UK) twice, removing it and then adding 5ml of 0.05% Trypsin-EDTA (GIBCO). Incubation at 37^{o}C/5% CO_2 for ~2 minutes allowed the adhered cells to detach from the flask. 10 ml of growth media containing 10% FBS was immediately added to neutralise the trypsin to stop digesting the cell surface. The cell suspension was then transferred into a 50 ml falcon tube and centrifuged at 180g for 5 minutes at room temperature to obtain a cell pellet. The supernatant was removed and the pellet was resuspended in 3 ml of growth media, which was followed by gentle up and down pipetting to obtain a homogenous suspension of cells. The cells were counted using an automated cell counter (ADAM; Digital Bio, Seoul, Korea). This technique implements the use of propidium iodide (PI) – a dye that stains cellular DNA. 20µl of the cell solution

was pipetted into two 600µl Eppendorf tubes, one containing AccuStain solution 'T' and the other solution 'N.' Solution T contained PI and a cell lysis solution which would thus stain all cells and thus represent the total cell count. Solution N only contained PI, and thus would only stained damaged cells without intact plasma membranes giving the non-viable cell count. After gently vortexing the tubes, 100µl of solution from each tube was loaded into a microchip, which was then inserted into the cell counter. By subtracting the total cell count from the non-viable cell count, the viable cell count was obtained and used to calculate the volume of cell suspension needed to plate at the desired cell density.

2.3 Characterisation of PASMCs

Confocal microscopy was used to characterise PASMCs by staining for the cytoskeletal smooth muscle markers, α smooth muscle actin (αSMA) and smooth muscle 22 α (sm22α) (Figure 4B-D). PASMCs were plated at a density of $1x10^4$ cells per well in eight-chambered slides (BD Bioscience, Oxford, UK) containing 500µl DMEM/F-12 with 10% FBS per well and grown for 2 days, at which time cells normally were at e60% confluency. To prepare for staining, cells were first fixed with 500µl of 4% paraformaldehyde (Sigma-Aldridge, Poole, Dorset, UK), prepared in PBS placed in the appropriate wells for 20 minutes. After this, cells were washed three times with 500µl PBS for 5 minutes, before being permeabilised with 500µl of 1% triton X-100 solution made up in PBS. The cells were washed three more times with 500µl of PBS, after which a blocking solution of 2% bovine serum albumin (BSA; Sigma-Aldridge, Poole, Dorset, UK) and

0.01% triton X-100 (Sigma-Aldrich, Poole, Dorset, UK) dissolved in PBS was added to the chambers and left to incubate at room temperature for 20 minutes. The blocking solution was then replaced with 100µl of primary antibody. Mouse monoclonal anti- αSMA (Sigma Aldridge, Poole, Dorset, UK) and mouse polyclonal anti-sm22α (Invitrogen, Paisley, UK) were prepared by diluting in fresh blocking solution both at a 1:500 dilution before being applied to the cells. The slides were then incubated at room temperature for 2 hours on a slow shaker. The cells were then washed 3 times with 500µl PBS, each for 5 minutes. 100µl of the fluorescent secondary antibody anti-mouse Alexa 555 (Invitrogen, Paisley, UK) and anti-sm22α, both prepared at 1:1000 dilution in blocking solution, were used for both α-SMA and sm22 staining. The slides were left to incubate with the secondary at room temperature for 2 hours on a slow shaker kept in the dark. The cells were washed for a final time with 500µl PBS in each well for 5 minutes, before the slides were detached from the chamber walls. 10µl of mounting reagent with 4', 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA), which binds strongly to the A-T rich regions in DNA and stains the nucleus blue, was pipetted onto each slide. A glass coverslip was placed on top and sealed in place with a layer of commercially available nail varnish (Boots, UK). The slides were stored at -20^{o}C in the dark until they were ready to be examined under a confocal microscope (Leica TCS SPE) preferably within 3 days. The negative control was acquired through exclusion of the primary antibody.

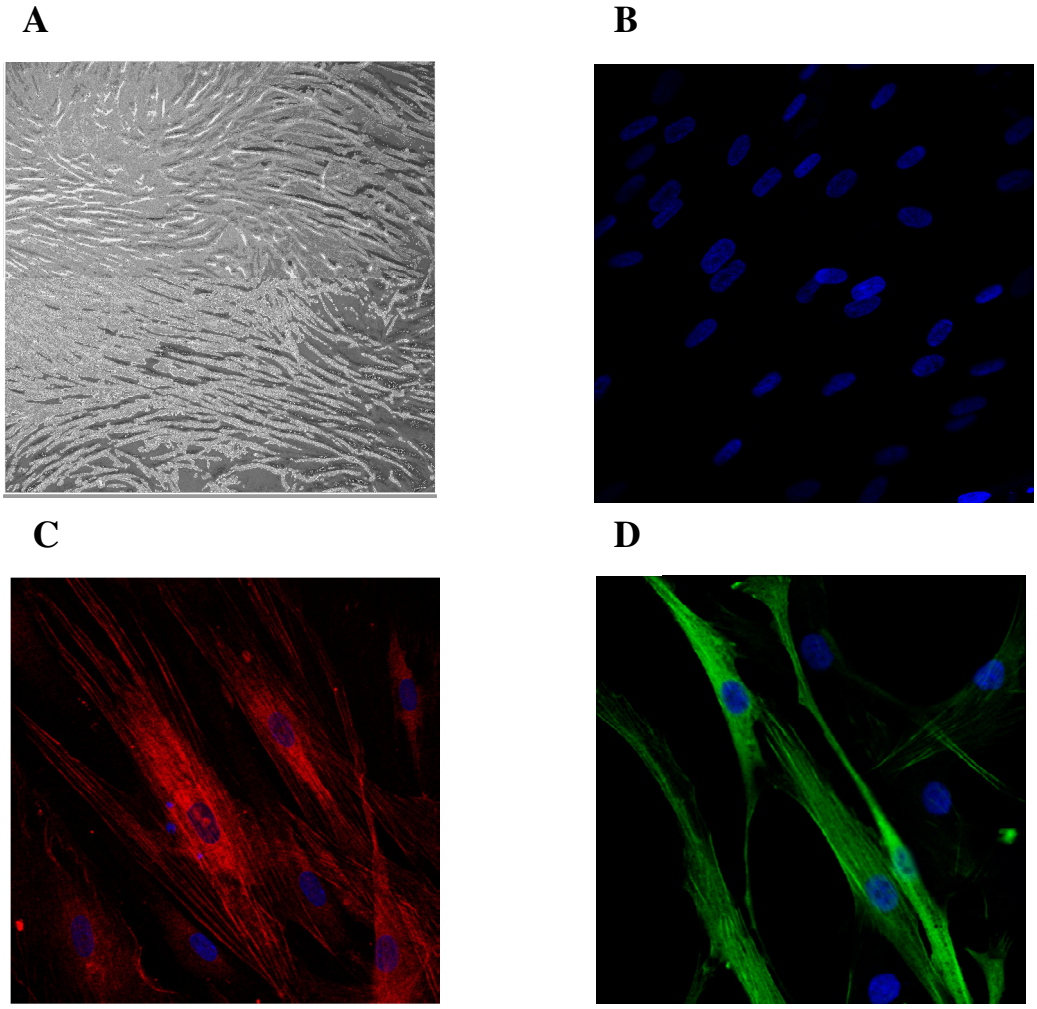

Figure 4. Confocal imaging of pulmonary arterial smooth muscle cells (PASMCs) stained with smooth muscle biomarkers

A) Human PASMCs isolated from distal pulmonary arteries from a child diagnosed with PAH were grown to 70% confluence in culture and observed under a phase contrast microscope. The classic hill and valley morphology characteristic of smooth muscle cells can be seen. **B)** PASMCs stained with only the nuclear marker, DAPI. **C)** PASMCs were stained with mouse monoclonal anti-αsmooth muscle actin (αSMA) primary antibody at a 1:500 dilution and visualised with the anti-mouse Alexa 555 secondary antibody (1:1000 dilution). Positive staining of αSMA is seen in red and the DAPI staining of the nucleus is in blue. **D)** PASMCs were stained with rabbit polyclonal anti-sm22α primary antibody at 1:500 dilution and visualised with anti-rabbit Alexafluor-488 secondary antibody (1:1000 dilution). Positive staining of sm22α is seen in green and the DAPI staining of the nucleus is blue.

2.4 Flow cytometric analysis of cell surface receptors

PASMCs and HUVECs were analysed by flow cytometry where cell surface receptors were labelled with antibodies conjugated to fluorescent dyes. This would serve two purposes: 1) It would allow the characterisation of the cells through the level of expression of various cell surface receptors and 2) the receptor expression profile on the cell surface plasma membrane. The latter would enable to define the profile of cell specific receptors that ought to be present on microparticle (MP) membrane from known derived primary cell lines of smooth muscle and endothelial cells. Such characterisation was utilised not only for MP detection in the culture supernatant, but also to confirm the cell origin of MPs cells in the whole blood.

The cells were grown in 6 well plates until 80% confluency. 0.5 ml trypsin-EDTA was added and the cells were left to incubate at 37^{o}C and 5% CO_2 for 1-2 minutes to detach the cells from the flask bottom. After detachment, growth media containing 10% serum was added to neutralise the action of trypsin. The cells were then collected into 15 ml falcon tubes and centrifuged at 180g for 5 minutes and the trypsin containing media was decanted. The cell pellet was resuspended in PBS (1 ml) by pipetting up and down several times. The cell suspension was then divided into 100µl aliquots in 600µl Eppendorf tubes for antibody labelling at a 1:50 dilution. Mouse monoclonal anti-human antibodies conjugated with phycoerythrin (PE) or allophocyanin Cy-7 (APC-Cy7) were used to stain for cell surface receptors. These antibodies were anti-platelet derived growth factor

receptor (PDGFR) α and β (R&D Systems Abingdon, UK), anti-endoglin/CD105 (BD Pharmingen), anti-neural glial antigen 2 (R&D Systems), anti-intracellelular adhesion molecule (ICAM1)/CD54 (BD Pharmingen), vascular cell adhesion molecule (VCAM)/CD106 (BD Biosciences, New Jersey, USA), anti-E-selectin (CD62E) (BD Biosciences), platelet endothelial cell adhesion molecule (PECAM)/CD31 (BD Biosciences), and mesenchymal cell adhesion molecule (MCAM)/CD146 (BD Pharmingen). Isotype control antibodies anti-mouse IgG1 PE (R&D), anti-mouse IgG1,k (BD Pharmingen), anti-mouse IgG1 FITC (R&D), and IgG1,k APC-Cy7 (BD Pharmingen) with equal protein:fluorochrome ratios were also used for cell staining to examine non-specific staining. The cells were pipetted up and down to mix and left to incubate at 4^{o}C for 30 minutes in the dark, after which they were centrifuged (Eppendorf Centrifuge 5415R, Stevenage, UK) at 3,000g for 3 minutes to decant unbound antibodies in the PBS. 200µl of PBS was then added into each tube and after pipetting up and down several times, the cells were transferred to a 96 well plate to be read by a FACSArray BioAnalyzer^{TM} flow cytometer. The gating was set by running unstained and isotype control stained cells through the cytometer and toggling the forward and side scatter settings on a linear scale (Figure 5A) while the colour channels were set to logarithmic (Figure 5B-C). A minimum of 5,000 gated cells were acquired through the cytometer to provide enough event count. Single colour controls ensured that compensation could be performed during analysis with the FlowJo software (version 8.3.3; Tree Star, Inc., OR, USA).

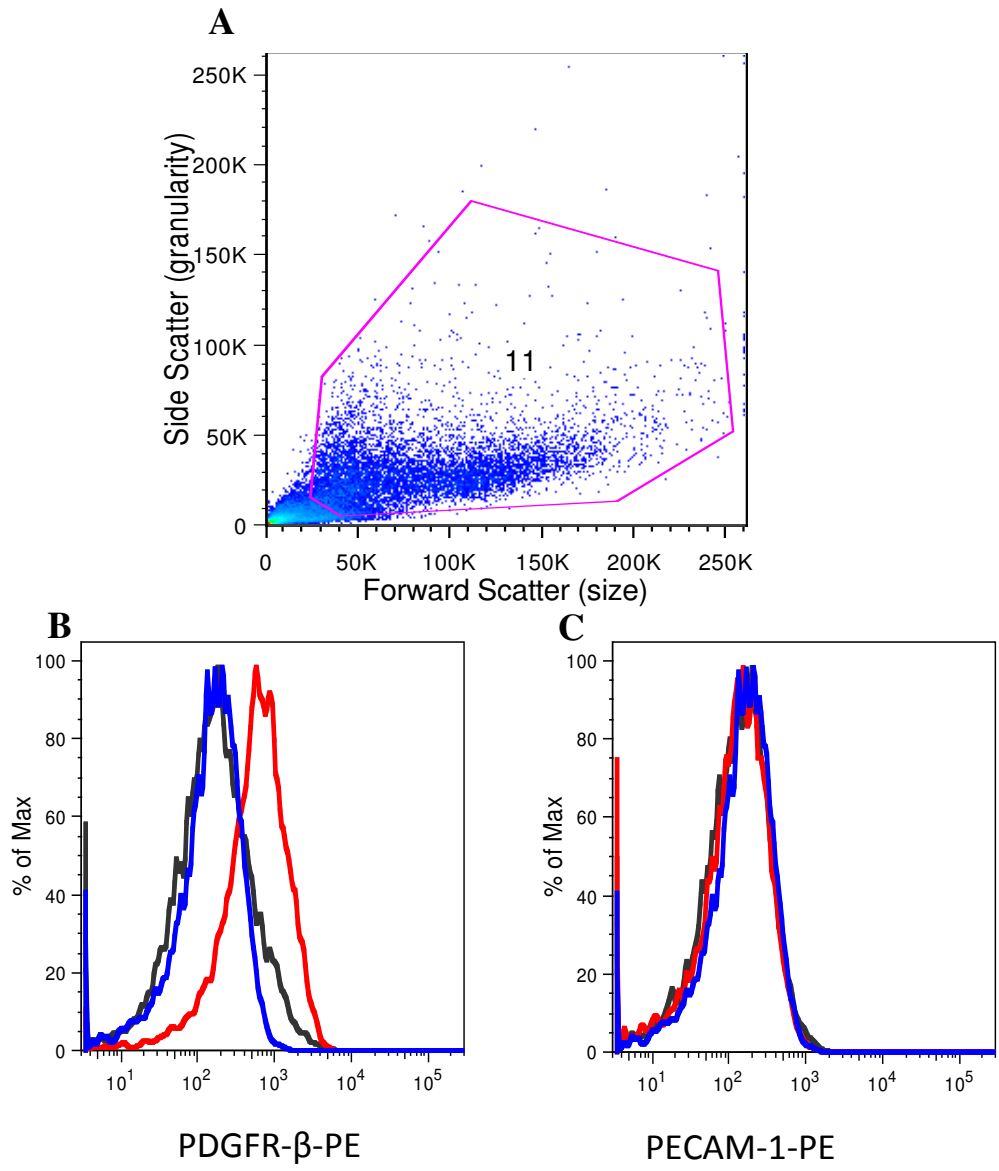

Figure 5. Flow cytometric analysis of PASMC surface receptor expression

PASMCs were prepared in PBS and characterised using fluorophore conjugated antibodies against cell surface receptors and analysed via flow cytometry. **A)** A dot plot was obtained with side scatter (granularity) and forward scatter (size) on linear scales established the gating of the smooth muscles. **B)** The number of events of cells were plotted against the fluorescent intensity of the phycoerythrin (PE) fluorescent colour. PASMCs stained with anti-platelet derived growth factor β (PDGFRβ) conjugated to PE showed a shift to the right of the red histogram compared to PASMCs labelled with the isotype control antibody (black) and unstained PASMCs (Blue). This indicates positive expression of PDGFRβ on the cell surface. **C)** Staining of PASMCs with phycoerythrin (PE) conjugated to PECAM1 did not result in a rightward shift (Red) indicating negative expression of PECAM1 on the cell surface of PASMCs when compared to either the isotype antibody staining (black) or non-staining (Blue).

2.5 Preparation of supernatants from cultured cells for microparticle analysis

HUVECs and PASMCs were plated at a density of 10^4 cells/ml in 6 well plates and grown in their respective growth media (endothelial basal medium with 10% FBS for HUVECs and DMEM/ F12 with 10% FBS for PASMCs) at 37^{o}C in a humidified atmosphere of 5% CO_2. When cells reached 70-80% confluence, either arrest media (0.1% FBS), growth media (10% FBS), or growth media in combination with either 20ng/ml tumour necrosis factor α (TNFα), 20ng/ml platelet derived growth factor (PDGF)-BB, 5ng/ml transforming growth factor (TGF)-β, or 10nM endothelin-1, were administered to each well, and the release of microparticles (MPs) from the plasma membrane assessed. 1ml of supernatant from cultured arrested to growing cells was collected from each 6 well plate and placed into autoclaved 1.5 ml Eppendorf tubes. This supernatant was spun at 2,000g for 5 minutes at room temperature. The top 600µl was collected, so as to avoid picking up intact cells and/or cell debris and immediately transferred to a -80^{o}C freezer for storage.

2.6 Identification of microparticles (MPs) from cell culture supernatants

Microparticles are released from the surface of various cells that are activated or undergoing apoptosis through a process known as “blebbing”. The MPs externalise phosphatidylserine on their surfaces in a calcium-dependent manner.

Annexin V is a protein that can be conjugated to a fluorescent dye and used experimentally to bind to the phosphatidylserine and thus identify microparticles in platelet poor plasma through flow cytometry.

To assess total microparticle count, anti-annexin V antibody conjugated to fluorescein isothiocyanate (FITC) or to phycoerythrin (PE) was diluted in annexin V buffer (BD Pharmingen) at a 1:10 dilution and 5µl was added to the appropriate wells of a 96-well U-bottom multiplate (Greiner Bio-One Ltd., Stonehouse, UK). Monoclonal mouse anti-human antibodies against cell surface markers were used to characterise MPs. These antibodies were anti-platelet derived growth factor receptor (PDGFR) α (R&D, Abingdon, UK), PDGFRβ (R&D), anti-endoglin/CD105 (BD Pharmingen, Oxford, UK), anti-neural glial antigen 2 (R&D), anti-intracellelular adhesion molecule (ICAM1)/CD54 (BD Pharmingen), vascular cell adhesion molecule (VCAM)/CD106 (BD Biosciences, Oxford, UK), anti-E-selectin (CD62E) (BD Biosciences), platelet endothelial cell adhesion molecule (PECAM)/CD31 (BD Biosciences), and mesenchymal cell adhesion molecule (MCAM)/CD146 (BD Pharmingen). Relevant isotype control antibodies included anti-mouse IgG1 PE (R&D), anti-mouse IgG1,k (BD Pharmingen), anti-mouse IgG1 FITC (R&D), and IgG1,k APC-Cy7 (BD Pharmingen) with equal protein:fluorochrome ratios and were used to examine non-specific staining. The 96 well-plate was covered with aluminium foil to shield from light and incubated at room temperature on a plate shaker for 15 minutes, after which 200µl of annexin V buffer was added to each well to dilute the samples and terminate the staining.

2.7 Microparticle analysis in patient blood

The levels of annexin V+ microparticles were studied in the blood taken from patients with PAH as well as other cardiovascular and inflammation driven diseases. Twenty patients presented with PAH from multiple centres in Rome, Italy were recruited with the approval from the local ethics committee of each centre (EC 340/12). Twenty-seven patients with coronary artery disease were recruited through Dr. Crysostomos Mavroudis and Dr. Sudheer Koganti at the Royal Free Hospital, with ethical approval granted from the local ethics committee at Royal Free Hospital (REC 14/LO/0387). Twenty-four patients with HIV were recruited at Queen Elizabeth Central Hospital (Blantyre, Malawi) and twenty-four HIV negative patients were recruited from the Voluntary Testing and Counselling clinic in Blantyre, Malawi through Dr. Christine Kelly. Ethical approval was granted from the independent scientific and ethics committee COMREC of the University of Malawi (REC P.02/12/1180). In all cases, informed written consent was provided by all patients.

2.7.1 Pulmonary arterial hypertensive patient recruitment for MP characterisation

The inclusion criteria were: diagnosis of PAH confirmed by haemodynamic assessment, either treatment naïve or already on prostacyclin analogue, endothelin atagonist, phosphodiesterase 5-inhibitor or a calcium channel blocker therapy, and planning to undergo therapy continuously for a minimum of 4 months. Of the

twenty patients recruited, ten were diagnosed with IPAH, seven with unclassified PAH, one with PAH associated with scleroderma, one with PAH associated with HIV, and one with pulmonary obstructive PAH. They were eleven male and nine female patients within the age of thirty and eighty-one years. At the time of recruitment, twelve out of twenty patients were not on any treatment (i.e. treatment naïve), while the rest were either on a PDE-5 inhibitor, endothelin antagonist or in combination with a prostacyclin analogue. After recruitment, they were put on a monotherapy either a prostacyclin (treprostinil or iloprost), an endothelin antagonist (bosentan or ambrisentan), a PDE-5 inihibitor (sildenafil or tadalfil), a calcium channel inhibitor (amlodipine) or a combination over a period of 4-26 months. Throughout the study, haemodynamic measurements such as the mean pulmonary artery pressure were recorded. 20 age and sex-matched healthy control volunteers were also recruited from whom blood was collected.

2.7.2 Preparation of platelet poor plasma (PPP) from PAH and control patients

As whole blood contains many different cells including erythrocytes, leukocytes, lymphocytes and platelets which can make the study of microparticles problematic, double centrifugation was applied to obtain platelet poor plasma (PPP) using an adapted protocol (Brogan et al., 2004)

Blood (4 ml) was collected from patients in lavender EDTA vacutainer K2EDTA tubes (BD, Oxford, United Kingdom). Within 2 hours, platelet poor plasma (PPP)

was obtained by a double centrifugation step. First the blood was centrifuged at 3,000g for 15 minutes to obtain plasma, which was then stored in 1.5 ml Eppendorf tubes at -80^{o}C until further use. For batch analysis, the plasma was rapidly thawed in a 37^{o}C water bath and centrifuged a second time at 5,000g for 5 minutes to remove most platelets. This would ensure that the PPP would mostly consist of microparticles from cell types other than platelets, enabling for clearer analysis via flow cytometry. 100µl aliquots of PPP of each sample were transferred to new 1.5 eppendorf tubes and centrifuged at 17,000g for 60 minutes. Most of the supernatant was decanted leaving approximately 20µl with the microparticle pellet. The MP pellet was then reconstituted in 490µl of annexin V buffer (BD Pharmingen, Oxford, United Kingdom), divided into 35µl aliquots and plated into a 96 well U-bottomed polypropylene plate.

2.7.3 Coronary heart disease patient recruitment

Ten patients with ST-elevated myocardial infarction (STEMI) and thirteen patients with non-STEMI were recruited for the study.

The diagnosis of STEMI was performed by following the standard diagnosis criteria according to current guidelines. All acute STEMI patients were treated with primary percutaneous coronary intervention (PCI), also known as coronary angioplasty, for treatment of myocardial infarction. These patients were given 300mg of the COX-inhibitor, aspirin by ambulance paramedics and 600mg of the antiplatelet drug, clopidogrel upon arrival to the catheterisation laboratory. Patients were given intravenous morphine to alleviate chest pain symptoms as

necessary and weight adjusted unfractionated heparin to maintain the activated clotting time (ACT) at 200-250 seconds.

The diagnosis of NSTEMI was based on the patients' history of cardiac chest pain at rest or without ECG changes and elevated 12 hour troponin levels >0.03 ng/l. All patients were treated with 300mg aspirin, 600mg clopidogrel and weight adjusted unfractionated heparin (enoxaparin 1mg/kg twice per day). Within 72 hours of chest pain, the patients underwent angiography. In the catheterisation laboratory before PCI, weight adjusted unfractionated heparin was administered to each NSTEMI patient to achieve an ACT at 200-250 seconds.

Patients with renal failure prior to undergoing coronary bypass graft, or those patients who received the anti-platelet agents glycoprotein IIb/IIIa inhibitors prior to sampling, were excluded.

2.7.4 Blood sampling and preparation of platelet poor plasma from coronary heart disease (CHD) patients

Blood samples were collected from the coronary artery and forearm vein from patients with CHD. Diagnostic coronary angiography and PCI were performed according to current guidelines. A 6F venous sheath was inserted into the femoral vein, which allowed the passage of a 5F multipurpose catheter (5F, Cordis®, internal diameter 0.22cm) to be placed in the right atrium. A Judkin's left 4 diagnostic catheter (Cordis®, internal diameter 0.11cm) and a Judkin's right 4

diagnostic catheter (Cordis®, internal diameter 0.11cm) were used to perform left and right coronary angiography, respectively. Blood samples were aspirated carefully through the catheters of similar internal diameter to minimise shear stress. Blood samples were also taken from the cephalic or antecubital vein on the arm using a 19G needle. All samples were taken before patients were treated with glycoprotein IIb/IIIa inhibitors.Whole blood (3.5 ml) was collected from the coronary artery and forearm vein in 3.2% tri-sodium citrate tubes (BD, Oxford, UK) and centrifuged at 5,000g for 5 minutes to isolate plasma. A second spin at 5,000g for 5 minutes was performed to obtain PPP, which was then stored at -80^{o}C until analysed.

2.7.5 Human immunodifficiency virus (HIV) patient recruitment and sample preparation

Twenty-four adult HIV-infected patients with low CD4 counts (<100 cells/ml blood) in the Queen Elizabeth Central Hospital (QECH), Blantyre Malawi, were recruited for the study. At recruitment, prior to initiating anti-retroviral therapy, blood was collected from the brachial vein together eand the carotid-femoral pulse-wave velocity (PWV) with patient history. The PWV was measured using a vicorder system, which involved placing a sensor on the patient's neck and a cuff around the thigh and the distance between the two areas measured. Age, sex and PWV-matched twenty-four HIV-negative control patients were also recruited from the Voluntary Testing and Counselling clinic for comparative blood samples.

Whole blood (7 ml) was collected in 3.2% tri-sodium citrate tubes, transferred to 1.5 ml Eppendorf tubes and centrifuged at 1,500g for 10 minutes at 4^{o}C to obtain plasma. This was stored at -80^{o}C in 1.5ml aliquots in cryo-vials until used for experiments. After rapidly thawing at 37^{o}C in a water bath, the plasma samples underwent a second centrifugation at 5,000g for 5 minutes to obtain the PPP which was stored in 1.5ml tubes and analysed immediately via flow cytometry.

2.8 Identification of microparticles from platelet poor plasma

Anti-annexin V antibody conjugated either to fluorescein isothiocyanate (FITC) or to phycoerythrin (PE), was diluted in annexin V buffer (BD Pharmingen) at a 1:10 dilution and 5 µl was added to appropriate wells of a 96-well U-bottom multiplate (Greiner) to assess total microparticle count. Monoclonal mouse anti-human antibodies against cell surface markers were used to characterise the origin of cells for MP derivation. For identifying of smooth muscle microparticles (SMMPs), samples were incubated with mouse PE-conjugated anti-human PDGFRβ, mouse PE-labelled anti-human endoglin/CD105, mouse PE-labelled anti-human neural glial antigen 2 (NG2), mouse PE-labelled anti-human intracellular cell adhesion molecule (ICAM)/CD54, and mouse (PE) anti-human vascular cell adhesion molecule (VCAM)/CD106. For identifying endothelial microparticles (EMPs), samples were incubated with mouse (PE) anti-human E-selectin/CD62E and mouse (APC-Cy7) anti-human platelet endothelial cell adhesion molecule (PECAM)/CD31 (BD Pharmingen). For identifying leukocyte microparticles

(LMPs), samples were incubated with mouse (PE) anti-human carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8)/CD66b (BD Pharmingen), mouse (FITC) anti-human tissue factor (CD142) (Sekisui Diagnostics, Lexington, MA, USA) and mouse (APC-Cy7) anti-human CD14. For identifying platelet microparticles, samples were incubated with mouse (PE) anti-human glycoprotein IX (GP9)/CD42a. Relevant isotype control antibodies were also used on all samples to distinguish non-specific staining (Figure 7). Single colour control staining by annexin V-FITC, PE-annexin V, and PECAM1-APC-Cy7 were used to compensate digitally during analysis on the FlowJo software after sample acquisition. The method of compensation is essential during multi-colour flow cytometric analysis, as it corrects for spillover, which happens when the fluorescent emission of a fluorochrome is detected by a detector designed to measure the signal of another fluorochrome. All antibodies were diluted in PBS containing 0.01% FBS and used at final dilutions (1:50 or 1:100) as listed in the table below. The 96 well plate was covered with aluminium foil to shield from light and incubated at room temperature on a plate shaker for 15 minutes, after which 200µl of annexin V buffer was added to each well to dilute the samples and terminate the staining.

Antibodies used for flow cytometric analysis

Specificity	Isotype	Conjugate	Company	Clone	Dilution
Annexin V	-	FITC	BD Pharmingen	--	1:100
Annexin V	-	PE	BD Pharmingen	--	1:100
PDGFRβ	IgG1	PE	R&D Systems	PRa292	1:50
Endoglin/CD105	IgG1,k	PE	BD Pharmingen	266	1:50
NG2	IgG1	PE	R&D Systems	11711	1:50
ICAM/CD54	IgG1,k	PE	BD Pharmingen	HA58	1:50
VCAM/CD106	IgG1,k	PE	BD Pharmingen	51-10C9	1:50
E-Selectin /CD62E	IgG1,k	PE	BD Pharmingen	68-5H11	1:50
PECAM/CD31	IgG1,k	APC-Cy7	BD Pharmingen	WM59	1:100
CD66b	IgM,k	PE	BD Pharmingen	G10F5	1:50
TF/CD142	IgG1	FITC	Sekisui Diagnostics	V1C7	1:50
CD14	IgG2b,k	APC-Cy7	BD Pharmingen	MϕP9	1:50
CD42a	IgG1,k	PE	BD Pharmingen	ALMA 16	1:50
CD146	IgG1,k	PE	BD Pharmingen	WM59	1:50
IgG1 Isotype control antibody	IgG1	PE	R&D Systems	11711	1:50
IgG1k Isotype control antibody	IgG1,k	PE	BD Pharmingen	MOPC-21	1:50
IgG1 Isotype control antibody	IgG1	FITC	R&D Systems	11711	1:50

IgG1,k Isotype control antibody	IgG1,k	APC-Cy7	BD Pharmingen	MOPC-21	1:50
IgGM,k Isotype control antibody	IgM,k	PE	BD Pharmingen	G155-228	1:50
IgG1,k Istoype control antibody	IgG2b,k	APC-Cy7	BD Pharmingen	MOPC-21	1:50

Table 3. Fluorochrome conjugated antibodies and reagents used for flow cytometry analysis of cells and microparticles. Antibodies conjugated to various fluorescent dyes were diluted in PBS containing 0.1% FBS at either a 1:50 or 1:100 dilution before incubating with cells or microparticles and running through the flow cytometry. As annexin V is a protein and not an antibody, FITC-annexin V does not have a clonal origin, unlike the antibodies used to label receptors and their respective isotype control antibodies.

Abbreviations:
FITC: Fluroscein isothiocyonate; PE: phycoerythrin; APC-Cy7: allophocyanin-Cy7.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PDGFRβ PECAM AnV+	Endoglin PECAM AnV+	NG2 PECAM AnV+	ICAM1 PECAM AnV+	VCAM1 PECAM AnV+	E-Selectin AnV	CD66b AnV+	TF CD14 AnV+	PECAM AnV+	MCAM PECAM AnV+	Un-stained	IgG1-PE Isotype
B											FITC control	IgG1k FITC Isotype
C											PE control	IgG1 FITC Isotype
D											APC-Cy7 control	IgG1k APC Cy7 Isotype
E												
F												
G												1.1uM beads
H												3uM beads

Table 4. 96 well plate plan of antibody staining of microparticles (MPs) from platelet poor plasma (PPP)

PPP was isolated after double centrifugation of whole blood at 5,000g x 5 min and MPs were obtained after a high speed centrifugation at 17,000g x 60 mins at 4^{o}C. The MPs were reconstituted in annexin V binding buffer and plated to appropriate wells. The fluorescent dye fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated to annexin V (AnV+) was used to label phosphatidylserine on MP surfaces to obtain total MP count. Fluorochrome conjugated antibodies were used to stain against surface receptors platelet derived growth factor β (PDGFRβ), platelet endothelial cell adhesion molecule (PECAM), Endoglin, neural glial antigen 2 (NG2), intracellular adhesion molecule 1 (ICAM1), vascular adhesion molecule 1 (VCAM1), E-selectin, carcinoembryonic antigen-relat cell adhesion 8 (CAECAM9)/CD66b, Tissue factor, CD14, glycoprotein IX (GP9)/CD42a, and melanoma cell adhesion molecule (MCAM)/CD146. Double and triple staining was used in the appropriate wells of the 96 well plate. The unstained well contained MPs without any labelling. Single colour controls for fluorochromes FITC, PE and APC-Cy7 were used for compensation. Isotype control antibodies were used to determine non-specific antibody binding. 1.1μM and 3μM latex beads were used for size gating and MP enumeration, respectively.

2.10 Flow cytometric analysis of microparticles from PH and CHD patients

Analysis of MPs was performed on the digital FACSArray^{TM} Bioanalyzer flow cytometer (BD Biosciences). To optimise the forward (FSC) and side scatter (SSC) settings, 1.1μm latex beads (Sigma-Aldridge, Poole, Dorset, UK) were run and logarithmic FSC and SSC plots obtained (Figure 6). The gates were set to obtain particles smaller than approximately 1.1 μm in diameter. When MPs were run through the cytometer, 100 μl of each sample was run at a medium flow rate of 2 μl/sec. As annexin V is a constitutive marker for MP, total MP were defined as particles <1.1 in size and annexin V+. Double and triple staining were used to define MP subpopulations to identify their cellular origin.

Data was collected and analysed with FlowJo computer programme (version 8.8.3; Tree Star, Inc., Oregon, USA). Optimal compensation was set for the appropriate channels detecting FITC, PE, and APC-Cy7 using single stained controls acquired on the analysis software as the data was collected digitally. 3μm beads (Sigma-Aldridge, Poole, Dorset, UK) were also run as an internal standard for enumeration (see next section).

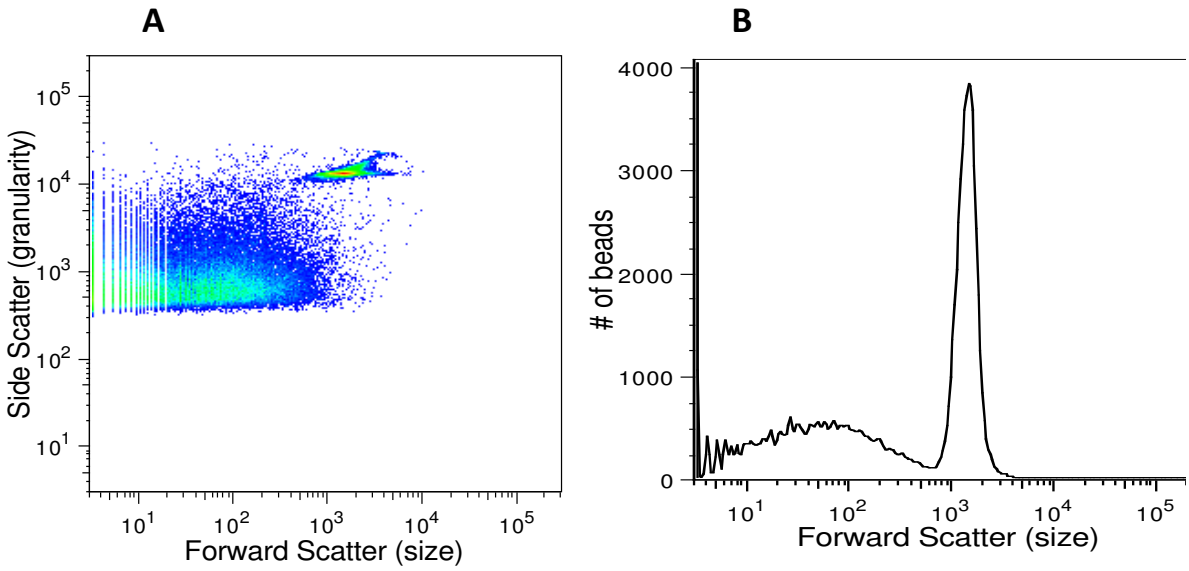

Figure 6. Optimisation of MP gating strategy using 1.1µm latex beads

A) 1.1µm beads were diluted in 0.22µm syringe filtered deionized water and analysed by flow cytometry. Data is presented as side scatter (depicting particle granularity) versus forward scatter (depicting particle size). **B)** Histogram indicates that the average size of the 1.1µm beads was $1.5x10^3$ as depicted by the peak on the forward scatter scale, which was used as the maximum size cut-off limit when gating for MPs in experiments.

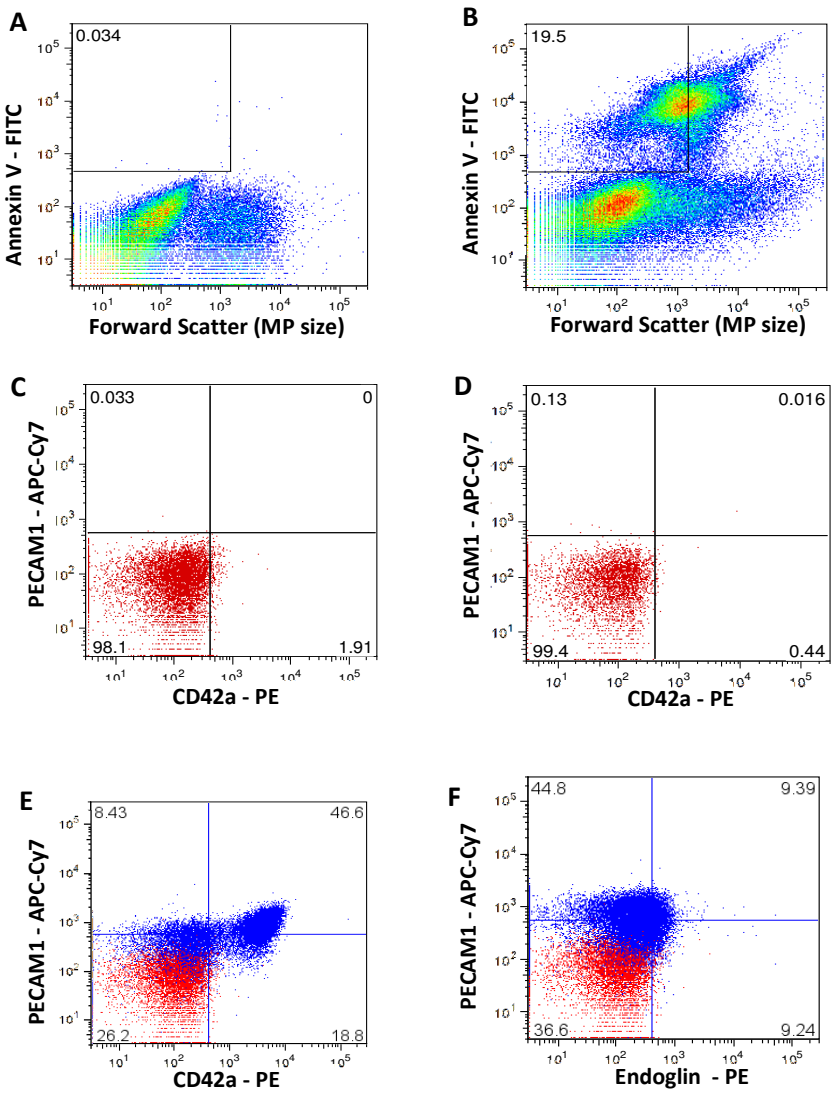

Figure 7. Flow cytometry analysis of MPs from platelet poor plasma (PPP)

A) Unstained control well shows events that were not stained for annexin V, thus representing background. This is useful to use in combination with 1.1µm latex beads for determining the gating of annexin V+ MPs. **B)** Annexin V+ MPs acquired from platelet poor plasma of a PAH patient. **C)** The IgG1k isotype control antibody conjugated to the allophocyanin Cy-7 (APC-Cy7) fluorochrome was used to determine nonspecific binding, indicated by low intensity values on the y-axis. **D)** The IgG1 isotype control antibody conjugated to the phycoerythrin (PE) fluorochrome was used to determine nonspecific binding, indicated by the low intensity values on the x-axis. **E-F:** Double antibody staining for PECAM1 with glycoprotein IX (CD42a) and endoglin (blue). A rightwards shift or upwards shift in intensity further away from the isotype control cut-off in intensity staining indicates presence of microparticles positive for the respective receptors.

2.11 Determination of microparticle number per ml of plasma

As the number of microparticle events analysed via flow cytometry could vary depending on variables such as the amount of plasma analysed, forward and side scatter parameters, and the type of cytometer used, it was important to enumerate the microparticle count in a standardised fashion. The use of latex beads as an internal standard was first introduced by Combes and colleagues (Combes et al., 1999), where the number of MPs per plasma or supernatant was determined by using the proportion of 3 μm beads counted and the volume of plasma from which the MPs were analysed. A predetermined number (200,000) of the 3 μm latex beads (Sigma-Aldridge, Poole, Dorset, UK) was calculated according to the manufacturer's recommendations (see below) and added to a well in the 96-well plate.

Equation for calculating number of latex beads:

Number of beads per ml = $(1.828 \times 10^{11})/d^3$

d=diameter of beads (μm)

Thus, to achieve this approximately, 6μl of 3μm latex beads was diluted in 2ml of deionised water (dH_20) that had first been filtered through a 0.22μl syringe filter. 10μl was added into a 96-well plate well with 240μl filtered dH_20 and run through the flow cytometer in the exact same manner as the MP analysis for PPP (Figure 8).

The latex beads were acquired using the flow cytometer and gated using the forward scatter (size) and side scatter (granularity) settings on a logarithmic scale. The total events count was recorded and used to calculate the number of microparticles per ml of plasma.

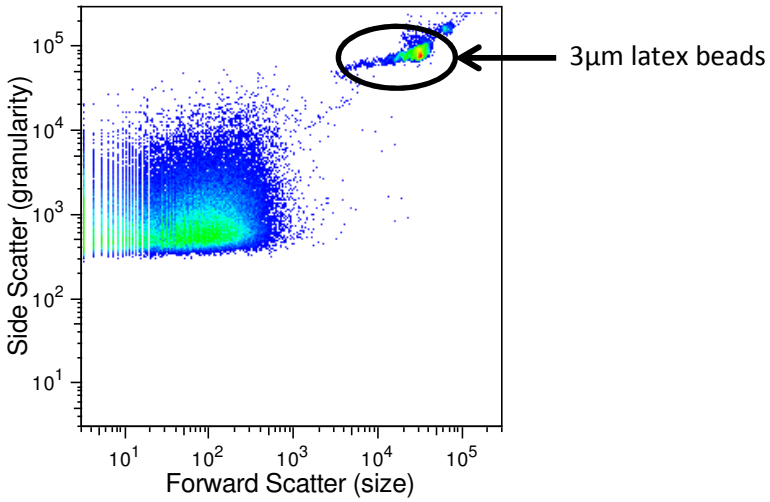

Figure 8. Detection of 3µm latex beads

A fixed number (200,000) of 3µm latex beads were prepared in filtered deionized water and added to wells of a polypropylene u-bottomed 96 well plate. They were acquired in the same manner as the MP samples via flow cytometry. The number of beads used and acquired was used as an internal standard for the conversion of the total number of MP events acquired to the number of MPs counted per ml of plasma.

The following equation (Figure 9) was adapted from Brogan et al. (Brogan et al., 2004), which converts flow cytometer events to an estimated count of MPs per ml of plasma.

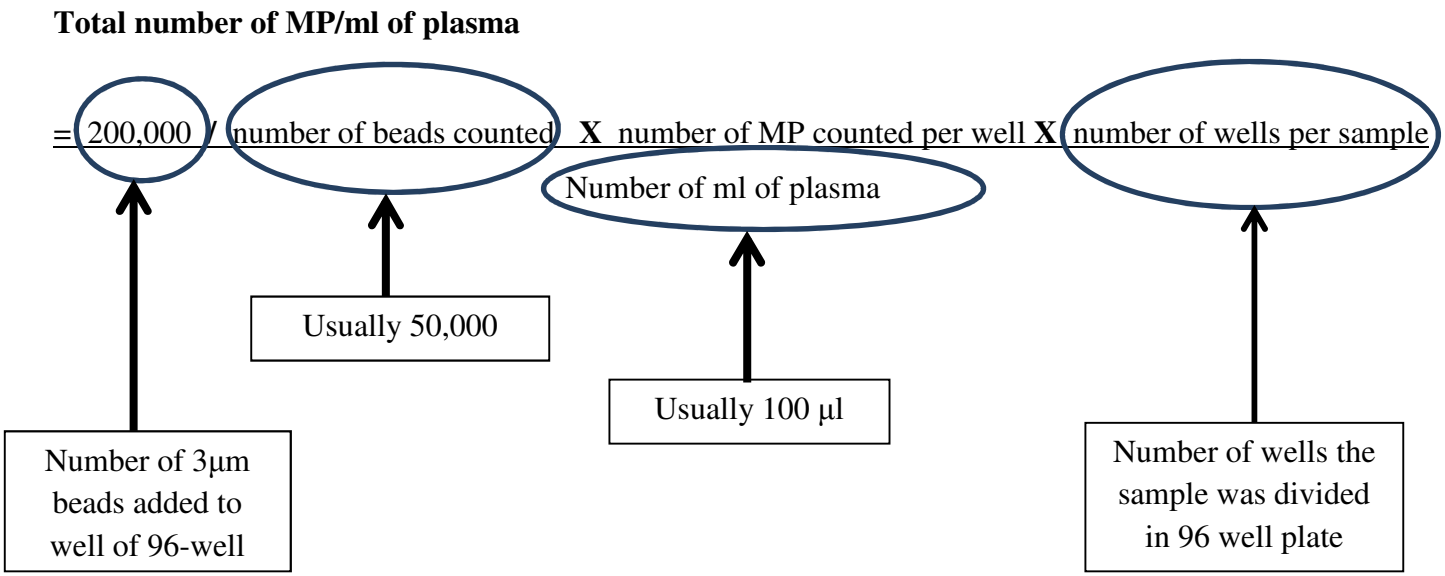

Figure 9. Conversion equation for MP number per ml of plasma calculated from flow cytomter event counts

The enumeration of microparticles from raw flow cytometry events count required a fixed number (200,000) of 3µm latex beads per well of the 96 well plate used, number of beads counted as raw events after acquisition, number of ml of plasma (100µl) used per platelet poor plasma to obtain the MPs, and the number of wells the sample was divided to during plating in the 96 well plate

2.12 Flow cytometric analysis of HIV-infected patient microparticles

Flow cytometric analysis of HIV-infected patient MPs were performed in a category III laboratory in the College of Medicine, Blantyre, Malawi using a CyAn ADP flow cytometer (Cyan^{TM} ADP Analyzer, BD). As this cytometer did not have a 96-well plate reading function, samples were read in individual polypropylene FACS tubes.

100 µl of PPP sample was pipetted into a 1.5 ml tube and centrifuged at 17,000g for 60 minutes at 4^{o}C. The MP pellet was isolated and prepared for staining using the identical method as stated above for PAH and CHD samples. The samples were then transferred from the wells of polypropylene 96-well U-bottomed plates to individual FACS tubes. An additional 400 µl of annexin V buffer was added to every tube prior to running the MPs through the flow cytometer, making a total volume of 650 µl per FACS tube. The samples were run at a medium speed and stopped after collecting a fixed volume of 300 µl. A fixed number (200,000) of latex beads was calculated using the manufacturer's instructions and pipetted into 650 µl of 0.22 µm syringe filtered distilled water. A fixed volume of 300 µl also was run through the cytometer in the same manner as the samples. Data was collected and compensation and analysis was performed digitally on the FlowJo 8.3.3 software.

2.13 The effect of prostacyclin on SMMP release

PASMCs isolated from child IPAH patients were grown to 70-80% confluency in 6 well plates. To assess the differential effects on microparticle release by different agents, 2ml of fresh growth media (DMEM/F12 containing 10% FBS) with and without 20ng/ml TNFα (Peprotech, Rocky Hill, New Jersey, USA) , 20ng/ml PDGFR-BB (Peprotech, Rocky Hill, New Jersey, USA), 5ng/ml transforming growth factor β (TGFβ) (Peprotech, Rocky Hill, New Jersey, USA), and 10nM endothelin-1 (ET-1) (Enzo Life Sciences, Exeter, UK) was administered to cells. The agents were also administered in the presence and absence 1μM treprostinil (gift from United Therapeutics, Chertsey, Surrey, UK) to assess the effect of the prostacyclin alalogue to inhibit SMMP release. A concentration- response study was also conducted whereby cells were treated with 10% FBS and 20ng/ml PDGF-BB alone and in the presence of either 1nM, 10nM, 30nM, 100nM, 1μM, and 10μM treprostinil. The role of the IP and EP_2 receptors were also studied by treating PASMCs with 20ng/ml PDGF-BB in combination 100nM treprostinil and 1μM of the EP_2 receptor antagonist PF-04418948 (Tocris, Bristol, UK) or 1μM of the IP receptor antagonist, RO1138452 (Tocris, Bristol, UK), Dallas, Texas, USA) or the two antagonists together. Given drugs were dissolved in the solvent DMSO, 0.03% DMSO (highest dilution that would otherwise be used) was added to all wells throughout the study.

After 24 hours of incubation, 1ml of supernatant was collected in 1.5 ml Eppendorf tubes from each well and centrifuged at 2,000g for 5 minutes at room temperature. The top 600μl was collected in new 1.5 eppendorf tubes and the bottom 400μl left to decant apoptotic bodies and cellular debris. The supernatants

were then spun at 17,000g for 60 minutes at 4^{o}C to obtain MP pellets. 350µl of annexin V was added to each tube, which was vortexed and labelled with annexin V-FITC. MPs were assessed through flow cytometry in a similar manner as explained previously and analysed by the FlowJo software version 8.3.3.

2.14 Thrombin Generation Assay (TGA)

MPs have phosphatidylserine (PS) rich areas on the surface membrane that are able to assemble and activate coagulation enzymes and give MPs their characteristic prothrombotic property (Morel et al., 2006). Moreover, some MPs externalise tissue factor on their surfaces, which is able to initiate blood coagulation (Gilbert et al., 1991; Sabatier et al., 2009). As thrombin is the endpoint of a series of proteolytic reactions in the coagulation cascade following vessel wall injury, and causes the conversion of fibrinogen to fibrin, the amount of active thrombin produced in plasma was measured by a thrombin generation assay (TGA) (Sabatier et al., 2009). When thrombin is generated, it cleaves a calcium-fluorogenic substrate (0.5 mM/L of Z-G-G-R-AMC and 7.5 mM/L of calcium final reagent concentrations, Pathway Diagnostics) which can be monitored by the assay (TECHNOTHROMBIN® TGA). The concentration of thrombin can be calculated with the aid of a calibration curve which was first obtained using the following protocol:

The thrombin calibrator was diluted with the TGA buffer into 1.5µl Eppendorf tubes in the following manner:

1st dilution (1:2) (STD 1): 200µl thrombin calibrator + 200µl TGA buffer

2nd dilution (1:4) (STD 2): 100µl 1st dilution + 100µl TGA buffer

3rd dilution (1:20) (STD 3): 20µl thrombin calibrator + 380µl TGA buffer

4th dilution (1:200) (STD 4): 20µl 3rd dilution + 180 µl TGA buffer

To obtain the calibration curve, 40µl of the calibrator dilutions (STD 1 – STD 4) and 50µl of the TGA substrate were pipetted into the wells of a microtiter plate in (NUNC Maxisorp 475515) in duplicates. The plate was run immediately after pipetting the substrate on a BMG Labtech FLUOstar OPTIMA fluorescence reader with filters 360nm and 460nm (excitation/emission). Thrombin was measured for 10 minutes in 30 second intervals for at 37°C (Figure 10).

Thrombin Generation Assay Calibration Curve

Dilution	nM Thrombin	Delta RFU/30 sec (W1)	ok	Delta RFU/30 sec (W2)	ok	AV Calculated
1:2	447.5	2197.2		2972.0		2584.6
1:4	223.75	1622.7		1578.4		1600.5
1:20	44.75	477.4		460.8		469.1
1:200	4.475	47		47.7		47.3

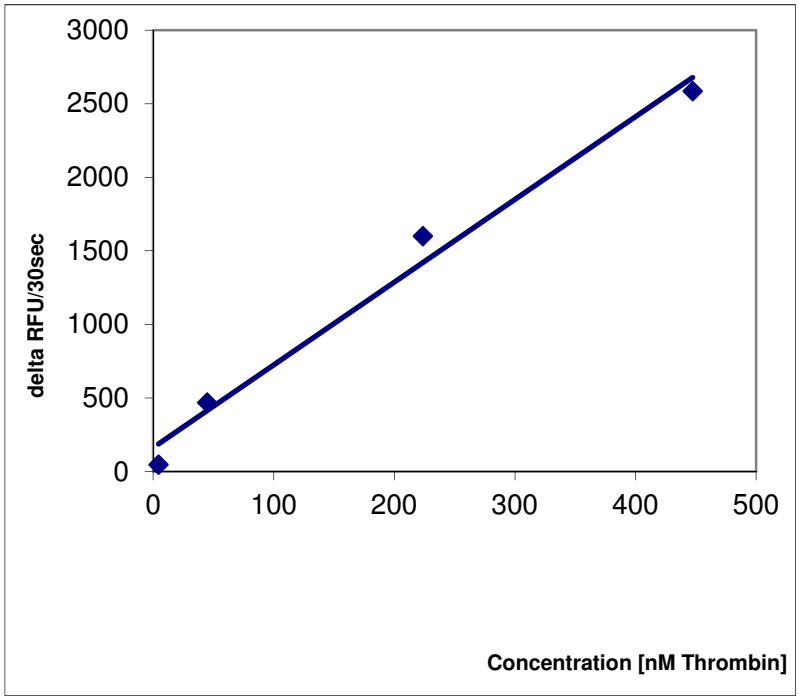

Figure 10. Thrombin assay calibration curve

A standard curve was generated for the thrombin assay using known thrombin concentrations (nM) as indicated and plotted as raw Δ relative fluorescence units (RFU) obtained by a fluorescence reader. A best fit line was generated to calculate thrombin concentration (nM) from Δ RFU values.

To conduct the TGA assay, 100µl of PPP samples were rapidly thawed at -37^{o}C and centrifuged at 17,000g to obtain MP pellets, as previously described. ~50ml blood from healthy volunteers was centrifuged twice at 5,000g for 5 minutes to obtain platelet poor plasma and centrifuged a second time at 17,000g to decant the remaining MP pellet and obtain MP free plasma. MP pellets were then resuspended in 80µl of microparticle free plasma (MPFP), which was obtained from healthy volunteers. 40µl of the MPs mixed in MPFP were then transferred to 2 plate wells so as to obtain duplicate readings per sample. The fluorogenic substrate was added and the plate was run immediately for 90 minutes in 1 minute measurement intervals. Measures of peak thrombin, lag time, velocity index, and endogenous thrombin potential were recorded (Figure 11).

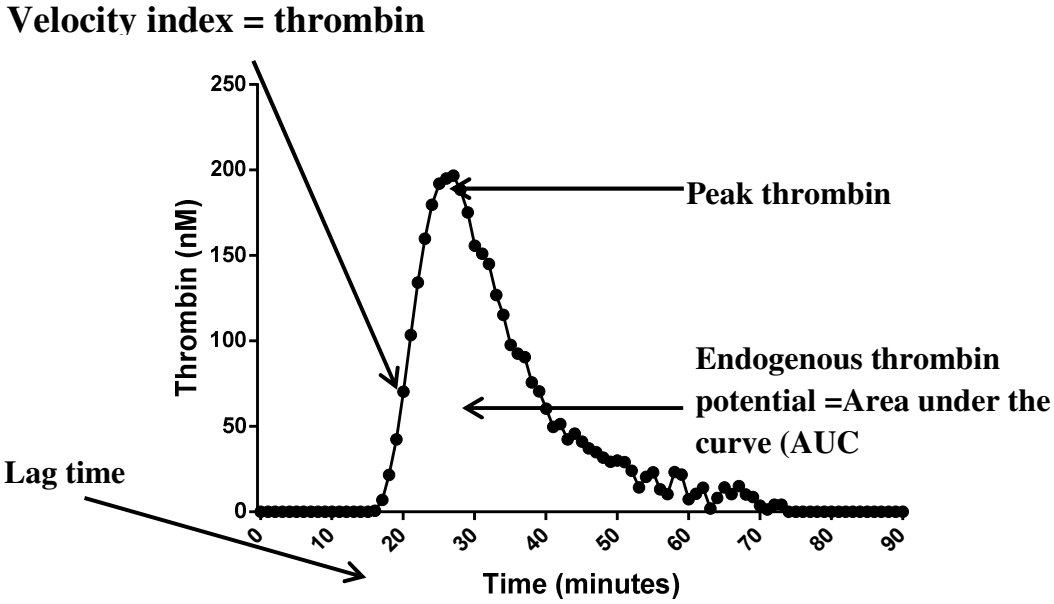

Figure 11. . Thrombin generation assay curve

Thrombin generation was determined and plotted against time to generate a curve. Measures of peak height in nM, lag time (=time required until thrombin generation onset) in minutes, velocity index (=rate of thrombin generated) in nM x min^{-1}, and endogenous thrombin potential (=area under the curve) in nM x min were recorded.

2.15 Proliferation Assay

To assess the effect of prostacyclin on mediating growth inhibition of PASMCs, a proliferation assay was conducted. This assay would serve two purposes: 1) examine the inhibition on cell proliferation caused by treprostinil and 2) investigate whether it may be doing so via inhibiting the nuclear factor of activated T-cells (NFAT)/calcineurin Aβ (CnAβ) pathway.

PASMCs from IPAH patients were plated into 6-well plates in growth medium (DMEM/F-12 containing 10% FBS, and 1% penicillin streptomycin) at a density of 10^4 cells per ml. After 24 hours of incubation at 37°C and 5% CO_2, cells were growth arrested in media DMEM/F12 containing 0.1% FBS for 48 hours. This period of time should be sufficient for the majority of the cells to reach the quiescent G0 phase of the cell cycle. Following this, appropriate drugs combinations were prepared in growth medium: 100nM treprostinil, 1μM treprostinil, 1μM of the calcineurin inhibitor cyclosporine, 1μM treprostinil + 1μM cyclosporine, 0.02% DMSO (Sigma-Aldridge, Poole, Dorset, UK). After addition of drugs the cells were incubated at 37°C and 5% CO_2 for 96 hours, after which they were washed with 1ml of warmed calcium and magnesium free PBS per well to remove residual serum. Cells were then trypsinised by replacing PBS with 0.5 ml of 0.05% trypsin-EDTA and incubating cells at 37°C for ~2 minutes. Growth media (0.5ml) was then immediately added to neutralise the trypsin and the cell suspension pipetted up and down several times to achieve a homogenous cell distribution. The cells were counted using the ADAM cell counter as already detailed. Data was analysed using GraphPad Prism (GraphPad Software, San Diego, CA) and cell proliferation expressed as the cell number per ml and as the % change in cell proliferation relative to the growth response induced by 10% FBS alone (100%).

2.16 Confocal microscopy of calcineurin Aβ

The mechanistic basis behind the effect of treprostinil on PASMC proliferation was examined through studying the roles of calcineurin Aβ (CnAβ) and PPARγ.

This was done via staining PASMCs for CnAβ and studying its expression and activation through nuclear translocation via confocal microscopy after treprostinil treatment.

To prepare the staining of calcineurin Aβ, the PASMCs cells were plated at a density of 2 x 10^4 cells/ml in eight chambered slides (BD Bioscience,) containing 500µl growth media (DMEM/Ham's F-12 with 10% FBS and 1% penicillin/streptomycin) for 2 days until reaching 50-60% confluence. The cells were then gently washed once with 500µl PBS in each well and serum starved with 500µl of DMEM/Ham's F-12 containing 0.1% FBS and 1% penicillin streptomycin for 48 hours. The cells in the different chambers were treated with the following drug treatments prepared in DMEM/F-12: Basal (0.1% FBS), 10% FBS, 1µM treprostinil with 10% FBS, 1µM rosiglitazone with 10% FBS, 1µM GW 9662 with 10% FBS, 1µM GW9662 with 1µM treprostinil and 10% FBS, 1µM T0070907 with 10% FBS, and 1µM T0070907 with 1µM treprostinil and 10% FBS. The cells were pre-treated with the PPARγ antagonists GW9662 and T0070907 in arrest media for 1 hour at 37^{o}C and 5% CO_2 prior to co-treatment with treprostinil and 10% FBS.

The PASMCs were prepared in the chamber slides for confocal microscopy in the same manner as explained in section for the characterisation of smooth muscle cells. 100µl of rabbit polyclonal anti-calcineurin (Cn) Aβ (Millipore, Watford, Hertfordshire, UK), diluted at 1:200 blocking solution consisting of 2% BSA and 0.01% triton x-100 dissolved in PBS, was added to each well and the cells

incubated for 2 hours at room temperature on a slow shaker. The cells were washed 3 times with 500µl PBS for 5 minutes each before a 100µl of the secondary antibody anti-mouse Alexa 488 (Invitrogen, Paisley, UK), prepared at 1:200 dilution in blocking solution, was used to stain CnAβ. After 2 hours of incubation at room temperature on a slow shaker in the dark, the slides were washed a final time with 500µl PBS per well and the walls of the chamber detached. 10µl of mounting reagent containing 4', 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA), which binds strongly to the A-T rich regions in DNA and stains the nucleus blue, was pipetted to the wells of each slide. A glass coverslip was placed on top and sealed in place with a layer of commercially available nail varnish. The slides were stored at -20^{o}C in the dark until they were ready to be examined under a Leica TCS SPE confocal microscope preferably within 3 days.

Confocal images of PASMCs were taken from a focal plane from the middle of the cell using a z-stack of 10 images with 0.10 µm spacing. Nuclear colocalisation of calcineurin Aβ was quantified using the ImageJ software where at least 8 different cells per treatment from 3 patients were analysed.

2.17 The effect of SMMP on proliferation

Normal control PASMCs were plated at a density of 1x10^{4} into the wells of a 96-well flat-bottomed plate in growth media (100µl) containing DMEM/F-12, 10% FBS, and 1% penicillin/streptomycin (50 units/ml) and incubated at 37^{o}C in a

humidified incubator for 24 hours. The cells were then washed with 200µl of PBS twice and growth arrested in 0.1% FBS in DMEM/Ham's F-12 media containing 1% penicillin/streptomycin for 48 hours. The media was replaced with fresh arrest which was added to each well in the absence and presence of 10% FBS, 20ng/ml platelet derived growth factor (PDGF-BB), 10^5 smooth muscle MPs derived from a? PAH patient(s) or 5ng/ml transforming growth factor β (TGFβ). The different treatments were administered into the wells in replicates of five. The cells were incubated for 96 hours and the changes in proliferation were compared to the cells in arrest media.

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Southhampton, UK) was used to examine the changes in proliferation of the smooth muscle cells caused by the different treatments. This assay utilises a colorimetric method to determine the number of viable cells. It is composed of two chemicals: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine methosulfate. MTS is cleaved by dehydrogenase enzymes into aqueous, soluble formazan in metabolically active cells. The quantity of formazan product measured by the absorbance at 490nm is directly proportionate to number of viable cells in culture media.

Twenty-one ml of Dulbecco's PBS was added to a container wrapped in foil for protection from light, into which 42mg of MTS reagent powder was added according to the manufacturer's instruction. The MTS was completely dissolved

by mixing on a magnetic stir plate for 15 minutes. The pH was adjusted to 6.0-6.5 and the mixture filter sterilised by passing through a 0.22µl filter into 15ml falcon tubes and stored at -20^{o}C in a container shielded from light. The MTS solution was thawed and 2ml was mixed with 100µl of PMS solution in a 15ml falcon using asceptic technique. After gentle swirling, the MTS/PMS solution was diluted in arrest media at a 1:6 dilution, and 120µl of the solution was used to replace the media containing the cells in the wells of the 96-well plate. The plate was incubated for 3 hours at 37^{o}C and 5% CO_2, after which absorbance was read at 490nm using an ELISA plate reader

2.18 Statistical analysis

All statistical analysis was carried out on GraphPad Prism 6 software (Jandel software, La Jolla, USA). The MP data is expressed as median ±interquartile range. The differences in MP levels between controls and diseased patient samples were examined using the Mann Whitney U test. The differences in MP levels in PAH patients before and after therapy was examined using the Wilcoxen matched pairs test. All in vitro assay data are expressed as mean ± standard error mean (S.E.M.) and the differences between more than two groups were determined using one-way analysis of variance (ANOVA) with Bonferroni, Dunnett's or Neuman Keuls multiple comparisons test where appropriate. Logistic regression analysis was used to examine the relationship between biomarker levels (ie. microparticle level vs. peak thrombin) and results are expressed using Spearman's rank correlation coefficient and odds ratios (OR) with corresponding 95%

confidence intervals (CI) and P-values. Receiver operator characteristic (ROC) curves, sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated to examine the diagnostic characteristics of indices described. The ROC curve is a valuable tool for evaluating diagnostic tests, in particular to this project, circulating microparticle levels and MP-induced thrombin generation. ROC curve were reported as area under the curve (AUC) and 95% confidence intervals (CI) to measure how well a parameter could distinguish between the two diagnostic groups (ie. diseased and normal). The plot is indicated as the true positive rate (=sensitivity) in function of the false positive rate (100%-specificity) for the different cut-off points of a parameter. The points on ROC curves represent sensitivity/specificity pairs corresponding to decision thresholds held at those specific points. Statistical significance was regarded when P values were less than 0.05 (two sided).

Chapter 3

3. Identification of smooth muscle microparticles

3.1 Introduction

PAH is a severe disease of the small pulmonary arteries characterised by narrowing of the lumen, increased inflammation, and vascular remodelling, which leads to elevated pulmonary artery pressure (PAP) and ultimately right heart failure. Due to nonspecific presenting symptoms, often delayed diagnosis by an average of 2 years, and an invasive definitive diagnosis via right heart catheterisation, the need for early biomarkers is much needed in PAH (Warwick et al., 2008). A wide array of biomarkers has been explored and can be divided into 5 major groups: markers of vascular dysfunction (e.g. endothelin-1, assymetric dimethylarginine (ADMA), angiopoietins, von Willebrand factor), markers of inflammation (e.g. pro-inflammatory cytokines such as IL-6, C-reactive protein, chemokines), markers of myocardial stress (BNP/NT-proBNP, ANP, troponins), markers of low carbon dioxide and/or tissue hypoxia (i.e. osteopontin, uric acid, growth factor 15 (GDF15), PCO_2), and markers of secondary organ damage (e.g. bilirubin, creatinine) (Galie et al., 2015b). Though this list is growing, BNP and NT-proBNP, biomarkers for myocardial dysfunction, are the only plasma markers used widely in PH centres and clinical trials. However, they are not necessarily specific for PH as they are elevated in most heart diseases and tend to have high variability, and thus should only be interpreted in the clinical context of the patients. A biomarker that plays a role in both vascular dysfunction leading to remodelling and inflammation are microparticles (MPs), which are submicron pro-inflammatory, thrombogenic vesicles released by activated or apoptotic cells

(Simak and Gelderman, 2006; Dignat-George and Boulanger, 2011). Circulating MPs of different cellular origin including platelets, erythrocytes, leukocytes, and endothelial cells are detectable in healthy subjects, but are elevated in a wide variety of cardiovascular diseases including atherosclerosis, heart failure, arrhythmias and inflammatory vascular diseases (Amabile et al., 2013).

Endothelial dysfunction plays a prominent role in the development and progression of PAH. An imbalance caused by increased proliferation and decreased apoptosis of endothelial cells has been reported in idiopathic PAH (IPAH) (Masri et al., 2007). Increased endothelial apoptosis at the initial stages of PAH and decreased apoptosis in later stages appears to contribute to the disease progression (Sakao et al., 2005). Additionally, primary pulmonary endothelial cells isolated from PAH lung specimens have a pro-proliferative, apoptotic-resistant phenotype (Eddahibi et al., 2006). Amabile and colleagues have shown that circulating endothelial MPs expressing the surface markers platelet and endothelial cell adhesion molecule (PECAM), vascular endothelial cadherin (VE-cadherin) and E-selectin were increased in subjects with PH compared to control subjects (Amabile et al., 2008). Levels of PECAM+/CD41- and VE-cadherin+ MPs correlated positively with mean PAP (mPAP), pulmonary vascular resistance (PVR), and mean right atrial pressure (mRAP) and inversely with cardiac index (CI). As PECAM is a surface marker that stains both endothelial cells and platelets, MPs with the heterodimeric integral membrane protein integrin alpha-IIbIIIa (CD41; GPIIbIIIa) were identified to distinguish endothelial specific PECAM+/CD41- MPs. Microparticles have been characterised on the basis of the

many surface markers that exist on the cells of origin. Endothelial microparticles (EMPs) have been extensively studied in their role as mediators or biomarkers of various vascular diseases including vasculitis, sickle cell anemia, and endotoxemia (Brogan et al., 2004; Dignat-George and Boulanger, 2011). EMPs have been shown to contain endothelial-derived proteins such as vascular endothelial cadherin, platelet endothelial cell adhesion molecule (PECAM) -1, intercellular cell adhesion molecule (ICAM)-1, endoglin, E-selectin, melanoma cell adhesion molecule (MCAM) or αv integrin (Chironi et al., 2009; Dignat-George and Boulanger, 2011). As aforementioned, since PECAM1 is expressed in both endothelial cells (ECs) and platelets, EMPs have specifically been defined by a CD31+/CD41- phenotype. CD41 is the platelet integrin glycoprotein IIBIIIa, a receptor for fibrinogen and von Willebrand factor (vWF) that is involved in platelet activation, although EMPs are also able to bind to vWF. Jimenez and colleagues conducted a study where growing renal and brain microvascular and coronary endothelial cells were deprived of growth factors to induce apoptosis (Jimenez et al., 2003).

The types of proteins detected on the surface of MPs may provide information on the vasculopathy in disease conditions. Endoglin is an accessory protein for transforming growth factor -β (TGF-β) and its expression is upregulated in endothelial cells during cell proliferation (Nassiri et al., 2011). Endoglin therefore has been suggested to be a marker for tumour-related angiogenesis and neovascularisation. Endoglin expression appears elevated in ECs of neoplastic tissue, which are more proliferative than ECs isolated from normal tissue. Conley and colleagues reported that cultured human arterial smooth muscle cells (SMCs)

also express endoglin, predominantly the L-isoform (Conley et al., 2000). High endoglin expression was also observed on SMCs in atherosclerotic plaques *in vivo*, though little or no expression was seen in smooth muscle within normal arteries. PECAM-1 is another molecule expressed on ECs as well as on platelets and various leukocyte subtypes. It has been shown to play a role in the transmigration of monocytes, neutrophils, natural killer cells and some sub-sets of lymphocytes (Woodfin et al., 2007). ICAM-1 and VCAM-1 are molecules that are expressed in both ECs and SMCs (Braun et al., 1999; Dignat-George and Boulanger, 2011) and contribute to the adhesion of leukocytes to the activated endothelium. Through the binding of lymphocyte function-associated antigen (LFA)-1 (CD18/11a) or Mac-1 (CD18/CD11b), ICAM1 is able to mediate the adhesion of neutrophils, monocytes and lymphocytes to the endothelium. ICAM-1 also acts as a receptor for fibrinogen and hyaluronic acid. VCAM-1 is able to bind to the very late antigen 4 (VLA_4; integrin α4/β1) on monocytes and lymphocytes (Faruqi and DiCorleto, 1993). E-selectin is a member of the selectin family of glycoproteins and is an endothelial-specific adhesion molecule. Its expression is rapidly induced by inflammatory cytokines such as TNF-α and IL-1β and is involved in the initial cell attachment and rolling of leukocytes at sites of endothelial activation during inflammation (Rahman et al., 1998). Like E-selectin, VE-cadherin is also an endothelial specific marker that is located at junctions between ECs and plays a role in controlling vascular permeability and leukocyte extravasation (Vestweber, 2007). Another adhesion molecule that is present on both endothelial cells and vascular smooth muscle cells is MCAM, which plays a role in cell-cell adhesion as a component of the endothelial junction associated with the actin cytoskeleton (Guezguez et al., 2007). MCAM is also expressed on

activated T-cells and in lymphoid tissues including the thymus and spleen. Indeed, many surface markers used to identify endothelial-derived microparticles as biomarkers of vascular diseases are highly involved in inflammation.

As medial thickening is the earliest known pathology in PAH, the identification of circulating smooth muscle MPs may be a valuable tool in prompting an earlier diagnosis of the disease. However, smooth muscle MPs have not been characterised unlike endothelial MPs. This may be due to the fact that SMCs in the past have classically been characterised by intracellular markers instead of surface markers. Thus, α-smooth muscle actin (αSMA) is frequently used to identify the smooth muscle phenotype, though it is not specific to SMCs as it is present in cultured fibroblasts as well (Hinz et al., 2001; Metz et al., 2012). Other intracellular markers of SMCs include myosin heavy chain, transgelin (SM22α), calponin, caldesmon, and the cytoskeletal protein smoothelin. SM22α is an intracellular SMC-specific protein that is related to the actin- and tropomyosin-binding protein calponin (Li et al., 1996). Wang and colleagues demonstrated that smooth muscle progenitor cells (SMPCs) were positive for certain surface markers such as platelet derived growth factor receptor β (PDGFRβ), carboxipeptidase M (CPM) and low-density lipoprotein receptor-related protein 1 (LRP1) (Wang et al., 2012). PDGFR-α and -β are receptors for PDGF, a growth factor that is elevated in PAH. mRNA expression of both receptor isoforms have been shown to be increased in small pulmonary arteries from patients with IPAH compared to control subjects (Perros et al., 2008). Protein expression of PDGFRβ is significantly increased in disease as well. Both PDGFR-α and –β receptors largely

stained pulmonary arterial SMCs and to a lesser extent endothelial cells. CPM is a protein in the family of carboxypeptidases and has a wide variety of roles physiologically including the regulation of blood coagulation/fibrinolysis, inflammation, food digestion, neuropeptide and prohormone processing (Deiteren et al., 2009). Though expressed on smooth muscle cells, greater CPM activity has been shown in cultured endothelial cells when both cell types were isolated from hog aorta (Palmieri et al., 1986). LRP1 is a protein expressed in various tissues including smooth muscles and has been shown to inhibit PDGF-induced mitogen-activated protein kinase (MAPK) activity and migration and proliferation of smooth muscles (Basford et al., 2009). It is also a clearance receptor for amyloid Aβ and has been shown to play a protective role in Alzheimer's disease (Kanekiyo et al., 2012).

Though smooth muscle microparticles have reportedly been identified in past studies, a full characterisation has not been initiated. Akker and colleagues showed that these MPs could bind potently to annexin V in the same manner as other microparticles, which allowed identification of the total MP count using flow cytometry (van den Akker et al., 2012). Essayagh and colleagues demonstrated that apoptotic rat aortic smooth muscle derived MPs were annexin V and expressed β3 integrin and contained low levels of tissue factor (Essayagh et al., 2005). Tissue factor on the surface of smooth muscle MPs were also confirmed by Brisset and colleagues, though this was also seen in endothelial MPs and monocytes (Jimenez et al., 2003; Stampfuss et al., 2006). In PH, circulating levels of PECAM+/CD41-, VE-cadherin+, and E-selectin+ EMPs as well as

CD45+ leukocyte derived MPs have been shown to be elevated compared with control subjects (Amabile et al., 2008). In PAH, Bakouboula and colleagues showed that procoagulant MPs bearing tissue factor and endoglin were elevated in patients and attributed it to endothelium damage (Bakouboula et al., 2008). With the intent of determining the level of smooth muscle MPs, I aimed to first characterise smooth muscle cells isolated from PAH cells and distinguish them from those from normal control SMCs and ECs.

3.2 Results

3.2.1. Characterisation of smooth muscle cells

Pulmonary arterial smooth muscle cells isolated from PAH patients expressed platelet derived growth factor receptor β (PDGFRβ), endoglin, neural glial antigen 2 (NG2), intracellular cell adhesion molecule (ICAM) and mesenchymal cell adhesion molecule (MCAM) (Figure 12). The level of fluorescence intensity representing receptor expression of endoglin, NG2, and MCAM were significantly higher than that of the isotype control antibody ($p=<0.001$, $p=<0.001$, and $p=0.05$, respectively; $n=3$). Expression levels of PDGFRα, vascular cell adhesion molecule (VCAM) 1, E-selectin, platelet endothelial cell adhesion molecule 1 (PECAM-1), and the cytoskeletal markers α-smooth muscle actin (αSMA), and transgelin (SM22α) were not elevated when their intensity was compared to the control antibody.

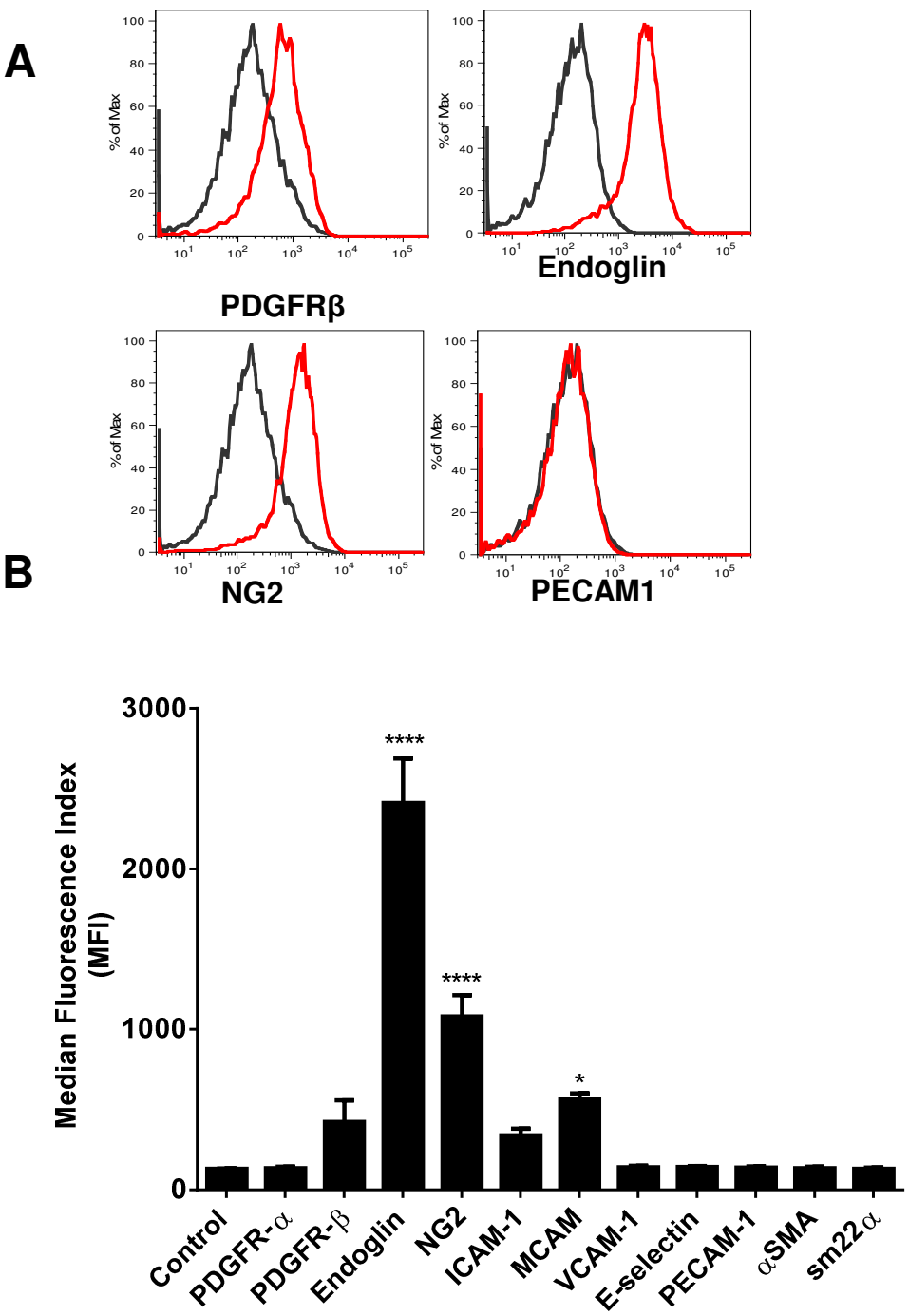

Figure 12. Measurement surface marker expression on PASMCs isolated from PAH patients

Measurement surface marker expression on pulmonary artery smooth muscle cells isolated from PAH patients and grown in 10% FBS. A) Histograms show flurescence intensity of surface markers platelet derived growth factor receptor-β (PDGFR-β), endoglin, neural glial receptor 2 (NG2), and platelet and endothelial cell adhesion molecule (PECAM-1). B) Data are presented as median fluorescence intensity ± S.E.M. (n=3); One-way ANOVA with Holm-Sidak's multiple comparisons test, with a single pooled variance was used.

3.2.2. Visualisation of intact microparticles

MPs were visualised in culture using green fluorescent probes and confocal microscopy (Figure 13). The fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) was used to permeate patient-derived pulmonary artery smooth muscle cells (PASMCs). This dye covalently binds via its succimidyl group to intracellular molecules and stains the cytosol fluorescent green. These cells were able to release microparticles after stimulation with 20ng/ml PDGF and 10% FBS for 24 hours. These microparticles were subsequently visualised as green globules when administered to growing smooth muscle cells and left to attach for 4 and 24 hours. The fluorescent staining of the MPs indicate that they are entities with intact membranes and are able to attach to the cell surface of PASMCs isolated from PAH patients and grown in culture. The number of visible particles was greater after 24 hours of administration to cells indicating that MPs are adhesive to cells and may be involved in the fusing of membranes and transporting intercellular material.

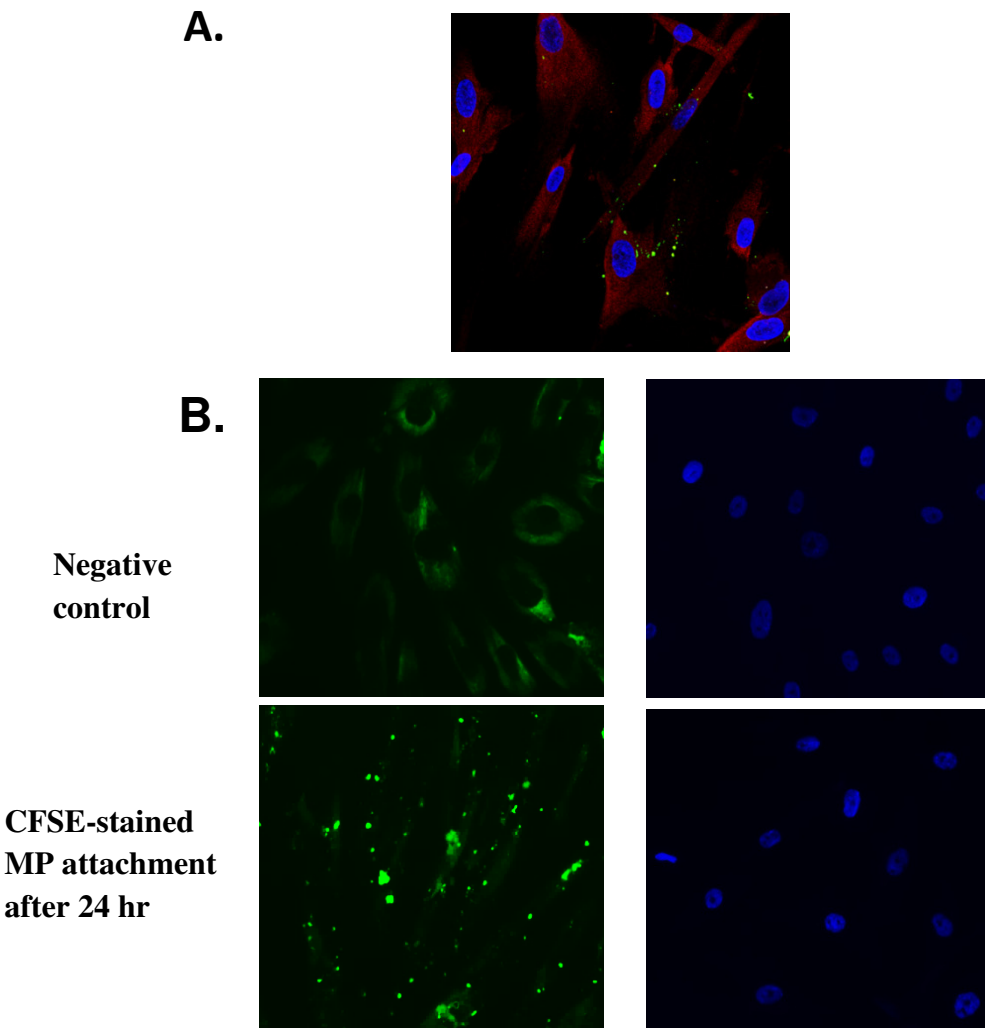

Figure 13. Green fluorescent staining of MPs attached to smooth muscle cells. A) PASMCs were grown in culture in the presence of the intracellular fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and then stimulated with 20ng/ml PDGF-BB and 10% FBS to cause the release of MPs. MPs were isolated and subsequently administered to smooth muscle cells for 24 hours. Cells were stained (red) with α-smooth muscle actin primary monoclonal antibody (1:500 dilution) and anti-mouse Alexa 555 (1:1000 dilution) and the nuclear marker DAPI (blue) and imaged via confocal microscopy. B) CFSE-stained MPs were administered to growing smooth muscle cells for 24 hours and imaged using a fluorescence microscope to assess level of attachment. DAPI was used for nuclear staining. Any un-attached MPs were washed away with PBS.

3.2.3. Microparticles derived from pulmonary artery smooth muscle cells

The release of MP derived from PAH smooth muscle cells (n=3) was studied over 48 hours (Figure 14). The level of MPs released gradually increased over time and was highest at 24 hours after stimulation with 10% serum and 20ng/ml PDGF-BB, declining thereafter. Total MP release was 3 fold greater with a combination of PDGF-BB and serum at 24 hours compared to serum alone. This decline may have been due to MP degradation or uptake by growing cells. MP release was minimal with 0.1% FBS, which stabilised after 6 hours. Serum was able to induce greater MP release compared to serum starvation (0.1% FBS), with peak levels that were 2.5 fold greater at 6 hours, though this did not reach significance.

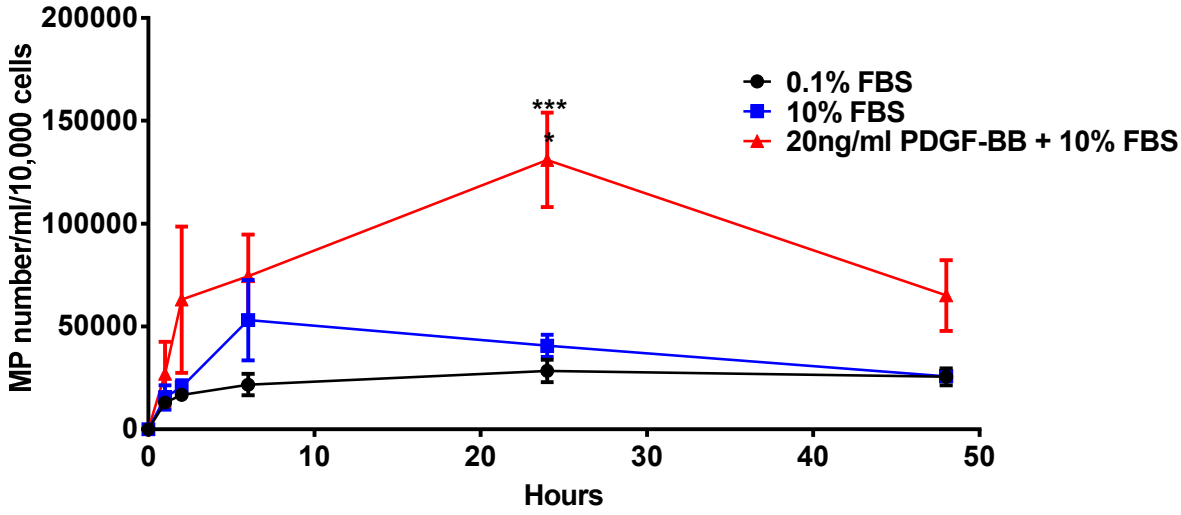

Figure 14. Time course of the total MPs released by SMCs isolated from PAH patients

Time course of the total MPs released by smooth muscle cells isolated from PAH patients and grown in culture with 0.1% FBS, 10% FBS, and 10% FBS + 20ng/ml PDGF-BB. Total MPs were Annexin+ and smaller than 1.1μm in size as assessed by side scatter using flow cytometry. Data is mean ± S.E.M. (n=3). Two-way ANOVA was conducted with Tukey's multiple comparisons test, where ***=P<0.001 when compared to 10% FBS.

3.2.4. PDGF-BB induced smooth muscle microparticle release

PDGF-BB induced MP release from SMCs isolated from PAH patients (n=3) in a dose-dependent manner (Figure 15). A visible, though not significant, increase was seen when 10^{-9} ng/ml PDGF-BB was administered with 10% FBS compared with serum alone. $3x10^{8}$ng/ml PDGF-BB caused the highest level of MP release from smooth muscle cells which was 2-fold greater than that caused by serum alone ($P<0.01$; n=3). There was a slight decrease in MP number at the highest dose $1x10^{7}$ng/ml, which could be due to PDGF-BB working via other pathways that counteract the MP shedding process.

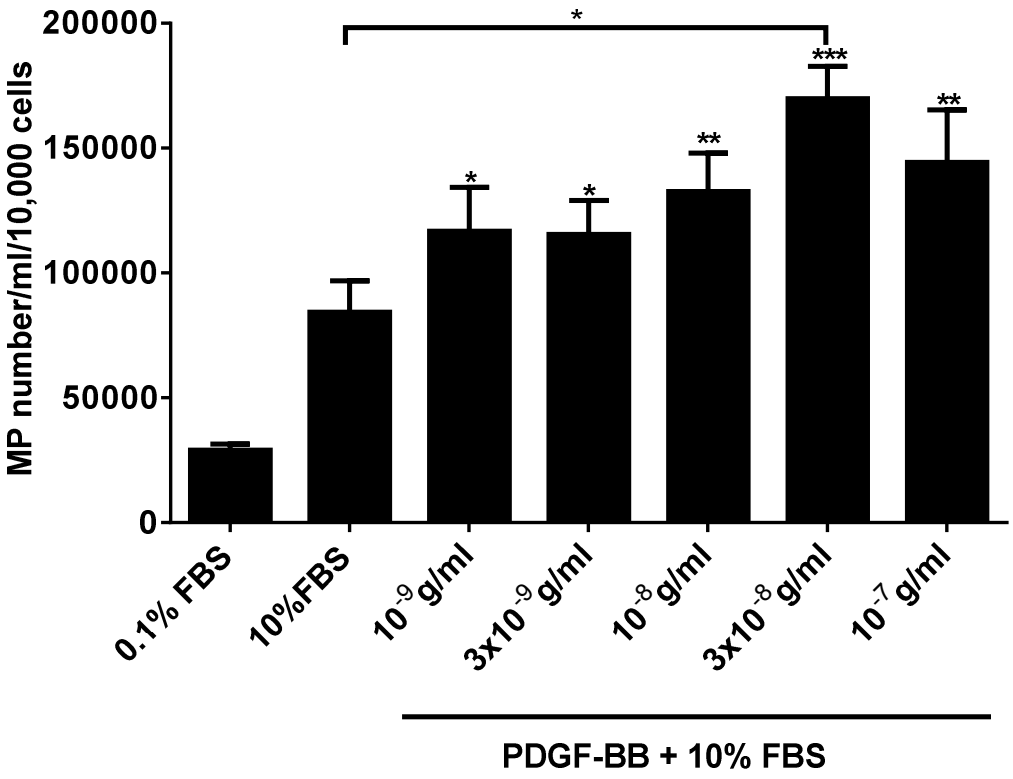

Figure 15. Effect of platelet-derived growth factor-BB (PDGF-BB) on MP release from growing smooth muscle cells.

PDGF-BB was administered for 24 hrs at concentrations 10^{-9}g/ml, $3x10^{-9}$g/ml, 10^{-8}g/ml, $3x10^{-8}$g/ml, 10^{-7}g/ml each in combination with 10% FBS and compared with 10% FBS and 0.1% FBS. Data are presented as mean ± S.E.M. (n=3). *=P<0.05; **=P<0.01; One-Way ANOVA with Tukey's multiple comparisons test was conducted when compared to 0.1% FBS.

3.2.5. Characterisation of smooth muscle microparticles from PAH cells

Pulmonary artery smooth muscle MPs from PAH cells (n=5) were further analysed and characterised. Cells were grown in 0.1% FBS, 10% FBS and 10% FBS with 20ng/ml PDGF-BB for 24 hours to stimulate MP release (Figure 16a). Low serum induced significantly less MP release compared to 10% FBS ($p=<0.05$), while 20ng/ml of PDGF-BB with serum induced significantly greater particle release ($p=<0.05$). For characterisation, MPs were co-stained for different cell surface markers (Figure 16b). High levels of PDGFRβ, endoglin, NG2, ICAM-1, and E-selectin positive MPs derived from smooth muscle (greater than 20,000 MPs/ml/10^4 cells) were detected after PDGF-BB administration, with significantly less generated with serum alone ($P=<0.05$; n=5) and accounted for 30%, 44%, 25%, 17% and 19% of the total MPs detected, respectively. Differences in the proportion of the MP subpopulations between treatments was not noticeable. Levels of PDGFRα+, MCAM+, +, αSMA+ and sm22α+ induced by PDGF were comparably lower, and few VCAM+-1 or PECAM-1+ MPs were detectable.

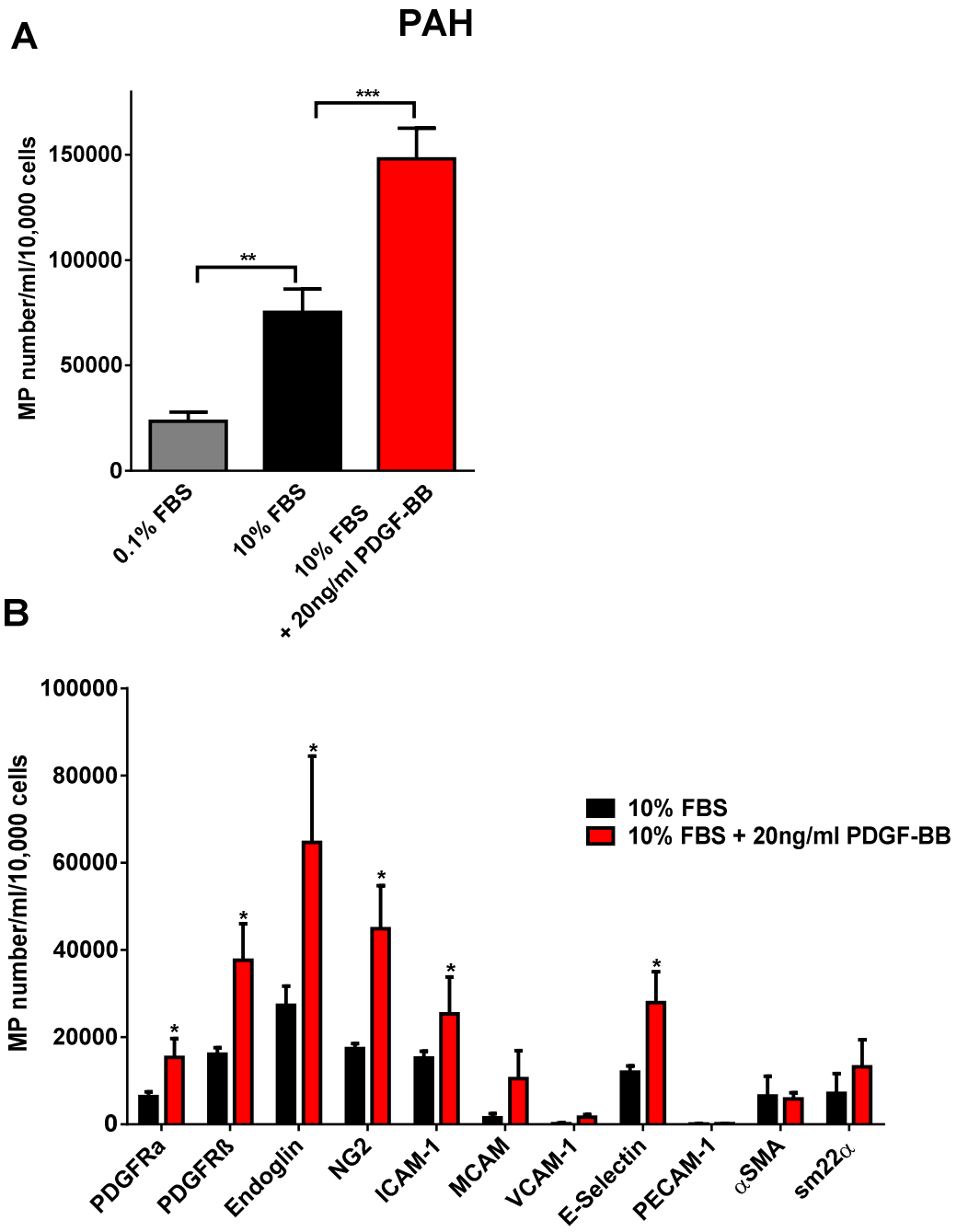

Figure 16. Characterisation of MPs released from cultured PASMCs isolated from PAH patients

A) Total MPs released by cells incubated for 24 hrs in the presence of either 0.1% FBS, 10% FBS or 10% FBS in combination with 20ng/ml PDGF-BB as indicated. **B)** Characterisation of MPs released from cultured cells stimulated with 10% FBS in the absence or presence of 20ng/ml PDGF-BB for 24 hrs. All MPs were stained for annexin V and a single cell surface/cytoskeletal marker. Data are expressed mean ± S.E.M (n=5). *=P<0.05; **=P<0.01; ***=P<0.001; One-Way ANOVA with respect to 0.1% FBS for A and unpaired t-test with respect to 10% FBS for B.

3.2.6 Smooth muscle microparticles released from growing cells from PAH patients

Levels of specific receptor positive MP subpopulations were studied over a 48 hour period to investigate their release from cells in proportion to the total number of MPs released (Figure 17). All three PDGFRβ+, endoglin+, and NG2+ MP subpopulations increased over time, peaking at 24 hours, with a combination of 20ng/ml PDGF-BB and 10% serum causing a significantly greater MP release than serum alone (**=P<0.01). During MP release over 24 hours, the proportion of the receptor positive subpopulations also increased relative to the total MP levels. At 6 hours and 24 hours respectively, the proportion of total MPs that were endoglin+ was 23.4% and 44.65%, for PDGFRβ+ was 15% and 32% and for NG2+ was 16% and 28%. At 48 hours, all three MP subpopulations decreased in number both in the presence of serum with and without PDGF-BB.

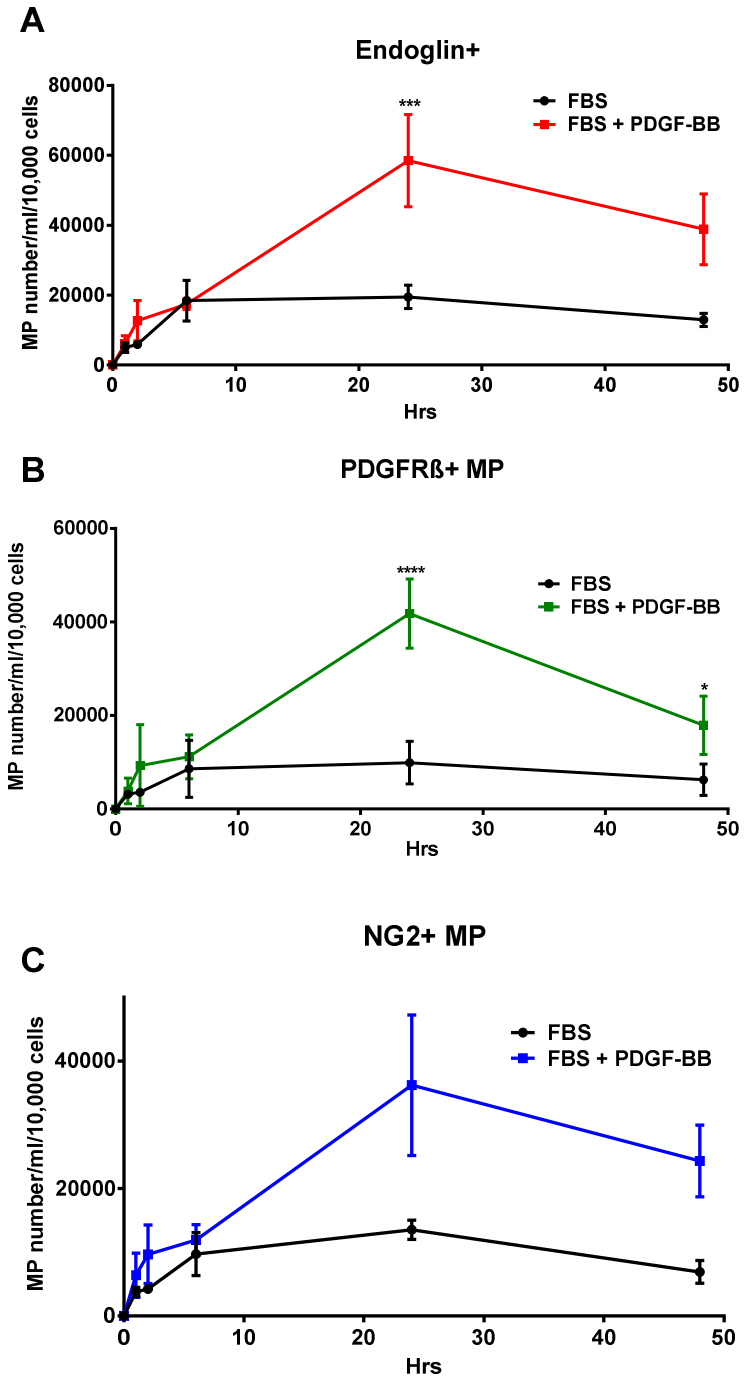

Figure 17. MPs released from growing PASMCs

MPs released from PASMCs grown in 10% FBS with and without 20ng/ml PDGF-BB over 48 hours. Smooth muscle MPs were stained for endoglin (A), PDGFRβ (B), and NG2 (C). Data are mean±S.E.M (n=5); *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001; Two-way ANOVA was conducted with Sidak's multiple comparisons test when compared to 10% FBS.

3.2.7. Characterisation of smooth muscle microparticles from normal cells

MPs released from control PASMCs (n=5) isolated from donor lungs were also analysed and characterised (Figure 18). 20ng/ml PDGF-BB in the presence of 10% FBS also induced a significantly greater release of MPs at 24 hours compared to serum alone (P=<0.05). MPs released by either serum alone or in combination with PDGF-BB contained a similar marker profile to that observed in PAH cells with PDGFRβ+, endoglin+, NG2+, ICAM-1+ and E-selectin+ MPs being considerably higher than other markers (figure 6b). MPs positive for these markers were all detected at levels greater than 20,000 MPs/ml/10^4 cells. Though PDGFRβ+ MP release was significant, in the presence of PDGF-BB and serum, levels for the receptor specific subpopulation were not significantly higher compared to serum alone. Moreover, total MP release and receptor specific subpopulations from normal control donor cells were slightly though not significantly lower than MP levels from PAH cells. PDGFRβ+ and endoglin+ MPs accounted for 17% and 33% of the total MPs, which are considerably lower than that seen in PAH cell-derived MPs. In contrast, E-selectin+ MPs accounted for 35% of the total count, which is comparably higher than in MPs from PAH cells. PDGFRα+, MCAM+, VCAM+, αSMA+, and sm22α+ MP numbers were noticeably low before and after PDGF stimulation (ie. ≤10000 MPs/ml/10^4 cells), with PECAM-1+ MPs virtually undetectable.

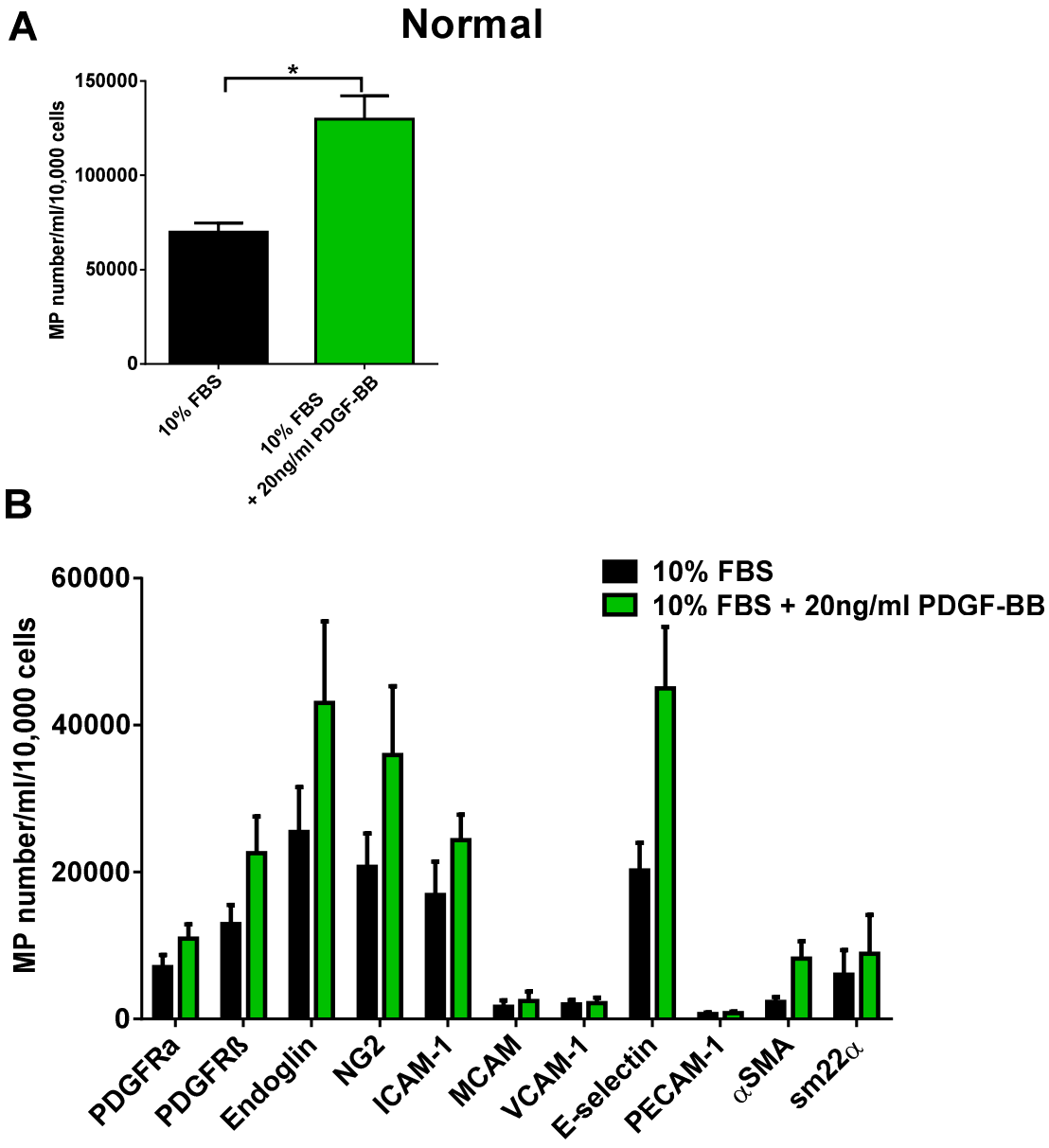

Figure 18. Characterisation of SMMPs released by PASMCs isolated from normal donor lungs

A) Total Annexin V+ MPs released by cells cultured in the following conditions: 10% FBS and 10% FBS + 20ng/ml PDGF-BB for 24 hours. Total MPs were positive for annexin V and smaller than 1.1µm in size. B) Annexin V+ MPs were also screened for cell surface and cytoskeletal markers. Data are expressed as mean±S.E.M. (n=5). Unpaired t-test was used when compared with 10% FBS.

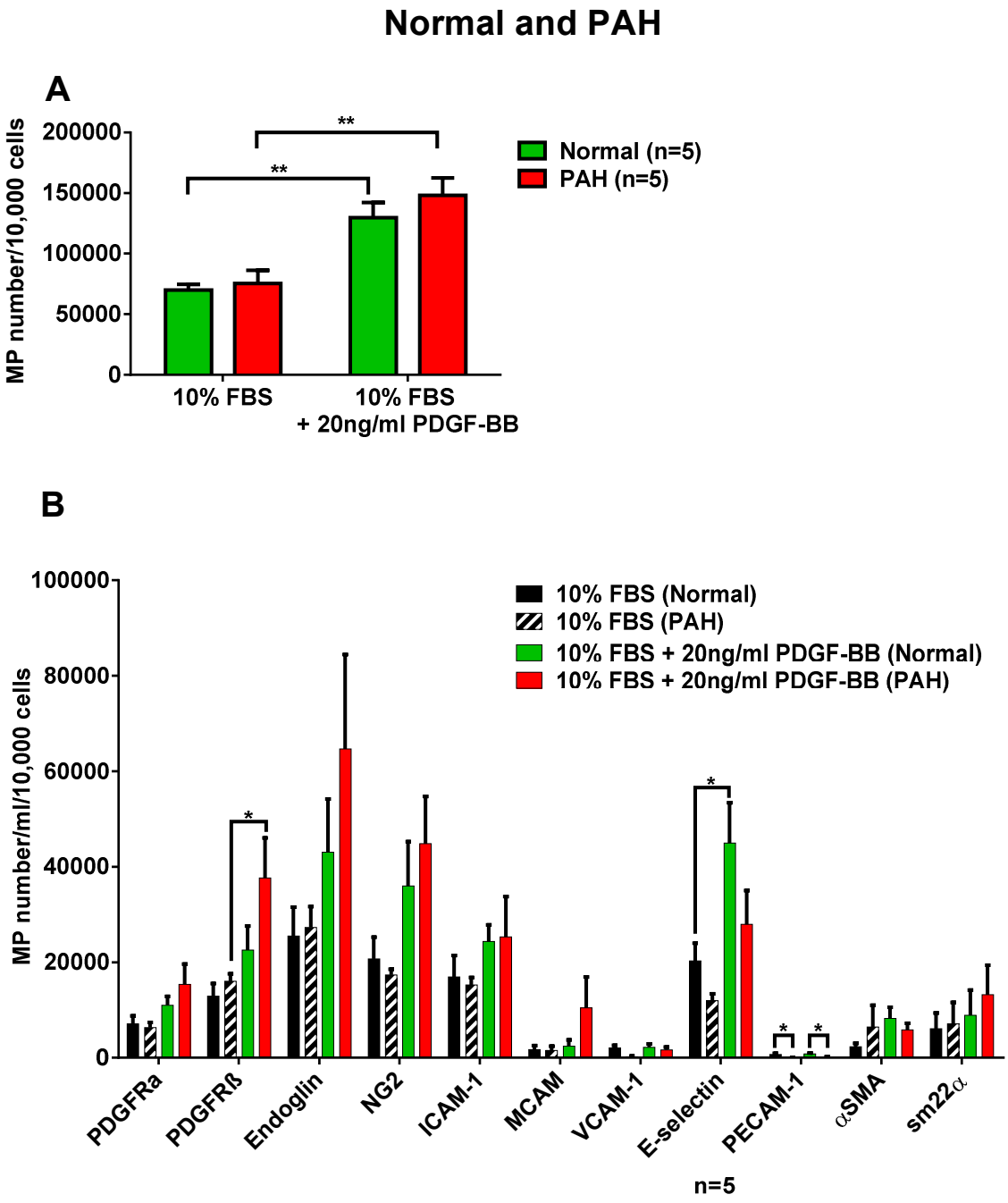

Figure 19. Characterisation of MPs released from cultured PASMCs from control and PAH patients.

A) Total Annexin V+ MPs released by cells cultured in the following conditions: 10% FBS and 10% FBS + 20ng/ml PDGF-BB for 24 hours. Total MPs were positive for annexin V and smaller than 1.1µm in size. B) Annexin V+ MPs were also screened for cell surface and cytoskeletal markers. Data are expressed as mean±S.E.M. (n=5). Two-Way ANOVA with Sidak's multiple comparison's test was used for A and One-way ANOVA with Tukey'smultiple comparison's test was used to compare MP levels.

3.2.8. The effect of inflammatory and proliferative mediators on MP release from PAH cells

I investigated the ability of different growth factors or proinflammatory cytokines that are known to be elevated in PAH. The efficacy of mediators causing smooth muscle MP release from PAH cells in the presence of 10% FBS was as follows: 20ng/ml TNFα < 20ng/ml PDGF-BB < 5ng/ml TGF-β < 10nM ET-1 (Figure 20). After 24 hours of stimulation, all four agents were able to increase MP release over and above that induced by serum alone. 20ng/ml PDGF-BB was able to cause 2 fold of the number of MPs compared to serum alone, while 5ng/ml TGFβ induced a 2.5 fold increase. The addition of 10nM ET-1 to serum was the most potent agent as it caused a 3.5 fold elevation compared to serum alone.

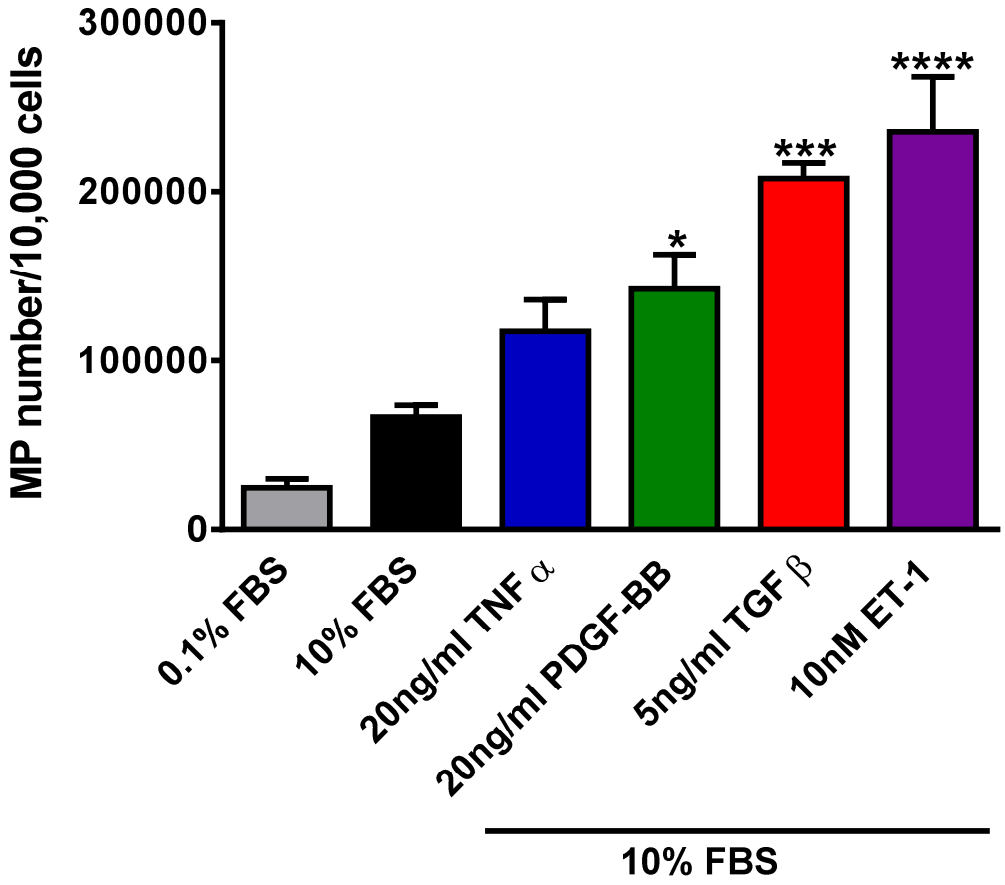

Figure 20. Total MPs released from cultured PASMCs after stimulation with growth factors and cytokines.

Growth arrested cells were stimulated with either 0.1% FBS with and without 20ng/ml TNFα, 20ng/ml PDGF-BB, 5ng/ml TGF-β, or 10nM ET-1 for 24 hours. Data are presented mean±S.E.M. (n=5). *=P<0.05; **=P<0.01; One-Way ANOVA with Bonferroni's multiple comparisons test was conducted when

3.2.9. Characterisation of human umbilical cord vein endothelial cells

Human umbilical cord vein endothelial cells (HUVECs) were isolated and characterised for the surface markers expressed (Figure 21). Similar to what was found in smooth muscle cells, these endothelial cells expressed high amounts of endoglin antibody binding on their cell surface as determined by fluorescence intensity using flow cytometry ($P<0.05$; $n=3$). Cells also expressed ICAM1 and low levels of MCAM, though the fluorescence intensities were not significantly different from that of the isotype control antibody. Unlike smooth muscle cells, HUVECs did express high levels of PECAM-1 ($P<0.05$; $n=3$) but did not express PDGFRβ, E-selectin or NG2.

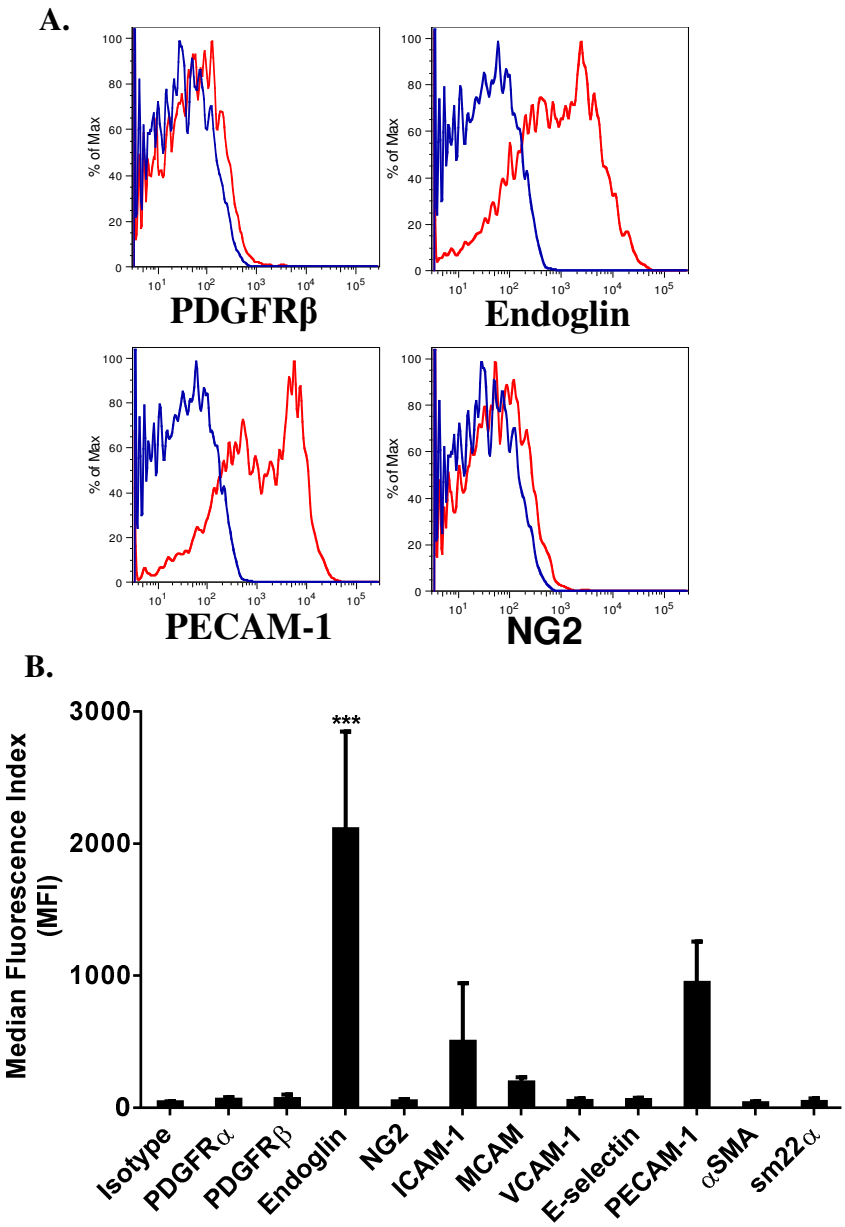

Figure 21. Surface marker expression on HUVECs

Measurement of surface marker expression on human umbilical cord vein endothelial cells isolated from female patients at term and grown in 10% FBS. **A)** Histograms show fluorescence intensity of surface markers in red for platelet derived growth factor receptor β (PDGFRβ), endoglin, neural glial receptor 2 (NG2), and platelet and endothelial cell adhesion molecule (PECAM-1). **B)** Data is presented as median fluorescence intensity ± S.E.M. (n=3) and analysed using one-way ANOVA with Holm-Sidak's multiple comparisons test, with a single pooled variance. ****=P<0.001 when compared to isotype control antibody.

3.2.10. Characterisation of endothelial microparticles

Endothelial microparticles were released from growing HUVECs in culture that were subjected to either of three conditions: 0.1% FBS, 10% FBS, or 10% FBS + 10ng/ml TNFα (Figure 22). The presence of serum increased MP release though this was not significantly different from 0.1% FBS. On the other hand, TNFα caused a significant 2-fold increase in MPs released from endothelial cells compared to serum alone ($p<0.01$; n=3). Numbers of microparticles positive for endoglin, ICAM, and E-selectin were considerably elevated after TNFα treatment, with the latter reaching significance ($p<0.01$; n=5). Unlike smooth muscle microparticles, PDGFRβ+ and NG2+ microparticle levels were low (<10,000 MPs/ml/10^4 cells). Interestingly, there was a high level of PECAM+ microparticles in the total endothelial microparticle population compared to the PECAM+ subpopulation that was almost undetectable in the smooth muscle microparticle population. Levels of PDGFRα+, αSMA+ and sm22α+ microparticles were also very low, while VCAM1+ MPS were significantly elevated by TNF-α (n=5).

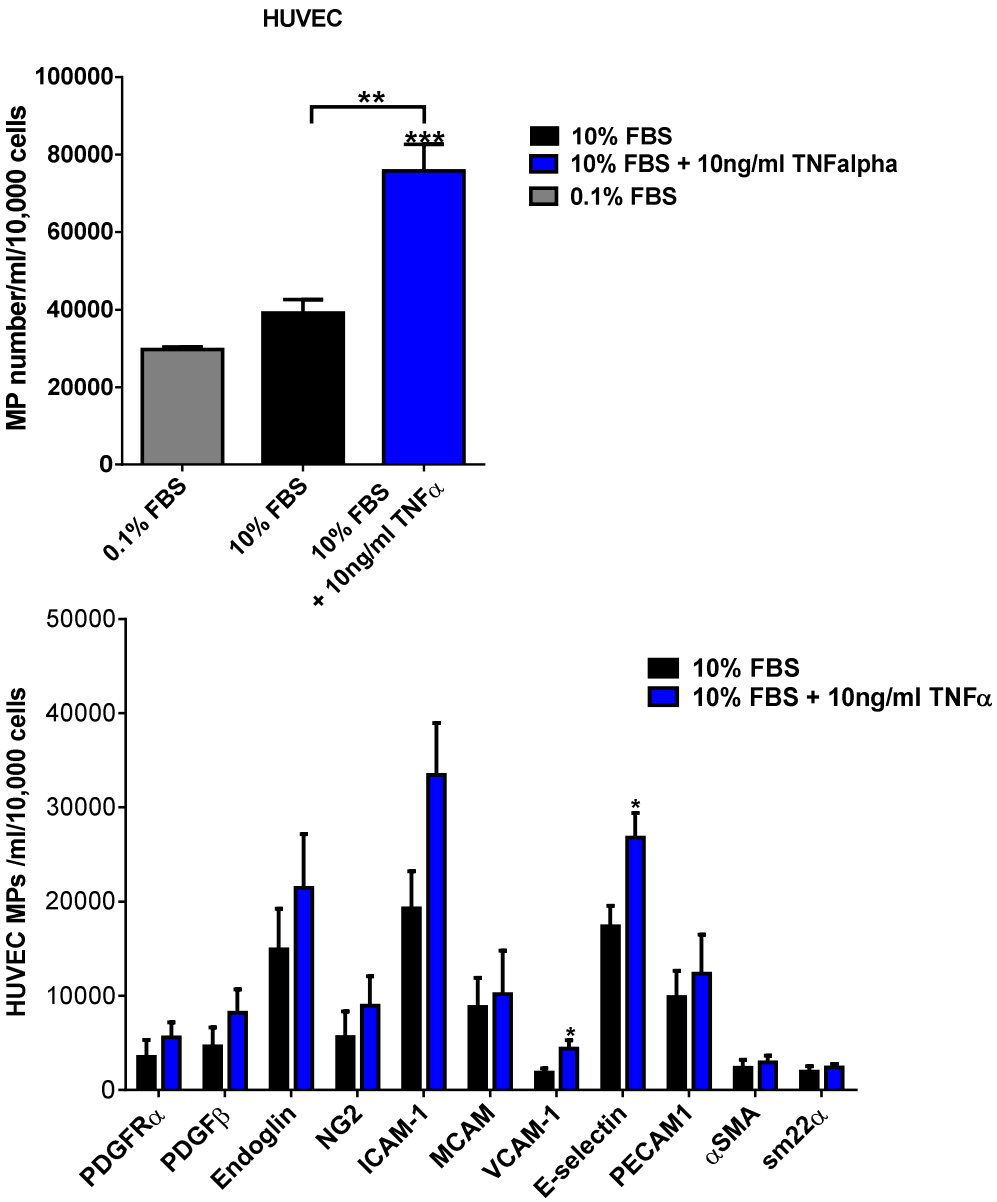

Figure 22. Characterisation of endothelial MPs released by HUVECs isolated from donor patients

Cells were cultured under the following conditions: 0.1% FBS, 10% FBS and 10% FBS + 20ng/ml PDGF-BB for 24 hrs. A) Total EMPs positive for annexin V and smaller than 1.1µm in size. B) Annexin+ MPs were screened for different cell surface markers. Data are presented as mean±S.E.M. (n=3 for A; n=5); *=P<0.05 and **=P<0.01; One-Way ANOVA was conducted when compared to to 0.1% FBS for A and unpaired t-test was conducted when compared to 10% FBS for B.

3.3. Discussion

The aim of the current chapter was to characterise SMMPs and distinguish them from EMPs by determining the surface markers present on these MP subtype, I first characterised culture smooth muscle cells that were growing in 10% FBS. In conjunction with studying cell surface protein expression, I investigated the presence of these markers on MPs from both smooth muscle and endothelial cells to elucidate smooth muscle specific surface markers.

Smooth muscle cell characterisation revealed high endoglin expression in growing cells *in vitro*, which was similarly seen in HUVECs. endoglin, a 95kDa homodimeric transmembrane glycoprotein, is classically known as a marker for angiogenesis that is abundantly expressed on actively proliferating endothelial cells (Conley et al., 2000). Observations of strong upregulation of endoglin in the endothelium of tumour tissues taken from the lung, breast, colon, brain, prostate, and cervical cancer, have suggested its involvement in tumour angiogenesis (Olsen et al., 2014). Loss of function in the human gene encoding for endoglin causes hereditary haemorrhagic telangiectasia (HHT1), a disease where abnormal formation of blood vessels in various organs including the skin and mucous membranes, lungs, liver and brain occurs (Li et al., 1999). In mice, loss of endoglin leads to disrupted angiogenesis and ultimately death, which is attributed to poor vascular smooth muscle development (Bourdeau et al., 1999; Conley et al., 2000). Expression of endoglin has been shown to be elevated in the smooth muscle of human atherosclerotic plaques and in smooth muscles responding to

vascular injury, though its expression in normal human carotid artery, as observed via immunohistochemistry, was minimal (Conley et al., 2000; Ma et al., 2000). Conley and colleagues demonstrated that the L-isoform of human endoglin was predominantly expressed in cultured vascular SMCs. Endoglin is an accessory receptor for the β1 and β3 isoforms of TGF-β, a multifunctional cytokine that is a part of a large superfamily of proteins that includes bone morphogenic proteins (BMPs) (Lebrin et al., 2004). The TGF-β family acts on a heterodimeric receptor complex comprised of a type II (TβR-II) and a type I receptor (TβR-I). There are two type I receptors: activin-like kinase 1 (ALK1), which is expressed in the endothelium, and ALK5, which is expressed ubiquitously (Lee et al., 2008). TβR-I is a superfamily serine/threonine kinase receptors that work downstream from TβR-II and phosphorylate effector proteins known as Smads. In endothelial cells, TGFβ can either signal through ALK1 to phosphorylate Smads1/5/8 and stimulate proliferation, migration and angiogenesis or signal through ALK5 to phosphorylate Smads2/3 and inhibit proliferation. Thus, endoglin can cause endothelial proliferation though TGFβ signalling via the ALK1 and also negatively regulate TGFβ/ALK5 signalling in the ECs (Goumans et al., 2002; Lebrin et al., 2004). In contrast, the bone morphogenic peptide (BMP) ligands such as TGF-β and BMP-2, 4 and 7 are able to activate the BMP recpeptor II (BMPR-II) receptor coupled with ALK1 to activate Smads 1, 5 and 8 to inhibit cell proliferation in normal PASMCs (Morrell et al., 2009). In contrast, TGFβ is able to bind TβR-II coupled to ALK5 which consequently can signal via Smads 2/3 and induce proliferation particularly in PASMCs derived from PAH patients (Yang, 2005; Morrell et al., 2009; Morrell, 2010). In the presence of mutation within the BMP receptor in heritable PAH, TGFβ signalling via the Smad2/3

pathway is dominant, and thereby induces SMC proliferation. The role of endoglin in smooth muscle cells has not been firmly established, though its high expression in cultured PAH smooth muscle cells may suggest that it plays a part in the regulation of cell proliferation. Moreover, high levels of endoglin+ smooth muscle MPs were detected following PDGF-BB stimulation compared to other MP subpopulations in both PAH and control cells. This may indicate that endoglin+ MPs detected in patient plasma may be smooth muscle- derived and not entirely endothelial derived as was concluded by Bakouboula and colleagues (Bakouboula et al., 2008).

The PASMCs also significantly expressed NG2, PDGFRβ, ICAM, and MCAM. NG2 is classically known as a pericyte marker. It is a membrane-spanning chondroitin sulfate proteoglycan expressed by mural cells during normal development and microvascular remodelling during tumour growth and wound healing. Murfee and colleagues demonstrated that NG2 expression was primarily confined to perivascular cells, including mature SMCs, immature SMCs and pericytes, along arterioles and capillaries but not along venules (Murfee et al., 2005). In my experiments, NG2 expression was not present on the cell surface of growing HUVECs. In previous studies, treatment of vascular smooth muscle cells from rat aorta with anti-NG2 immunoglobulins decreased DNA synthesis and cell migration in response to PDGF-AA, but not to PDGF-BB (Grako and Stallcup, 1995). Likewise, I also found high levels of NG2+ MPs were released by activated SMCs but only low levels by HUVECs following PDGF-BB and TNF-α stimulation, respectively. Thus, NG2+ MPs may account for smooth muscle derived microparticles from activated cells.

Similar to NG2, I demonstrated that PDGFRβ was expressed on the cell surface of smooth muscle cells and not on HUVECs, and that high amounts of PDGFRβ+ MPs were released by smooth muscle cells compared to HUVECs. PDGFRβ is known to be expressed by developing smooth muscle cells and pericytes, and plays a key role in the signalling of its ligand PDGF-B in inducing cell proliferation and migration (Hellström et al., 1999). Lack of signalling though this pathway leads to pericyte loss as well as changes to the endothelium leading to capillary dilatation and rupture. Upon ligand binding, the receptor is able to undergo phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis through phospholipase Cγ binding, and subsequently generate diacylglycerol and inositol triphosphate (IP_3). Through IP_3-mediated calcium release from intracellular compartments which increases intracellular calcium levels and mitogen activated protein kinase activation, PDGFRβ is able to promote cell proliferation (Bornfeldt et al., 1995). Additionally, transfection of Notch1 and Notch3 intracellular domain into primary human VSMCs has been shown to potentiate PDGF-induced activation of extracellular signal-regulated kinases (ERK1/2) and Akt, which leads cell proliferation. PDGF has also been shown to signal through the protein phosphatase calcineurin through its activation of transcription factor nuclear factor of activated T-cells (NFAT) leading to a proliferative phenotype in aortic smooth muscle cells (Jabr et al., 2007). Thus, increase in PDGFRβ+ MPs released from PASMCs may be an indicator of proliferating smooth muscles (Weber, 2008).

ICAM expression was seen on both growing SMCs and HUVECs. The number of ICAM+ MPs released by SMCs from both PAH and control patients were high (>20,000 MPs/ml/10,000 cells) after PDGF-BB stimulation and high in HUVECs (>10,000MPs/ml/10,000 cells) following TNFα stimulation. ICAM is an

immunoglobulin-like cell adhesion molecule expressed by a variety of cells including endothelial cells, leukocytes and smooth muscle cells (Lawson and Wolf, 2009). It is involved in the transendothelial migration (diapedesis) of leukocytes to sites of inflammation and the interaction between antigen presenting cells and T lymphocytes (immunological synapse). Thus, it plays an important role in both the innate and adaptive immune responses. TNFα has been known to increase ICAM-1 expression on human vascular endothelial and lung epithelial cells in a tyrosine kinase-dependent manner (Burke-Gaffney and Hellewell, 1996). Combes and colleagues first demonstrated that the expression of ICAM-1 and the release of ICAM+ MPs could be elevated by administering TNFα to growing HUVECs, albeit by a high concentration (100ng/ml) of TNFα (Combes et al., 1999). Moreover, TNFα- and interleukin 1-α-induced ICAM -1 expression was significantly upregulated by pretreatment of HUVECs with the adenylyl cyclase activator forskolin, thus indicating that TNFα may act via the adenylyl cyclase pathway during EC activation (Bernot et al., 2005). In other studies, ICAM-1 mRNA was very low in cultured human aortic smooth muscle cells grown in the presence 5% foetal calf serum, but expression was induced (Couffinhal et al., 1993) in a dose- and time-dependent manner following incubation with 10 ng/ml TNFα. Furthermore, administration of TNFα also increased adhesiveness of the SMCs to monocytes (Couffinhal et al., 1993). Thus, ICAM+ MPs may be indicative of an inflammatory activation of vascular cells. Although MCAM was expressed on the smooth muscle surface, MCAM+ microparticles derived from cultured smooth muscle cells were low in number after PDGF-BB stimulation. MCAM expression was also present on HUVECs, thereby confirming its lack of selectivity for smooth muscle cells. Though it is often associated as a constitutive

endothelial marker independent of vessel size or site, the transmembrane glycoprotein has been shown to be expressed on other cells such as smooth muscle cells, melanoma cells, follicular dendritic cells and subpopulations of activated T-lymphocytes (Schrage et al., 2008). Strong expression of MCAM on bone marrow mesenchymal stem cells was associated with vascular smooth muscle cell lineage commitment (Espagnolle et al., 2014). Thus, MCAM may be more of a marker of cell and vascular development than vascular pathology.

Cultured smooth muscle cells under growing conditions did not express PECAM-1, whereas growing HUVECs did express high levels. Likewise, PECAM-1+ MPs were not detected from SMCs following PDGF-BB stimulation, but was detected on EMPs after serum and TNF-α stimulation. PECAM1 is a member of the immunoglobulin gene superfamily comprised of 6 extracellular Ig folds, that has a molecular weight of 130kDa and is differentially glycosylated with N-linked and O-linked glycosylated sites (Woodfin et al., 2007). Ligands for the receptor include PECAM-1, as well as a ligand expressed on transfected L cells, the integrin αvβ3 (domains 1 and 3), ADP-ribose cyclase, and CD177 (domain 6) located on a subset of neutrophils (Deaglio et al., 1998; Woodfin et al., 2007). Its 2 immunoreceptor tyrosine inhibitory motifs (ITIMs) in the cytoplasmic (intracellular) domain serve as docking sites for signalling molecules including protein tyrosine kinase phosphatases. PECAM1 ligation/dimerization of the protein can induce phosphorylation of the tyrosine and serine/threonine residues of the ITIMs, which leads to the recruitment of molecules including SH2-containing phosphatases and C-γl that collectively lead to downstream signalling pathways. These intracellular events regulate PECAM-1-mediated responses such as leukocyte transmigration, endothelial cell motility and permeability, and the

expression and activation state of integrins (Newman, 2003). PECAM1 is expressed on the cell surface of haematopoietic and immune cells including endothelial cells, platelets, neutrophils, monocytes, megakaryocytes, natural killer cells and some subsets of T-lymphocyte. More recently, platelet PECAM has been found to be involved in the negative regulation of platelet aggregation in vitro and thrombus formation in vivo (Falati et al., 2008).

A surprising finding of my current study was that a high number of E-selectin+ MP were released by both PAH and control patient derived cells. This occurred despite a lack of expression on the surface of SMCs. E-selectin is a 115-kDa cell surface glycoprotein expressed by cytokine-activated ECs and regulates the adhesion of neutrophils to the endothelial surface. It is a member of the selectin gene family which includes P-selectin and L-selectin, adhesion molecules expressed on platelets and leukocytes, respectively. The expression of E-selectin by endothelial cells can be rapidly induced using cytokines such as TNF-α in vitro reaching maximum expression at 4-6 hours of activation followed by a rapid decline (Leeuwenberg et al., 1992). Its inducible expression is similar to ICAM1, which reaches maximum expression for approximately 48 hours. This might explain why E-selectin was not expressed in growing HUVECs from control subjects without the administration of cytokine for stimulation. Though E-selectin expression is classically thought to be endothelial specific, Chen and colleagues demonstrated *in vitro* that human aortic SMCs do have the potential to express E-selectin after TNF-α and lipopolysaccharide stimulation via nuclear factor κB signalling (Chen et al., 1997). To my knowledge, this was the first study to show a non-endothelial cell expressing E-selectin and suggests that smooth muscles have the capability of releasing MPs containing E-selectin.

PDGF-BB, TNFα, TGFβ, and ET-1, in the presence of 10% serum, significantly increased the level of total MPs released from PASMCs over and above that induced by serum alone. Perros and colleagues demonstrated that PDGF-A, PDGF-B, PDGFRα, and PDGFRβ mRNA expression was elevated in small pulmonary arteries from idiopathic PAH patients compared with control subjects (Perros et al., 2008). PDGF-BB is able to bind to both PDGFR-α and –β on SMCs to induce proliferation and migration, though the former was not found to be expressed on the smooth muscle cells derived from PAH patients. PDGF-BB was shown to upregulate the expression of phosphorylated c-Jun NH2-terminal kinase 1/2 (JNK1/2), a member of the mitogen-activated protein kinases (MAPKs). Additionally, PDGF-BB-induced proliferation was weakened following antagonism of the JNK pathway or JNK knockdown by siRNA, thus suggesting that the mitogen may act via the JNK pathway (Zhao et al., 2014).

In PAH, TNFα is elevated along with other pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and CCL5/RANTES (Rabinovitch et al., 2014). TNFα is mainly produced by macrophages but also by other cells including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue (Wajant et al., 2003). It can exert its effects through the TNF receptor family TNF receptor (TNF-R)1, which is constitutively expressed in most tissues. TNF-R2 on the otherhand has a more restricted expression, typically being expressed in cells of the immune system. TNFα is able to signal via the nuclear factor kappa B (NFkB) transcription factors to activate inflammatory related genes, as well as cause cellular TNF-R1-mediated apoptosis (Wajant et al., 2003).

My experiments showed that TGF-β and endothelin are highly potent growth factors and induced the release of high levels of smooth muscle MPs. TGFβ is produced by macrophages as well as lymphocytes and dendritic cells (Letterio and Roberts, 1998). As aforementioned, due to the BMPRII mutation, TGFβ in PAH binds to TβR-II, which dimerises with ALK5 and signals through Smads2/3 to induce proliferation (Morrell, 2006). Endothelins are a family of naturally occurring peptides that consist of endothelin (ET)-1, ET2, and ET3 (Shao et al., 2011). They are largely expressed by endothelial cells and to a lesser extent by smooth muscle cells, fibroblasts, macrophages, cardiac myocytes, airway epithelial cells, brain neurons, and pancreatic islet cells. By acting on two receptors, ET_A and ET_B, ET1 is able to act as a potent vasoconstrictor and mitogen, as well as a mediator of fibrosis and aspects of inflammation (Hall et al., 2011). Thus, ET-1 plays a key role in vascular remodelling seen in PAH.

Future experiments would be characterisation of endothelial microparticles isolated from pulmonary arterial endothelial cells as that would provide more accurate information as to pulmonary specific endothelial markers and their quantities.

Chapter 4

4.1 Introduction

MPs are released by budding and fission of the plasma membrane of various cells including endothelial cells (ECs), smooth muscle cells (SMCs), leukocytes and platelets (Simak and Gelderman, 2006). They can be detected in a variety of biological fluids, peripheral blood, urine, asceptic fluids and synovial fluids (Budaj et al., 2012). The site and cellular origin of the MPs determine their biological function. For example, skeletal cell-derived MPs initiate bone mineralisation, whilst endothelial derived MPs (EMPs) have been associated with angiogenesis (Morel et al., 2004). The vesicularisation of MPs, known as blebbing, is triggered or enhanced during cellular activation or apoptosis during pathological conditions including inflammation, injury, vascular dysfunction, or cancer. The externalisation of phosphatidylserine (PS) to the outer membrane leaflet is specific to sites where MP shedding occurs while the topology of the membrane proteins remains intact (Hugel et al., 2005; Lima et al., 2009). As PS binds annexin V with high affinity, its externalisation is useful in detecting MPs and distinguishing them from exosomes, which are smaller vesicles (50-100 nm in diameter) and have no/low annexin V binding capacity as a result of very low levels of surface PS (Budaj et al., 2012).

MPs have been detected via multiple methods including solid-phase capture assay, enzyme-linked immunosorbent assay (ELISA), and flow cytometry, the latter of which is the preferred method in the majority of studies. This is due to the ability of flow cytometry to quantitate MP number and multicolour analysis attributes, thus allowing simultaneous detection of several markers on the MP surface

(Chironi et al., 2009; Baj-krzyworzeka et al., 2013). Solid-phase capture assays rely on the fact that MP membranes contain externalised PS which can activate prothrombinase to generate thrombin. Thus, by measuring prothrombinase activity in the coagulation process, MP levels can be estimated (Hugel et al., 2004). ELISAs are able to accurately measure MP levels, though their lack of ability to detect multiple markers simultaneously, thus limiting their ability to provide information of specific MP subpopulations in the circulation (Nomura, 2004).

The study of circulating MP *in vivo* from blood samples *via* flow cytometry involves several steps that ensure accuracy in MP measurement and characterisation. The determination of the centrifugation speed used to isolate MPs is an important step in MP collection. Prior to this, unwanted/contaminating cells from cells/tissue media or bodily fluids must be removed with an initial centrifugation speed of 200-500g for 5-20 minutes (Orozco and Lewis, 2010; Baj-krzyworzeka et al., 2013). To obtain a MP pellet, centrifugation at 10,000-17,000g for 30 minutes to 1 hour is required. As exosomes are much smaller than MPs (50-100nm as opposed to 100nm-1μm the ultracentrifugation at 100,000-150,000g for 1 hour is required (Baj-krzyworzeka et al., 2013; Colombo et al., 2014). The determination of total MPs is another step, and is made possible *via* the detection fluorescently conjugated annexin V bound to microparticles, whereas exosomes will not bind to annexin V because they have no/low externalised PS. Polychromatic flow cytometric analysis of MP subpopulations is the crucial step determining the cellular origin of the different circulating MPs. The final step in MP measurement is the enumeration of the particles.

In the past, endothelial cells have been characterised as expressing endoglin, intracellular adhesion molecule (ICAM-1), E-selectin, platelet endothelial cell adhesion molecule type 1 (PECAM-1), vascular cell adhesion molecule type 1 (VCAM-1) and vascular endothelial (VE)-cadherin on their outer membrane surface (Chironi et al., 2009; van der Heyde et al., 2011). On the other hand, leukocytes express CD45, while monocytes express CD14 and CD11, granulocytes express CD66b, T helper cells express CD4, cytotoxic T cells express CD8, and B cells express CD20. CD4 has also been shown to be expressed on monocytes and CD11b on granulocytes. All platelets express CD41/CD61 (glycoprotein IIb/IIIa) on their surface (French and Seligsohn, 2000; van der Heyde et al., 2011). CD42a is also a ubiquitously expressed platelet marker (Van Velzen et al., 2012). CD42b is an activation marker that is proteolytically cleaved after platelet activation, while CD42c is a constitutively expressed. In addition to being expressed on endothelial cells, PECAM-1 is also expressed on platelets, so care must be taken to further characterise the origin of the cell type expressing this marker. Erythrocytes express glycophorin A (CD235) on their cell surfaces, which can be used to detect red blood cell-derived MPs. Using polychromatic flow cytometric analyses, different surface marker combinations have been used to more accurately characterise specific MP subsets. EMPs have been characterised as PECAM-1+/CD42b-, PECAM-1+/CD41-, PECAM-1+/CD62E+, endoglin+/CD45-, VE-cadherin+/endoglin+, and MCAM+/endoglin+ in a number of studies (Boulanger et al., 2007; Dey-Hazra et al., 2010; Dignat-George and Boulanger, 2011; Huica et al., 2011). Similarly, PMPs have been characterised using several marker combinations such as

CD31+/CD41+ and CD31+/CD42b+ (Chirinos et al., 2005; Angelillo-Scherrer, 2012).

Circulating MPs have been shown to be increased in animal models and patients with pulmonary hypertension. Total MPs were elevated two-fold in male Wistar rats exposed to chronic hypoxia for 3 weeks in a controlled hyperbaric chamber compared to normoxic rats (Tual-Chalot et al., 2010a). Rats with PH had elevated PMPs (CD61+) and erythrocyte-derived MPs compared to normoxic rats, though no difference was seen in leukocyte (CD45+) and endothelial (CD54+) MPs (Tual-Chalot et al., 2010b). In contrast, MPs positive for the endothelial markers PECAM-1, VE-cadherin, E-selectin, and the leukocyte marker CD45 were significantly elevated in patients with pulmonary hypertension (n=24) compared to healthy age- and sex-matched controls (Amabile et al., 2008). This elevation was seen in total annexin V+ MPs as well as in PECAM+/CD41+ PMPs. Moreover E-selectin+ MPs positively correlated with high-sensitivity C-reactive protein, confirming that endothelial MPs may be a marker of inflammation in PAH ($r=0.51$, $P=0.035$). Both VE-cadherin+ and PECAM-1+ MPs positively correlated with haemodynamic measurements including mean pulmonary artery pressure (mPAP), pulmonary vascular resistance, cardiac index and right atrial pressure as well as haemoglobin levels in PH patients.

In this chapter, I aimed to measure MP levels in plasma originating from a variety of cell types including smooth muscle cells, endothelial cells, leukocytes, and platelets in the venous blood from patients with pulmonary arterial hypertension

compared with sex- and age-matched controls. Additionally, I aim to determine whether these MPs could be used to determine therapeutic impact on disease. Finally, I aim to determine whether circulating MPs may be elevated in PAH and how levels might compare to other vasculopathies, potentially making MPs a viable biomarker to distinguish between PAH and other inflammatory disease states.

4.2. Results

4.2.1. Total circulating annexin V+ microparticles in patients with pulmonary arterial hypertension

Total annexin V+ microparticles (MPs) were measured from the forearm venous blood collected from patients (n=18) with pulmonary arterial hypertension and compared to age- and sex-matched controls (n=20) (Figure 23). In PAH patients, MP's were assessed before and after treatment for a minimum and maximum of 4 months and 18 months, respectively. The median total MP count in PAH was $2.93x10^6$ MPs/ml in PPP, which was significantly $p<0.0001$) higher (almost 100-fold) than the median count observed in control subjects, $3.13x10^4$ MPs/ml PPP. After long-term therapy for a minimum of 4 months, which consisted of either a prostacyclin analogue, phosphodiesterase (PDE)-5 inhibitor, endothelin-1 receptor antagonist (ETRA), calcium channel blocker, or combination therapy consisting of a PDE-5 inhibitor with an ETRA and/or a prostacyclin analogue, total MP count decreased by a third in pulmonary arterial hypertensive patients to a median of 2.13×10^6 MPs/ml PPP ($P<0.01$).

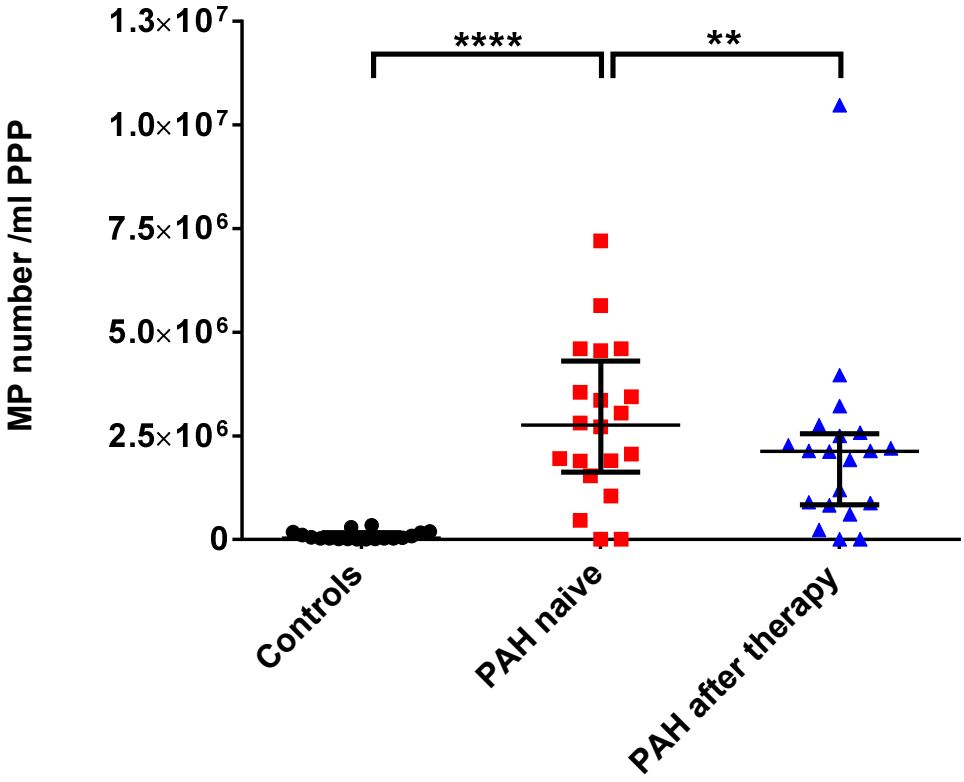

Figure 23. Total annexi V+ MPs in forearm venous blood taken from PAH patients before and after long-term drug therapy

Total annexin V+ microparticle levels in forearm venous blood taken from pulmonary arterial hypertensive patients before and after drug therapy (n=18) for a minimum of 4 months, compared with and age- and sex-matched controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE)-5 inhibitor, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. Data is presented median with interquartile range. **=P<0.01; *=P<0.0001; The Mann-Whitney test was performed.

4.2.2. Sensitivity/specificity analysis of total annexin V+ microparticles for identification of pulmonary arterial hypertension recurrence

Receiver operator characteristic curve analysis was performed to examine the sensitivity and specificity of total annexin V+ microparticle quantification in diagnosing PAH in patients (figure 24). Total annexin MP levels in venous blood from patients with PAH were plotted alongside age- and sex-matched healthy controls. The diagnostic performance test of identification of disease recurrence was significant with an area under the curve (AUC) of 1, standard error of mean of 1.000-1.000 and $P<0.0001$. As the AUC is a measure of how well a parameter is able to distinguish between disease and control, this would suggest that total annexin V+ MPs are capable of PAH difference.

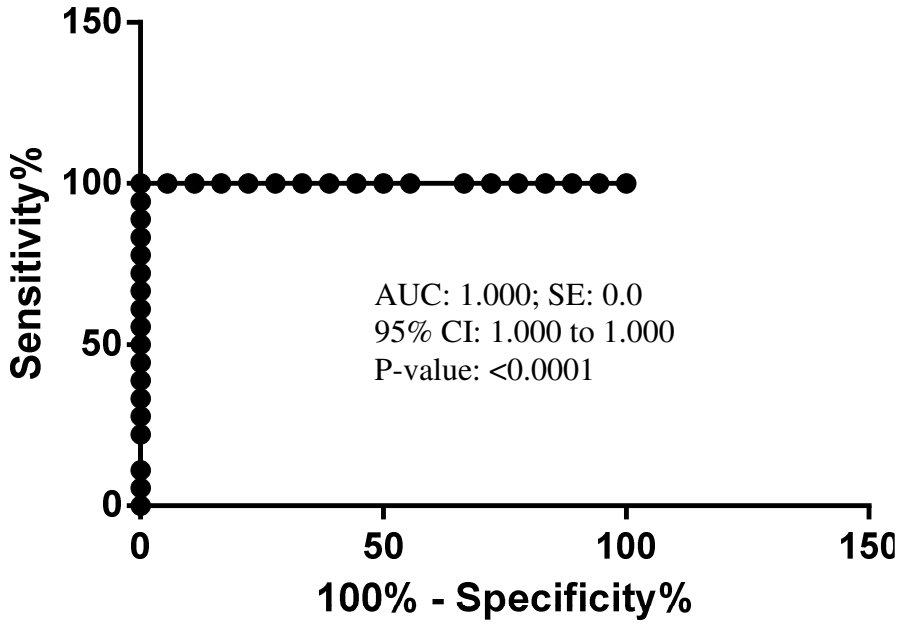

Figure 24. Receiver operator characteristic curve for total annexin V+ MPs for the identification of PAH recurrence

(n=18 PAH vs n=18 controls). ROC analysis was significant with the area under the curve (AUC) of 1.000, confidence interval (CI) of 1.000-1.00, P<0.0001.

4.2.3. Circulating smooth muscle microparticles in pulmonary arterial hypertensive patients

Smooth muscle microparticles (SMMPs) were measured in forearm venous blood collected from patients with PAH before and after long term therapy (n=18) and compared with normal control subjects (Figure 25). Polychromatic flow cytometric analysis was performed using four marker combinations to characterise the SMMPs. Platelet derived growth factor receptor β (PDGFRβ)+/PECAM-1-/Annexin V+ SMMPs were significantly elevated in PAH with a median of median $1.38x10^5$ MPs/ml of PPP compared to levels in control subjects with a median of $7.10x10^3$ MPs/ml of PPP ($P<0.0001$). After therapy, these levels significantly reduced by half to 69580 MPs/ml PPP ($P<0.01$). Endoglin+/PECAM-1-/Annexin V+ MPs displayed a similar trend as their levels were elevated significantly in PAH patient blood (median $3.80x10^5$ MPs/ml of PPP) compared to those seen in control subjects (median $1.49x10^4$ MPs/ml of PPP; $P<0.001$). These levels reduced by 40% to a median of $2.64x10^5$ MPs/ml of PPP after long-term therapy, though this difference was not significant. NG2+/PECAM1-/Annexin V+ SMMPs were also significantly elevated in PAH patients with a median of $2.31x10^5$ MPs/ml PPP) compared to control subjects which were 10 fold lower with a median of $1.44x10^4$ MPs/ml PPP ($P<0.0001$). The MP count dropped by 25% after long-term therapy to a median of $1.74x10^5$ MPs/ml PPP, though this was not significant. Levels of ICAM+/PECAM1-/Annexin V+ SMMPs revealed significant elevation in PAH patients with median of $2.37x10^5$ MPs/ml PPP compared to control subjects $1.28x10^4$ MPs/ml PPP ($P<0.0001$) and a significant 48% decrease after long-term therapy ($P<0.05$).

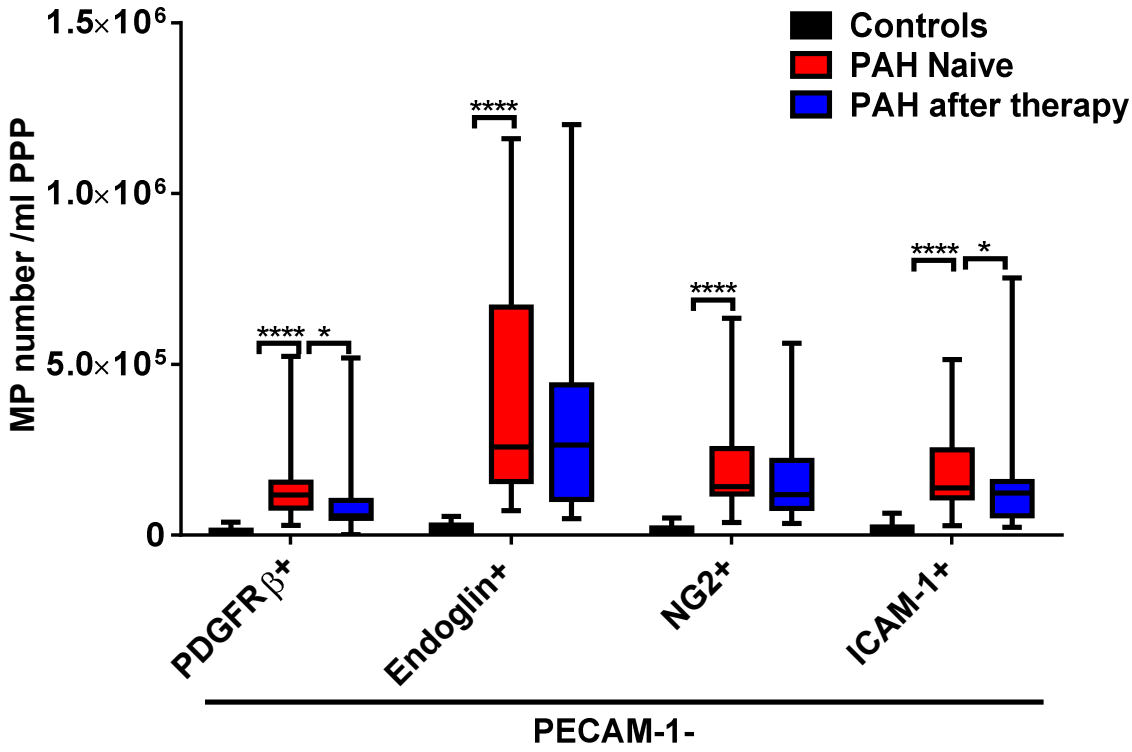

Figure 25. Smooth muscle microparticle levels in forearm venous blood taken from AH patients before and after long-term therapy

(n=18) for a minimum of 4 months, and healthy controls (n=18) were measured. The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. Annexin V+ SMMPs were characterised as platelet derived growth factor receptor β (PDGFRβ)+/PECAM-1-, endoglin+/PECAM-1, neural glial antigen 2 (NG2)+/PECAM1-, and ICAM-1+/PECAM-1-. Data is presented as median with min and max. *=P<0.05; **=P<0.01; *=P<0.0001; The Mann-Whitney test were performed

4.2.2. Sensitivity/specificity analysis of smooth muscle microparticles for identification of pulmonary arterial hypertension recurrence

Receiver operator characteristic curve analysis was performed to examine the sensitivity and specificity of annexin V+ SMMP quantification in diagnosing PAH in patients (figure 26). SMMP levels in venous blood from patients with PAH were plotted alongside age- and sex-matched healthy controls. The diagnostic performance test of identification of disease recurrence was significant for all four SMMP subpopulations: PDGFR+/PECAM- (AUC of 0.9907, standard error of 0.01128 to 1.007, $P<0.0001$), endoglin+/PECAM-1- (AUC of 1.000 with standard error of 0.0, 95% CI of 1.000-1.000, and $P<0.0001$), NG2+/PECAM-1- (AUC of 0.997 with a standard error of 0.0051, 95% CI of 0.969-1.013, and $P<0.0001$), and ICAM-1+/PECAM-1- (AUC of 0.9907 with a standard error of 0.0113, 95% CI of 0.969 to 1.013, and $P<0.0001$). Thus, this would suggest that the SMMPs are capable of distinguishing PAH patients from normal subjects.

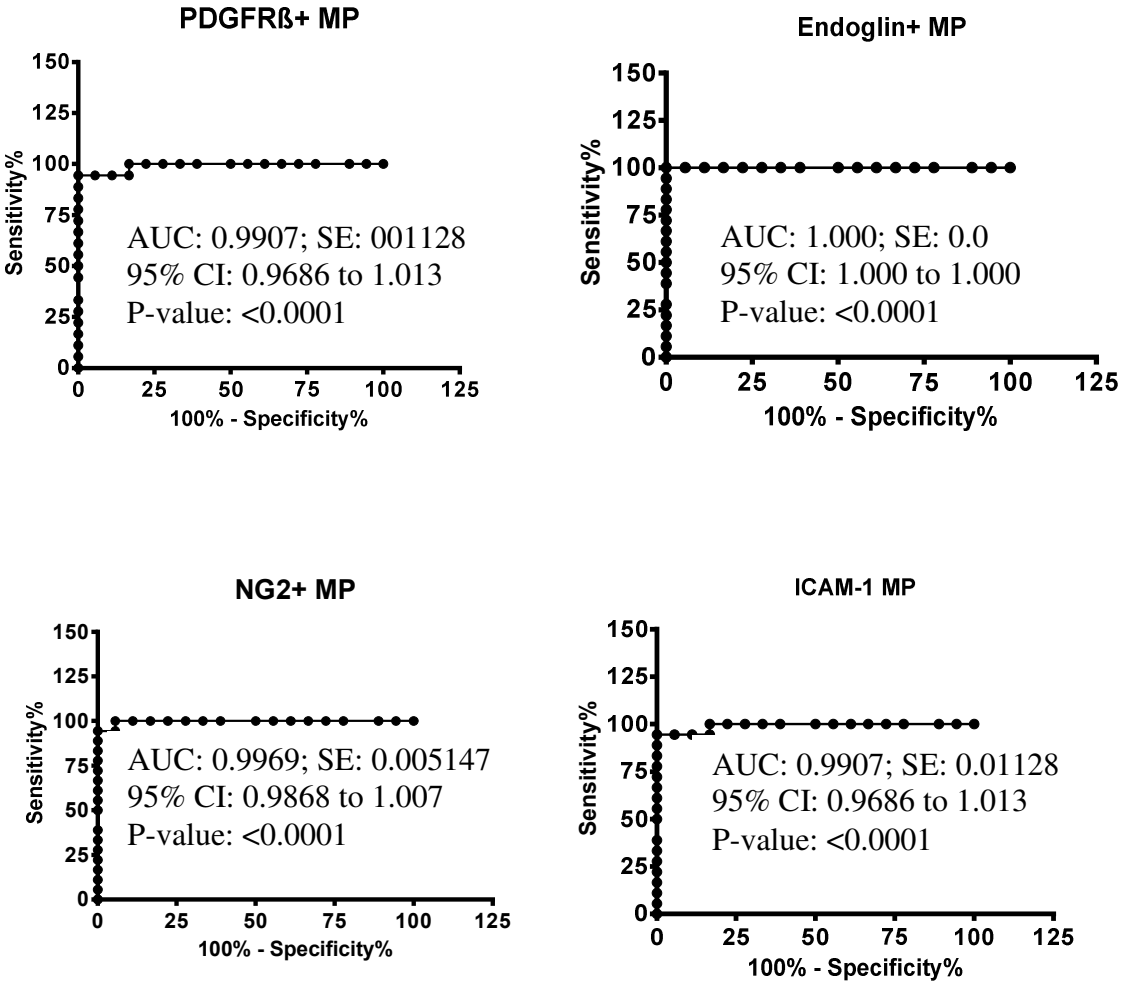

Figure 26. Receiver operator characteristic curve for smooth muscle annexin V+ MPs

for smooth muscle annexin V+ MPs that were PDGFRβ+/PECAM-1-, Endoglin+/PECAM-1-, NG2+/PECAM-1-, and ICAM-1+/PECAM-1- for the identification of pulmonary arterial hypertension recurrence (n=18 PAH vs n=18 controls). ROC analysis was significant for each SMMPs subpopulation as P<0.001 and the area under the curve (AUC) with confidence interval (CI) were close to 1.

4.2.5. Circulating endothelial microparticles in pulmonary arterial hypertensive patients

Endothelial microparticles (EMPs) in forearm venous blood collected from patients with pulmonary arterial hypertension before and after long term therapy (n=18) and normal control subjects (n=20) were measured (Figure 27). E-selectin+/PECAM-1+/Annexin V+ EMPs were s elevated in PAH patients with a median of $1.83x10^5$ MPs/ml PPP compared to control subjects with a median of $8.22x10^3$ MPs/ml PPP ($P<0.001$). Long-term therapy decreased these EMP levels by 24.5% to a median of $1.38x10^5$ MPs/ml PPP, though this was not significant. Similarly, PECAM-1+/CD42a+/Annexin V+ levels were significantly higher in PAH patients with a median of $1.751x10^5$ MPs/ml of PPP compared to control subjects with a median of $1.21x10^4$ MPs/ml PPP. Long-term therapy decreased by these EMP levels by 27%, though not significantly.

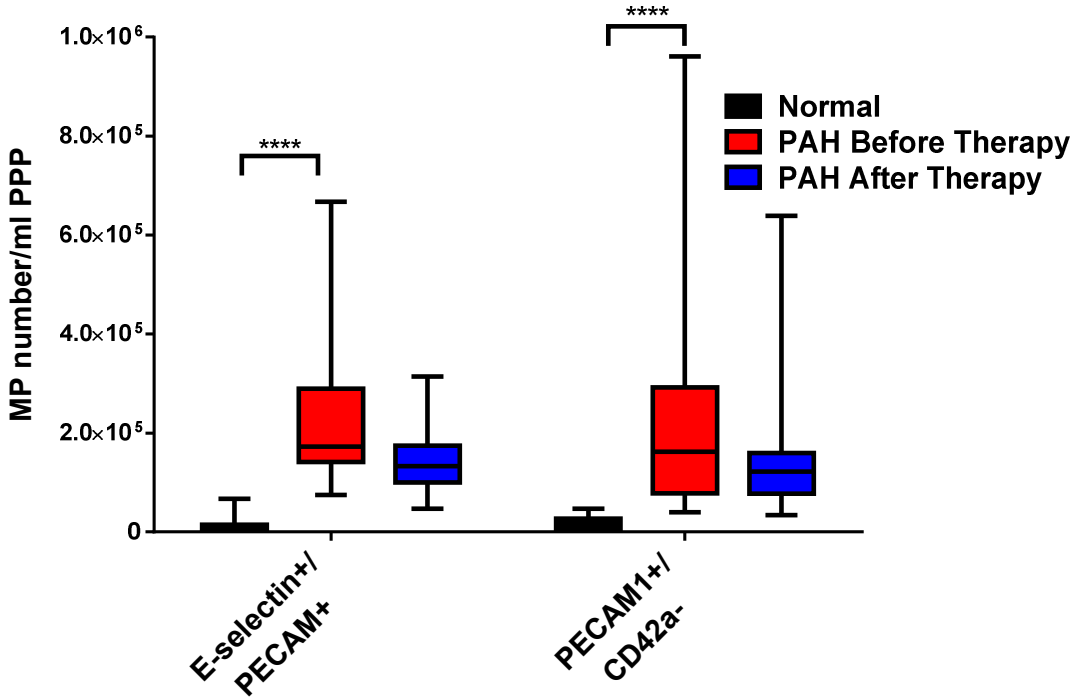

Figure 27. Circulating endothelial MP levels in PAH patients before and after long-term therapy.

Endothelial MP (EMP) levels in forearm venous blood taken from pulmonary arterial hypertensive patients before and after therapy (n=18) for a minimum of 4 months compared with healthy controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. Annexin V+ EMPs were characterised as E-selectin+/PECAM-1+ and PECAM-1+/CD42a-. Data is presented median with min and max****=P<0.0001; Mann-Whitney and Wilcoxen tests were performed.

4.2.6. Circulating leukocyte microparticles in pulmonary arterial hypertensive patients

Leukocyte microparticles (LMPs) were measured in forearm venous blood collected from patients with pulmonary arterial hypertension before and after therapy (n=18) and from normal control subjects (Figure 28). Median CD66b+/Annexin V+ LMP levels in PAH were 1.086910^5 MPs/ml in PPP, which was 24 fold greater compared to levels in control subjects who had a median of $4.489x10^3$ MPs/ml PPP ($P<0.0001$). These LMP levels decreased by 54% after long term therapy, though due to variability in patient levels of MPs this just failed to reach significance($P=0.072$). Tissue factor+ LMP levels in PAH were also significantly elevated with median of $1.15x10^5$ MPs/ml PPP compared to control subjects ($P<0.001$). These MP levels were maintained and did not decrease after long-term therapy.

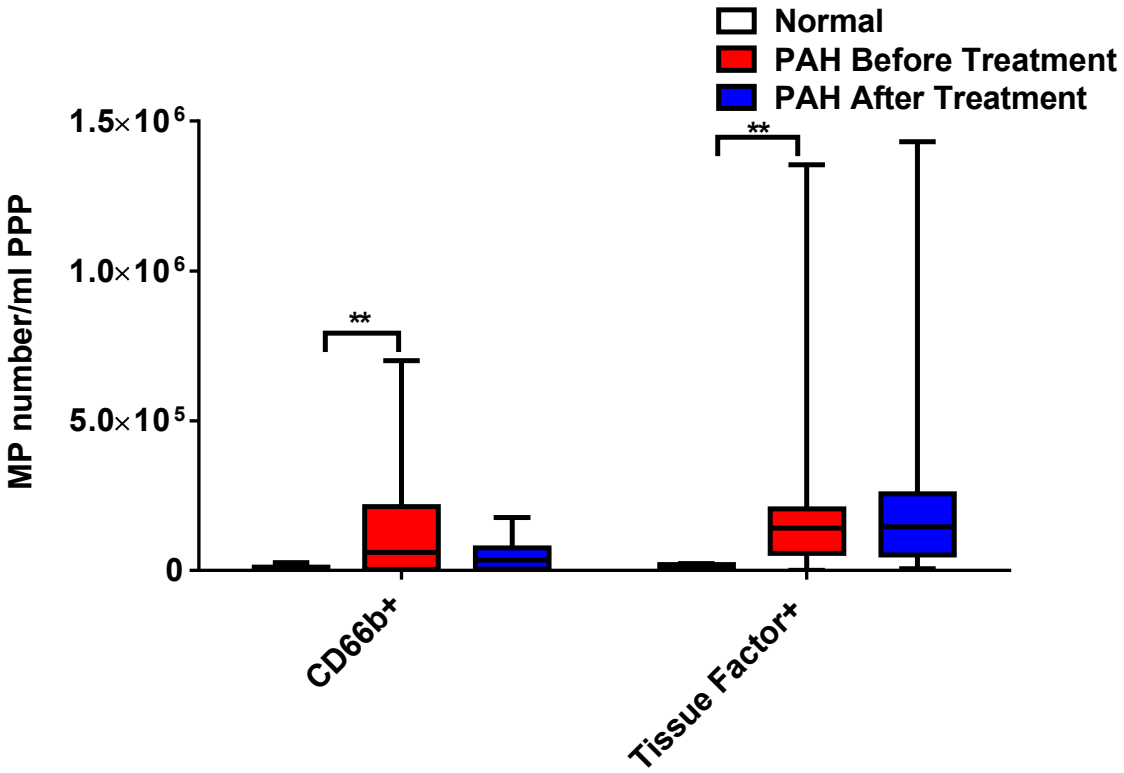

Figure 28. Circulating leukocyte MPs in PAH before and after long-term therapy

: Leukocyte MP levels in forearm venous blood was taken from pulmonary arterial hypertensive patients before and after therapy (n=18) for a minimum of 4 months compared to healthy controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. Annexin V+ LMPs were characterised as CD66b+ and Tissue Factor+. Data is presented as median with min and max. *=P<0.05; ****=P<0.0001; Mann-Whitney were performed.

4.2.7. Circulating platelet microparticles in pulmonary arterial hypertensive patients

Platelet microparticles (PMPs) were measured in forearm venous blood collected from patients with pulmonary arterial hypertension before and after long term therapy (n=18) and in normal control subjects not on any drug treatment (Figure 29). Median CD42a+/Annexin V+ PMP levels were 100-fold greater in PAH patients with a median of $1.71x10^6$ MPs/ml in PPP compared to control subjects who had a median of $1.704x10^4$ MPs/ml PPP. PMP levels were not significantly different after long-term therapy in PAH patients. After therapy, though the median decreased to $1.49x10^6$ MPs/ml PPP, the variability measured by the range in PMP levels increased by 2-fold in PAH patients.

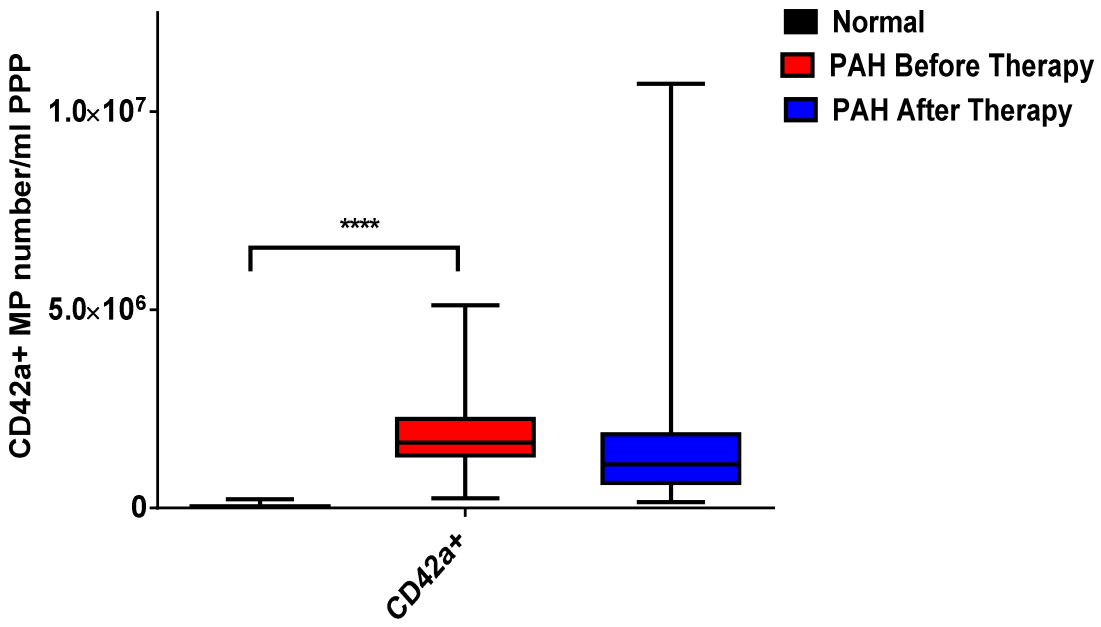

Figure 29. Circulating PMP levels in PAH before and after long-term therapy

Platelet MP levels in forearm venous blood taken from pulmonary arterial hypertensive patients before and after long term therapy (n=18) for a minimum of 4 months and compared with age- and sex-matched controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. LMPs were characterised as CD42a+. Data is presented as median with min and max. *=P<0.05; ****=P<0.0001; The Mann-Whitney test were performed.

4.2.8. Effect of drugs on circulating microparticle levels in pulmonary arterial hypertension

The impact of different drug treatments over a minimum period of 4 months on the number of total circulating annexin V+ MPs released was assessed in PAH patients (figure 30). The median MP level in patients who were on a prostacyclin analogue (treprostinil or iloprost) with or without a phosphodiesterase (PDE)-5 inhibitor (sildenafil or tadalafil; n=6) was $3.82x10^6$ MPs/ml in PPP prior to treatment, which was significantly higher than in control subjects (n=18; $P<0.001$). After long-term therapy, total MP levels decreased significantly by 44%. In patients who were on a PDE-5 inhibitor with or without an endothelin-1 receptor antagonist (ETRA; bosentan or ambrisentan; n=6), the median MP level prior to long-term treatment was $2.00x10^6$ MPs/ml PPP, which was significantly higher than we observed in control subjects ($P<0.001$). After therapy, the median MP count decreased by only by 12%.

Patients under prostacyclin therapy of a prostacyclin analogue with or without a PDE-5 inhibitor also contained higher median levels of smooth muscle microparticles than in patients on PDE-5 inhibitor with or without ETRA therapy (Figure. 32). Prostacyclin therapy signficantly decreased levels of PDGFRβ+/PECAM1-, Endoglin+/PECAM1-, and NG2+/PECAM1- SMMPs by more than 50% after long-term therapy ($P<0.05$). Such a decrease was not seen after non-prostacyclin therapy. Similarly, significant reductions were seen in E-selectin+/PECAM1+ EMPs ($P<0.05$), CD66b+ LMPs ($P<0.05$), and CD42a+

PMPs ($P<0.0001$) after long-term prostacyclin therapy, which was not seen after non-prostacyclin therapy (Figures 31 and 32).

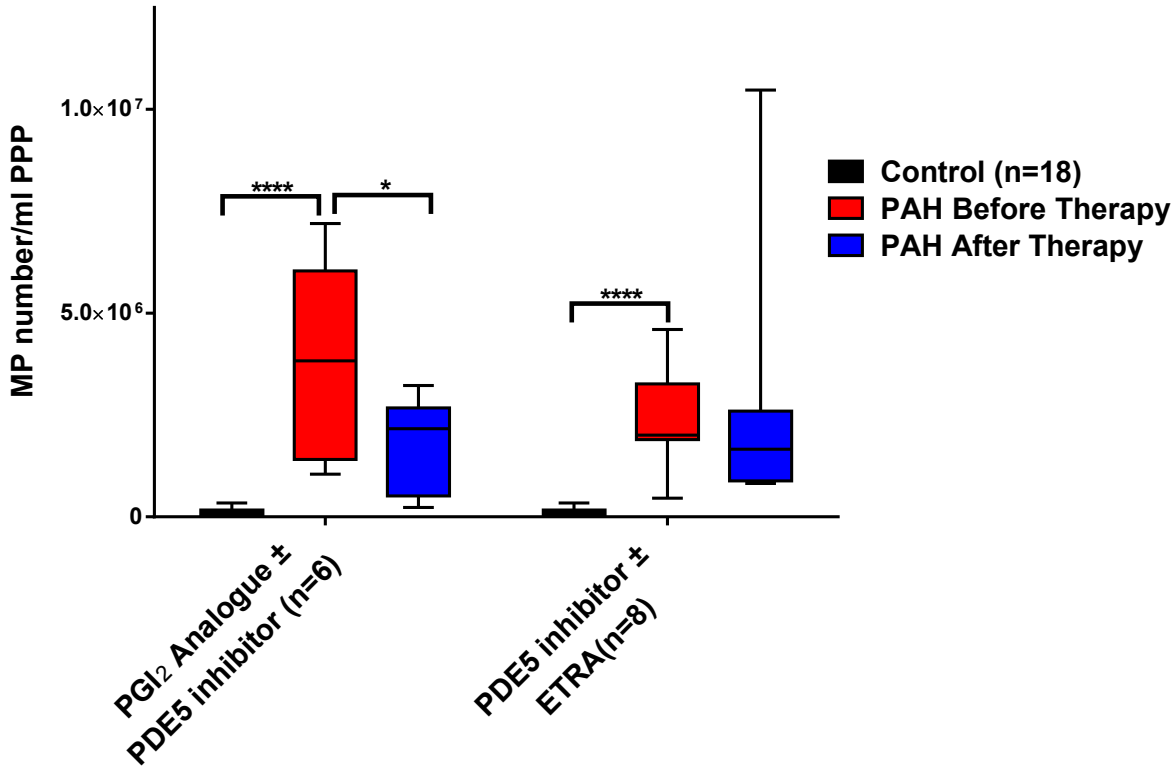

Figure 30. Effect of different long-term PAH therapies on total annexin V+ MPs in PAH patients.

Total annexin V+ MPs were measured in blood taken the forearm vein of PAH patients before and after long term therapy (prostacyclin analogue with/without a phosphodiesterase 5 (PDE5) inhibitor (n=6) and a PDE5 inhibitor with/without an endothelin-1 receptor antagonist (ETRA) (n=8). Microparticle levels of age- and sex-matched healthy controls were also used for comparison (n=18). Data is presented median with min and max. *=P<0.05; ****=P<0.0001; The Wilcoxen and Mann-Whitney tests were performed.)

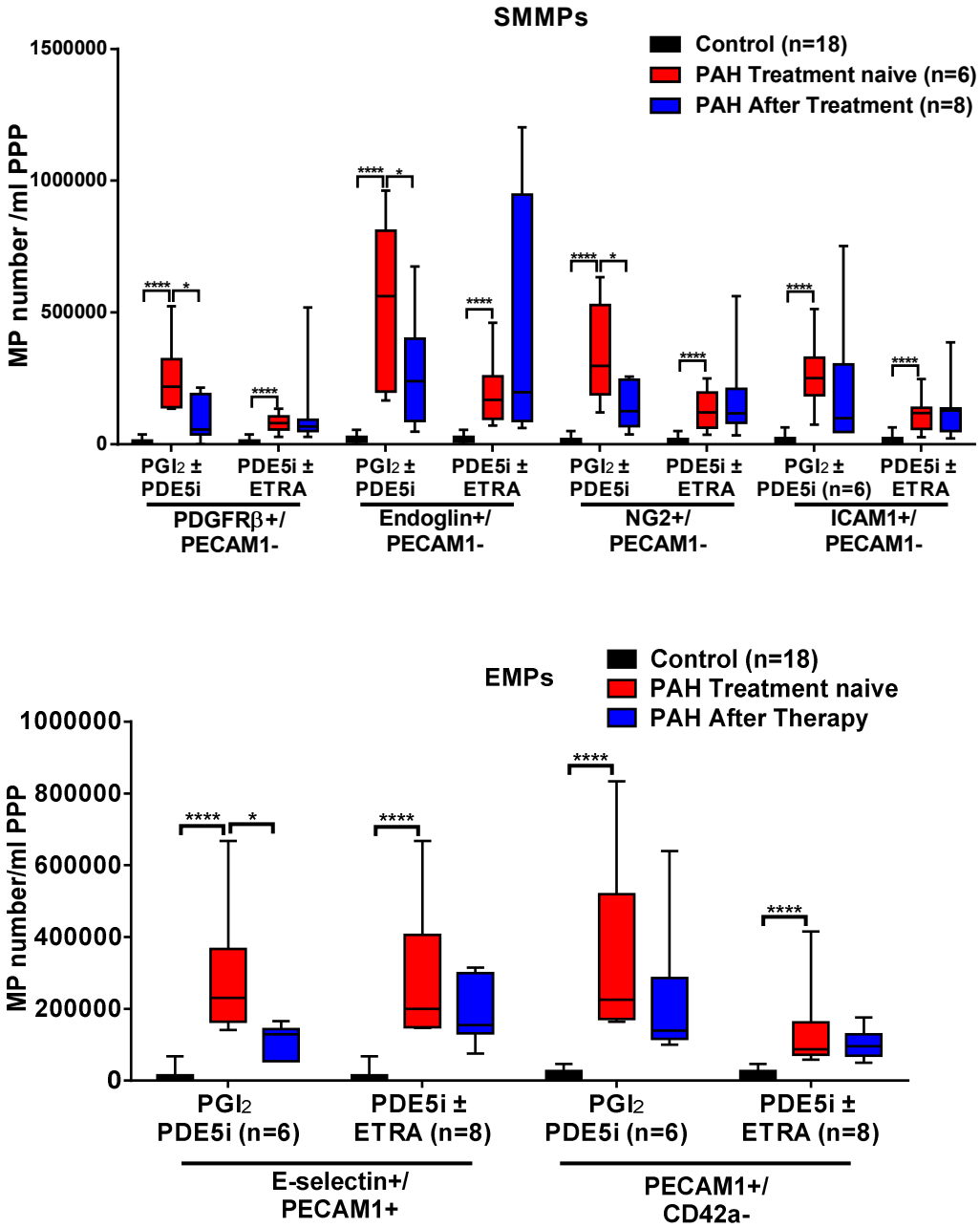

Figure 31. Effect of different long-term PAH therapies on total annexin V+ smooth muscle and endothelial MPs in PAH patients.

Annexin V+ MPs derived from smooth muscle (SMMPs; top) and endothelial cells (EMPs; bottom) were measured in blood taken the forearm vein of PAH patients before and after long term therapy (prostacyclin analogue with/without a phosphodiesterase 5 (PDE5) inhibitor (n=6) and a PDE5 inhibitor with/without an endothelin-1 receptor antagonist (ETRA) (n=8). Microparticle levels of age- and sex-matched healthy controls were also used for comparison (n=18). Data is presented median with min and max. *=P<0.05; ****=P<0.0001; The Wilcoxen and Mann-Whitney tests were performed.

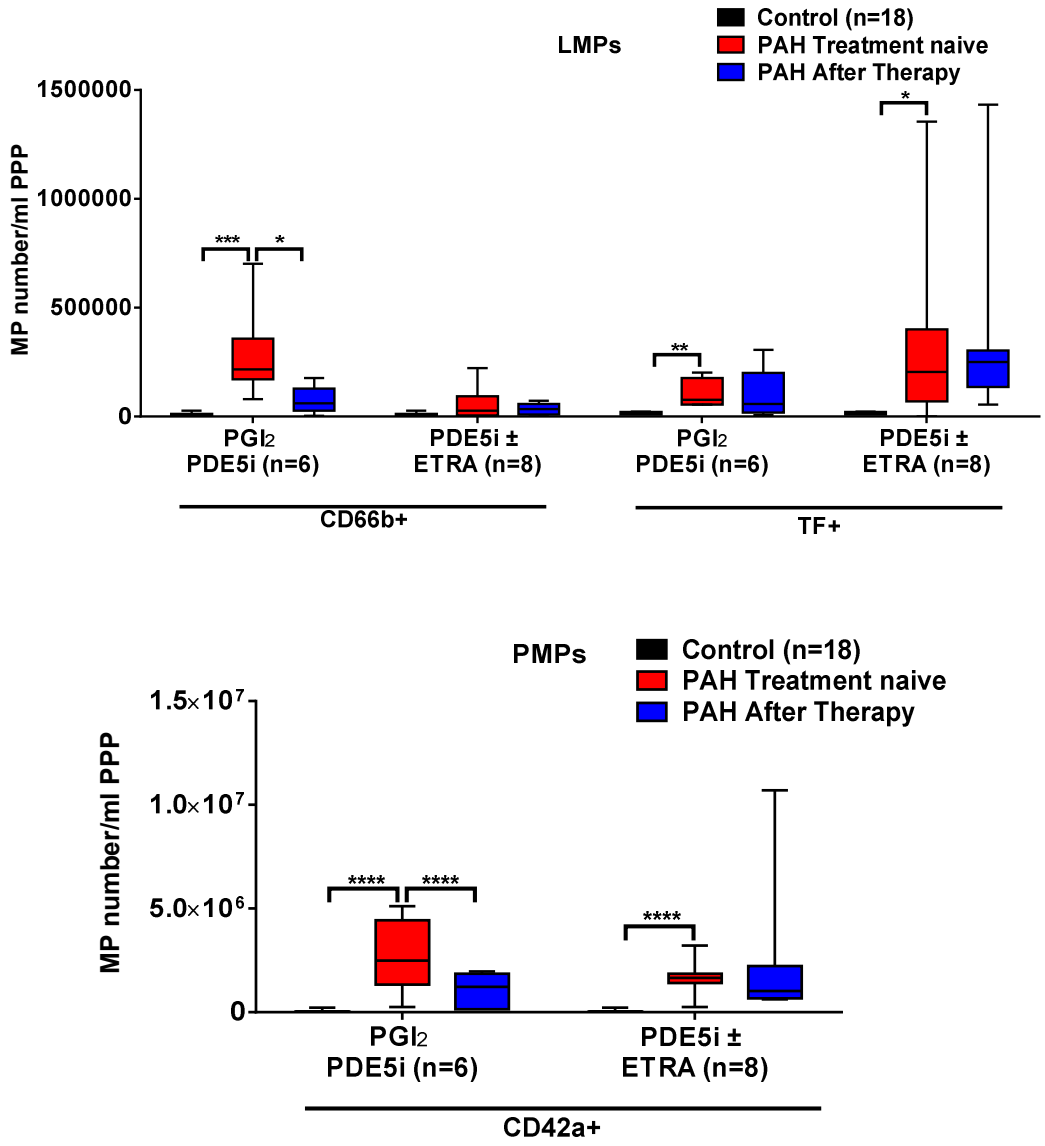

Figure 32. Effect of different long-term PAH therapies on total annexin V+ smooth muscle MPs in PAH patients.

Annexin V+ MPs derived from leukocytes (LMPs; top) and platelets (PMPs; bottom) were measured in blood taken the forearm vein of PAH patients before and after long term therapy (prostacyclin analogue with/without a phosphodiesterase 5 (PDE5) inhibitor (n=6) and a PDE5 inhibitor with/without an endothelin-1 receptor antagonist (ETRA) (n=8). Microparticle levels of age- and sex-matched healthy controls were also used for comparison (n=18). Data is presented median with min and max. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001; The Wilcoxen and Mann-Whitney tests were performed.

4.2.9. Total Annexin V+ microparticles in patients with coronary artery disease

The total number of annexin V+ MPs was assessed in patients with coronary artery disease (figure 33). Circulating total MPs in coronary arterial blood collected from patients with ST-elevated myocardial infarction (STEMI) was a median of $2.927x10^6$ MPs/ml PPP, which was significantly higher than those from control subjects ($P<0.0001$) but significantly lower than those from PAH patients ($P<0.05$). Total MPs in coronary artery blood collected from patients with Non-ST-elevated myocardial infarction (NSTEMI) was slightly lower than in STEMI with a median of $1.14x10^6$ MPs/ml PPP, but was nonetheless still significantly higher than in control subjects ($P<0.05$). Forearm venous blood from STEMI patients had a median MP count of $1.02x10^6$ MPs/ml PPP, which was significantly higher than in control subjects ($P<0.01$), and while lower than in coronary blood from STEMI patients, was not significantly different. Forearm venous blood from NSTEMI patients also contained a low median MP level of $1.32x10^6$ MP/ml in PPP, this was not significantly higher than levels in control subjects. Thus, total MP levels in both coronary artery and venous forearm blood in both STEMI and NSTEMI patient groups were significantly lower than in PAH patients.

Receiver operator characteristic curve analysis was performed to examine the sensitivity and specificity of annexin V+ quantification in distinguishing PAH from coronary artery disease in patients (figure 34). MP levels in venous blood from patients with PAH were plotted alongside coronary arterial blood from patients with STEMI. The diagnostic performance test of identification of PAH

recurrence was significant with an AUC of 0.739, a standard error of 0.104, a 95% CI of .534 to 0.944, and P=0.039. This would therefore suggest that measuring total annexin V+ MPs in forearm venous blood is capable of distinguishing PAH patients from STEMI patients.

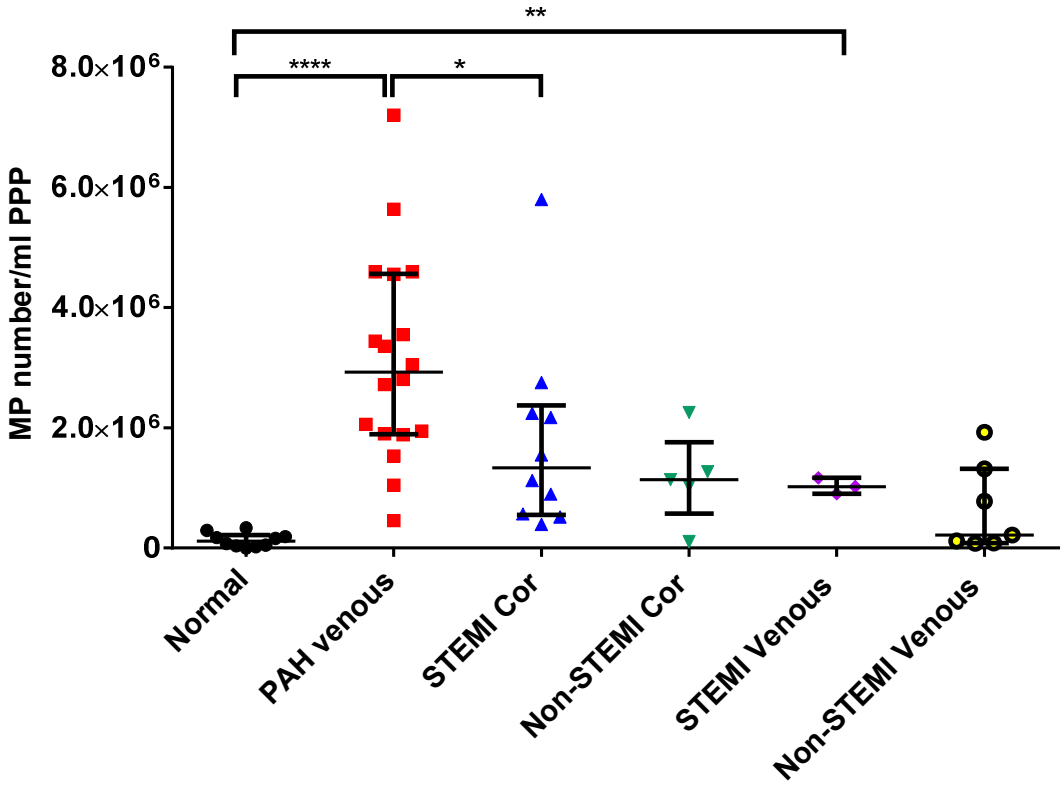

Figure 33. Total circulating annexin V+ MPs in coronary artery disease

Total Annexin V+ MP levels in coronary artery blood (Cor) from patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and in forearm venous (FV) from STEMI patients (n=3) and non-STEMI patients were measured. MP values were compared with PAH patients before start of therapy (n=18). Data are presented as median with interquartile range. *=P<0.05; **=P<0.01; ****=P<0.0001; The Mann-Whitney test was performed.

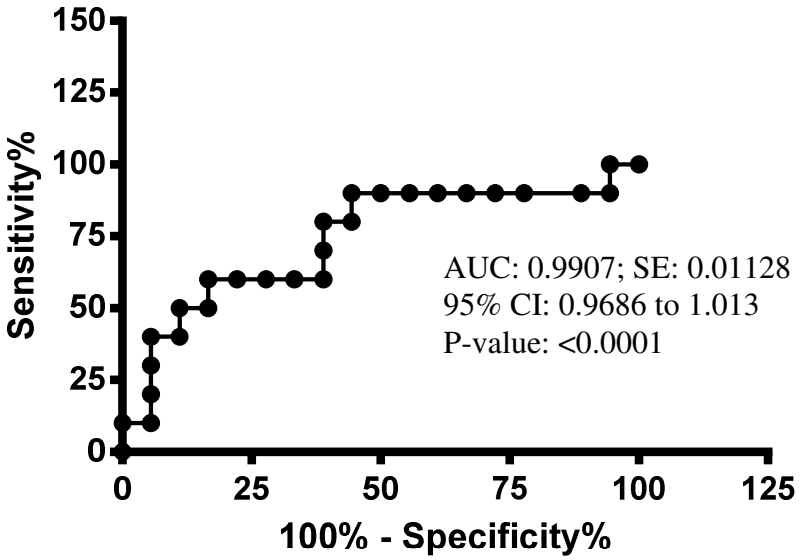

Figure 34. Receiver operator characteristic curve for total annexin V+ MPs for identification of PAH patients from STEMI patients

Receiver operator characteristic curve for total annexin V+ MPs for the identification of patients with PAH (n=18) from patients with ST-elevated myocardial infarction (n=10). ROC analysis was significant with the area under the curve (AUC) of 0.739 with a standard error (SE) of 0.104, a confidence interval (CI) of 0.534 to 0.944 and P<0.0001.

4.2.10. Smooth muscle microparticles in coronary artery disease

SMMPs in CAD patient blood was measured and compared to healthy control subjects (Figure 35). PDGFRβ+/PECAM-1-/Annexin V+ MP levels in coronary arterial blood from STEMI and NSTEMI patients had a median of $1.48x10^5$ MPs/ml and $4.76x10^4$, respectively in PPP, but were significantly higher than forearm venous blood from control subjects who had a median of $7.19x10^3$ MPs/ml of PPP ($P<0.001$ and $P<0.01$, respectively). Forearm venous blood collected from both STEMI and NSTEMI patients had low PDGFRβ+/PECAM1-/Annexin V+ SMMP levels that were not significantly different from those in control subjects, with medians of $2.86x10^3$ MPs/ml and $4.48x10^3$ MPs/ml of PPP, respectively. These SMMPs were significantly elevated in STEMI patient-derived coronary blood compared to STEMI forearm blood ($P<0.01$) and NSTEMI coronary blood ($P<0.01$), suggesting the dependence of SMMP levels on disease severity and site of measurement. Levels of PDGFRβ+/PECAM1-/Annexin V+ SMMPs in PAH forearm venous blood (median of $1.38x10^5$ MPs/ml PPP) was similar to STEMI coronary artery blood and significantly higher than in NSTEMI coronary blood ($P<0.01$).

Endoglin+/PECAM-1-/Annexin V+ SMMPs from STEMI coronary artery blood was elevated to a median of $2.84x10^5$ MPs/ml in PPP, which was very close to levels seen in PAH (median $3.00x10^5$ MPs/ml PPP) but was ~20 fold higher than in forearm venous blood from control subjects ($1.298x10^4$ MPs/ml in PPP; $P<0.001$). SMMPs were also elevated in NSTEMI Coronary blood ($1.63x10^5$ MPs/ml in PPP) compared to controls ($P<0.01$) but were significantly lower than PAH forearm blood ($P<0.05$). STEMI forearm venous blood contained $4.20x10^4$

MPs/ml in PPP Endoglin+/PECAM1-/Annexin V+ SMMPs, which were higher than in control subjects though not significant. NSETMI forearm venous blood contained very low SMMPs with a median of $7.84x10^4$ MPs/ml PPP, which was not significantly different from the number in controls. Though a trend was seen, there was no significant difference between coronary artery- and forearm-derived blood as well as between STEMI- and NSTEMI-derived blood.

NG2+/PECAM1-/Annexin V+ SMMPs in STEMI coronary artery blood was significantly higher than in controls with a median of $1.91x10^5$ MPs/ml PPP compared to $1.22x10^4$ MPs/ml PPP, respectively ($P<0.01$). The SMMPs in NSTEMI coronary blood was lower, though not significantly, with a median of $5.68x10^4$ MPs/ml PPP and was also significantly higher than in controls ($P<0.05$). Forearm derived blood from STEMI patients had a low median of $5.33x10^3$ MPs/ml in PPP and were significantly lower than coronary derived blood ($P<0.05$). NSTEMI forearm blood also had a low median of $6.16x10^3$ MPs/ml in PPP. STEMI and non-STEMI forearm bloods did not contain elevated NG2+/PECAM1-/Annexin V+ SMMPs compared to healthy control blood.

ICAM+/PECAM-/Annexin V+ SMMPs was also elevated in STEMI coronary artery blood compared to controls with a median of $1.31x10^5$ MPs/ml ien PPP compared to $1.163x10^4$ MPs/ml PPP, respectively ($P<0.001$). Levels of these SMMPs were lower in NSTEMI coronary blood with a median of $4.22x10^3$ MPs/ml PPP ($P<0.05$), but was also significantly higher than in controls ($P<0.01$). In STEMI patients, the median ICAM+/PECAM-/Annexin V+ SMMPs in the forearm venous blood were $2.953x10^4$ MPs/ml PPP and were also significantly lower than coronary artery blood ($P<0.05$). Similarly in NSTEMI patients, the

median SMMPs in the forearm venous blood with a median of $8.4x10^3$ MPs/ml PPP were significantly lower than in coronary artery blood ($P<0.05$). The SMMPs levels in PAH venous blood containing a median of $1.238x10^5$ MPs/ml PPP was significantly higher than in both forearm venous STEMI and STEMI blood ($P<0.01$).

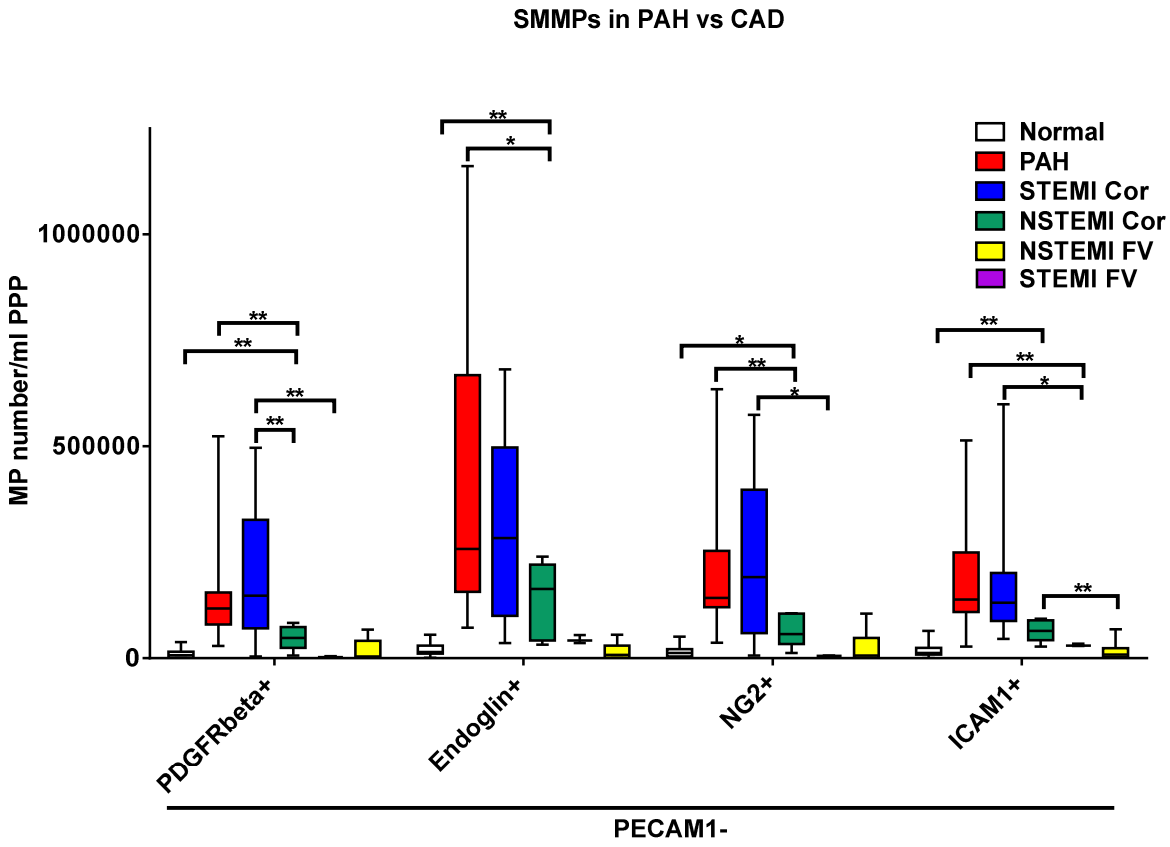

Figure 35. Circulating smooth muscle microparticles in coronary artery disease patients

SMMP levels in blood taken from the coronary artery of patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and from the forearm vein from STEMI patients (n=3). SMMP values were compared in pulmonary arterial hypertensive patients before and after drug treatment (n=18) for a minimum of 4 months, and in age- and sex-matched controls (n=18). Therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. SMMPs were characterised as platelet derived growth factor receptor β (PDGFRβ)+/PECAM-1-/Annexin V+, endoglin+/PECAM-1-/Annexin V+, neural glial antigen 2 (NG2)+/PECAM-1-/Annexin V+, and ICAM1+/PECAM-1-/Annexin V+. (*=P<0.05; **=P<0.01; Mann-Whitney and Wilcoxen tests were performed. Results are expressed as median and range.)

4.2.11. E-selectin+ microparticles in coronary artery disease

E-selectin is classically known to be an endothelial surface marker and was used to label EMPs in blood from patients with coronary artery disease. E-selectin+ MPs were 106-fold higher in coronary blood from STEMI patients with a median of $8.87x10^5$ MPs/ml PPP compared to venous blood in control subjects ($8.351x10^3$ MPs/ml PPP; $P<0.001$; Figure 36). Coronary NSTEMI blood contained only 35919 MPs/ml PPP, which was 96% less E-selectin+ MPs than coronary STEMI blood ($P<0.01$) and the MP count was not significantly different from that seen in controls ($P=0.0553$). E-selectin+ MPs in STEMI forearm venous blood were a median of $3.296x10^4$ MPs/ml PPP and were significantly higher than in control subjects ($P<0.05$). NSTEMI forearm venous blood contained much lower levels of these EMPs (median of $1.12x10^4$ MPs/ml PPP) which were comparable to levels in controls. E-selectin+ MP levels in PAH venous blood (median $4.00\ x10^5$ MPs/ml PPP) was not significantly different from levels in STEMI coronary artery blood but was significantly higher than levels in NSTEMI coronary artery blood ($P<0.01$).

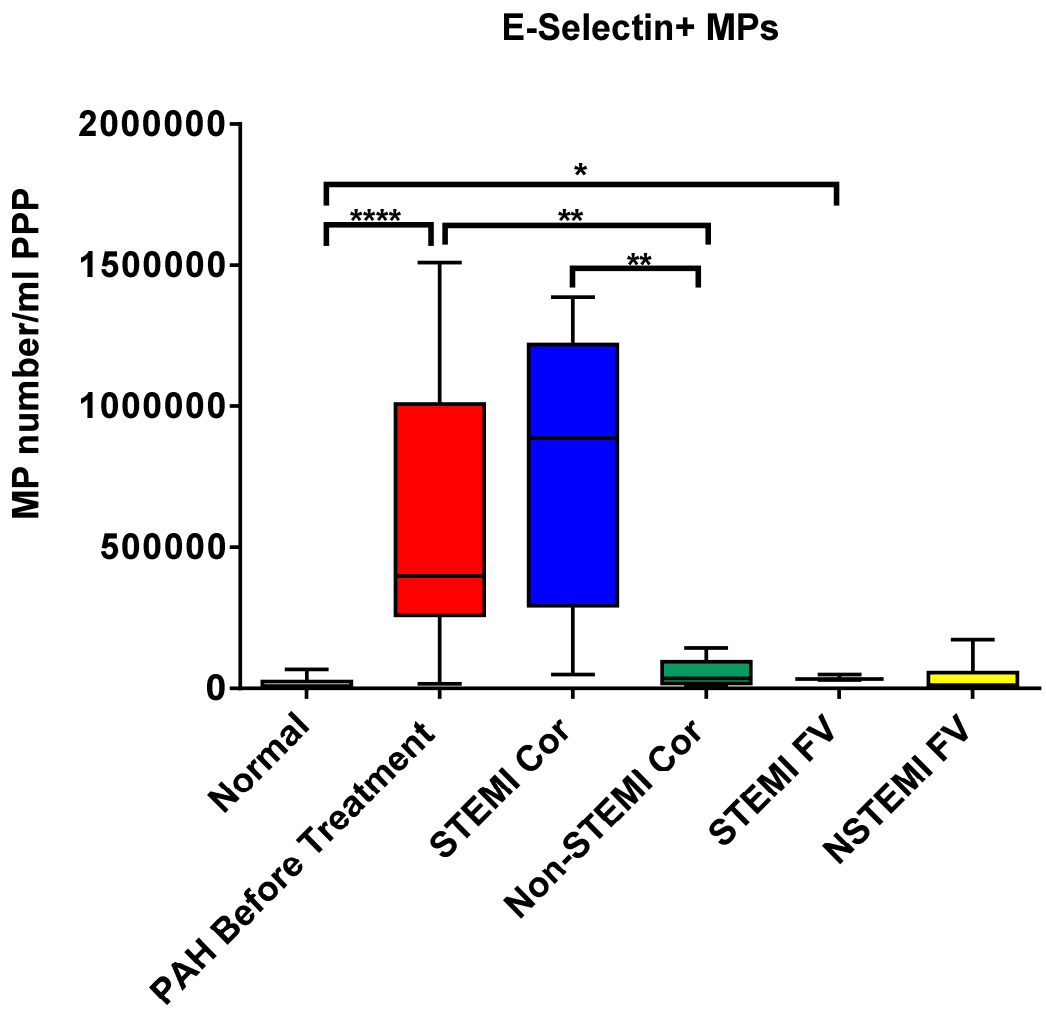

Figure 36. Circulating E-selectin+ microparticles from CAD patients

E-selectin MP levels in blood taken from the coronary artery of patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and from the forearm vein from STEMI patients (n=3). SMMP values were compared with pulmonary arterial hypertensive patients before and after long term therapy (n=18) for a minimum of 4 months and compared to age- and sex-matched controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. MPs were E-selectin+/Annexin V+. (**=P<0.01; ***=P<0.001; ****=P<0.0001; Mann-Whitney and Wilcoxen tests were performed. Results are expressed as median and range.)

4.2.12. Leukocyte microparticles in coronary artery disease

STEMI coronary artery blood contained very high levels of CD66b+ leukocyte MPs (LMPs; $1.32x10^5$ MPs/ml PPP), which were significantly greater than in control venous blood ($P<0.0001$) and more than two-fold greater than in PAH venous blood ($6.07x10^4$ MPs/ml PPP; $P<0.05$; Figure 37). Levels of CD66b+ MPs in NSTEMI coronary artery blood was had a median of $2.53x10^4$ MPs/ml in PPP, which was significantly lower than in STEMI coronary artery blood ($P<0.05$) and but higher than in control venous blood ($P<0.05$). Forearm venous blood from both STEMI and NSTEMI patients did not contain CD66b+ levels that were significantly different from levels seen in control venous blood (3.62x103 MPs/ml PP and $1.12x10^3$ MPs/ml PP, respectively). CD66b+ were significantly higher in PAH forearm venous blood compared to NSTEMI forearm venous blood ($P<0.01$).

TF+ MPs were elevated in STEMI coronary artery blood (median of $4.34x10^5$ MPs/ml PPP) compared to control venous blood ($P<0.01$) and STEMI venous blood (median of $1.103x10^5$ MPs/ml PPP; $P<0.05$), and was three-fold higher than in PAH venous blood ($1.41x10^5$ MPs/ml PPP; $P<0.05$). NSTEMI coronary artery blood contained significantly less TF+ MPs compared to STEMI coronary artery blood with a median of $1.103x10^5$ MPs/ml PPP ($P<0.05$). Levels of TF+ MPs in the forearm venous blood from STEMI and NSTEMI patients were low and not significantly different from control subjects ($2.477x10^3$ MPs/ml PPP and $3.920x10^3$ MPs/ml PP, respectively). Forearm venous blood from PAH patients contained significantly higher levels of TF+ MPs than in forearm venous blood from STEMI and NSTEMI patients ($P<0.01$ and $P<0.001$, respectively).

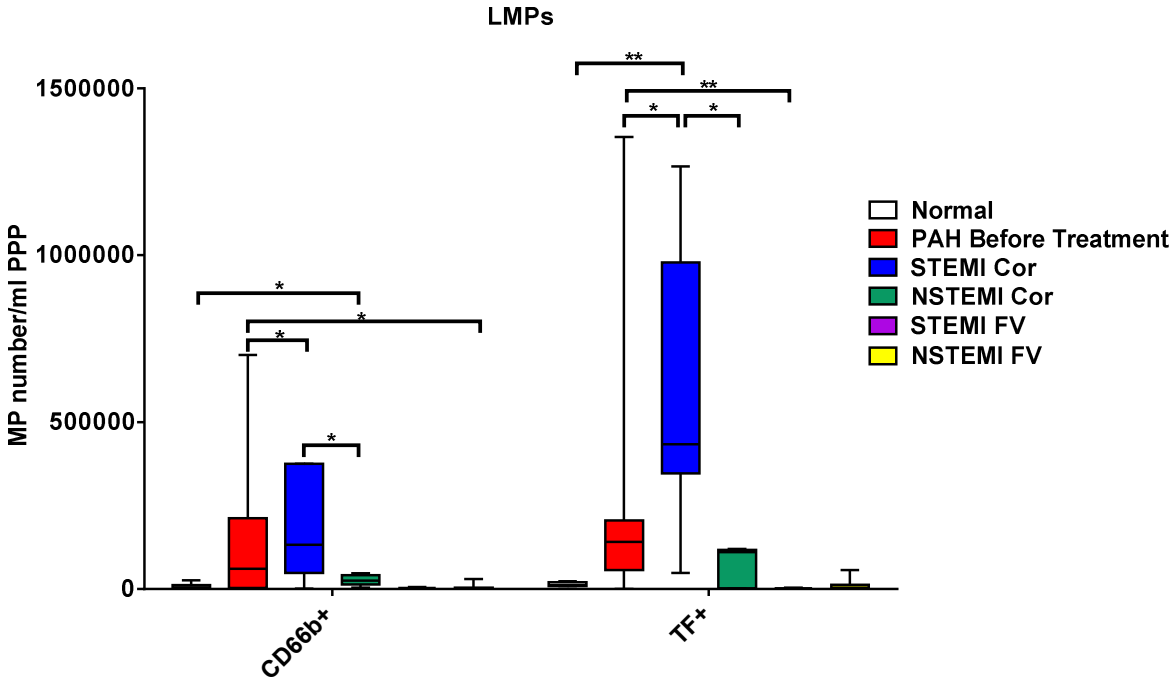

Figure 37. Circulating leukocyte microparticles in blood taken from CAD patients

LMP levels in blood taken from the coronary artery of patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and from the forearm vein of STEMI patients (n=3). SMMP values were compared with pulmonary arterial hypertensive patients before and after therapy (n=18) and age- and sex-matched controls (n=18). The drug therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. LMPs were characterised as CD66b+/Annexin V+ and TF+/Annexin V+. (*=P<0.05; Mann-Whitney and Wilcoxen tests were performed. Results are expressed as median and range.)

4.2.13. Platelet microparticles in coronary artery disease

CD42a+ platelet MPs (PMPs) were elevated 100 fold in STEMI coronary artery blood (median 1.57 x 10^6 MPs/ml PPP) compared to control venous blood (median 1.704x10^4 MPs/ml PPP; P<0.0001; Figure 38). STEMI coronary artery blood contained levels similar to PAH forearm venous blood (median 1.651x10^6 MPs/ml PPP) and was significantly higher than STEMI forearm venous blood (median 1.555x10^5 MPs/ml PPP; P<0.05). NSTEMI coronary artery blood contained significantly higher PMP than control venous blood but lower CD42a+ PMPs (median 3.962x10^5 MPs/ml PPP) than control venous blood, though this difference did not reach significance. Forearm venous blood from STEMI and NSTEMI patients (median 1.12x10^5 MPs/ml PPP) contained lower PMPs compared to coronary artery blood samples, but were both found containing PMPs significantly higher than in control venous blood (P<0.05 and P<0.01, respectively).

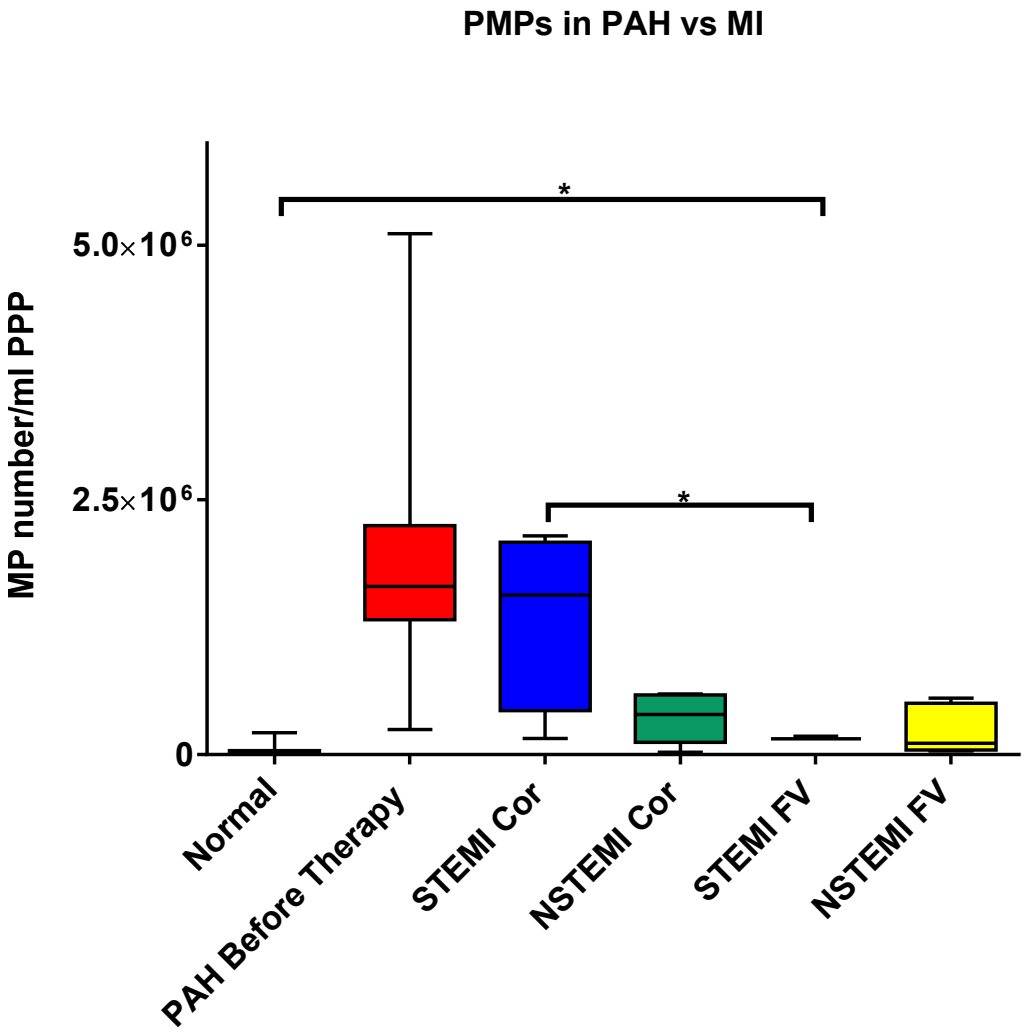

Figure 38. Circulating platelet microparticles in coronary artery disease

PMP levels in blood taken from the coronary artery of patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and from the forearm vein from STEMI patients (n=3). SMMP values were compared with pulmonary arterial hypertensive patients before and after therapy (n=18) and age- and sex-matched controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. PMPs were characterised as CD42a+/Annexin V+. (****=P<0.0001; Mann-Whitney and Wilcoxen tests were performed. Results are expressed as median and range.)

4.2.14. Total circulating annexin V+ microparticles in human immunodifficiency virus infected patients

Total circulating annexin V+ MP levels in forearm venous blood from Malawian patients with human immunodeficiency virus (HIV) were a median of $2.11x10^5$ MPs/ml of PPP and were not significantly different from Malawian control patients (median $2.66x10^5$ MPs/ml of PPP), though the range of MPs in HIV blood was broader and had a higher maximum count ($1.03x10^6$ MPs/ml of PPP) compared to controls ($6.224x10^5$ MPs/ml PPP; Figure 39). Both Malawian patient and control bloods contained significantly higher levels of total MPs than in control subjects from the UK who had a median of $3.13x10^4$ MPs/ml in PPP ($P<0.001$). Total MPs in forearm venous blood from PAH patients were significantly higher levels than blood from HIV-infected ($P<0.0001$) and non-infected controls from both Malawi ($P<0.0001$) and the UK ($P<0.0001$).

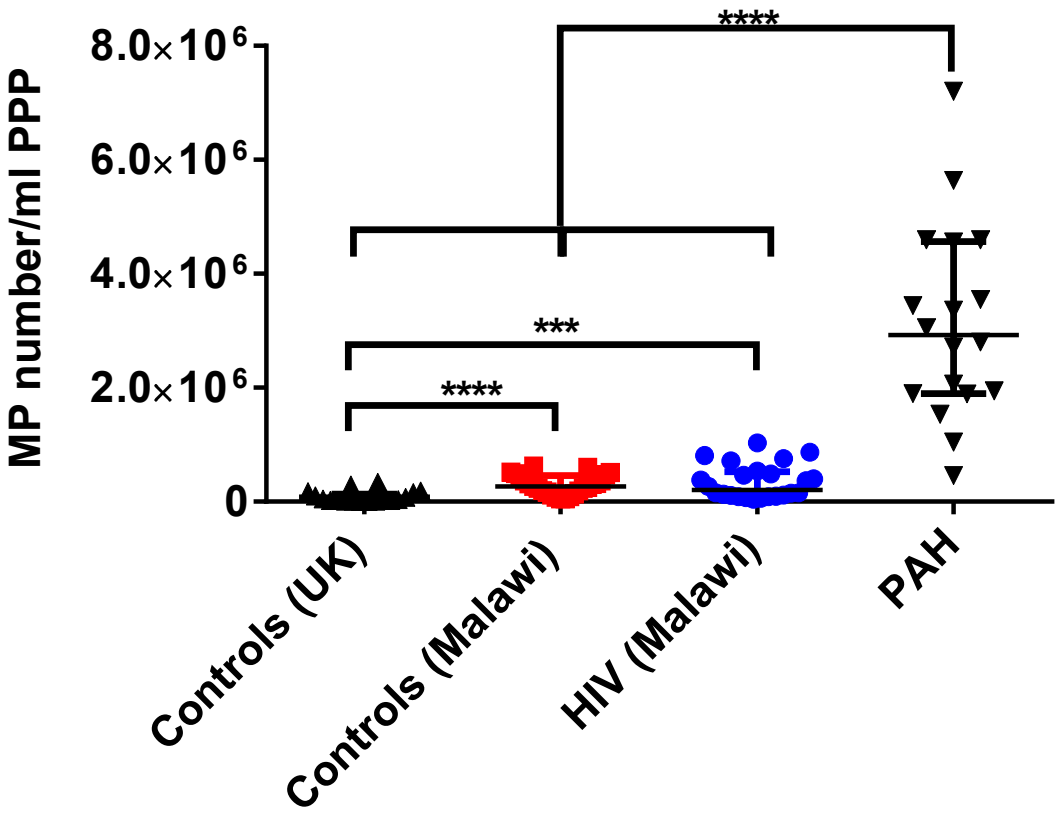

Figure 39. Total circulating microparticles in human immunodeficiency virus-infected patients

Total annexin V+ MPs were measured in blood samples from HIV infected Malawian patients (n=24), HIV non-infected Malawian control subjects (n=25), and control British subjects (n=18). (***=P<0.001; ****=P<0.0001; The Wilcoxen test was performed. Results are expressed as median and interquartile range.)

4.2.15. Smooth muscle microparticles in human immunodifficiency virus infected patients

PDGFRβ+/PECAM-/Annexin V+ SMMPs were significantly lower in HIV venous blood (median $4.34x10^3$ MPs/ml of PPP) than in Malawian venous controls (median $1.00x10^4$ MPs/ml of PPP; Figure 40). Venous blood from UK control subjects contained these SMMPs at levels that were lower than Malawian control venous blood by 28% (median of $7.19x10^3$ MPs/ml of PPP) but not significantly different from Malawian control and HIV bloods.

Endoglin+/PECAM-1-/Annexin V+ SMMPs was ~5-fold lower in HIV venous blood (median $5.47x10^3$ MPs/ml PPP) compared to both Malawian control venous blood ($1.251x10^4$ MPs/ml PPP) and UK controls ($1.423x10^4$ MPs/ml PPP; both $P<0.001$). Levels of these SMMPs were not significantly different between Malawian and UK control bloods.

NG2+/PECAM-1-/Annexin V+ SMMPs were lower, though not significantly ($P=0.0604$) in HIV venous blood (median of $3.478x10^3$ MPs/ml PPP) compared to Malawian control blood (median of $9.067x10^4$ MPs/ml PPP) and UK control blood (median of $1.22x10^4$ MPs/ml PPP). Though the range of these SMMP counts was wider, and the maximum level higher in the Malawian control blood (median of $1.83x10^5$ MPs/ml PPP) compared to UK controls (median of $5.04x10^4$ MPs/ml in PPP), the latter had a higher median, though the difference was not significant.

ICAM1+/PECAM-/Annexin V+ SMMPs levels in HIV venous blood (median of $4.18x10^3$ MPs/ml of PPP) was significantly lower than in Malawian (median of $9.85x10^3$ MPs/ml of PPP) by 57% and UK venous control bloods (median of

$1.25x10^4$ MPs/ml in PPP) by 66% ($P<0.01$ and $P<0.001$, respectively). There was no significant difference in the SMMP counts between Malawian and UK control bloods.

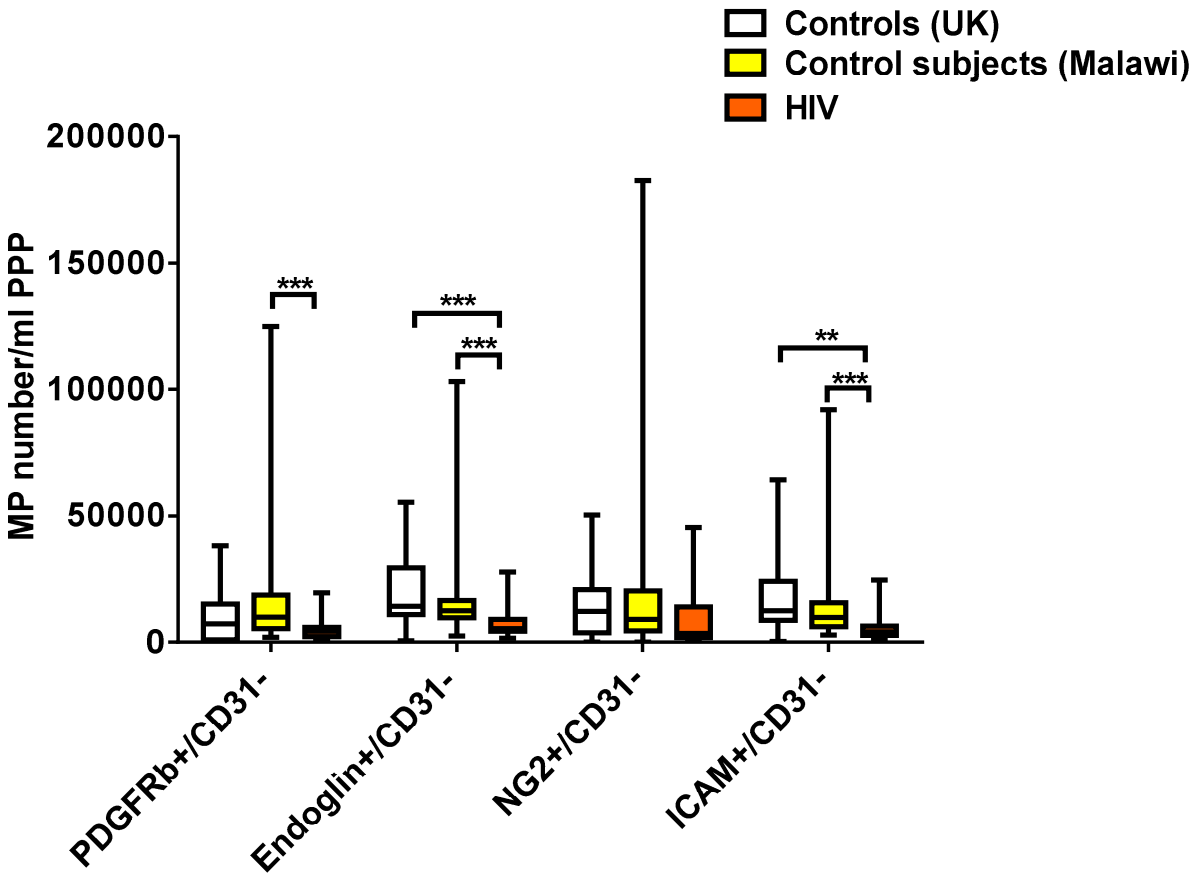

Figure 40. Circulating smooth muscle microparticles in HIV-infected patients

SMMPs were measured in blood samples from HIV infected Malawian patients (n=24), HIV non-infected Malawian control subjects (n=25), and control British subjects (n=18). SMMPs were characterised as platelet derived growth factor receptor β (PDGFRβ)+/PECAM1-/Annexin V+, endoglin+/PECAM1-/Annexin V+, neural glial antigen 2 (NG2)+/PECAM1-/Annexin V+, and ICAM1+/PECAM1-/Annexin V+. (***=P<0.001; ****=P<0.0001; The Wilcoxen test was performed. Results are expressed as median and range.)

4.2.16. Endothelial microparticles in human immunodifficiency virus infected patients

Levels of E-selectin+ MPs in HIV patients were slightly though not significantly higher than in UK controls and 67% lower than in Malawian controls though this was not significant (Figure 41). E-selectin+ MPs were significantly higher in Malawian control blood compared to UK controls.

Circulating levels of PECAM-1+/CD42a- EMPs in HIV patients (median of $2.85x10^3$ MPs/ml PPP) were 64% lower than in Malawian controls (median of $7.97x10^3$ MPs/ml PPP) and 76% lower than in control subjects ($1.21x10^4$ MPs/ml PPP; $P<0.001$ and $P<0.01$, respectively). There was no significant difference in PECAM1+/CD42a- EMP levels between Malawian and UK control blood samples.

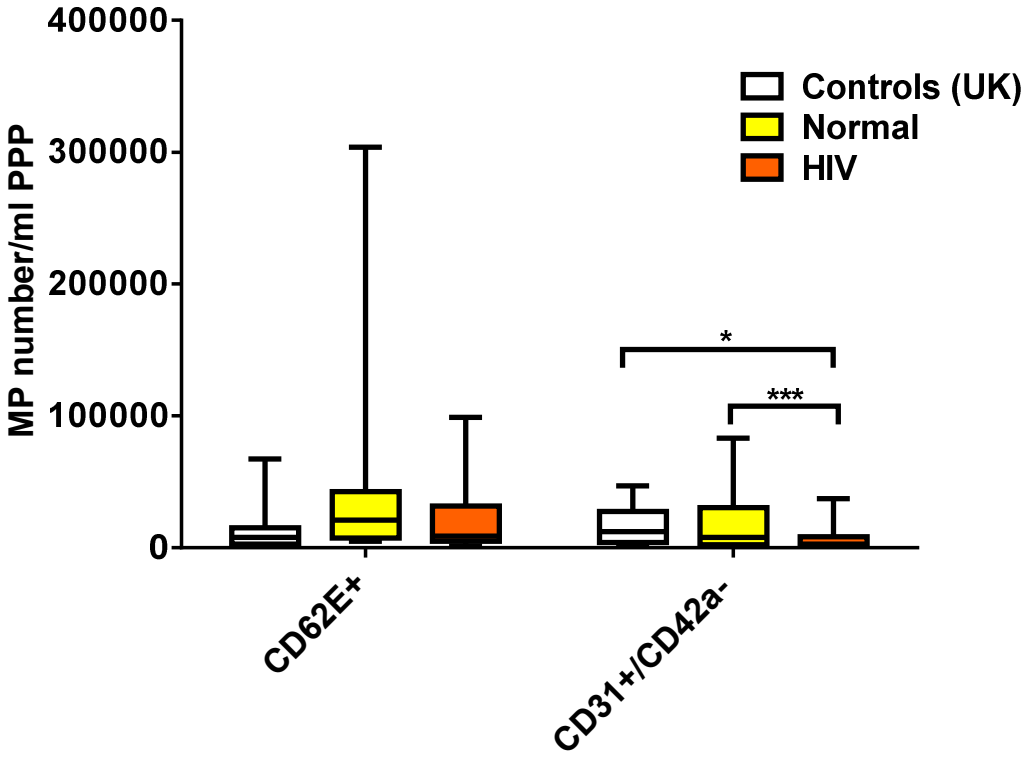

Figure 41. Circulating endothelial microparticles in HIV-infected patients

EMPs were measured in blood samples from HIV infected Malawian patients (n=24), non-infected Malawian control subjects (n=25), and non-infected British subjects (n=18). EMPs were characterised as E-selectin+/PECAM-1+/Annexin V+ and PECAM-1+/CD42a-/Annexin V+. (*=P<0.05; ***=P<0.001; ****=P<0.0001; The Wilcoxen test was performed. Results are expressed as median and range.)

4.2.17. Leukocyte microparticles in human immunodifficiency virus infected patients

Circulating CD66+ LMP levels in HIV patients measured at $2.46x10^3$ MPs/ml in PPP and were lower than, though not significantly different, from levels in control venous blood from both Malawian (median of $5.63x10^3$ MPs/ml PPP) and UK (median of $4.49x10^3$ MPs/ml PPP) subjects (Figure 42). However, there was no difference in the levels of LMPs between the two control groups.

HIV venous blood contained significantly lower amounts of TF+ MPs (median of 1. $6x10^4$ MPs/ml in PPP) compared to Malawian controls (median of $3.86x10^4$ MPs/ml in PPP; $P<0.01$) but were similar to levels in UK controls (median of $1.13x10^4$ MPs/ml in PPP). Circulating TF+ MPs were significantly higher in Malawian controls than in UK controls ($P<0.05$).

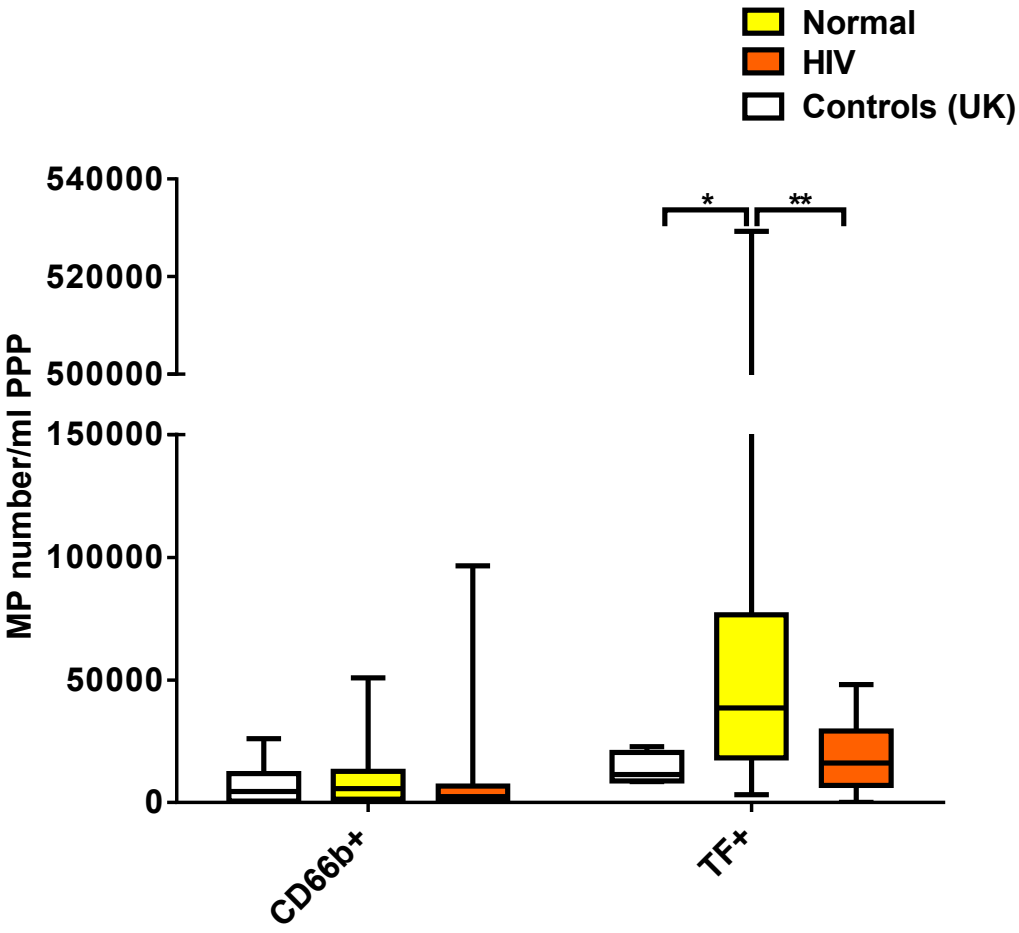

Figure 42. Circulating leukocyte microparticles in IHV-infected patients

LMPs were measured in blood samples from HIV infected Malawian patients (n=24), non-infected Malawian control subjects (n=25), and non-infected British subjects (n=18). LMPs were characterised as CD66b+/Annexin V+, tissue factor+/Annexin V+, CD16+/Annexin V+, and CD14+/Annexin V+. (**=P<0.01; The Wilcoxen test was performed. Results are expressed as median and range.)

4.2.18. Platelet microparticles in human immunodifficiency virus infected patients

Circulating CD42a+ PMPs were elevated, two-fold in HIV venous blood (median $6.75x10^4$ MPs/ml in PPP) compared to Malawian control venous blood (median $3.533x10^4$ MPs/ml in PPP) though this was not significant (Figure 43). However, PMPs were significantly higher in HIV patients compared to UK control venous blood (median $1.70x10^4$ MPs/ml PPP; $P<0.05$). There was no difference in CD42a+ PMP levels between the two control groups.

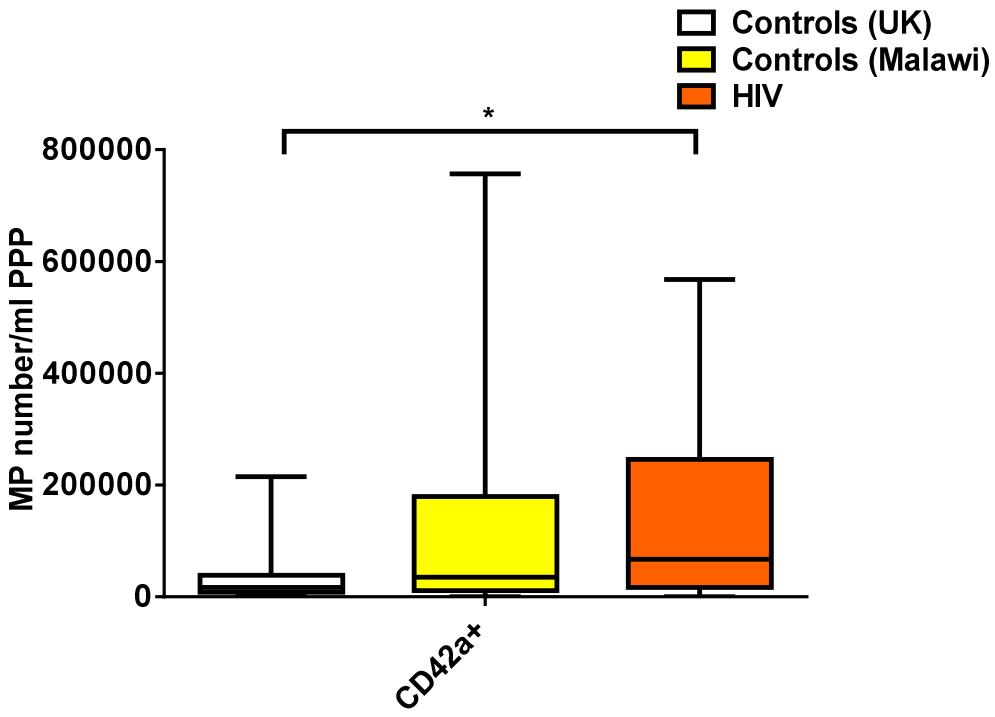

Figure 43. Circulating platelet microparticles in HIV-infected patients.

PMPs were measured in blood samples from HIV infected Malawian patients (n=24), non-infected Malawian control subjects (n=25), and non-infected British subjects (n=18). PMPs were characterised as CD42a+ /Annexin V+. Results are expressed as median and interquartile range; and the Wilcoxen test performed where *=P<0.05.)

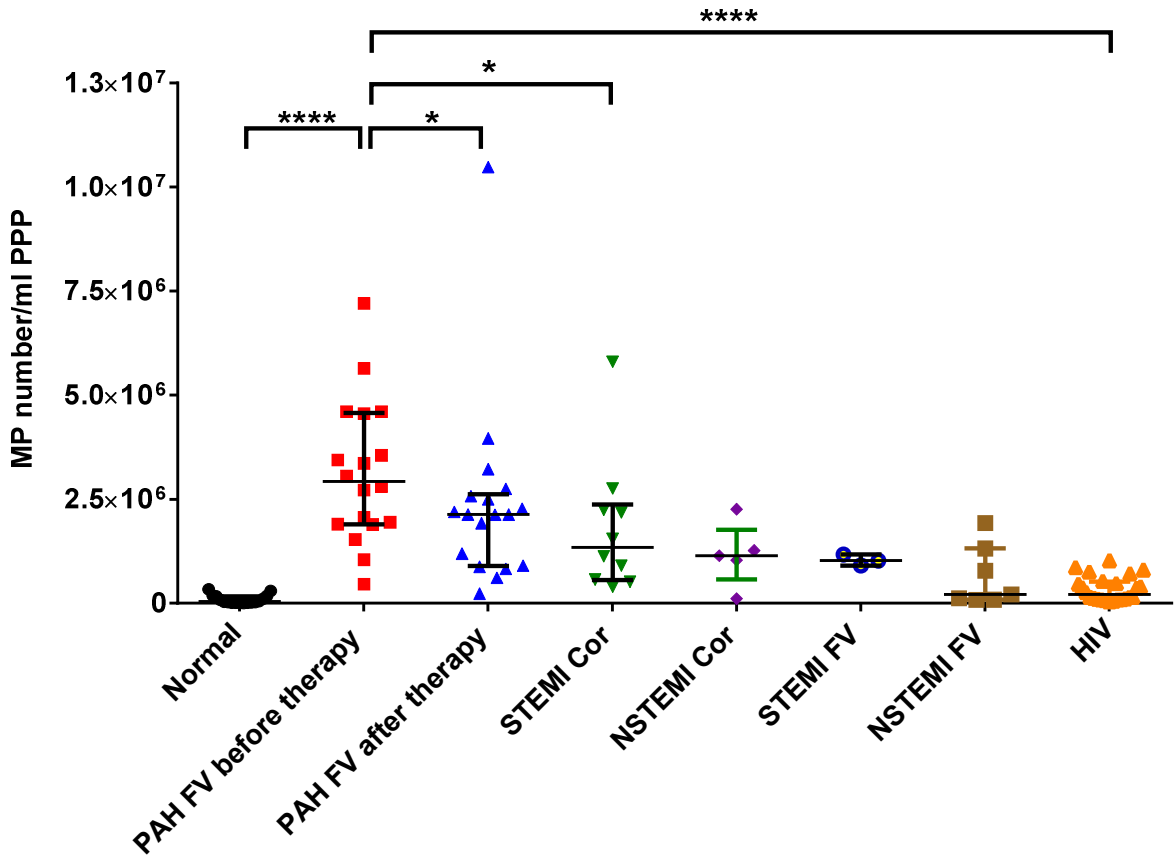

Figure 44. Summary of total Annexin V+ in PAH, CAD, and HIV

Summary of total annexin V+ microparticle levels in forearm venous blood taken from pulmonary arterial hypertensive patients before and after drug therapy (n=18) compared with age and sex-matched controls (n=18). The therapy consisted of prostacyclin analogues, phosphodiesterase (PDE-5) antagonist, endothelin-1 receptor antagonist (ETRA), calcium channel blocker or combination therapy of PDE-5 antagonist with ETRA and/or a prostacyclin analogue. MP levels were also measured in blood taken from the coronary artery of patients with ST-elevated myocardial infarction (STEMI; n=7), non-ST-elevated myocardial infarction (NSTEMI; n=5), and from the forearm vein of STEMI patients (n=3). Total Annexin V+ MPs were measured in blood samples from HIV infected Malawian patients (n=24), HIV non-infected Malawian control subjects (n=25), and non-infected British subjects (n=20).. Results are expressed as median and interquartile range. The Mann-Whitney and Wilcoxen tests were performed with *=P<0.05 and ****=P<0.0001;)

4.3. Discussion

In this chapter I have shown that circulating total annexin V+ MPs were significantly elevated in PAH patients compared to healthy control subjects and that these levels were considerably higher in PAH than in other diseases affecting the vasculature such as coronary artery disease and HIV. Total MP levels in forearm venous blood in PAH were more than two-fold greater compared to coronary artery blood of patients with STEMI and almost 3-fold greater compared to coronary artery blood from patients with NSTEMI. This may suggest a higher level of vascular damage, cell activation and apoptosis in PAH than in other vascular diseases such as myocardial infarction. A study conducted in 2008 by Amabile and colleagues also showed that total annexin V+ MP levels in PH patients were higher than in healthy controls though a significant difference was not seen (Amabile et al., 2008). In this study, the level of annexin V+ MPs were considerably elevated in control subjects compared to diseased, potentially indicating pre-existing cellular activation and underlying inflammation within the vasculature (Amabile et al., 2008). A lower total MP count would presume to reflect a healthier control group. Hong et al. showed this as their control group, consisting of both adults and children contained, less than 10^5 MPs/ml PPP in venous blood, which was more similar to my results (Hong et al., 2012). The method needed only 100µl of platelet poor plasma for the flow cytometric analyses to determine that MPs in venous blood were considerably higher in PAH than in coronary artery blood in coronary artery disease. This suggests that MPs may be a viable biomarker to use for PAH screening as a less invasive procedure compared to right heart catheterisation that requires minimal sample collection.

Through detecting MPs, I was also able to assess the severity of cell activation in coronary artery blood and forearm venous blood samples from STEMI and NSTEMI patients. The level of circulating annexin V+ MPs detected in blood from the different vascular complications in order of least to higher magnitude was as follows: HIV forearm venous<NSTEMI forearm venous<STEMI forearm venous<NSTEMI coronary artery<STEMI coronary artery<PAH forearm venous.

Smooth muscle MPs were defined as PDGFRβ+/PECAM-1-, endoglin+/PECAM-1-, NG2+/PECAM-1- and ICAM1+/PECAM-1-. All four MP subpopulations were significantly elevated in PAH venous blood significantly compared to normotensive control venous blood samples, coronary artery disease coronary and forearm venous blood, and HIV venous blood, possibly as a result of increased hyperproliferation and activation of smooth muscle cells in PAH. Of the four SMMPs sub-populations analysed in PAH blood samples, PDGFRβ+/PECAM1- MPs had the lowest median while endoglin+/PECAM- MPs had the highest median Nevertheless, all SMMP subpopulations were all significantly elevated in STEMI coronary artery-derived and NSTEMI coronary artery-derived blood compared to healthy controls, but not in forearm venous blood from STEMI and NSTEMI patients. In PAH forearm venous blood as well as STEMI and NSTEMI coronary artery blood, the endoglin+/PECAM1- and NG2+/PECAM1- SMMP subpopulations were detected at higher levels compared to PDGFRβ+/PECAM1- and ICAM1+/PECAM1-, with levels of endoglin+/PECAM1- being the highest. Increased circulating endoglin+ MPs in PAH compared to healthy controls have been reported though levels were higher in the pulmonary artery compared to the

jugular vein (Bakouboula et al., 2008). PDGFRβ+/PECAM- SMMPs were elevated significantly higher in STEMI coronary blood compared to NSTEMI coronary and STEMI forearm venous blood, thereby suggesting that the level of circulating SMMPs present is affected by disease severity and site of blood sampling. The difference in coronary arterial and forearm venous blood was further seen as the former had significantly higher levels of NG2+/PECAM-1- and ICAM-1+/PECAM- SMMPs. A similar trend was seen of endoglin+/PECAM- SMMPs, though the difference was insignificant. Unlike in PAH and CAD, HIV forearm venous blood contained lower SMMPs of each of the four supopulations compared to both control subjects from Malawi and the UK.

Other MPs such as endothelial, leukocyte and platelet MPs were also studied alongside smooth muscle MPs in PAH, coronary artery disease and HIV. E-selectin+/PECAM-1- and PECAM-1+/CD42a- EMPs were significantly elevated in PAH venous blood compared to controls with levels in the similar range as for SMMPs, though the range of total SMMPs was wider for the endogolin+/PECAM- subtype as opposed to the PECAM+/CD42a subtype. E-selectin+ MPs were highly elevated in STEMI coronary blood compared to NSTEMI coronary blood and such levels were comparable with levels in PAH venous blood. That levels of E-selectin+ MPs were significantly elevated in STEMI coronary and forearm bloods and non-significantly elevated in NSTEMI coronary and forearm bloods may result from coronary endothelial damage in coronary (Mutin et al., 1999). Indeed, elevated CD31+/Annexin+ microparticles, thought to be an indicator of endothelial damage correlated with impairment of

coronary endothelial dilatation as assessed by measuring the coronary luminal diameter (Werner et al., 2006). Though this would go against the current dogma my results presented in the previous chapter, which provide evidence that the increase of E-selectin+ MPs may result from activated smooth muscle cells.

CD66b+ LMPs were significantly elevated in PAH compared to normotensive controls indicating increased leukocyte activation in the disease though total numbers of these LMPs were lower than SMMPs and EMPs. TF+ MPs were also significantly elevated, as reported by Bakouboula and colleagues(Bakouboula et al., 2008). These LMPs may be viable biomarker for severe coronary artery disease as their levels in STEMI coronary blood were significantly elevated compared to NSTEMI coronary blood. Interestingly, TF+ leukocyte-derived MPs values were also significantly higher than I found in PAH venous samples, suggesting that leukocyte involvement maybe more active in coronary artery disease within the disease site compared to PAH. However we cannot rule out that MP levels in PAH patients would be higher if pulmonary artery blood samples were taken instead of venous blood, although MP numbers were considerably higher in PAH venous blood than in venous blood from patients with coronary artery disease. Levels for both CD66b+ and TF+ MPs were half as low in HIV patients compared to Malawian non-infected control groups. Tissue factor may also be expressed in multiple cell types including smooth muscle cells and activated monocytes and endothelial cells, thus the number of TF+ MPs may reveal a broader picture of disease pathophysiology than just leukocyte activation (Steffel, 2006). Indeed, agents elevated in PAH such as platelet derived growth

factor and TNFα have been shown to increase TF levels in the medium of growing smooth muscle cells (Schecter et al., 2000). In other studies, it has been reported that CD4+ lymphocyte microparticles and CD11a+ monocytic MPs released from human atherosclerotic placques also expressed TF on the surface of MPs thus adding to the dynamic role of the TF molecule in different vasculopathies (Mallat et al., 1999).

CD42a+ platelet microparticles were elevated in PAH forearm venous blood, STEMI coronary and forearm venous blood, and NSTEMI coronary and forearm venous blood compared to control venous blood. Platelets were highly elevated in PAH and represented 57% of the total Annexin V+ MPs. Nadaud and colleagues showed increased levels of CD31+/CD41+ PMPs in idiopathic, heritable, and associated PAH though to a similar extent in each distinct form of PAH (Nadaud et al., 2013). The CD42a+ PMP levels in coronary blood of STEMI patients were significantly higher than in NSTEMI patients, potentially indicating enhanced platelet activation within the coronary arteries during disease.

The effect of drug therapy on circulating MP count of PAH patients was examined. This study revealed that patients who underwent long-term therapy for a minimum of 4 months (consisting of various combinations of prostacyclin analogues, PDE-5 inhibitor, ETRA or calcium channel blocker) had significantly decreased total annexin V+ MP count compared to prior to therapy. The median levels after therapy were 27% lower than before therapy, but still significantly higher than in control venous blood. Despite this, the lowering of the MP count

still allowed the assessment of the impact of therapy on the patients of different sub-populations. Isolated SMMPs showed there to be a similar trend, with PDGFRβ+/PECAM1- and ICAM1+/PECAM1- SMMP levels being significantly decreased after long-term therapy. This might be expected given that prostacyclin analogues, PDE5 inhibitors and ETRAs have been shown to inhibit PASMC cell proliferation in various studies (Wang et al., 2008; Davie et al., 2009; Falcetti et al., 2010). The number of E-selectin+/PECAM-1- and PECAM-1+/CD42a- EMPs also lowered after therapy. Consistent with this, prostacyclin, and in particular iloprost, is capable of inhibiting the expression of selectins (P and E) and the adhesion molecules ICAM and VCAM in endothelial or inflammatory cells of patients with PAH, systemic sclerosis and peripheral vascular disease (Sakamaki et al., 2000; Zardi et al., 2005; Rehberger et al., 2009). Though the decreases in my studies were not significant, it may lack power due to a limited sample size. Likewise, the CD66+ LMPs and CD42a+ PMPs count decreased after long-term therapy but did not reach significance. This may also have been due to a limited sample size. Interestingly, the level of tissue factor+ MPs remained the same after long-term therapy. The level of CD42a+ PMPs also decreased by 34% after long-term therapy, though this was non-significant. Tamburrelli et al. have shown than the eproprostenol was able to inhibit CD42b PMP release after a stimulating blood with a mixture of collagen and ADP. The synthetic prostacyclin was also capable of inhibiting the formation of platelet mixed conjugates with polymorphonuclear or monocytes as well inhibiting the expression of the adhesion molecule P-selectin and PAC-1 (activated glycoprotein IIb/IIIa) on platelets (Tamburrelli et al., 2011).

Taking a closer look at the specific therapies, prostacyclin analogue treatment with/without a PDE-5 inhibitor induced a significant 44.5% reduction in the level of total annexin V+ MPs, whilst a PDE-5 inhibitor with/without an ETRA induced 17% reduction that was not significant. To note, patients on a prostacyclin analogue with/without a PDE-5 inhibitor had a higher median MP level of $3.826x10^6$ MPs/ml PPP compared to patients on a PDE-5 inhibitor with/without an ETRA (median of $2.003x10^6$ MPs/ml PPP). The addition of the PDE-5 inhibitor sildenafil to long-term intravenous epoprostenol therapy in patients with PAH was studied in the PACES trial, which resulted in greater changes in mean pulmonary arterial pressure, cardiac output, and shorter time to clinical worsening compared to epoprostenol monotherapy (Simonneau et al., 2008). Thus patients with this combination therapy may have resulted in a further decline in MP levels after long-term therapy.

Chapter 5

5.1. Introduction

MPs are submicron vesicles shed into the circulation from the plasma membrane of a variety of cells, including platelet, endothelial, leukocyte and smooth muscle cells, in response to cell activation, injury and/or apoptosis (Simak and Gelderman, 2006; Chironi et al., 2009; Amabile et al., 2013). They were first described as "platelet dust" and were found to be capable of inducing thrombin generation in a similar manner to platelets (Wolf, 1967). MPs are able to have a role in coagulation within the vasculature as their phospholipid surface is rich with phosphatidylserine (PS), a procoagulant anionic aminophospholipid, which is translocated to the external leaflet of the MP membrane during MP formation (Morel et al., 2006). PS is able to activate circulating blood factors, including enhancing the activity of tissue factor (TF; CD142), the main cellular initiator of blood coagulation. Shielding phosphatidylserine rich surfaces decreases the catalytic efficiency of both extrinsic tenase and prothrombinase complexes by 200 and 1000-fold, respectively (Nesheim et al., 1979; Ruf et al., 1991; Morel et al., 2006).

The coagulation cascade is a series of stepwise enzymatic conversions involving the activation of inactive precursors called zymogens required for the formation of fibrin, the ultimate product in the process (Adams and Bird, 2009; Figure 1). There are two pathways involved in the cascade that later converge downstream to produce coagulation: the intrinsic and extrinsic pathways. The intrinsic pathway is dependent on contact activation by a negatively charged surface that is seen on damaged areas within the vasculature and involves coagulation factors XII, XI,

IX, VIII and V. This pathway starts with the formation of factor XIIa, which can cleave prekallikrein to produce kellikrine (McLaughlin et al., 2009). This in turn activates factor XII, which becomes activated when it comes in contact with negatively charged damaged surfaces by undergoing a conformational change to generates factor XIIa (Long et al., 2015). Factor IIa, in the presence of high molecular-weight kininogen, converts factor XI to factor XIa, which then is able to convert factor IX to factor IXa. In presence of calcium and membrane phospholipids, factor IXa binds its cofactor protein factor VIIIa to form the tenase complex (Adams and Bird, 2009), which activates factor X to Xa. Factor Xa is able to bind to its cofactor protein factor Va in the presence of calcium to form the prothrombinase complex and convert prothrombin (factor II) to thrombin (factor IIa), which is the essential component that converts fibrinogen (factor I) to fibrin (factor Ia). The extrinsic pathway also leads to the production of thrombin, but also initiates the expression of TF following vascular trauma. TF is an intrinsic membrane protein located on a variety of cells such as monocytes, endothelial cells, and smooth muscle cells. It is a 47 kDa cell-bound membrane glycoprotein, a member of the class II cytokine superfamily, and functions as both a receptor involved in downstream signalling promoting inflammation, apoptosis, embryonic development and cell migration well as a cofactor for the factors, VII/VIIa (Key et al., 2007; Adams and Bird, 2009). The TF/VIIa complex is able to activate factor X to Xa as well as activate factor IX to IXa (Ruttmann, 2006), thus promoting the generation of thrombin, and ultimately fibrin. Thrombin is able to rapidly convert soluble plasma fibrinogen to an insoluble fibrin polymer as well as activate plasma tranglutaminase (factor XIII), which after being converted to factor XIIIa cross links the fibrin polymers to form a fibrin clot (Lewis et al., 1985).

The generation of thrombin is a central biochemical reaction that is important in the normal homeostasis of the blood and during thrombosis. The thrombin produced can engage in a positive feedback loop where it amplifies its own generation through multiple mechanisms. It can activate factor XI upstream in the intrinsic pathway and generate small amounts of factor XIa which can amplify through its own positive feedback mechanism and increase coagulation. Activation of factor XIa plays an important role when the initiation phase of thrombin generation is prolonged due to low TF levels or elevated levels of coagulation inhibiting proteins (Cawthern et al., 1998). Thrombin can also activate factor VIII, which can be converted to factor VIIIa and form a tenase complex with factor IXa. The tenase complex activates factor X at a 50-100-fold higher rate than the TF/factor VIIa complex, thus giving rise to a "thrombin burst" (Lawson and Mann, 1991). Both thrombin and factor Xa, with the latter being 40-fold more potent, are also able to activate factor VII in the extrinsic pathway, thereby further driving the coagulation process (Radcliffe and Nemerson, 1975). Thrombin is also able to activate factor V, which is important for both extrinsic and intrinsic pathways, further potentiating its generation. Factor V has shown to play an important physiological role as deficiency in platelet factor V is associated with decreased factor Xa binding and bleeding diathesis (Tracy et al., 1984).

There are two types of inhibition that exist for the regulation of thrombin. The first is circulating levels of activated inhibitors of thrombin such as antithrombin III (ATIII), α2-macroglobulin, and Protein C. ATIII inhibits target enzymes by forming complexes and blocking the active site of the enzyme. These target

enzymes including factors like IXa, Xa, Xia and XIIa. α2-macoglobulin is also able to inhibit multiple serine proteases such as thrombin, factor Xa, plasmin and kallikrein. Protein C, a vitamin K-dependent protein that is generated when thrombin is converted to activated protein C is also a coagulation inhibitor. The second type of inhibition is a negative feedback mechanism that involves the TF pathway inhibitor (TFPI), an important regulator of serine proteases that inhibits factor Xa and the factor VIIa/TF/Factor Xa complex (Broze et al., 1988).

MPs contain multiple functional membrane or cytoplasmic effectors such as selectins, glycoprotein IIIa, glycoprotein IIb, von Willebrand factor, arachidonic acid and thromboxane A_2, which can promote pro-thrombotic pathways (Morel et al., 2006). The presence of microparticles as well as activated endothelial cells and other cells enables the formation of the tenase complex, which in turn is able to activate factor X at a high rate, resulting in a thrombin burst (Lawson and Mann, 1991). Furthermore, endothelial MPs have been shown to interact with monocytes and promote TF mRNA expression and TF-dependent procoagulant activity, suggesting that MPs are also able to transfer their procoagulant potential to target cells (Sabatier et al., 2002). Thus, MPs are able to activate both intrinsic and extrinsic pathways in the coagulation cascade as well as pathways involving other target cells, which give rise to their procoagulant phenotype.

MPs induce inflammation in the vascular wall through contact with vascular cells and can upregulate cytokine expression in monocytes and endothelial cells as well as promote leukocyte recruitment and aggregation (Forlow et al., 2000; Nomura et

al., 2001). Consequently, leukocyte MPs can activate endothelial cells to release interleukin-6 and MCP-1 as well as upregulate tissue factor expression through the JNK-1 signalling pathway (Mesri and Altieri, 1998, 1999). In conditions of oxidative stress, EMPs contain oxidised phospholipids that can promote monocyte-endothelial interactions (Huber et al., 2002). PMPs have also been shown to increase adhesion and interactions between the endothelial layer and monocytes (Barry et al., 1998). Of note, PMPs have also been shown to contain IL-1β, which contributes to endothelial inflammation (Lindemann et al., 2001). In addition to providing a proinflammatory stimulus, PMPs have been shown to induce angiogenesis *in vitro* and *in vivo* (Brill et al., 2005). Moreover, PMPs are able to deliver platelet adhesion receptors to hematopoietic stem cells, promoting chemotaxis towards endothelial cells, thereby causing cell adhesion, proliferation and survival (Baj-Krzyworzeka et al., 2002).

Several therapies for cardiovascular disorders in past studies have shown to decrease the level of circulating MPs in disease. Statins have shown to be able to target MPs. Previous studies *in vitro* showed that fluvastatin reduced EMP release on growing human coronary artery endothelial cells stimulated with TNF-α, partially via inhibiting Rho the small GTPases that regulate cytoskeletal remodelling (Tramontano et al., 2004). In patients with type 2 diabetes mellitus, pravastatin caused the decrease of glycoprotein IIb/IIIa (GPIIbIIIa)+ PMPs (Sommeijer et al., 2005). In hypertensive patients, co-administration of simvastatin and the angiotensin II receptor antagonist, losartan, reduced levels of monocyte-derived MPs (Nomura et al., 2004). Anti-platelet treatments such as

GPIIbIIIa antagonists (*i.e.* abciximab and eptifibatide) and thienopyridines (adenosine diphosphate receptor inhibitors) have shown to lower circulating PMP levels (Goto et al., 2003).

Classically, PGI_2 acts through the membrane prostacyclin (IP) receptor, which is expressed on multiple tissues including blood vessels, leukocytes and thrombocytes (Anderson and Nawarskas, 2010). This receptor is coupled to the G stimulatory protein, Gs, which through the activation adenylyl cyclase can generate cyclic adenosine monophosphate (cAMP). This second messenger can exert multiple biological effects, with protein kinase A being a major target protein. PGI_2 and its stable analogues are able to induce vasodilatation, inhibit platelet activation and inhibit cell proliferation, giving a strong rational for using this class of drugs to treat PAH patients (Takubowski et al., 1994; Montani et al., 2013; Clapp and Gurung, 2015; Galie et al., 2015b).

Whilst the mechanism behind the anti-proliferative effects of PGI_2 analogues on normal smooth muscle cells are driven by the cAMP pathway, the anti-proliferative effects of these agents on idiopathic PAH cells were shown to be driven in a cAMP independent manner and seemingly not involving the IP receptor (Falcetti et al., 2010). A 2007 study from our laboratory demonstrated that PGI_2 analogues could inhibit cell proliferation in human embryonic kidney 293 (HEK-293) cells by signalling through the IP-receptor and activating the peroxisome proliferator-activated receptor γ (PPARγ) pathway (Falcetti et al., 2007). Moreover, the PPARγ antagonist GW9662 was able to significantly reverse

the inhibition of proliferation caused by the prostacyclin analogue treprostinil in normal PASMCs but to a greater extent in cells derived from IPAH patients (Falcetti et al., 2010). PPARs are a family of nuclear transcription factors, of which there are three isoforms (α, β, and γ), that are commonly activated by binding to ligands including prostaglandins (ie. 15-Deoxy-delta 12, 14-PGJ_2), fatty acids, lipoxygenase metabolites and a variety of synthetic ligands (Forman et al., 1997). Classically, PPARs are known to bind to retinoid X receptor (RXR) and form a heterodimer which can associate with coactivators and bind to peroxisome proliferator response elements (PPRE) in the nucleus to regulate the expression of target genes involved in cell processes such as inflammation, cell growth and cell differentiation (Nisbet et al., 2007). In addition to directly regulating gene expression, PPARγ is also able to transrepress transcription factors like nuclear factor-κB (NFκB), activated protein-1 (AP-1), nuclear factor of activated T-cells (NFAT) and signal transducer and activator of transcription (STAT) (Abdelrahman et al., 2005). Precipitation experiments in T-cells revealed a direct interaction between NFAT and PPARγ, which led to the suppression of T cell proliferation and activation (Yang, 2000). NFAT is activated by binding to the heterodimeric serine/threonine phosphatase enzyme calcineurin, which is dependent on elevated intracellular calcium for its activation. Upon activation, the NFAT/calcineurin complex is able to translocate into the nucleus and initiate transcription of genes that regulate smooth muscle and cardiac muscle, the immune response and allergic pulmonary inflammatory response (Said et al., 2010). The calcineurin Aβ (CnAβ) isoform was shown to be activated by PDGF-BB and to contribute to smooth muscle cell proliferation in rat aorta by inducing calcineurin-dependent translocation of NFATc3 into the nucleus from the cytosol

(Jabr et al., 2007). PDGF-induced CnAβ activation/nuclear translocation and smooth muscle proliferation were abrogated by the adenylyl cyclase activator forskolin, suggesting that cAMP may also be involved in regulating the NFAT/calcineurin activation (Jabr et al., 2007). Thus, I aimed to investigate whether the mechanism underlying the antiproliferative effects of prostacyclin analogues involved activation of PPARγ and the subsequent inhibition of the translocation of calcineurin Aβ to the nucleus because of its ability to inactivate (transrepress) NFAT.

Results

5.2.1. Thrombin generation by MPs in plasma from healthy patients

Thrombin generation induced by microparticles in healthy microparticle-free plasma was measured over a period of 90 minutes (Figure 45). MPs were isolated from 100µl of platelet poor plasma obtained from treatment naïve patients with a diagnosis of PAH (n=7) or who subsequently had been treated with a combination of a prostacyclin with/without a phosphodiesterase-5 (PDE-5) inhibitor (n=4), PAH patients treated with a PDE-5 inhibitor with/without an endothelin-1 antagonist (n=4). Effects on thrombin generation were compared against MPs isolated from patients with non-ST-elevated myocardial infarction (NSTEMI) and from healthy control subjects (n=10). Peak thrombin levels caused by MPs from PAH treatment naïve patients and from control subjects was 225 nM (range 123-428nM) and 38 nM (range 19-190nM, respectively ($P<0.001$). Thrombin levels in PAH patients after long-term treatment with PGI_2 analogue ± PDE-5 inhibitor 151.8nM (85-173nM; $P<0.05$) and NSTEMI patients 151.8nM (103-222nM; $P<0.05$) were lower than MPs from PAH. Treatment of PAH patients with a PDE-5 inhibitor±ETRA 170nM (114-186nM) caused lower peak thrombin levels compared to treatment naïve PAH patients, though failed to reach significance ($P=0.07$). There was a trend to increased thrombin levels with MPs derived from the venous blood with NSTEMI compared to control subjects, though this was not significant ($P=0.08$).

The endogenous thrombin potential (ETP) as measured by the area under the curve (AUC) was elevated significantly by MPs from PAH treatment naïve

patients 3.72 x 10^4 nM x min (2.3-4.4x10^4nM x min) compared to MPs from control subjects 2.08 x 10^4 nM x min (0.90-3.06 x 10^4 nM x min; P<0.0001) and PAH patients treated with prostacyclin analogue therapy 2.9 x10^4nM x min (1.77-3.13 x10^4nM x min; P<0.05). MPs derived from Non-STEMI patients 2.9 x10^4nM x min (2.43-4.13 x10^4nM x min) also had significantly elevated ETP compared to control subjects (P<0.01), though this was significantly less than in PAH treatment naïve patients (P<0.05). MPs derived from PAH patients treated with a PDE-5 inhibitor ± ETRA 3.22x10^4nM x min (2.45-3.58x10^4nM x min) also had decreased ETP compared to PAH naïve, though this was not significantly different, largely due to the increase in length of time thrombin remained elevated under these conditions (P=0.119).

The time taken to reach the peak level of thrombin generated was significantly lower with MP samples taken from treatment naïve PAH compared to control subjects patients being 25 minutes (15.5-28.5 min) to peak as opposed to 31.8 minutes (28.5-41 min; P<0.0001). After treatment, the time taken for thrombin generation to reach peak levels in both treatment groups of PAH patients treated with either a PGI_2 mimetic ± PDE-5 inhibitor 152 minutes (85.2-173.4 min; P<0.01) or PDE-5 inhibitor ± ETRA 170 minutes (114.6-185.5 min; p<0.05) was lengthened. The peak time was significantly greater for MPs from non-STEMI patients 33.5 minutes (28.5-38.5 min) compared to PAH patients (P<0.0001).

The rate of thrombin generated in healthy plasma was elevated 3 fold for MPs from PAH treatment naïve patients 32.13nM/min (14.89-91.21nM/min) compared

to control subjects 10.28nM/min (3.75-26.52nM/min; P<0.001) and was double that seen with MPs isolated from non-STEMI patients (P<0.001). In MPs isolated from PAH patients treated with PGI_2 mimetic ± PDE-5 inhibitor therapy, this significantly fell to 14.67nM/min (10.66-20nM/min; P<0.05) compared to untreated PAH patients. While the rate of thrombin generation for MPs isolated from PAH patients after long-term PDE-5 inhibitor ± ETRA therapy also fell to 20.02nM/min (12.19-21.36nM/min; P<0.001), this decrease just failed to reach significance (P=0.055). The velocity index for MPs derived from the venous blood of non-STEMI patients was not significantly different from control subjects (P=0.313).

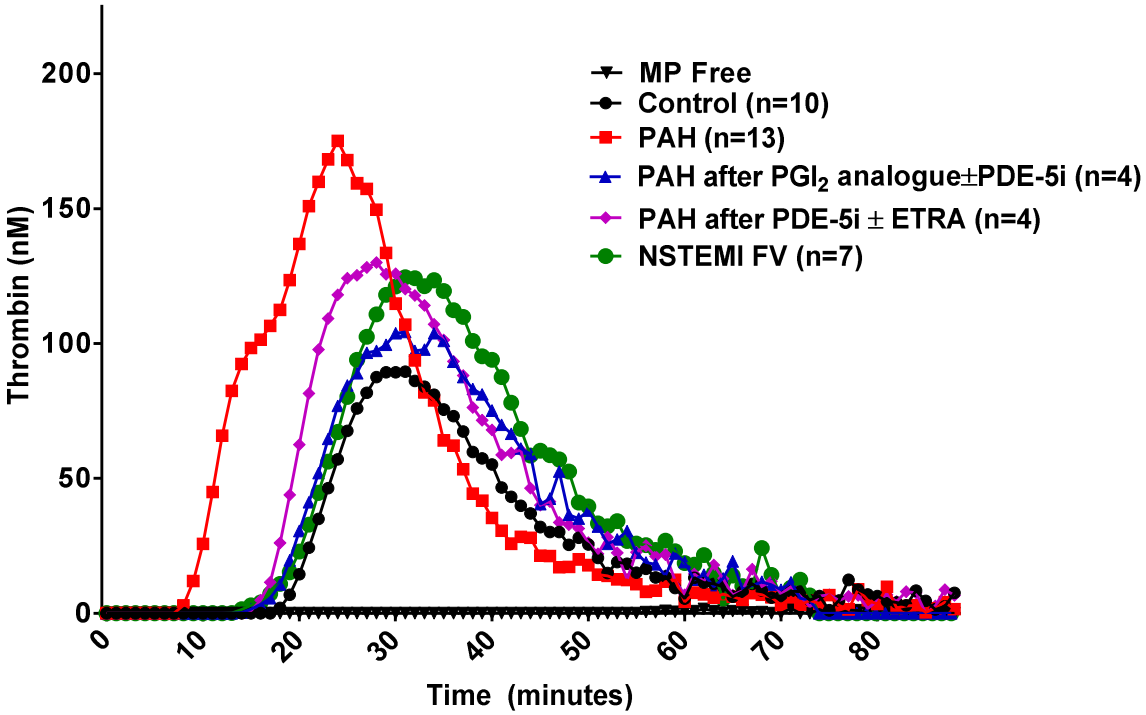

Figure 45. Representation of thrombin generation curves induced by circulating MPs

Representation of thrombin generation curves induced by microparticles derived from the venous blood of patients with pulmonary arterial hypertension (naive or treated) and non-ST-elevated myocardial infarction (STEMI). Isolated microparticles were administered in equal volume (100 µl plasma) to microparticle-removed plasma from healthy volunteers. Thrombin generation (nM) measured over 90 minutes using a calcium fluorogenic substrate which is cleaved during thrombin generation and detected using a fluorescence reader. Therapy included 1) a prostacyclin mimetic with/without a phosphodiesterase-5 inhibitor (PDE-5i), or 2) a PDE-5i with/without an endothelin-1 receptor antagonist (ETRA). Data is presented as mean values.

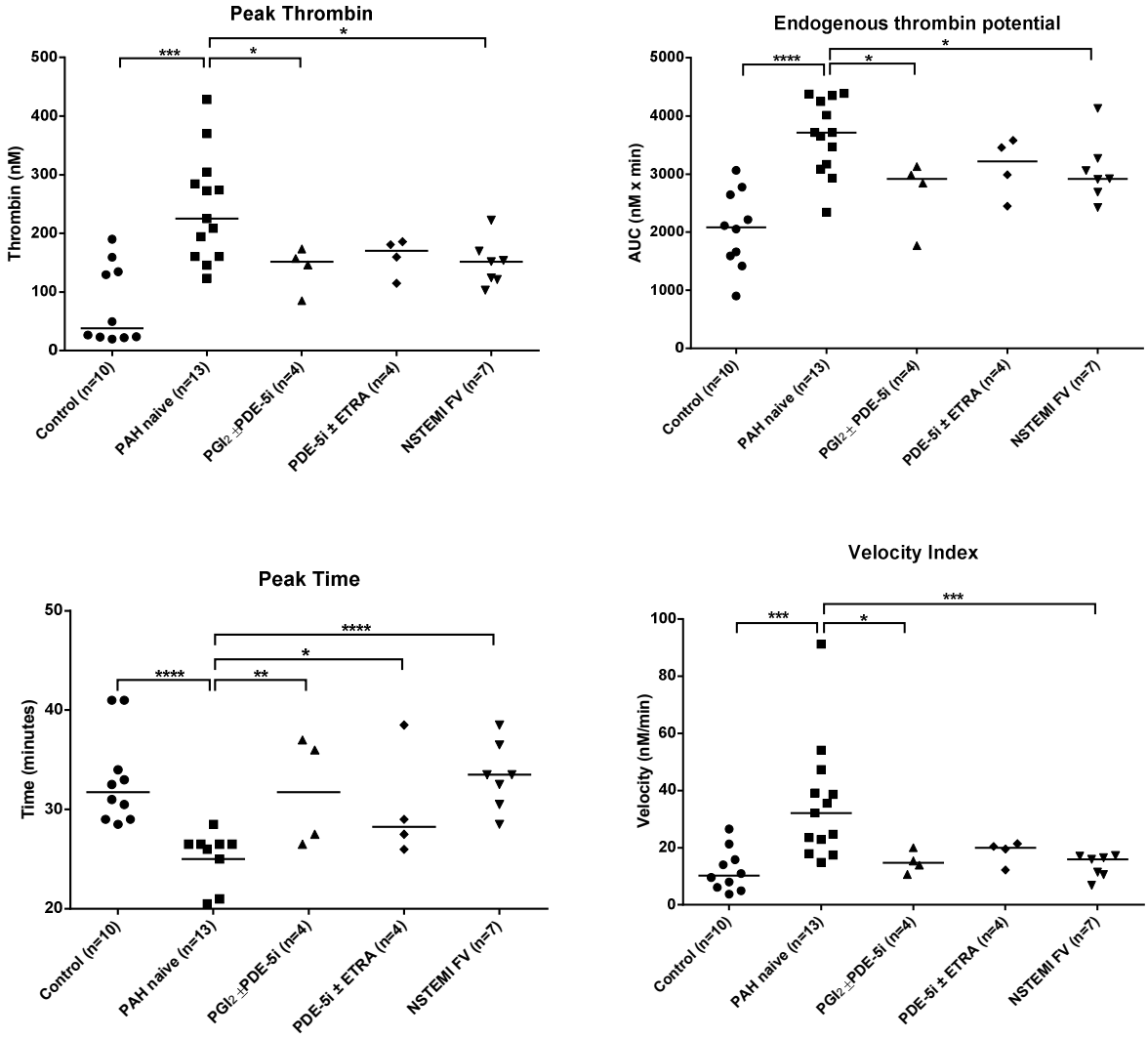

Figure 46. Analysis of circulating microparticle-induced thrombin generation in PAH and Non-STEMI patients

Microparticle-induced thrombin generation in patients diagnosed with pulmonary arterial hypertension (n=7) and non-ST-elevated myocardial infarction (STEMI; n=7). Microparticles were isolated from the venous blood and administered to microparticle-free plasma obtained from healthy volunteers (n=10). Thrombin generation (nM) was measured over 90 minutes. Therapy included 1) an prostacyclin analogue with/without a phosphodiesterase-5 (PDE-5i; n=4), or 2) a PDE-5i with/without an endothelin-1 receptor antagonist (ETRA; n=4). Levels of peak thrombin (nM), endogenous thrombin potential (AUC), time to peak (minutes) and velocity index (nM x min^{-1}) were recorded and analysed. Data are represented as median; *=P<0.05; **=P<0.01; ***=P<0.001, with Mann-Whitney U Test.

5.2.2. Sensitivity/specificity analysis of peak thrombin and endogenous thrombin potential for identification of pulmonary arterial hypertension recurrence

Receiver operator characteristic curve analysis was performed to examine the sensitivity and specificity of peak thrombin and endogenous thrombin potential (ETP) of patient-derived microparticles in the diagnosis of pulmonary arterial hypertension (Figure 47). Peak thrombin and ETP levels of microparticles from PAH patients (n=13) were compared with healthy controls (n=10). The diagnostic performance test for identifying disease recurrence with peak thrombin levels was significant with area under the curve (AUC) 0.9385, standard error (SE) 0.0469, 95% confidence interval (CI) 0.846-1.031, and P=0.0004. The diagnostic performance test for identifying disease recurrence was with endogenous thrombin potential levels was significant with area under the curve (AUC) 0.9592, standard error (SE) 0.031, 95% confidence interval (CI) 0.9092-1.029, and P=0.0001567.

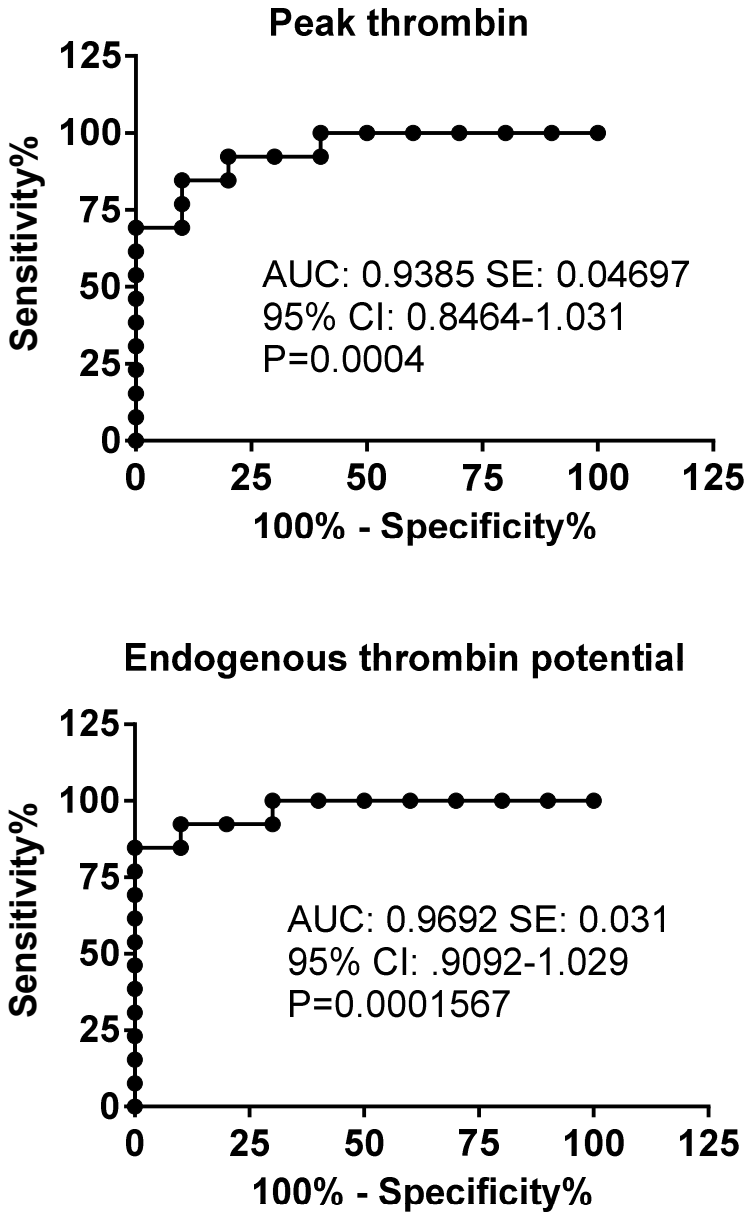

Figure 47. Receiver operator characteristic curve for peak thrombin and endogenous thrombin potential for identification of PAH recurrence

Receiver operator characteristic curve for peak thrombin and endogenous thrombin potential for the identification of pulmonary arterial hypertension recurrence (n=13 PAH vs n=10 controls). ROC analysis was significant for endogenous thrombin potential with the area under the curve (AUC) = 0.9692, standard error (SE) = 0.031, 95% CI = 0.9020-1.029, and P=0.0001567. The ROC analysis was also significant for peak thrombin with the AUC = 0.9385, SE=0.04697, 95% CI = 0.8464-1.031, and P=0.0004.

5.2.3. Peak thrombin and endogenous thrombin potential correlated with total number of annexin V+ microparticles

Peak thrombin and endogenous thrombin potential levels of PAH patient-derived microparticles were correlated with total annexin V+ MP numbers (n=13; Figure 48). Peak thrombin levels positively correlated with total annexin V+ MP levels and was significant with $r=0.705$ and $p<0.0137$ using the Spearman correlation test. The ETP of total microparticles also correlated positively and was significant with $r=0.5714$ and $p<0.045$.

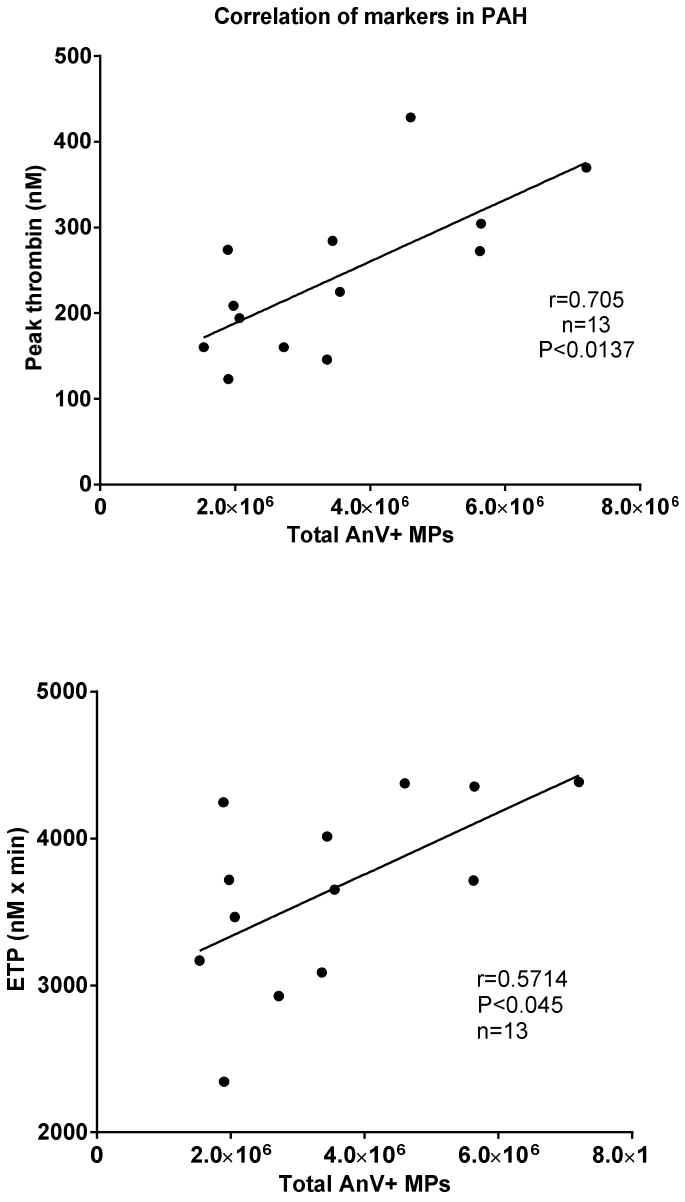

Figure 48. Total annexin V+ microparticles correlated with peak thrombin and endogenous thrombin potential in PAH

Levels of peak thrombin (top) and endogenous thrombin potential (bottom) of microparticles from patients with pulmonary arterial hypertension (n=13) plotted against total annexin V+ microparticle number. There was a significant positive correlation between peak thrombin and total microparticle number (r=0.75 and P<0.0137 using the Spearman correlation test). The endogenous thrombin potential, measured as the area under the curve of the thrombin generation profile was also significantly correlated with total microparticle number with r=0.57 and p<0.045.

5.2.4. Generation of thrombin in healthy plasma by smooth muscle microparticles and human umbilical cord vein endothelial cell microparticles

Thrombin generation was compared between a fixed number of microparticles (10^5 MPs) derived from cultured smooth muscle cells isolated from PAH patients and from human umbilical vein endothelial cells (HUVECs). The peak thrombin level, total thrombin generated over time (endogenous thrombin potential), time taken to reach peak thrombin levels (peak time) and rate of thrombin generation (velocity index) were measured. The PAH patient derived SMMPs caused a significantly greater (by 31%) peak thrombin level (203±15.54nM) compared to the endothelial microparticles (140.3 ± 3.31nM; $P<0.05$). SMMPs had significantly elevated endogenous thrombin potential (2515±77.29 nM x min) compared to the EMPs (2103 ± 53.11nM x min; $P<0.05$). The time taken to reach peak levels was also significantly lower in SMMPs (8. 7±0.601 min) than EMPs (16.3 ±0.667 min). The rate of thrombin generation caused by SMMPs (47.38±9.68 nM x min^{-1}) was two-fold higher compared to that of EMPs (21.72±2.60 nM x min^{-1}).

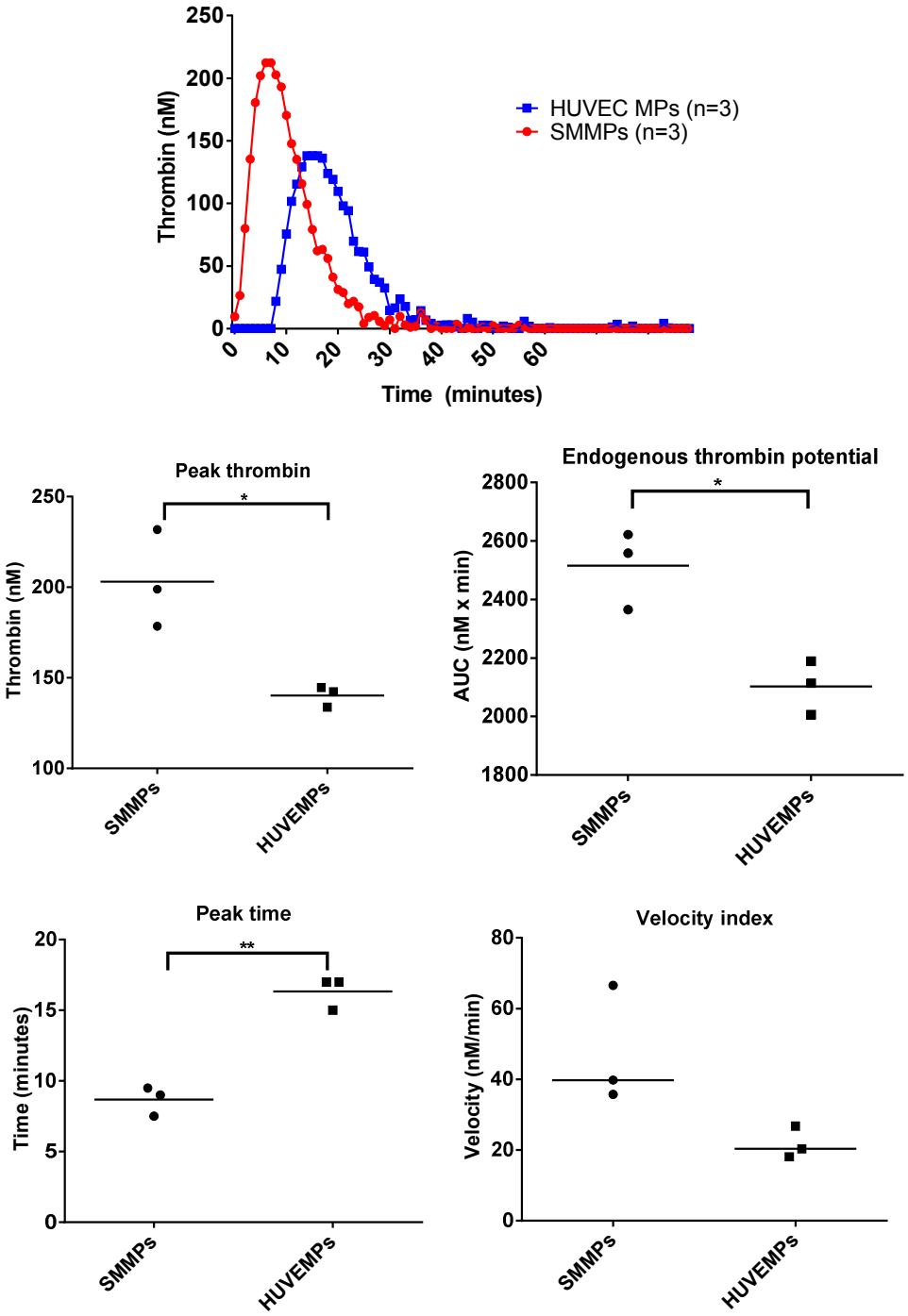

Figure 49. Analysis of thrombin generation by SMMPs from PAH cells and EMPs from HUVECs

Thrombin generation was compared between smooth muscle cells of PAH patients and human umbilical vein endothelial cells (HUVECs). The peak thrombin level (nM), total thrombin generated over time (endogenous thrombin potential), time taken to reach peak thrombin levels (peak time), and rate of thrombin generation (velocity index) were measured. (Data is represented as mean±S.E.M; *=P<0.05; **=P<0.01; n=3.)

5.2.5. Prostacyclin analogue-mediated inhibition of microparticle release by pulmonary arterial smooth muscle cells

Given that certain growth factors and cytokines are elevated in PAH which lead to raised MP levels, I wished to assess the impact of antiproliferative prostacyclin analogue treprostinil on inhibiting MP. Smooth muscle cells were grown in culture until about 70% confluence and were then stimulated with 20ng/ml PDGF-BB, 20ng/ml TNF-α, 5ng/ml TGF-β, and 10nM ET-1 alone and in combination with 1μM treprostinil. Cells were stimulated for 24 hours in growth media containing 10% foetal bovine serum plus growth factors and drugs, the supernatants collected, and total annexin V+ MPs measured. 1μM treprostinil was able to inhibit microparticle release by smooth muscle cells induced by various growth factors though the degree to which it did, depended on the growth stimulus (Figure 50).

The addition of 20ng/ml PDGF-BB to 10% serum significantly increased SMMP numbers (142670±20046 MPs/ml/10^4 cells) compared to serum alone (66521±6958 MPs/ml/10^4 cells; $P<0.01$; n=4). Treprostinil completely abolished the PDGF-BB and 10% FBS-induced MP release to 49062±8005 MPs/ml/10^4 cells ($P<0.01$; n=4) back to slightly below levels seen by serum alone.

Administration of 20ng/ml TNF-α to growing cells caused ~1.75-fold increase in MP number (117306±18731 MPs/ml/10^4 cells) compared to serum alone (66521±6958 MPs/ml/10^4 cells), though this failed to reach significance. Treprostinil inhibited the TNF-induced release by 44% to 77741±27363

MPs/ml/10^4 cells, though the difference was not significant due to a large variation in the response to treprostinil.

5ng/ml TGF-β in combination with serum significantly increased MP number by 3-fold (216879±2554 MPs/ml/10^4 cells) compared to serum alone (69572±8844 MPs/ml/10^4 cells; $P<0.001$; n=3). This increase was significantly inhibited by 1μM treprostinil by 56% (121705±18518 MPs/ml/10^4 cells).10nM ET-1 in the presence of 10% serum caused nearly a 3.5-fold increase in MP count (235522±32627 MPs/ml/10^4 cells) compared to serum alone (66521±6958 MPs/ml/10^4 cells; $P<0.01$; n=4). The addition of 1μM treprostinil significantly inhibited the release MPs by 66% to 102469±25833 MPs/ml/10^4 cells ($P<0.01$; n=4).

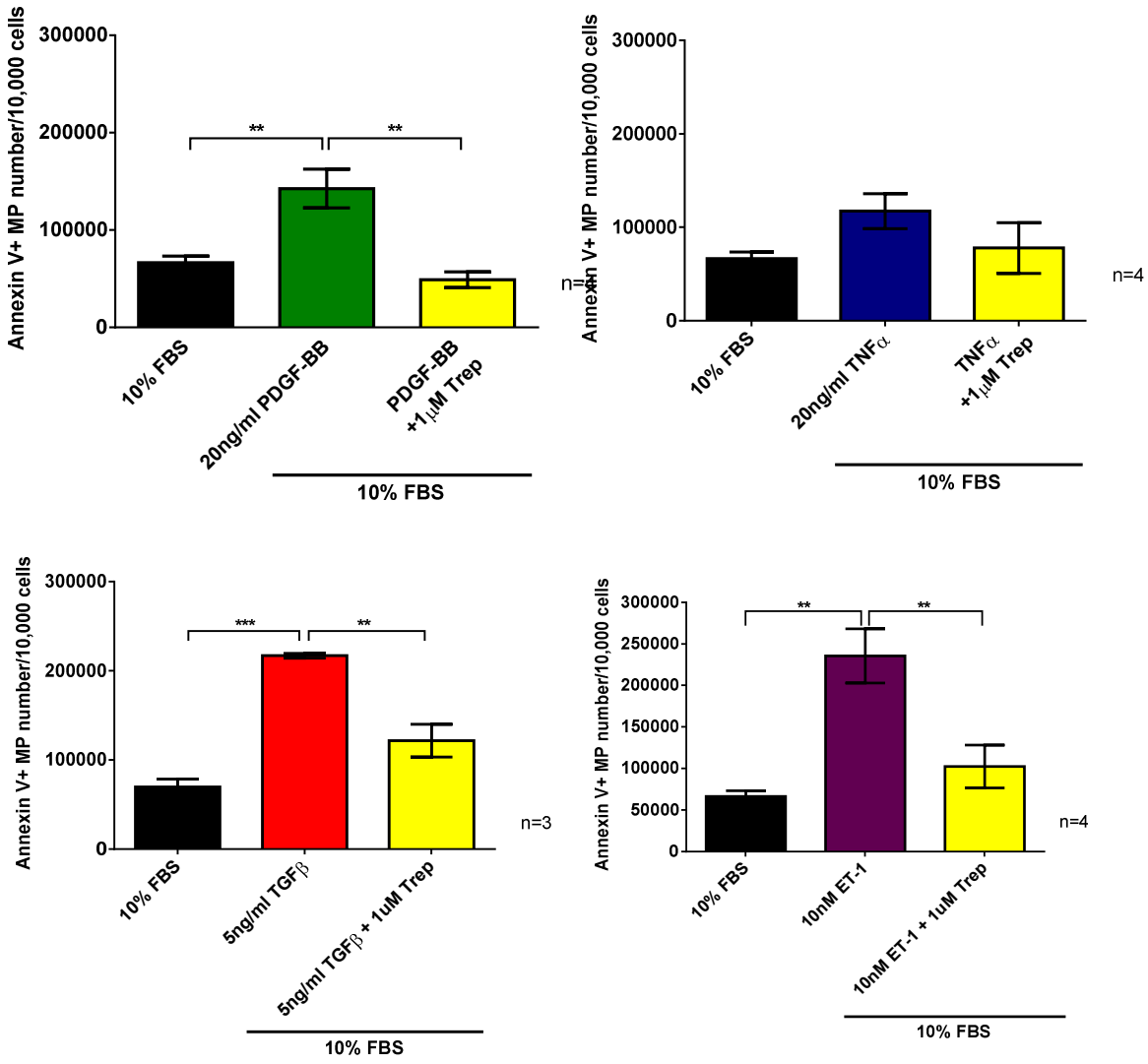

Figure 50. The prostacyclin analogue treprostinil inhibited microparticle release by PASMCs from PAH patients after cell stimulation.

Smooth muscle cells were grown in culture until 70% confluence and were stimulated with 20ng/ml PDGF-BB, 20ng/ml TNF-α, 5ng/ml TGF-β, and 10nM ET-1 alone and in combination with 1μM treprostinil in growth media with 10% foetal bovine serum. Cells were stimulated for 24 hours, supernatants were collected, and total annexin V+ MPs were measured. (Data is represented as mean±S.E.M (n=3-4); **=P<0.01; ***=P<0.001; One-way ANOVA was performed.

5.2.6. Dose-dependent of treprostinil on smooth muscle microparticle release

The prostacyclin analogue treprostinil inhibited MP release by PDGF-BB in a dose-dependent manner (Figure51). Pulmonary artery smooth muscle cells from PAH patients were grown in culture until 70% confluent and stimulated for 24 hours with 0.1% foetal bovine serum (FBS), 10% FBS ± 20ng/ml platelet-derived growth factor (PDGF) with increasing concentrations of treprostinil). Supernatants were collected and total annexin V+ MPs were measured and normalised to cell number.

Total annexin V+ SMMP levels were 3-fold higher after 10% serum stimulation compared to 0.1% serum (77329±15097 MPs/ml/10^4 cells). The total SMMP number significantly increased by 2-fold after the administration of 20ng/ml PDGF+10% FBS to 152672±102677 MPs/ml/10^4 cells compared to serum alone (77329 MPs/ml/10^4 cells; $P<0.01$). The SMMP number started to decline at concentrations above 1nM and this increased in a dose-dependent manner. Co-administration of 100nM treprostinil significantly ($P<0.05$, n=5) decreased SMMP numbers (92767±12886 MPs/ml/10^4 cells) by 39% compared to 20ng/ml PDGF+10% FBS The addition of 1μM treprostinil induced the greatest inhibition in MP release by 61% (59916±18896 MPs/ml/10^4 cells) compared to 20ng/ml PDGF+10% FBS ($P<0.001$). Unexpectedly, SMMP levels were higher in the presence of 10μM treprostinil compared to lower (0.03-1μM), indicating a weaker inhibition in SMMP release at this higher concentration, perhaps indicative of activation of opposing pathways.

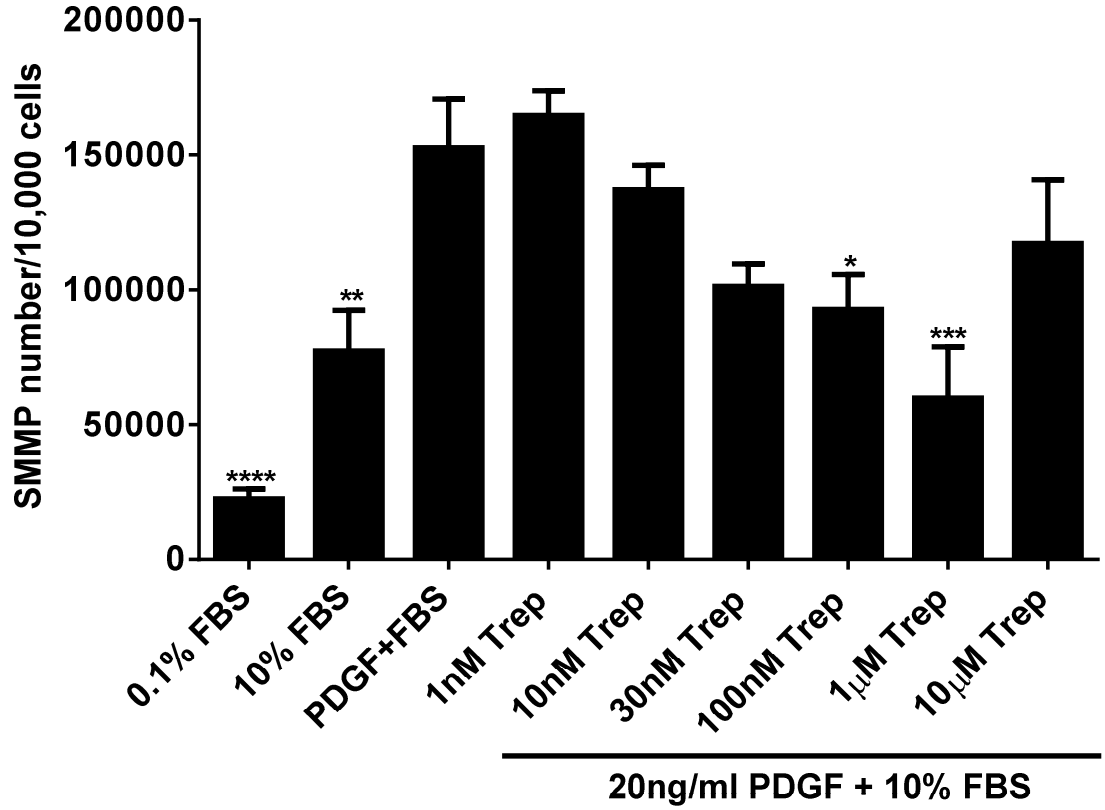

Figure 51. The prostacyclin analogue treprostinil inhibits microparticle release by PDGF-BB in a dose-dependent manner.

Pulmonary artery smooth muscle cells from PAH patients were grown in culture until 70% confluent and stimulated for 24 hours with 0.1% foetal bovine serum (FBS), 10% FBS ± 20ng/ml platelet-derived growth factor (PDGF) with increasing concentrations of treprostinil (0.001-10μM). Supernatants were collected and total annexin V+ microparticles were measured. Data is presented as mean ± S.E.M. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001, one-way ANOVA with Holm-Sidak's multiple comparisons test with respect to 20ng/ml PDGF+10% FBS (n=5).

5.2.10. Smooth muscle microparticle-induced proliferation of normal smooth muscle cells

Next I wished to assess if microparticles could stimulate or enhance the proliferation of PASMCs derived from control patients. 10^5 MPs derived from PASMCs from PAH patients were used to stimulate 10^4 growth arrested cells per well in a 96-well plate.

10% FBS induced a two-fold increase in proliferation of smooth muscle cells compared to 0.1% FBS ($P<0.0001$; Figure 52). Likewise 20ng/ml PDGF-BB significantly increased cell proliferation compared to 0.1% FBS alone by 80% ($P<0.0001$). The addition of smooth muscle MPs significantly increased cell proliferation by 37% compared to 0.1% FBS ($P<0.05$), though to a lesser extent. In contrast, administration of TGF-β plus 0.1 FBS% did not induce cell proliferation over and above what was observed under basal conditions (0.1% FBS).

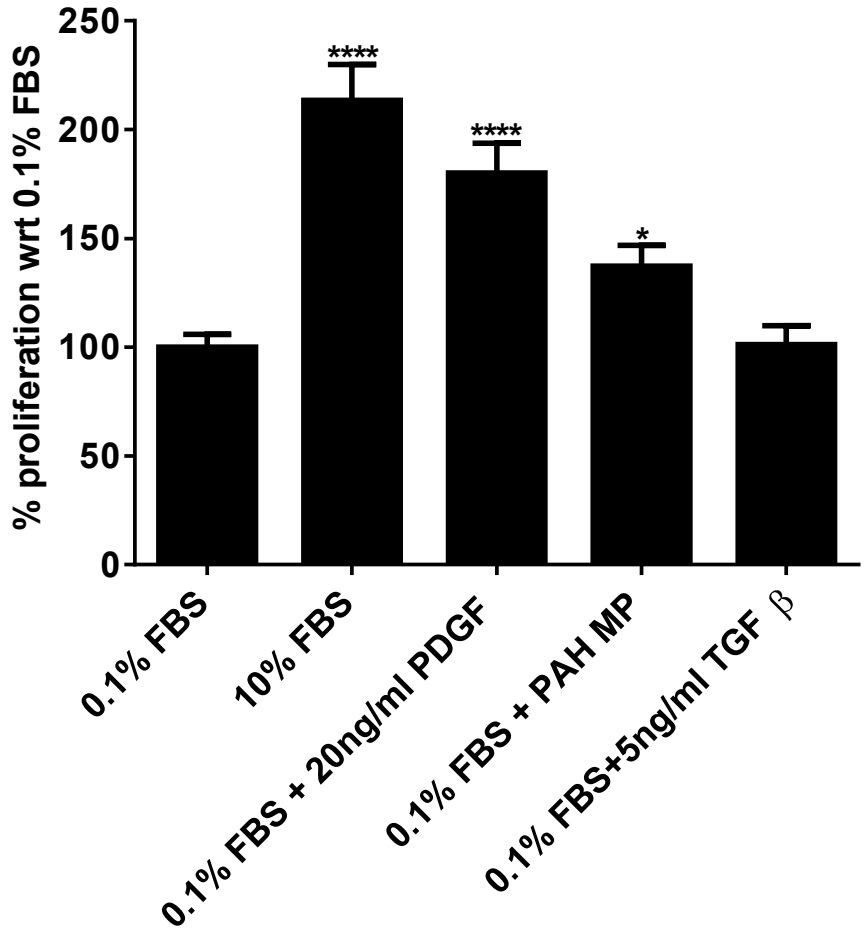

Figure 52. Smooth muscle MPs induced PASMC growth *in vitro*.

: A fixed number (10^5) of MPs derived from cultured PASMCs of PAH patients induced proliferation of growth arrested PASMCs from healthy donors. PASMCs were plated into a 96-well plate at a density of 10^5 cells/ml, arrested in 0.1% FBS for 48 hours and treated with 0.1% FBS, 10% FBS, 0.1% FBS+20ng/ml PDGF-BB, 0.1% FBS+ PAH MPs, and 0.1%+5ng/ml TGF-β. The cells were left to grow for 96 days and the % proliferation with respect to 0.1% FBS was measured. Data are presented as mean ± S.E.M. *=P<0.05; ****=P<0.0001, with respect to 0.1% FBS (One-way ANOVA with Holm-Sidak's multiple comparisons test was used; n=3 patient isolates with 5 repeats).

5.2.7. Effect of prostacyclin receptor and prostaglandin E_2 receptor antagonists prostacyclin-mediated inhibition of microparticle release

Treprostinil is known to potently bind, not only to the IP receptor, but EP_2 receptors as well (Whittle et al., 2012), with both receptors shown to contribute to the antiproliferative effects of treprostinil (Falcetti et al., 2010; Patel et al., 2015). Thus microparticle release was examined in PASMCs isolated from PAH patients that were grown in culture until 70% confluent and stimulated for 24 hours with 10% foetal bovine serum (FBS) alone and with 20ng/ml (PDGF in combination with 100nM treprostinil, 1μM of the IP receptor antagonist (IPRA), R01138452, 1μM of the EP_2 receptor antagonist (EP2RA), PF04418948 or a combination of the two antagonists. The supernatants were collected, and total annexin V+ microparticles measured. Inhibition of PDGF and serum-induced microparticle release by 100 nM treprostinil was reversed with either a prostacyclin or a prostaglandin EP_2 receptor (Figure 53). Microparticles release was increased 2-fold by the addition of 20ng/ml PDGF-BB and 10% FBS compared to serum alone. The addition of 100nM treprostinil significantly inhibited MP release caused by 20ng/ml PDGF+10% FBS ($P<0.05$). When treprostinil and 1μM IPRA was added in combination with 20ng/ml PDGF with 10% FBS, it reversed the inhibitory effects of treprostinil on MP release, though this just failed to reach significance ($P=0.074$). Likewise, the EP2RA at 1μM reversed the effect of treprostinil, though not significantly ($P=0.251$). The combination of both antagonists did however significantly ($P<0.01$) reverse the inhibitory effects of treprostinil on MP release.

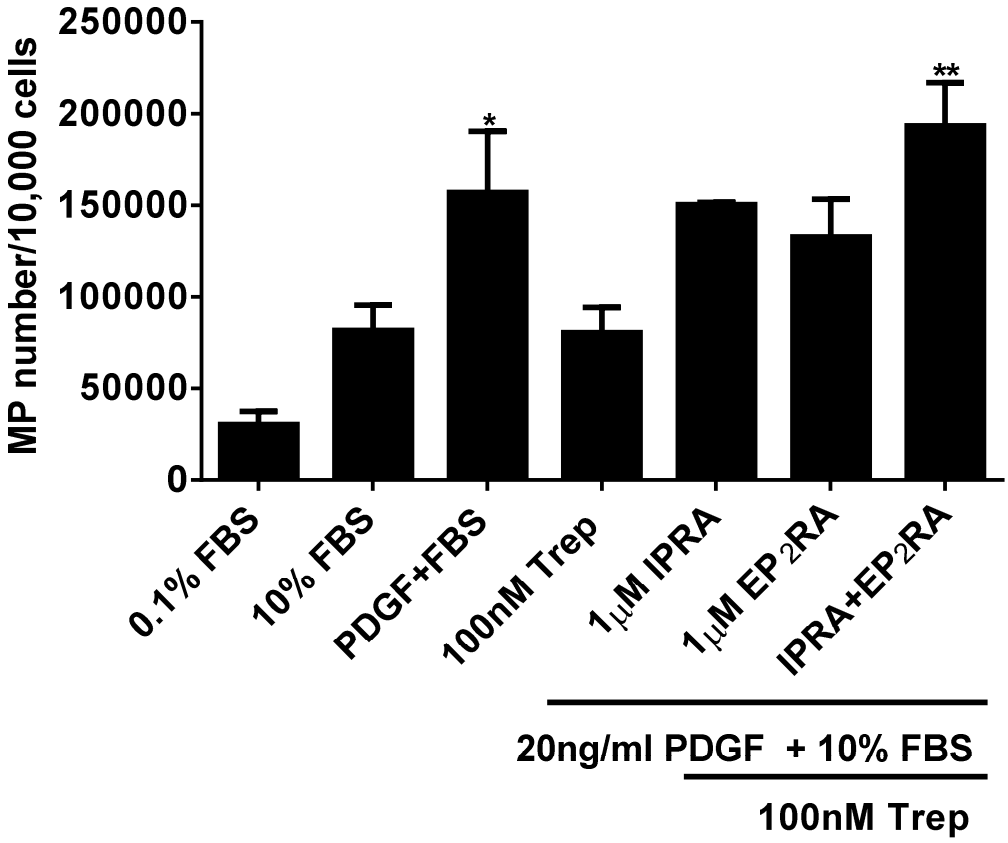

Figure 53. Treprostinil-induced smooth muscle microparticle release inhibition through the IP_2 and EP_2 receptors.

The inhibitory effects of treprostinil on PDGF-mediated microparticle release, was reversed by a combination of a prostacyclin and prostaglandin EP_2 receptor antagonist. Pulmonary artery mooth muscle cells from PAH patients were grown in culture until 70% confluent and stimulated for 24 hours with 10% foetal bovine serum (FBS) alone and with 20ng/ml platelet-derived growth factor-BB (PDGF) in combination with 100nM treprostinil in the absence and presence of 1μM R01138452 (IP receptor antagonist; IPRA), 1μM PF04418948 (EP_2 receptor antagonist; EP2RA) and a combination of both antagonists. Cell culture supernatants were collected and the total annexin V+microparticles were measured. Data are presented as mean±S.E.M. **=P<0.01, ***=P<0.001 (n=4). One-way ANOVA with Dennett's multiple comparisons test was used with respect to 100nM Trep+20ng/ml PDGF-BB+10% FBS

5.2.8. Expression and activation of calcineurin Aβ in pulmonary arterial smooth muscle cells

Calcineurin-Aβ (CnAβ) activation plays an important role in driving PASMC proliferation and is known to be inhibited by PPARγ signalling. As prostacyclin has been shown to act through PPARγ to inhibit cell growth, I wanted to investigate whether the prostacyclin analogue treprostinil may be signalling through PPARγ to inhibit CnAβ, and in turn suppress PASMC proliferation. The expression and nuclear translocation in PASMCs from PAH patients was investigated. Cells were growth arrested for 48 hours and treated for 24 hours with 0.1% foetal bovine serum (FBS), 10% FBS alone and in combination with 1μM treprostinil, the PPARγ activator, rosiglitazone (1μM) and the PPARγ antagonists GW9662 (1μM) and T00701 (1μM) as shown (Figure 54).

The expression of CnAβ as assessed by the intensity of fluorescent staining outside the nucleus was increased after treatment with 10% FBS, as was the presence of CnAβ within the nucleus. The addition of the PPARγ antagonist GW9662 at 1μM increased the nuclear presence of CnAβ compared to 10% FBS, as did 1μM of the selective PPARγ antagonist T00701 though to a slightly lesser extent. 1μM treprostinil treatment abolished the effect of serum and dropped CnAβ expression and its nuclear presence to levels lower than by 0.1% FBS. Blockade of PPARγ by GW9662 and T00701 increased the CnAβ expression and nuclear presence, though not to levels of the antagonists alone. CnAβ expression

was slightly lower with 1μM rosiglitazone compared to serum alone, as were its presence in the nucleus.

The nuclear occupancy was quantified through confocal image analysis to study calcineurin activation in the PASMCs (n=3; 24 cells total per treatment; Figure 55). Nuclear occupancy of CnAβ was increased in cells growing in 10% FBS (22.64%) compared with cells grown in 0.1% serum (11.06%) for 24 hr. 1μM treprostinil significantly ($P<0.01$) reduced the nuclear occupancy by 67% (7.53%) compared to 10% serum. Rosiglitazone also reduced nuclear occupancy to 15%, though not significantly. There was some increase in CnAβ nuclear occupancy with GW9662 (30.17%) compared to 10% serum alone, suggesting basal PPARγ activity maybe affecting calcineurin activation. Co-administration of 1μM GW9662 and 1μM treprostinil significantly reversed the effect of treprostinil by doubling the nuclear occupancy to 20.77% ($P<0.05$). Similarly, 25.14% he combination of T00701 and 1μM treprostinil significantly reversed the effect of treprostinil by increasing the nuclear occupancy to 19.79% ($P<0.05$), slightly below that observed in the presence of T00701 alone (25.1%).

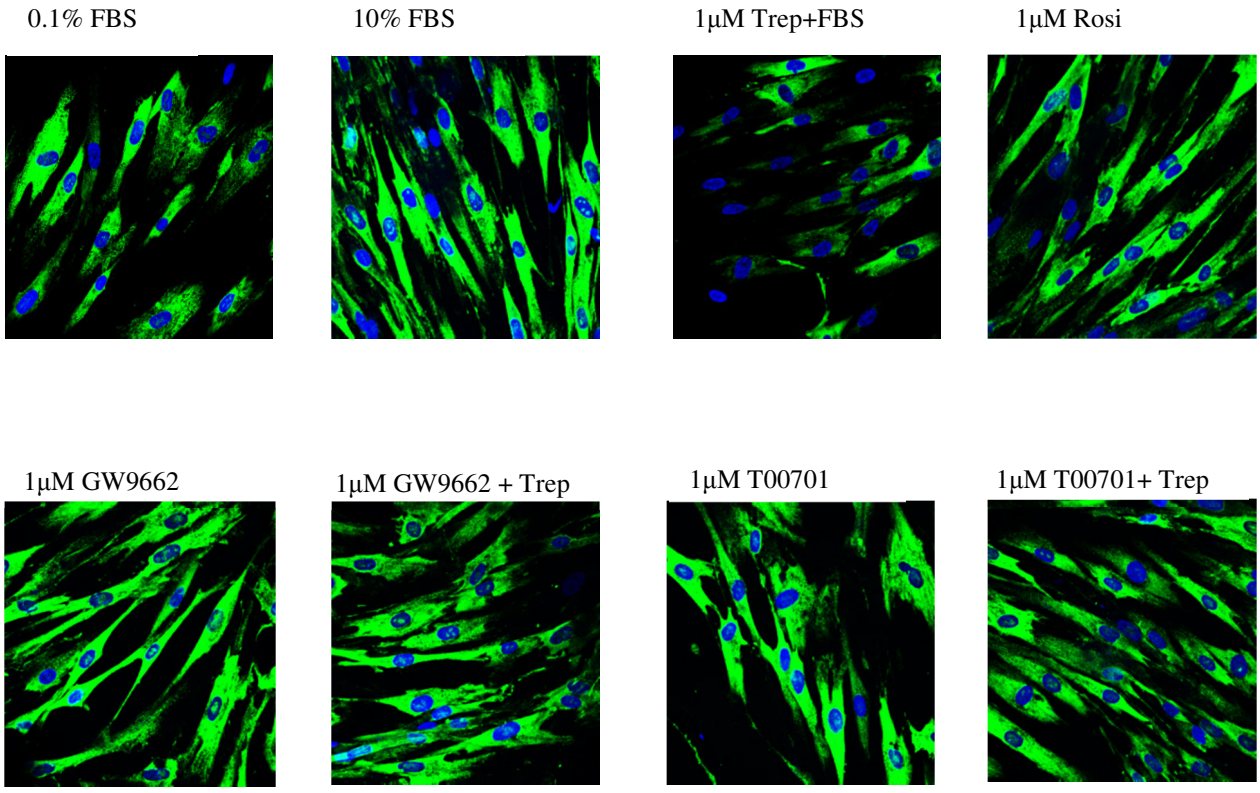

Figure 54. Calcineurin Aβ exression and activation in PASMCs from PAH patients by treprostinil involves activation of the PPARγ pathway.

Cells were plated in 8-well chambered and growth arrested for 48 hours, then treated for 24 hours with 0.1% foetal boviane serum (FBS), 10% FBS alone and in combination with 1μM treprostinil, 1μM rosiglitazone, 1μM GW9662±1μM treprostinil and 1μM T00701±1μM treprostinil. Cells were immunostained for calcineurin Aβ and imaged via confocal microscopy. The images were analysed using the ImageJ software and the % of nuclear occupancy of calcineurin Aβ representing calcineurin-Aβ activation was calculated. (Data is represented as mean±S.E.M; *=P<0.05; **=P<0.01; One-way ANOVA with Tukey's multiple comparisons test was used; n=3).

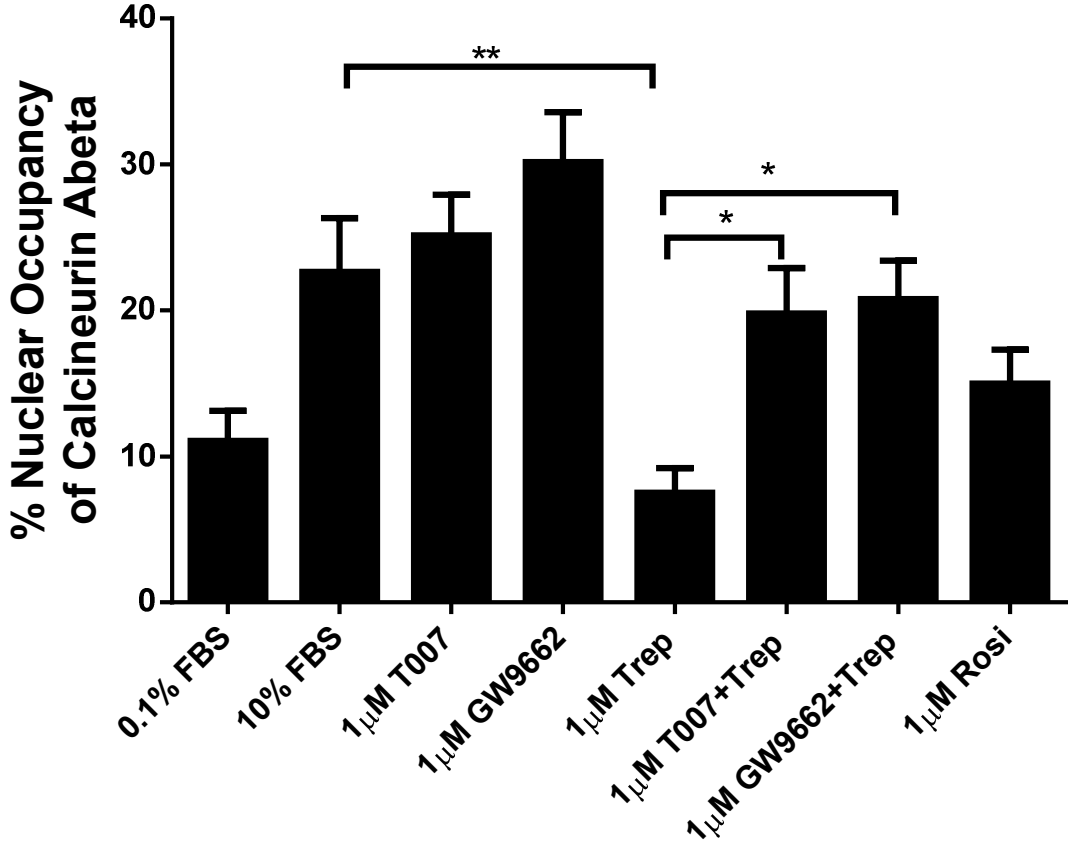

Figure 55. Analysis of nuclear occupancy of Calcineurin Aβ in PASMCs from PAH patients by treprostinil

Inhibition of calcineurin-Aβ expression and activation in pulmonary arterial smooth muscle cells from PAH patients by treprostinil involves activation of the PPARγ pathway. Cells were plated in 8-well chambered and growth arrested for 48 hours, then treated for 24 hours with 0.1% foetal boviane serum (FBS), 10% FBS alone and in combination with 1μM treprostinil, 1μM rosiglitazone, 1μM GW9662±1μM treprostinil and 1μM T00701±1μM treprostinil. Cells were immunostained for calcineurin Aβ and imaged via confocal microscopy. The images were analysed using the ImageJ software and the % of nuclear occupancy of calcineurin Aβ representing calcineurin-Aβ activation was calculated. (Data is represented as mean±S.E.M; *=P<0.05; **=P<0.01; One-way ANOVA with Tukey's multiple comparisons test was used; n=3).

5.2.9. Inhibition of proliferation of pulmonary arterial smooth muscle cells by treprostinil and cyclosporine A

Given the above results showing that treprostinil inhibited not only CnAβ expression but its nuclear translocation, I wished to assess the role of calcineurin in regulating cell proliferation in in PASMCs isolated from PAH patients. Proliferation induced by serum in the presence of DMSO was significantly and similarly inhibited by 100 nM treprostinil or by the calcineurin inhibitor, cyclosporine A at 1μM (Figure 56). When the two agents were combined, cell proliferation was further inhibited, though not significantly. At the higher dose of treprostinil (1μM), a greater inhibition of cell proliferation was observed with further inhibition when combined with cyclosporine. The enhanced antiproliferative effects of treprostinil in the presence of cyclosporine were however less than the effect of either agent alone, suggesting some crossover of mechanism of inhibition of cell growth.

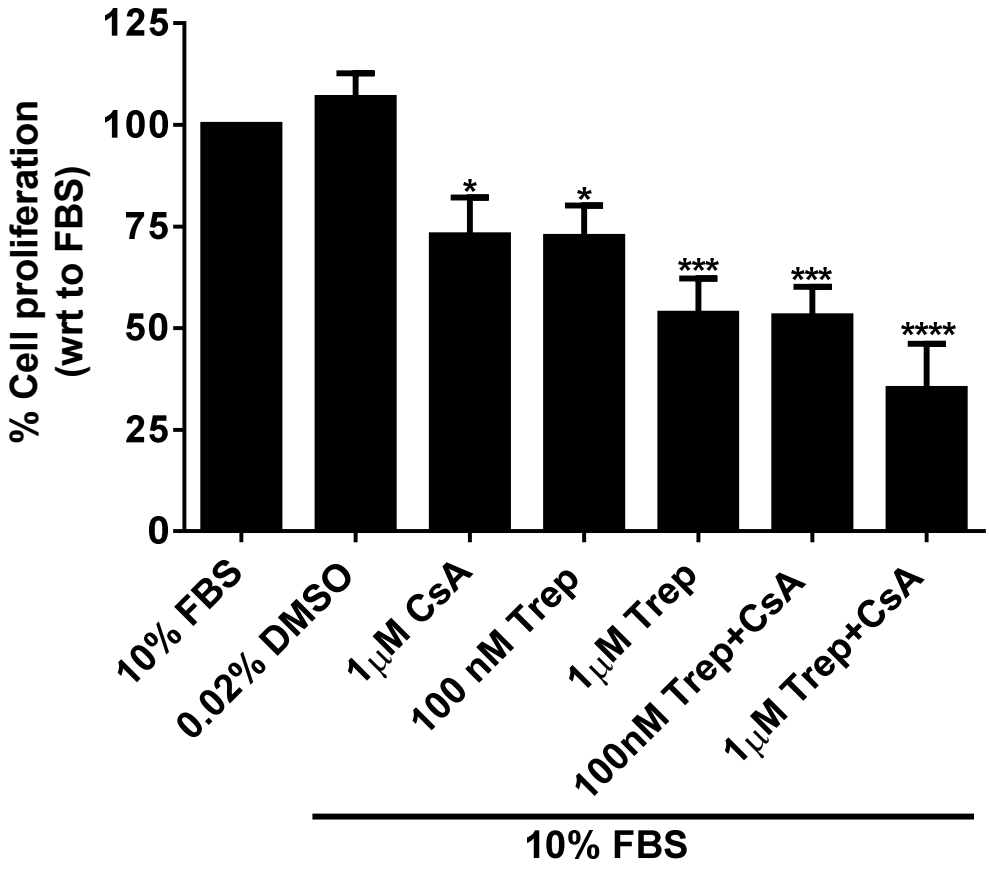

Figure 56. : Proliferation of PASMCs from PAH patients was inhibited by treprostinil and the calcineurin inhibitor cyclosporin A.

Cells were plated at 10^4 cells/ml density and growth arrested for 48 hours, then treated with 0.1% foetal boviane serum (FBS), 10% FBS alone and in combination with 1μM treprostinil (Trep), 100nM treprostinil+1μM cyclosporine A (CsA), 1μM treprostinil+1μM cyclosporine A, 1μM cyclosporine A, and 0.02% DMSO as the solvent control. (Data is represented as mean±S.E.M; *=P<0.05; **=P<0.01; ***=P<0.001, with respect to 0.1% FBS in graph A and with respect to 10% FBS in graph B; One-way ANOVA with Tukey's multiple comparisons test was used; n=6).

5.3. Discussion

A major finding of the current finding was that MPs isolated from the plasma of PAH patients induced a higher peak thrombin level compared to both controls and NSTEMI patients. Given that the same volume of plasma was used in this study, suggests this was caused by the significantly higher number of MPs. Furthermore, peak thrombin levels, endogenous thrombin potential, and the rate of thrombin generation were lower in MPs isolated from PAH patients after therapy, being more reduced in PAH patients treated with PGI_2 analogue with/without a PDE-5 inhibitor compared to treatment with a PDE-5 inhibitor with/without an ETRA. Thrombin generation was also significantly higher for MPs from NSTEMI patients compared to control subjects, though these levels were still lower compared to MPs from PAH patients. As MP number positively correlated with peak thrombin and endogenous thrombin potential in PAH patients, the high total number of MPs seen in PAH patients is likely to account for higher thrombin levels generated compared to that seen in NSTEMI patients. As total MPs in the forearm blood in PAH patients were 13–fold higher than in the forearm of NSTEMI patients, but I observed only a 33% higher median peak thrombin level and a 21% higher median ETP, may indicate that the thrombin generation potential per MP is lower in PAH than in NSTEMI. This could be due to a greater thrombogenicity of the MPs in myocardial infarction or that the maximum thrombin generation was reached by PAH MPs. For clinical relevance, a fixed volume of MPs of 100µl plasma was able to show that MPs in PAH potentiate

greater coagulation than MPs in NSTEMI and that this could be damaging throughout the vasculature. This was confirmed by ROC analyses for peak thrombin and ETP between PAH and NSTEMI were significant.

MP elevation in myocardial infarction was shown in the previous chapter and has also been shown in past studies. Elevated circulating procoagulant platelet and endothelial PECAM-1+ and glycoprotein Ib+ MPs have been reported in ST-elevated myocardial infarction (STEMI) compared to healthy volunteers (Morel et al., 2005). Elevated MP levels have also been indicated in other thromboembolic diseases such as thrombocythemia and acute pulmonary embolism (Trappenburg et al., 2009; Bal et al., 2010). Essayagh and colleagues reported that patients with acute myocardial infarction, who had undergone thrombolysis treatment, had lower levels of TFPI on TF+ MPs compared to before treatment, though such a decrease was not seen in TF+ MP levels in the patient groups who had undergone stenting (Essayagh et al., 2005). The high amounts of thrombin produced by MPs in the blood from PAH patients may be partially due to SMMPs from being highly thrombogenic compared to endothelial MPs. Indeed, SMMPs induced a higher peak thrombin, ETP and velocity index and a lower peak time compared to EMPs from HUVECs, suggesting SMMPs may cause greater coagulation and damage to the vasculature. This may be due to greater numbers of SMMPs being TF+ as TF is expressed constitutively in smooth muscle cells as opposed to being induced with an inflammatory stimulus in endothelial cells (Zwicker et al., 2011).

Treprostinil was effective at inhibiting the release of MPs caused by growth factors and pro-inflammatory agents elevated in PAH in combination with serum.

Treprostinil was particularly effective at decreasing MP levels induced by 20ng/ml PDGF-BB. Such results may not be surprising, given that previous studies in normal human PASMCs have shown that PGI_2 analogues can inhibit the mitogenic responses to PDGF and serum in a largely cAMP-dependent manner (e.g. Wharton et al, 2000; Clapp et al, 2002). Furthermore, PGI_2 itself is known to be a potent inhibitor of growth factor released from platelets and leukocytes, in particular platelet-derived growth factor (PDGF), a key driver of smooth muscle cell proliferation and neointimal formation in atherosclerosis (Fredrich and Muller, 1992) as well as in PAH (Hassoun, 2009; Clapp & Gurung, 2015).

MP levels were not elevated as much by TNFα, and the administration of treprostinil while not significantly lowering total MP levels, a trend was nonetheless visible. A number of studies have shown that PGI_2 analogues can downregulate pro-inflammatory cytokine production (e.g. TNF-α, IL-1 IL-6 and interferon-γ) in a variety of inflammatory cells types via suppression of NF-κB activity (see Clapp & Gurung) and studies in patients show iloprost to inhibit plasma TNFα levels in critical limb ischemia (Di Renzo et al., 2005).

Treprostinil significantly lowered the release of MP levels caused by TGFβ and was even more effective in lowering ET-1-induced MP release How treprostinil may reduce MP release induced by these two mitogens is unclear. PGI_2 analogues have been reported to reduce ET-1 synthesis stimulated by mitogens in PASMCs (Wort et al., 2001; Davie et al., 2002) and to reduce elevated ET-1 plasma levels in patients with systemic sclerosis (Rehberger et al., 2009). Furthermore,

treprostinil inhibited the proliferative effects of TGF-β in PASMCs harbouring a BMPRII mutation cells by reducing SMAD3 phosphorylation (Ogo et al., 2013).

Based on the effects of two relatively selective IP (Falcetti et al., 2007) and EP_2 receptor antagonists (Birrell and Nials, 2011), treprostinil is likely to be working via the activation of both these receptors to inhibit MP release by PASMCs derived from PAH patients. When 1μM of the IP receptor antagonist was used in combination with treprostinil, MP release was restored to levels similar to that by PDGF-BB with serum. Similarly, blocking with the EP2 antagonist also reversed the inhibiting effects of treprostinil on MP release by smooth muscle cells. Interestingly, the combination of both increased the MP number to levels higher than by 20ng/ml PDGF-BB and serum. This may suggest that treprostinil may have another target that increases MP release. That treprostinil is likely to have functional effects at other prostanoid receptors besides the IP receptor was confirmed in functional assays where this agent was assessed as a vasorelaxant in several isolated smooth muscle preparations. In such studies, it was found to be equipotent with PGE_2 against EP_2 receptors (EC_{50} 4-5 nM) in mouse trachea and only 3–4 times less potent than PGD_2 at DP_1 receptors in rabbit saphenous vein and vena cava (Syed et al, 2015).

The impact on normal smooth muscle proliferation by smooth muscle MPs from PAH patients was assessed. 0.1% FBS instead of 10% FBS was used as the control for these experiments as we were interested in subtle changes in proliferation. A fixed number of MPs (100,000 MPs) were able to stimulate cells

and increase proliferation by 37% over four days, with the ratio of MPs to cells being 10 MPs:1 cell. In contrast, 5ng/ml TGF-β was not able to stimulate any growth, confirming that the normal cells, unlike diseased, were unresponsive in proliferation to TGF-β, suggesting a functional BMP/BMPRII/Smad1/Smad5/Id gene axis that is growth suppressive (Morrell, 2010). This increase in proliferation seen caused by SMMPs was similarly seen suggest that the MPs may play a role in the remodelling seen in PAH. Similarly, PMPs have been shown to increase proliferation, adhesion and survival of normal stem cells and progenitor cells, and activate various intracellular cascades including MAPK p42/44, PI3-AKT, and STAT proteins and other extracellular kinases (Kim et al., 2002). With the addition of being able to activate the coagulation cascade due to its high phosphatidylserine and tissue factor rich surface, smooth muscle MPs may play an important role in driving inflammation and remodelling in PAH.

Calcineurin Aβ expression and activation in pulmonary arterial smooth muscle cells were assessed using confocal microscopy. CnAβ expression and nuclear translocation was low in arrested cells, while 10% serum was able to increase expression and nuclear translocation. Treprostinil decreased both the expression and nuclear translocation of CnAβ to levels lower than seen with 0.1% FBS, while rosiglitazone did not affect the expression of CnAβ but did reduce nuclear translocation, though the difference did not reach significance Both PPARγ antagonists GW9662 and T00701 increased nuclear translocation compared to 10% serum, suggesting that the PPARγ pathway may be active in growing PASMCs from PAH patients.. Indeed, PPARγ expression was enhanced in the

medial layer of distal pulmonary arteries taken from the lungs of children with endstage PAH (Falcetti et al, 2010). Furthermore, PPARγ expression was reported to be increased in the smooth muscle layers from asthmatic patients (Benayoun et al., 2001) as well as in atherosclerotic lesions (Hamblin et al., 2009).

Blocking PPARγ with both GW9662 and T00701 when given in combination with treprostinil significantly reversed the effect of treprostinil and increased CnAβ nuclear translocation. However, nuclear occupancy levels were still lower when the PPARγ antagonists were given in combination with treprostinil than treprostinil treatment alone. These results may suggest that treprostinil may be partially acting via PPARγ and inhibiting CnAβ activity. Consistent with this notion, PPARγ negatively regulates store-operated Ca^{2+}entry through the down regulation TRPC1 and TRPC6 (Wang et al, 2013), which in turn is likely to reduce calcineurin activity and thus its interaction with NFAT. Administration of GW9662 has been shown to reverse the effects of the prostacyclin analogue cicaprost inhibiting PDGF-induced cell proliferation (Falcetti et al., 2010). Protein kinase A has been reported to be able to phosphorylate and activate PPARγ, suggesting a potential cross-talk between PPARγ and prostacyclin (Hamblin et al., 2009). However, the activation of the ligand binding domain of PPARγ by prostacyclin analogues has been shown to be independent of the cAMP pathway (Falcetti et al., 2007).

The CnAα and CnAβ isoforms of calcineurin have been shown to contain very different cellular distribution of expression in rat aortic smooth muscle cells.

CnAα was found to be evenly expressed throughout the nucleus and cytosol, while CnAβ was scarce in the nucleus and predominantly expressed in the perinuclear region of the cytosol in growth arrested cells (Jabr et al., 2007). PDGF-BB was able to induce nuclear translocation of CnAβ and NFATc3 but not CnAα or NFATc1. This may suggest that PDGF may be a common pathway for CnAβ and NFATc3 in driving the proliferative phenotype. CnAα may still play a role in SMC proliferation as it can activate calcium-dependent Cl^- channels and inhibit ATP-sensitive K+ channels, which are also both involved in promoting agonist induced and/or spontaneous vascular smooth muscle contractions (Greenwood et al., 2004; Orie et al., 2009; Chan et al., 2012). NFATc2 levels have been shown to be increased in PASMCs in IPAH patients, while NFATc3 levels and smooth muscle actin, a marker for cell proliferation, were increased by chronic hypoxia in mice. This was thought to be calcium/calcineurin driven as cyclosporine A was able to inhibit NFATc3 nuclear translocation and smooth muscle actin expression (de Frutos et al., 2007b).

Treprostinil was able to decrease proliferation significantly compared to serum alone. The calcineurin inhibitor, cyclosporine A was also able to decrease proliferation compared to serum alone. As 100nM treprostinil concentration is considered just within therapeutic range, that cyclosporine A was able to decrease proliferation to levels similar levels may indicate that calcineurin activation may play a role. The combination of 1μM treprostinil and cyclosporine A caused an increased inhibition of cell proliferation, which may indicate that treprostinil may also be acting via another pathway, such as downstream the EP_2 receptor.

Treprostinil could have been inhibiting calcineurin activation and suppressing PASMC proliferation through the cAMP-driven pathway, as it has shown to increase cAMP production by 2-3-fold in PASMCs derived from both IPAH and normal cells (Davie et al., 2002; Falcetti et al., 2010). Cyclic AMP is able to decrease intracellular calcium levels through a variety of mechanisms that may lead to decreased activation of calcineurin/NFAT. cAMP could inhibit the formation of inositol-1,4,5-triphosphate (IP_3) through inhibiting phospholipase Cβ, causing the inhibition calcium release from the sarcoplasmic reticulum, as well as inhibiting calcium entry into the cell and stimulating calcium uptake and extrusion (Cogolludo et al., 2007). Two major types of calcium channels have been demonstrated to be responsible for the regulation of calcium influx: 1) voltage-dependent calcium channels (VDCC), such as the L-type calcium channel, and 2) voltage-independent calcium channels, such as the receptor-operated channels and store operated calcium channels. Prostacyclin analogues have also been shown to act via plasma membrane potassium channels to reduce vascular tone (Schubert et al., 1996, 1997; Clapp et al., 1998). These channels are powerful and sensitive regulators that are able to inhibit VDCC-mediated calcium entry. In normal PASMCs, such potassium channels include the TASK1 channel, which is inhibited by hypoxia and endothelin-1, and the large conductance calcium activated potassium channel (BK_{Ca}) (Li et al., 2012). The ATP-sensitive potassium channel (K_{ATP}) and the calcium activated potassium channel (K_{Ca}) were also found to play a role in iloprost-mediated pulmonary vessel dilatation in rat lung (Dumas et al., 1997).

The store operated calcium entry (SOCE) appears to play an important role in PAH as calcium influx through this mechanism is enhanced in both humans and animal models. The transient receptor potential channels (TRPC) 3 and 6 were reported to be upregulated in pulmonary arterial smooth muscle cells from IPAH patients compared to healthy, normotensive, and non-PH patients (Zhang et al., 2007). Moreover, iloprost was shown to be able to decrease TRPC3 expression and SOCE-associated calcium influx, while the adenylyl cyclase activator, forskolin was able to inhibit IPAH smooth muscle proliferation. This may help explain the observed decrease in PASMC proliferation and calcineurin activity caused by treprostinil.

Future experiments that would be useful to understand the impact on inflammatory cells by the SMMPs would be to conduct experiments measuring the levels of cytokines (eg. IL-1β, TNFα, IL-6) after stimulating cells such as pulmonary arterial endothelial cells as well as to measure adhesion molecules expressed (eg. ICAM-1 and E-Selectin). I also would like to measure ROS production by cells after being treated with MPs. Platelet aggregation assays would provide further information on the effect of SMMPs on coagulation as it would be interesting to see if it correlates with thrombin generated in normal MP free blood.

Chapter 6

6 General Discussion and Conclusion

Pulmonary arterial hypertension (PAH) is a debilitating and fatal disease with nonspecific presenting symptoms, making diagnosis often delayed for two or more years, during which time the disease becomes irreversible (Galie et al., 2015b). Untreated, median survival is 3 years in adults and 10 months in children, highlighting the particularly aggressive nature of this disease in children (D'Alonzo et al., 1991; Takatsuki and Ivy, 2013). The diagnostic approach for PAH is based on the patient's history and physical exam screened by echocardiogram and confirmed by right heart catheterisation (RHC), which is considered the gold standard for haemodynamic evaluation although it is highly invasive (Bazan and Fares, 2015). Although echocardiography is less invasive, it has limited accuracy for estimating hemodynamic measures such as pulmonary artery pressure (Fisher et al., 2009). When considering treatment decisions for PAH, echocardiography alone is not sufficient and RHC is required (Galie et al., 2015b). Thus, biomarkers detecting early disease through less invasive means but with high accuracy for disease severity and assessment of therapeutic impact would prompt more immediate intervention with the hope of stabilizing and possibly reversing the disease.

Various biomarkers that are closely associated with cardiovascular injury have been established for PAH but are not used clinically for the purpose of diagnosis and assessing treatment. Brain natriuretic peptide (BNP) and NT-pro-BNP have been confirmed as biomarkers for PAH relating to ventricular dysfunction but are "late" markers relating to ventricular dysfunction (Warwick et al., 2008). Cardiac

troponins are biomarkers of myocardial injury that are also elevated in PAH but do not represent a sensitive marker of early disease (Neuhold et al., 2008).

PAH initially develops from endothelial damage, which is set off by sheer stress, hypoxia and genetic factors. As a result, the adhesion and migration of circulating inflammatory cells leads to the structural remodelling of small blood vessels, which is characteristic of the disease (Rabinovitch, 2008; Hassoun et al., 2009). Thus, inflammation coupled with vascular remodelling thus play important roles in driving the progression of the disease. Indeed, levels of numerous cytokines including interleukin (IL)-1β, -6, -8, monocyte chemoattractant protein (MCP)-1, fractaline, CCL/RANTES, and tumour necrosis factor (TNF)-α are abnormally elevated in pulmonary hypertension, some of which correlate with clinical worsening (Rabinovitch et al., 2014).

As markers of both inflammation and vascular remodelling, microparticles (MPs) have shown to be increased in a variety of cardiovascular diseases including acute coronary syndrome, venous thromboembolism and pulmonary embolism (Chironi et al., 2009). These MPs in the circulation are elevated in PAH and appear to correlate with severity of disease, though their origin has only been characterised for endothelial cells and leukocytes (Amabile et al., 2008, 2013). As the earliest pathology is medial thickening involving the abnormal proliferation of smooth muscle cells, I took a special interest in smooth muscle derived MPs as potential early markers of PAH. During disease progression, as the proliferation of the adventitial and intimal layers take over, the formation of plexiform lesions also

start to appear (Rabinovitch, 2012). The endothelial damage and formation of vascular lesions could potentially allow blood to come into direct contact with the smooth muscle layer, thereby allowing smooth muscle MPs (SMMPs) to be released into the pulmonary circulation. Through this body of work, I aimed to show how elevated MPs derived from various cells, with a particular interest to SMMPs, may be used as biomarkers for early PAH diagnosis, disease severity, and assessment of therapeutic impact.

6.1. Characterisation of smooth muscle microparticles

The characterisation for SMMPs had not been extensively investigated prior to my work in this project. Brisset and colleagues reported that Fas ligand was able to induce annexin V+ MPs from rat aortic smooth muscle cells growing in culture, of which 26.6±5.8% were positive for TF (Brisset et al., 2003). Similarly, TF+ MPs were also shown by Schecter and colleagues to be released by smooth muscle cells from human coronary artery grown in 10% foetal bovine serum (FBS) and concentrated on a 60% sucrose bed (Schecter et al., 2000). The α5 and β1 integrin subunits were also found on the SMMP surfaces as well as on the smooth muscle cells plasma membrane. Both tissue factor and the integrin subunits are expressed in multiple cell types apart from smooth muscle cells, including endothelial cells and leukocytes, and even satellite/skeletal muscle cells, carcinoma cells and nervous tissue for the latter (Hirsch et al., 1994; Dingemans et al., 2010; van der Flier et al., 2010; Wang et al., 2011; Zwicker et al., 2011). Thus, the challenge of characterising SMMPs was to identify combinations of cell surface markers that could allow detection of specific SMMPs subpopulations in blood.

Classical markers of smooth muscle cells include α-smooth muscle actin, transgelin (sm22α), smooth muscle-myosin heavy chain, smoothelin A/B, smooth muscle calponin, and H-caldesmon which are all intracellular markers involved in either cytoskeletal support, cell proliferation or cellular contraction (Rensen et al., 2007). Because MP detection using flow cytometry relies on the externalised plasma membrane markers on the MP surface, other less classical markers had to be investigated. In order to determine the specificity of markers on pulmonary arterial smooth muscle cells (PASMCs), their expression was also examined on growing human umbilical vein endothelial cells. PASMCs isolated from PAH patients and normal donors expressed platelet derived growth factor receptor-β (PDGFR-β), endoglin, neural glial antigen 2 (NG2), and intracellular adhesion molecule-1 (ICAM-1), which were also present on their MPs. It must be noted that high amounts of SMMPs positive for E-selectin was also detected via flow cytometry, though the adhesion molecule was not detected on the smooth muscle surface. Classically, endothelial cells are known to express endoglin, ICAM-1, E-selectin and PECAM-1, which was confirmed by my experiments. These markers were also present in a high proportion of endothelial MPs.

Though endoglin is classically thought to be expressed primarily on endothelial cells (ECs), expression of endoglin on growing human aortic smooth muscle cells in culture has also been seen in past experiments (Conley et al., 2000). High expression was also seen in human atherosclerotic plaques following vascular injury. In contrast to my results, Gore and colleagues reported that cultured

pulmonary arterial smooth muscle cells from IPAH patients had low endoglin expression, while pulmonary endothelial cells expressed it at high levels (Gore et al., 2014). Elevated levels of TGF-β and ALK-5 mRNA levels were also seen in PASMCs and pulmonary ECs (PECs). As endoglin is a transmembrane accessory protein for TGF-β, the TGF-β/ALK1/endoglin signalling pathway may be important in the endothelial activation seen in PAH. Through this axis, the activation of smooth muscle cells may also occur as the incubation of PECs with TGF-β led to Smad1/5/8 phosphorylation and fibroblast growth factor 2, PDGFb and ET-1 expression, all of which promote smooth muscle proliferation. Endoglin may also play a role in TGF-β signalling via ALK-5 and Smads 2/3 to induce smooth muscle cell proliferation (Morrell, 2010). This could be supported by reported data showing that endoglin deficiency in mice was found to be protective during hypoxic conditions (Gore et al., 2014). Soluble endoglin has also been shown to be elevated in PAH compared to controls and was shown to predict survival and functional class (Malhotra et al., 2013). Thus, endoglin+ smooth muscle MPs may be an important indicator of vascular remodelling and disease severity in PAH.

PDGFRβ+ and NG2+ smooth muscle MPs may also indicate the hyperproliferative phenotype seen in PAH. PDGF receptors are made of two receptor subunits, α and β, that homo- (αα or ββ) or hetero-dimerise (αβ) to form a functional receptor for PDGF (dimeric isoforms PDGF-AA, -BB, -AB, CC, and –DD). Mice experiments have shown that most mutations in PDGFRβ involve the disruption of phosphoinositide 3 (PI3)-kinase and phospholipase C kinase

pathways which lead to reduced vascular smooth muscle development in various tissues, while mutations in PDGFR-α lead to defects in the function of a wide variety of cells including chondrocytes, neural crest cells, leydig cells, intestinal mesenchymal cells, and kidney interstitial fibroblasts (Tallquist and Kazlauskas, 2004). Expression of PDGFRβ has been shown to be significantly higher in PAH lungs compared to healthy donors (Schermuly et al., 2005). Additionally, the addition of the PDGFR inhibitor imatinib reversed advanced pulmonary vascular disease in monocrotaline-induced PH in rats and hypoxia-induced PH in mice. Similarly, NG2 has also been shown to be involved in the proliferation of smooth muscles. Schatteman and colleagues demonstrated that microvascular smooth muscle cells expressing PDGFRα depended on NG2 to potentiate cellular responses to PDGF-AA and progress in cell development (Stallcup, 2002; Schatteman et al., 2005). NG2 blockage using antibodies also inhibited PDGF-AA-induced cell mitosis and migration (Grako and Stallcup, 1995).

The increase of ICAM-1+ and E-selectin+ microparticles released from SMCs may be indicative of SMMPs as inflammatory markers. The role of inflammation on SMMP release was further supported by increased MP numbers following TNFα stimulation. Rolfe and colleagues showed that ICAM-1 expression was very low in rat aortic smooth muscle cells and increased with stimulation with cytokine IL-1β (Rolfe et al., 2000). Though I did not observe E-selectin expression on smooth muscle cells, E-selectin+ MPs were detected after PASMC stimulation with PDGF-BB. Although classically E-selectin is known to be expressed on endothelial cells, aortic SMCs have also shown to have the

capability of E-selectin expression. Nuclear factor kappa B was shown to be involved in the expression of E-selectin by human aortic SMCs, as its inhibitor pyrrolidinedithiocarbamide was able to prevented a protein synthesis inhibitor, cyclohexamide, from inducing a TNF-α- and LPS-mediated E-selectin upregulation (Chen et al., 1997). It may be possible that E-selectin+ MPs may be located on selective cell surface areas of the MP blebbing process after cell activation, though more studies would have to take place to confirm this.

Validation experiments were also carried out in vitro to test whether the smooth muscle MPs detected represented markers of cell activation and inflammation. The MPs were quickly released by growing cells after PDGF-BB stimulation, with maximum levels reached after approximately 24 hours, in a dose-dependent manner. Apart from PDGF-BB, other agents elevated in PAH also were capable of increasing MP levels, with the order of potency being: TNFα<PDGF-BB<TGF-β<ET-1. The activation of PASMCs by TGF-β would support the role of TGF-β as a mitogen in PAH due to the activation of the TGF/Alk5/Smads 2 and 3 signalling (Morrell, 2006). As the most potent vasoconstrictor and mitogen for smooth muscle cells, ET-1 can act on endothelin receptors A and B to induce the phenotypic response (Cacoub et al., 1997; Dupuis and Hoeper, 2008). Interestingly, ET1-1 requires the presence of serum for its proliferative impact to be effective (Lambers et al., 2013). As an inhibitor of cAMP production via ETB receptors, ET-1 may have inhibited the cAMP-mediated suppression of intracellular calcium levels, thereby leading to a large increase in MP release (Davie et al., 2002). It has also been reported that TGF-β and ET-1 in PAH appear

to have a synergistic effect on PASMC proliferation as they both work via the ERK1/2 MAPK pathway (Lambers et al., 2013). Weigand and colleagues showed that ET-1-induced contractions in chronic hypoxic rats was mediated by the activation of Rho-kinase, which is also crucially involved in the process of blebbing during MP release (Weigand et al., 2006).

6.2. Circulating microparticles in PAH

Having characterised smooth muscle microparticles, they were measured in blood taken from the forearm vein of patients with PAH that were treatment naïve and after long-term therapy. Smooth muscle microparticles were defined as PDGFRβ+/PECAM-1-, Endoglin+/PECAM-1-, NG2+/PECAM-1-, and ICAM+/PECAM-1-. These SMMPs as well as total, EMPs, LMPs, PMPs and TF+ MPs were all increased significantly in PAH compared to age- and sex-matched control venous blood. Total MP levels in normal blood was very low, confirming low inflammation and cellular activation and apoptosis. After long-term therapy of a minimum of 4 months, all microparticle subpopulation levels except TF+ MPs were decreased compared to prior to therapy. Prior to going on long-term therapy, PAH patients about to go on prostacyclin therapy with/without a phosphodiesterase (PDE-5) inhibitor had higher total MP levels than patients about to go on therapy with PDE-5 inhibitor with/without endothelin receptor antagonist. The former group had a significantly decreased total annexin V+ MP level post-long-term therapy, while the latter group did not.

The increased MP levels in PAH may be due to high amounts of pro-inflammatory cytokines and mitogens within the circulation as well as blood vessel damage. As explained above, the combination of highly potent proliferative agents such as TGF-β and ET-1 may partially help explain the extremely high levels of MP levels seen in PAH compared to healthy controls and even coronary artery disease and HIV. Additionally, the vascular injury and remodelling caused by high blood pressure, increased coagulation, medial thickening, endothelial damage and the formation of plexiform lesions may account for the increased number of both smooth muscle MPs and endothelial MPs. Increased CD66b+ MPs in PAH was be indicative of activated eosinophils and neutrophils in severe inflammation compared to control patients. Similarly, increased TF+ MPs in PAH would be indicative of three major processes: 1) the inflammatory status, as it would signify monocyte and macrophage activation and their release of cytokines including TNF-α and IL-1, 2) vascular remodelling, as TF+ MPs may represent increased smooth muscle and endothelial activation/proliferation, and 3) coagulation, as TF is the key player in the intrinsic coagulation pathway. As coagulation increases, the increase in thrombin would help stimulate large numbers of already activated and aggregating platelets, thereby leading to high amounts of platelet microparticles being released in the circulation. My results showed that TF+ MP levels did not decrease after long-term PAH therapy. This was not dissimilar to the findings of Steppich et al. who observed that TF+ MPs in patients with acute myocardial were unchanged after thrombolytic treatment and stenting (Steppich et al., 2005).

The decrease in circulating MPs after long-term therapy may be due to a variety of factors including, largely involving the decrease in cell proliferation. Indeed, all three major classes of drugs, prostacyclin analogues, endothelin antagonists, and PDE-5 inhibitors) have shown to decrease smooth muscle proliferation (Rhodes et al., 2009; Galie et al., 2015b). However, only prostacyclin analogues are also anti-inflammatory and powerful inhibitors of platelet aggregation, which may help explain why microparticles were lower in PAH treated with prostacyclin analogue with or without an PDE-5 inhibitor compared to non-prostacyclin therapy. Inhibition of platelet aggregation by prostacyclin has been shown to be cAMP driven (Kelton and Blajchman, 1980). The combination of prostacyclin analogue with a PDE-5 inhibitor may also be an effective combination therapeutically as the PACES trial revealed that the addition of sildenafil to long-term intravenous epoprostinil therapy on patients with PAH resulted in greater mean pulmonary artery pressure, cardiac output, and longer time to clinical worsening compared to epoprostinil treatment on its own (Simonneau et al., 2008).

Total annexin V+ MP levels were significantly higher in PAH venous blood than in STEMI coronary blood, which contained the highest number of MPs among the acute coronary syndrome groups. Coronary blood contained higher SMMPs, EMPs, LMPs, and PMPs than forearm venous bloods in STEMI and non-STEMI patients, possibly indicating the high level of inflammation present within the coronary vasculature. In coronary blood, STEMI patients had significantly higher circulating MPs of all subpopulations compared to non-STEMI, which may be an indication that MPs may be markers of disease severity. However, in venous

blood, the difference in MP levels was not as evident, as noticeable differences were seen only in SMMPs and not on other MP subpopulations. Interestingly, LMPs and TF+ MPs were elevated in STEMI coronary artery blood to levels higher than in PAH venous blood. The origination of SMMPs in myocardial infarction may come from thrombus formation as well as vascular damage. Indeed smooth muscle cells may be exposed to the blood, particularly during and after plaque rupture though the presence of more metalloproteinase-secreting macrophages appears to be more prominent during plaque development (Davies et al., 1993; Filardo et al., 2000). This may help explain elevated TF+ MPs and SMMPs. The elevation of CD66b+ MPs may be explained by the activation of neutrophils as they have been shown to accumulate in ischemic and reperfused myocardium after acute myocardial infarction and release thromboxane B_2 and leukotriene B_4 and induce vasoconstriction and platelet aggregation (Frangogiannis et al., 2002). The significantly higher levels of PAH venous blood compared to STEMI and non-STEMI venous blood suggests that MP measurement may be a biomarker that involves a quick, convenient, and far less invasive method compared to right heart catheterisation to aid in the early diagnosis of PAH. Though MP detection may not lead to direct diagnosis of the disease, it may help in the initial screening of patients who are more likely to suffer from PAH.

The presence of microparticles was also investigated in venous blood from HIV-infected Malawian patients and compared with blood from control subjects to study the effects of chronic inflammation. The level of total annexin V+ MPs in

HIV blood were not significantly different compared to Malawian age- and sex-matched controls, though they were higher than in British healthy controls. SMMPs, EMPs and LMP levels were lower in HIV-infected patients than in Malawian control subjects, with British control subjects having higher MP numbers of the three groups. This was contrary to past work performed by da Silva et al. who showed that CD51+ EMPs were elevated compared to controls (da Silva et al., 2011). I did observe that E-selectin MPs were slightly elevated in HIV compared to British controls, though this difference was not significant. Mayne and colleagues reported that TF+ microparticles were also elevated, which was not seen in my results (Mayne et al., 2012). However, similar to the latter study, my results in chapter 4 showed that PMP levels in HIV-infected patient blood was higher than in Malawian controls, and was lowest in British controls. This may suggest that MPs may be a specific indicator of increased risk of thrombosis predisposed to HIV infected patients than inflammation at a wide spectrum. Several difficulties were faced when measuring HIV-infected blood samples. The measurement of samples using the only flow cytometer located in Blantyre, Malawi where I collected blood samples meant that I had to 1) use a different FACS machine with a different software, 2) could not process samples in the same way as my other PAH and coronary artery disease samples, and 3) had to use Malawian subjects for HIV non-infected controls, many who also likely had other inflammatory conditions.

6.3. Microparticle function and response to pulmonary arterial hypertension therapy

MPs from a fixed volume of plasma from PAH patients were highly thrombogenic compared to microparticles from control patients and non-STEMI patients. Thrombin generation by MPs in PAH were lower after long-term therapy than prior to treatment. Thrombin generation was inhibited to a greater extent in patients treated with prostacyclin analogues with/without a PDE-5 inhibitor than in patients treated with a PDE-5 inhibitor with/without ET-1 antagonists. The correlation between thrombin generation and total MP count may suggest that the degree of thrombin generation may be, in part, due to number of MPs. A large part of the high thrombogenicity seen in PAH-derived MPs may also be due to elevated SMMP levels as my in vitro experiments showed that they were able to significantly induce greater amounts of thrombin in a shorter amount of time compared to EMPs from HUVECs. SMMPs Smooth muscle have also been shown to express CD154, which when bound to its receptor CD40 on leukocytes can also induce greater TF expression (Schonbeck et al., 2000). During coagulation, SMCs can be stimulated by thrombin and PDGF and in response release IL-6 (Libby and Simon, 2001). In response, IL-6 is able to increase plasma fibrinogen, plasminogen activator inhibitor-1 as well as the inflammation marker C-reactive protein. To add, were also found to be proliferation-inducing in normal smooth muscle cells, indicating that they may promote the pro-proliferative disease phenotype and may be involved to some degree in the remodelling that occurs within disease.

The impact of drug treatment on MP release by smooth muscle cells was also assessed in vitro. Treprostinil was able to decrease levels of MP release during cell activation after stimulation by platelet derived growth factor-BB (PDGF-BB), tumour necrosis factor-α (TNF-α), transforming growth factor –β (TGF-β), and endothelin-1 (ET-1). Moreover, the inhibition of treprostinil on PDGF-BB-induced MP release was dose-dependent and appeared to act through both the prostacyclin and prostaglandin E2 receptors. It has been shown that prostacyclin can act via the cAMP-driven pathway in reducing intracellular calcium levels (Clapp et al., 2002). I wanted to investigate whether it could also be working via another pathway, the PPARγ pathway involving calcineurin/NFAT signalling which may be activated during vascular remodelling. Calcineurin Aβ expression and activation as measured by nuclear occupancy after translocation from the cytosol was decreased slightly with rosiglitazone and significantly with treprostinil. The partial reversal of the effect of treprostinil by the PPARγ antagonist GW9662 as well as the PPARγ-selective antagonist T00701 suggest that treprostinil may be working partially via the PPARγ-calcineurin/NFAT pathway. Indeed, inhibiting calcineurin with cyclosporin did cause an inhibition in cell proliferation compared to serum alone. When treprostinil was administered in combination with cyclosporin, proliferation was significantly lower, though this difference was not additive suggesting that treprostinil and cyclosporine may be working via a common pathway. Still other pathways may be involved in prostacyclin signalling. In human PASMCs, prostacyclin has shown to also activate the TWIK-related acid-sensitive K channel 1 (TASK1) and calcium-dependent potassium channel (KCa), which may also account for the vasorelaxation effect of prostacyclin (Li et al., 2012). Similarly, the Kv1.5 channel

may be involved in the apoptotic-resistant pro-proliferative phenotype of smooth muscles in PAH as the calcineurin inhibitor cyclosporine A and NFAT blocker VIVIT were able to restore reduced Kv1.5 expression and function in PAH cells (Bonnet et al., 2007). Protein kinase A has been shown to be able to phosphorylate NFAT, thus there may also be an integration of the cAMP and NFAT/calcineurin pathways (Chow et al., 1999).

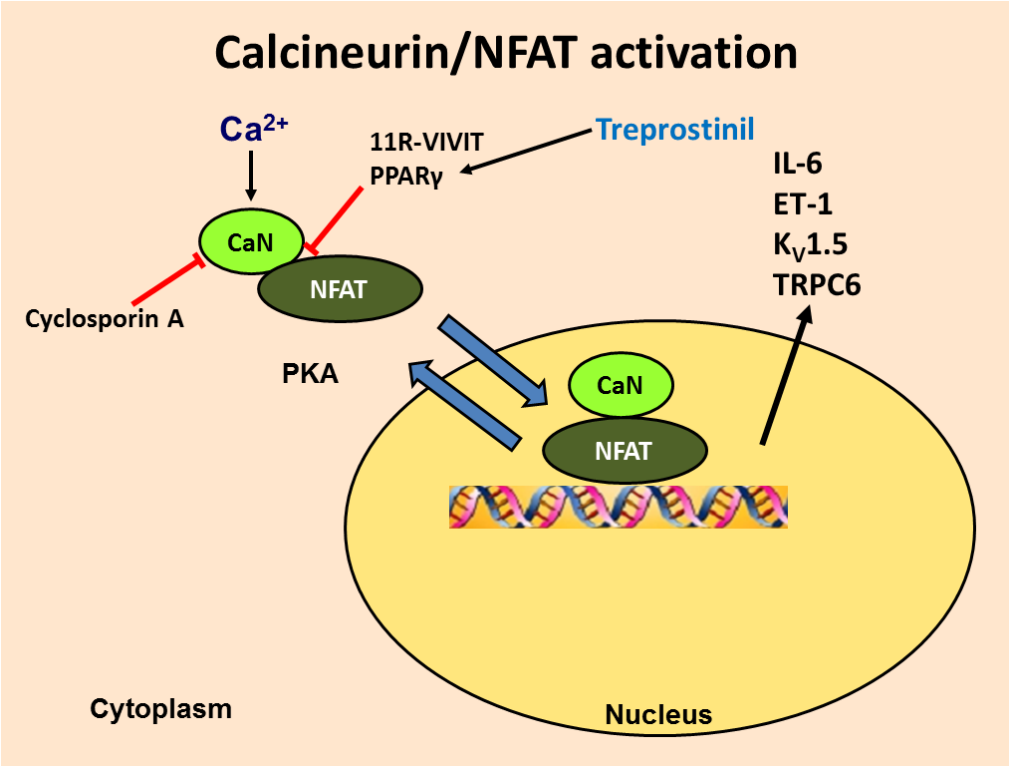

Figure 57. Calcineurin/NFAT activation

Calcineurin (CaN) activates nuclear factor of activated T-cells (NFAT) through dephosphorylates and allows its nuclear translocation where it can bind to specific regions of the DNA and induce the expression of pro-proliferative and pro-inflammatory mediators. CaN/NFAT is phosphorylated by kinases such as protein kinase A (PKA) causing it to return from the nucleus.

Abbreviations: Ca2+=calcium; PPARγ=peroxisome proliferator activated receptor γ; IL-6=interleukin 6; ET-1=endothelin-1; Kv1.5=Volage gated potassium channel 1.5; TRPC6= transient receptor potential cation channel 6

The Rho kinase pathway plays an important role in the formation of EMPs (Distler et al., 2005; Dignat-George and Boulanger, 2011). Rho GTPases (RhoA, B, and C) are intracellular signalling molecules that play a role in actin cytoskeletal regulation (Distler et al., 2005). After activation, Rho proteins can exchange GDP for GTP and signal to downstream effector proteins, and finally hydrolyse the bound GTP to return to its inactive GDP-bound state. Coleman and colleagues showed that the Rho-associated kinase I (ROCK-1) as an important element in Rho signalling that causes myosin light-chain phosphorylation and coupling of actin-myosin filaments to the plasma membrane, which contributes to the cytoskeletal restructuring leading to MP blebbing from mouse fibroblasts (Coleman et al., 2001). The small molecule inhibitor of ROCK activity Y-27632 reduced of myosin light-chain phosphorylation and MP formation caused by TNFα. Additionally, the caspase inhibitor z-VAD-fmk blocked the cleavage of ROCK-I and MP release, suggesting that caspases could play a role in ROCK-I activation. Similarly, *in vitro* experiments showed that the cholesterol lowering drug fluvastatin was also shown to decrease TNFα-induced endothelial MP release from human coronary artery endothelial cells in a Rho-kinase mediated fashion (Tramontano et al., 2004). Another study showed that simvastatin could also inhibit chronic hypoxia-induced PAH and ROCK I and II expression (Girgis et al., 2007).

The rho-kinase pathway may play a role in the pro-proliferative phenotype of PASMCs in PAH, vascular remodelling and pulmonary vascular contraction (Fukumoto et al., 2007). ROCK inhibition attenuates chronic hypoxia-induced

PAH in mice and rats, monocrotaline and high flow-induced PAH in rats, reduces susceptibility to PAH in fawn-hooded rats, and plays a role in the beneficial vasodilating effect of the PDE-5 inhibitor sildenafil in PAH (Abe, 2004; Fagan et al., 2004; Hyvelin, 2005; Nagaoka et al., 2006). The ROCK inhibitor fasudil has shown to decrease pulmonary artery pressure and pulmonary vascular resistance in PH patients (Ishikura et al., 2006; Xiao et al., 2015). Interestingly, prostacyclin does not regulate pulmonary vasodilatation through ROCK inhibition, though the combination of the prostacyclin analogue beraprost with fasudil has shown to be more effective than monotherapy with either drug (Abe et al., 2005). In bovine PASMCs, Rho kinase may be activated by the serotonin receptor 5-HT_{1B} and mediate the nuclear translocation of phosphorylated ERK1/2 (Lee et al., 1999). These can in turn increase DNA binding of transcription factors including GATA4, Elk-1, Egr-1 and express proteins involved in cell proliferation, though the phosphorylation and nuclear translocation mechanism for ERK1/2 may be different for humans (Liu et al., 2004; Dempsie and MacLean, 2008). The vasoconstricting agent and mitogen serotonin that is elevated in PAH is able to signal via the 5-HT_{1B} receptor on smooth muscle cells and fibroblasts and induce proliferation through signalling involving reactive oxygen species and ERK1/2 mediated pathways, and may be working via ROCK as well (Dempsie and MacLean, 2008). ROS generation may also be a result of serotonin breakdown shown in human PASMCs or serotonin-induced NADPH oxidase activation shown in bovine PASMCs (Liu and Folz, 2004; Dempsie and MacLean, 2008).

My experiments showed that PDGF-BB induced MP release from smooth muscle cells, which may have possibly been through the Rho/Rho kinase pathway amongst others. Indeed, PDGF-BB is able to upregulate Rho A expression in vascular SMCs (Kamiyama et al., 2003). Moreover, inhibiting Rho kinase using Y-27632 suppresses PDGF-BB-induced ERK1/2 activation and SMC proliferation, suggesting that Rho A may be an important mediator of SMC activation and MP release. Apart from Rho, PDGF has shown to promote the proliferation of cells via the activation of calcineurin/NFAT (Jabr et al., 2007). Additionally, calcineurin was shown to be inhibited by the protein kinase A activator forskolin, suggesting how the cAMP pathway may be capable of antagonising the action of PDGF-BB. Bonet et al. has shown that PASMCs from PAH cells and lungs unlike normal contained activated NFATc2 (Bonnet et al., 2007). NFATc3 is also increased in hypoxia-induced pulmonary hypertensive adult and neonatal mice but not in NFATc3 knockout adult mice, suggesting its significance in vascular remodelling (Bierer et al., 2011). To add, elevation of the highly potent vasoconstrictor and mitogen endothelin-1 was seen in PAH may also activate NFATc3 dependent on ROCK activation (de Frutos et al., 2011). Like PDGF, TNFα has also been shown to act via the rho kinase pathway in activating pulmonary microvascular endothelial cells to release JNK-mediated interleukin-6 secretion (Mong et al., 2007). However, unlike in SMCs, the Rho/ROCK pathway does not seem to play a role in endothelial contraction (Hunter et al., 2003; Garofalo and Surmacz, 2006). Another cytokine as well as a growth factor, TGFβ activity which has been implicated as an important driver of the remodelling process in PAH has also been shown to be regulated to an extent by Rho. Deficiency of the prostaglandin E3 receptor, which normally mediates

vasoconstriction of human arteries and is upregulated in human and mouse PASMCs in response to hypoxia, was shown to attenuate PH through suppression of TGFβ signalling via Rho/ROCK signalling. Thus, understanding the role of Rho in the pathogenesis of PAH and development of MPs may help explain why MPs may be a valuable marker for PAH.

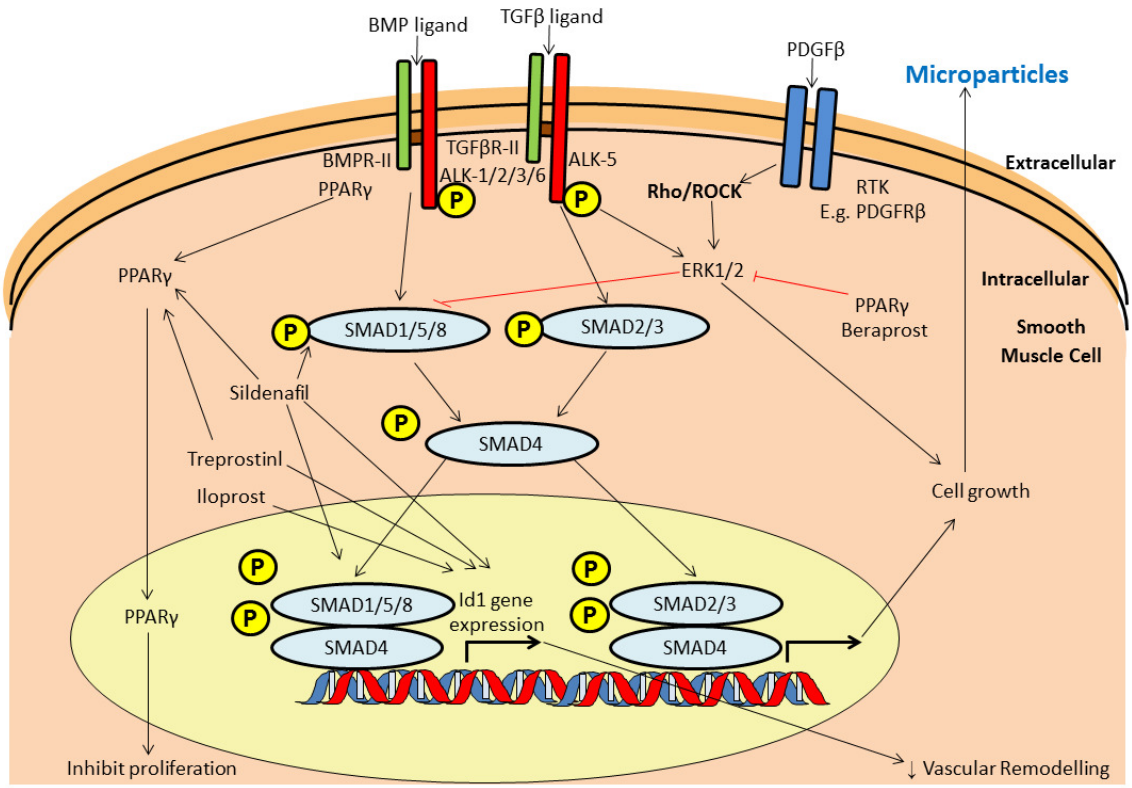

Figure 58. TGFβ, PDGFRβ, Rho, and PPARγ pathways in smooth muscle

Schematic of the transforming growth factor β (TGFβ), platelet derived growth factor receptor β (PDGFRβ), Rho, and peroxisome proliferator activating receptor γ (PPARγ) pathways in smooth muscle

Abbreviations: ROCK=rho associated kinase; ALK=activing like kinase; ERK=extracellular signal regulated kinase; RTK=receptor tyrosine kinase; BMP=bone morphogenetic protein; BMPRII=bone morphogenetic protein type 2

Another pathway that may also be involved in the activation of cells and release of MPs is the NOTCH pathway, which is involved in various aspects of vascular development such as vascular remodelling, angiogenesis, vascular SMC development and differentiation (Weber, 2008). As shown by Jin and colleagues, notch receptor activation leads to upregulation of PDGFRβ expression and activity in vascular SMCs, which would lead to increased cellular development and proliferation (Jin et al., 2008). TGFβ administered to mesenchymal stem cells was shown to induce expression of the Notch ligand Jagged 1 and SMC markers such as α-smooth muscle actin, calponin 1 and myocardin, which were dependent on Smad3 and Rho kinase activity (Kurpinski et al., 2010). Moreover, notch activation led to decreased endothelial markers PECAM-1 and VE-cadherin, suggesting that notch signaling via TGFβ may induce differentiation of mesenchymal stem cells into SMCs. Li et al reported greater levels of NOTCH3 mRNA and protein levels in PASMCs from PAH patients compared to normotensive controls (Li et al., 2009). Constitutive expression of NOTCH3 by cultured PASMCs increased proliferation, while knocking out NOTCH3 in mice led to resistance in PH development.

Future studies

- The characterisation of pulmonary artery endothelial cells and their microparticles after stimulation by pro-inflammatory agents such as TNFα would help clarify the distinction between smooth muscle and endothelial MPs involved in PAH.
- Using multiple pro-inflammatory and/or pro-remodelling agents to stimulate cells and measure their impact on different receptor expression on the cell surface as well as on microparticle surface may help identify specific MP subpopulations that may act as viable markers in certain disease conditions.
- Comparing levels of pro-inflammatory cytokines (e.g. IL-1, TNFα, and IL-6) and circulating endothelial cells in the plasma of PAH patients and controls could serve as indicators of vascular inflammation and damage. Levels in patients could subsequently be used to correlate with microparticle levels in patients to assess inflammatory status.
- The inflammatory impact of SMMPs on activating vascular cells such as smooth muscle and endothelial cells could be investigated in vitro by measuring the release of proinflammatory cytokines and expression of adhesion molecules such as ICAM-1 and E-selectin by cells in culture.
- ROS production by pulmonary artery smooth muscle cells after treatment with microparticles could also be measured in vitro using the indicator H2DCFDA, which can be converted to fluorescent 2', 7'-di-chlorofluorescein (DCF) after oxidation and intracellular removal of acetate groups.

- The role of PPARγ in its ability to inhibit MP release in smooth muscle cells could be further studied. Using the PPARγ agonist rosiglitazone, the inhibition of SMMP release could be studied in vitro.
- Other PPARs (α and β) in their ability to inhibit pulmonary artery smooth muscle proliferation and contribute to the anti-proliferative effect of treprostinil could be investigated.
- Platelet aggregation assays could be performed to provide further information on the effect of SMMPs on coagulation and assess correlation with thrombin generation in normal MP-free blood. The role of Rho kinase pathway in SMMP formation in vitro could be identified. The small molecule inhibitor of ROCK activity Y-27632 could be used to study MP release inhibition by cells. The activation of ERK1/2 by RhoA could also be studied after the stimulation of smooth muscle cells by PDGF-BB in inducing MP release. The activation of NFAT by ROCK activation could also be studied in the process of cell proliferation and the release of SMMPs.
- The notch pathway is implicated in various vascular processes such as vascular remodelling, angiogenesis and smooth muscle development and differentiation, and may be a novel pathway worth investigating in the mechanism of MP release. The activation of the notch pathway may potentially be a common pathway shared by multiple stimulants such as PDGF-BB and TGF-β leading to MP release.

6.4. Conclusion

I propose that my work supports the innovation in quantifying microparticles for the detection of early PAH in patients. This body of work shows that MPs derived from smooth muscle cells classified as PDGFRβ+/PECAM1-, Endoglin+/PECAM1-, NG2+/PECAM1-, and ICAM1+/PECAM1- are elevated in PAH compared to healthy controls as well as other vascular diseases such as myocardial infarction and HIV. This was similarly seen in levels of microparticles derived from endothelial cells, leukocytes and platelets as well. As prothrombotic, pro-inflammatory, and pro-proliferative mediators, SMMPs could be an important player in the development of vascular remodelling. Furthermore, MPs may be able to allow assessment of the impact of therapy on PAH patients, making it a valuable tool in studying disease progression and patient clinical status. Prostacyclin was shown to be a potent inhibitor of smooth muscle microparticle release in vitro, and was also potent in reducing smooth muscle, endothelial, platelet and leukocyte circulating microparticles in PAH patients. Right-heart catheterisation may still be the gold-standard in PAH diagnosis but MP detection could potentially improve early patient screening for later more invasive diagnostic testing. Thus, as microparticles are a marker of disease severity and a quick, cost-effective, and less invasive alternative to the current diagnostic methods, this work could make a valuable contribution to the research of early biomarkers in PAH.

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