Investigating Pim kinase inhibition as a novel anti-thrombotic endothelium targeting strategy

UNIVERSITY OF LEEDS

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy.

The University of Leeds

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I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Background and Purpose: Atherothrombosis, a key driver of cardiovascular disease, involves complex interactions between endothelial cells and circulating platelets. While current anti-platelet therapies are effective, they offer limited secondary prevention and are associated with significant adverse effects. Pim kinases have emerged as novel regulators of platelet function and thrombosis and are expressed in endothelial cells. Their inhibition may therefore offer additional therapeutic benefit on thrombosis. This study investigates the effects of PIM1 deletion in mice and pan-Pim kinase inhibitors AZD1208, PIM447, and TP3654 on human coronary artery endothelial cell (HCAEC) function and their potential to modulate thrombogenic responses.

Methodology: Constitutive PIM1$^{-/-}$ mice were used to investigate the impact of PIM1 deletion on coagulation parameters and endothelial phenotype and function. Inhibitor studies were performed to investigate the effect of Pim kinase inhibition on coronary arterial endothelial function. HCAECs were treated with AZD1208, PIM447, and TP3654 (0.1–10μM) and endothelial survival, viability, migration, and angiogenesis assessed using LDH release, MTS assay, wound healing, and tube formation assays, respectively. Expression and release of inflammatory and thrombotic markers were quantified via RNA-Seq, RT-qPCR, and a Luminex multiplex assay. Finally, the impact of Pim inhibitor-conditioned media on platelet adhesion and coagulation was evaluated using *in vitro* thrombosis and clotting assays.

Key Results: PIM1 deletion in mice was associated with a decrease in PIM2 and PIM3 gene expression in endothelial cells, along with decreased endothelial angiogenesis, and a delay in intrinsic clotting time. In human endothelial cell experiments, expression of the three Pim kinase isoforms was upregulated by TNFα and IL-6 treatment, and Pim kinase inhibitor treatment did not impair HCAEC viability, proliferation, or migration, indicating a lack of cytotoxicity at physiologically achievable concentrations. However, angiogenic capacity of HCAECs was significantly reduced. RNA-Seq analysis demonstrated alterations in expression of endothelial mediators of inflammation and thrombosis following 24-hour inhibitor treatment. Additionally, platelet and leukocyte deposition on confluent treated HCAECs was attenuated under flow and releasate from treated HCAECs delayed intrinsic coagulation pathways.

Conclusion and Implications: Pan Pim kinase inhibitors exhibit anti-inflammatory and anti-thrombotic effects in HCAECs without compromising endothelial integrity or function, supporting further investigation into Pim kinase inhibition as a novel strategy for the prevention of atherothrombosis.

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Abbreviations

ACS	Acute coronary syndrome
ANOVA	Analysis of Variance
BAD	Bcl-2-associated death promoter
BBB	Blood-brain barrier
BSA	Bovine Serum Albumin
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CNP	C-type natriuretic peptide
$CoCl_2$	Cobalt chloride
COL	Collagen
COX	Cyclooxygenase
Ct	Cycle threshold
CVD	Cardiovascular disease
DAPI	4,6'-diamidino-2-phenylindole
DiOC6	3,3'-Dihexyloxacarbocyanine iodide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOAC	Direct oral anti-coagulant
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDHF	Endothelium-derived hyperpolarizing factor
EDTA	Ethylenediaminetetraacetic acid
EMP	Endothelial microparticle
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase-1
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting

FBS	Foetal bovine serum
FDP	Fibrin degradation products
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate desidrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
HCAEC	Human coronary artery endothelial cells
HSP90	Heat shock protein 90
H_20_2	Hydrogen peroxide
ICAM-1	Intercellular adhesion molecule-1
IC50	Half-maximal inhibitory concentration
IL	Interleukin
IMS	Industrial methylated spirits
JAK	Janus kinase
JAM	Junctional adhesion molecule
Km	Michaelis constant
KO	Knockout
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MFI	Median fluorescence intensity
MI	Myocardial infarction
MW	Molecular weight
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
PAD	Peripheral artery disease
PAF	Platelet-activating factor
PAR	Protease-activated receptor
PDGF	Platelet-derived growth factor

PECAM	Platelet endothelial cell adhesion molecule
PG	Proteoglycan
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
Pim	Proviral Integration Site for MuLV
PLA	Platelet-leukocyte aggregates
PMA	Phorbol 12-myristate 13-acetate
PPP	Platelet poor plasma
PSGL-1	P-selectin glycoprotein ligand-1
RT-qPCR	Reverse transcription Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SERPIN	Serine protease inhibitor
SMC	Smooth muscle cell
SS	Shear stress
STAT	Signal transducer and activator of transcription
SUMO	Small Ubiquitin-like Modifier
TEM	Trans endothelial migration
TEER	Trans endothelial electrical resistance
TF	Tissue factor
THBD/TM	Thrombomodulin
TNFα	Tumour necrosis factor alpha
TP	Thromboxane receptor
TXA2	Thromboxane A2
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VKA	Vitamin K antagonist

VSMC	Vascular smooth muscle cell
VWF	Von Willebrand factor
WPB	Weibel-Palade bodies
WT	Wild type

Conferences and Presentations

2023

- British Society of Haemostasis and Thrombosis (BSHT), Birmingham (poster presentation)
- British Society for Cardiovascular Research (BSCR), Manchester (poster presentation)
- Joint EUPLAN/Platelet Society meeting, Bristol (poster presentation)
- Northern Cardiovascular Research Group (NCRG) meeting, Manchester (poster presentation)

2024

- British Society of Haemostasis and Thrombosis (BSHT), Belfast (poster presentation)
- British Heart Foundation (BHF) Fellow's meeting, Coventry (poster presentation)

2025

- British Society of Haemostasis and Thrombosis (BSHT), Newcastle (poster presentation)
- International Society of Thrombosis and Haemostasis (ISTH), Washington D.C., US (oral presentation)

Publications

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Chapter 1. General introduction

1.1 Cardiovascular disease

Cardiovascular disease (Nelson et al.) describes conditions that affect the heart or circulation, including conditions that narrow or block blood vessels (BHF, 2024). CVD is associated with a build-up of fatty deposits inside the arteries and an increased risk of blood clots and remains the leading cause of disease burden and death globally (Global Burden of Cardiovascular and Risks, 2025). Up-to-date statistics from the British Heart Foundation (BHF) in January 2026 state that currently there are more than 8 million people living with heart and circulatory diseases in the UK, and the burden of healthcare costs relating to heart and circulatory diseases are estimated at £12 billion each year (BHF, 2026). Coronary artery disease (CAD), the most common type of heart and circulatory disease, is one of the UK's leading causes of death, responsible for around 63,000 deaths in the UK annually (BHF, 2026).

Cardiovascular events such as CAD are often caused by blockage of the blood vessels due to the development of atherosclerosis and thrombus formation narrowing the arteries (Ashorobi et al., 2021). Components of the cardiovascular system play a key role in the pathogenesis of vascular thrombosis and inflammation (thromboinflammation) forming occlusive blood clots in response to atherosclerotic plaque rupture and/or erosion that are particularly rich in platelets (Yau et al., 2015b).

Anti-platelet therapy is therefore currently the main treatment strategy for the secondary prevention of myocardial infarction (MI). Over 7 million people in the UK are prescribed anti-platelet drugs annually, primarily for CVD prevention and post-surgical care for cardiovascular conditions including heart attacks, strokes, and peripheral artery disease (PAD) (NHSEngland, 2025). In England alone, around 30 million prescriptions are issued for aspirin annually and around 10 million for Clopidogrel (NHSEngland, 2025). Despite their widespread use, most anti-platelet therapies are associated with variable outcomes in patients, and

adverse side effects such as severe bleeding (Hankey and Eikelboom, 2004). While effective in some patients, aspirin produces significantly variable responses between individuals where some patient groups such as those with Type 2 Diabetes mellitus and obesity demonstrate high platelet reactivity on aspirin, which has been shown to associate with an increased risk of acute coronary syndromes (ACS) (Cattaneo, 2009, Cattaneo, 2013). Furthermore, aspirin targets platelet activation and aggregation via inhibition of cyclooxygenase (Cox and Ng) 1 and thromboxane A2 signalling (Figure 1.1). However, aspirin also demonstrates dose-limiting, off-target effects on COX-2 expressed in the endothelium (Figure 1.1). Inhibition of COX-2 leads to a reduction in the synthesis of endogenous inhibitors of platelet function and can, in some individuals adversely result in an increased thrombotic and cardiovascular risk (Warner et al., 2011, Desborough and Keeling, 2017). This is supported by the results of a large meta-analysis study of aspirin use for the prevention of vascular disease, which suggested that in low-risk individuals, the harms of aspirin may outweigh the cardiovascular benefits (Antithrombotic Trialists et al., 2009). A novel approach targeting the endothelium, a key player in cardiovascular events, achieving an appropriate balance between thrombosis and bleeding is therefore necessary.

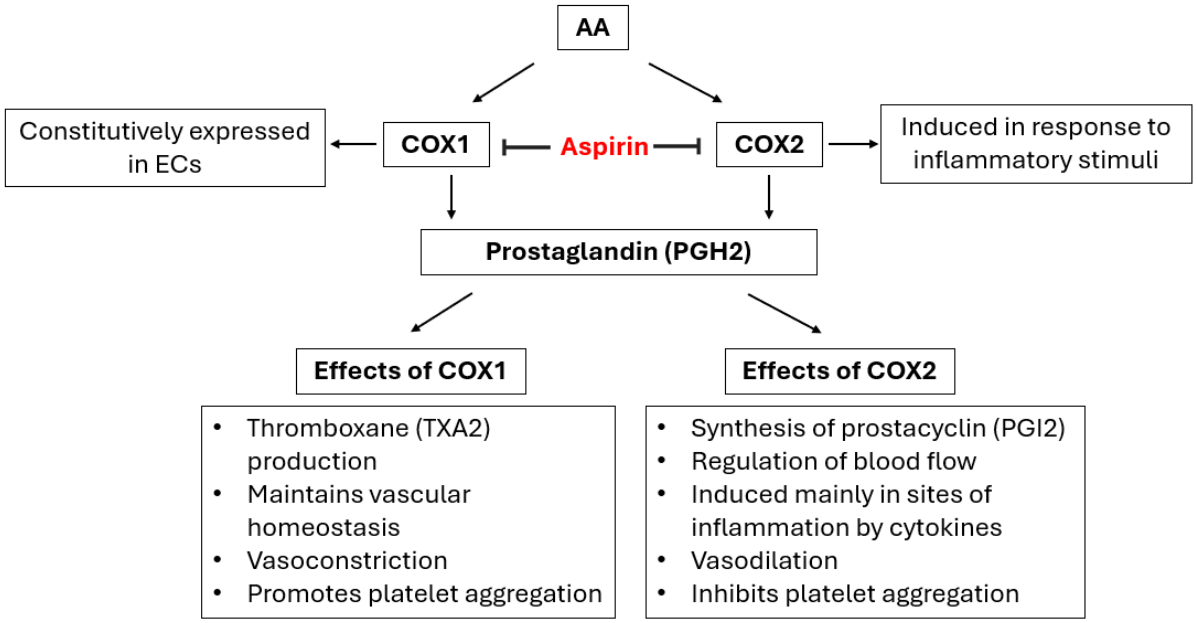

Figure 1.1: Effects of Aspirin targets cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in the endothelium. Aspirin irreversibly inhibits COX-1 and COX-2 by inhibiting prostaglandin biosynthesis. The effects of COX-1, which is constitutively expressed in endothelial cells, include thromboxane production, the maintenance of vascular homeostasis, vasoconstriction, and promoting platelet aggregation. The effects of COX-2, which is induced in response to inflammatory stimuli, include the synthesis of prostacyclin, regulation of blood flow, vasodilation, and the inhibition of platelet aggregation. Created in BioRender and PowerPoint.

1.2 Biology of the vasculature

The vascular system consists of a network of blood vessels including the aorta and its branches (arteries), arterioles, capillaries, venules and veins, which perform many physiological functions. The structure and function of each segment of the vascular system vary depending on the organ it supplies (Tucker et al., 2023).

1.2.1 Structure

Aside from capillaries, blood vessels are made of three layers (Figure 1.2) (Higashi, 2024, Tucker et al., 2023). The general three-layered structure of arteries and veins is comprised of:

- The tunic intima, the innermost layer, consists of the lumen lined by a single layer of vascular endothelial cells (ECs), a sub-endothelial collagen-rich basement membrane and internal elastic lamina with ECM components (Tucker et al., 2023).
- The tunica media is a thick, muscular middle layer of the vessel composed of smooth muscle cells (SMCs) and elastic tissue (made up of collagen and elastin fibres) which regulates the internal diameter of the vessel via vasoconstriction and vasodilation (Tucker et al., 2023).
- The adventitia, the outer layer composed primarily of collagen fibres, elastic tissue, connective tissue and fibroblasts. Many large arteries and veins possess a microvasculature in their adventitial layers (Heistad and Marcus, 1979), a network of small blood vessels which supply the vascular wall with essential nutrients and oxygen (vasa vasorum) (Khurana et al., 2005). The adventitia provides structural support for the vessels.

The vessels are strengthened by connective tissue. Within each layer, the amount of muscle and collagen fibrils varies, depending on the size and location of the vessel (Tucker et al., 2023).

In contrast to arteries and veins, capillaries are thin-walled vessels composed of a single layer (tunica intima) consisting of simple squamous epithelium (endothelium), a basement membrane (composed of type IV collagen, laminin, nidogen, and perlecan), and scattered connective tissue cells (pericytes) (Godwin et al., 2023), a structure that is optimised for the exchange of materials between the blood and surrounding tissues.

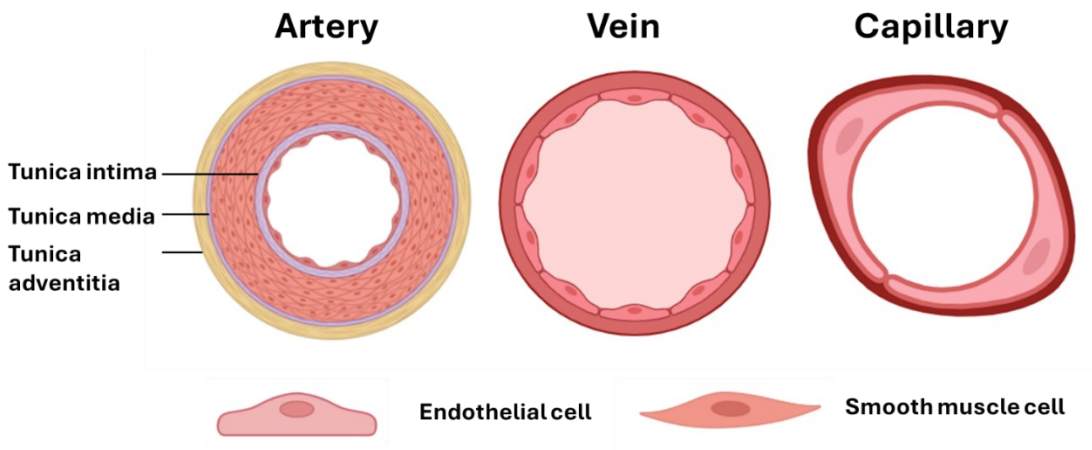

Figure 1.2: Basic artery, vein and capillary structure. Arteries and veins are comprised of three distinct layers; tunica intima (inner layer), tunica media (middle layer), and tunica adventitia (outer layer). Capillaries are composed of a single layer (tunica intima). Endothelial cell (EC) and smooth muscle cell (SMC) are highlighted. Created in BioRender and PowerPoint.

1.2.2 Function

Blood vessels transport nutrients to tissues and play a significant role in the removal of waste (Tucker et al., 2023). A primary purpose and significant role of the vasculature is its contribution in oxygenating the body. Deoxygenated blood from the peripheral veins is transported back to the heart from capillaries, to venules, veins (vena cava returns blood to the right atrium), before it is transported to the lungs via the pulmonary artery through the right ventricle. The pulmonary vein transports oxygenated blood from the lungs to the left atrium before it is pumped around the body by the left ventricle into the aorta, then to arteries, arterioles, and finally capillaries where the exchange of nutrients occurs. Loading and unloading of oxygen and nutrients occurs mostly in the capillaries (Tucker et al., 2023). The main physiological role of veins is to regulate circulating blood volume (Higashi, 2024), holding a large volume of blood at relatively low pressures. Conversely, arteries play a major role in sustaining organs with blood and nutrients at high systemic pressure and low volumes (Tucker et al., 2023).

1.2 Structural organisation of the endothelium

1.2.1 Vascular endothelium structure and physiology

ECs are in direct contact with the flowing blood forming the boundary between the bloodstream and the vascular wall. Their morphology and functions are influenced by their interaction with the blood flow (Watanabe and Fan, 2025), with haemodynamic forces directly affecting the health of blood vessels (Baratchi et al., 2017).

The endothelium, a monolayer of ECs, is an endocrine organ, the inner-most structure that coats the intima of arteries, veins, and capillaries. Although the morphology of ECs varies along the vascular tree and depending on the type of vessel, ECs are generally thin, flat, and slightly elongated with average dimensions of 30-50μm length, 10-30μm width, and 0.1-1μm height (Theofilis et al., 2021). Healthy ECs show a characteristic cobblestone pattern *in vitro* (Kruger-Genge et al., 2019a), although their phenotype is altered when cultured under flow (Kroon et al., 2017). In linear parts of the arteries, ECs align in the direction of flow (accompanied by actin cytoskeleton remodelling resulting in rearrangement of filamentous actin (f-actin) into bundles of stress fibers) leading to EC elongation, whilst in atherosclerotic lesions, which mostly develop near branch points and curvatures of the arterial tree, ECs are uniformly polygonal, and no longer elongated (Pan, 2009).

Despite their flattened morphology, ECs exhibit pronounced polarity. The apical (luminal) glycocalyx forms the lumen of the vessel facing circulating blood and contains glycocalyx structures that demonstrate sensitivity to shear due to blood flow transmitted through the cytoskeleton, therefore regulating shear stress sensing and molecular interactions (Vittum et al., 2024). The cytoskeleton, composed of actin filaments, intermediate filaments (vimentin), and microtubules, provides mechanical stability while allowing morphological remodelling in response to shear stress and inflammation (Zeng and Tarbell, 2014). The basal glycocalyx, at the smooth muscle cell interface, is anchored to the basement membrane and interacts with pericytes and ECM components

(Vittum et al., 2024). The endothelial glycocalyx encompasses the entire EC, transducing extracellular signals and regulating vascular permeability and barrier functions (Vittum et al., 2024).

1.2.2 Endothelial junctions

Intercellular junctions connect ECs into a contiguous monolayer to restrict the transport of proteins across the endothelial barrier in a size-selective manner and maintain the integrity of the endothelium (Dejana, 2004). Intercellular junctions are the main structures maintaining tissue-fluid homeostasis and are critical for vascular integrity and barrier function. ECs have three types of junctions (Figure 1.3):

- **Tight junctions**: Composed of claudins, occludins, and junctional adhesion molecules (JAMs).
- **Adherens junctions**: Mainly VE-Cadherin.
- **Gap junctions**: Formed by connexins.

Tight junctions and adherens junctions form pericellular zipper-like structures along EC borders through the adhesion of distinct adhesive proteins (Hartsock and Nelson, 2008). Platelet endothelial cell adhesion molecule (PECAM) is an endothelial junctional component that is located outside adherens and tight junctions (Dejana, 2004). In contrast, gap junctions are intercellular channels enabling direct electrical and chemical communication and providing pathways between ECs through the passage of ions and signalling molecules (Scemes et al., 2007, Dhein, 2002).

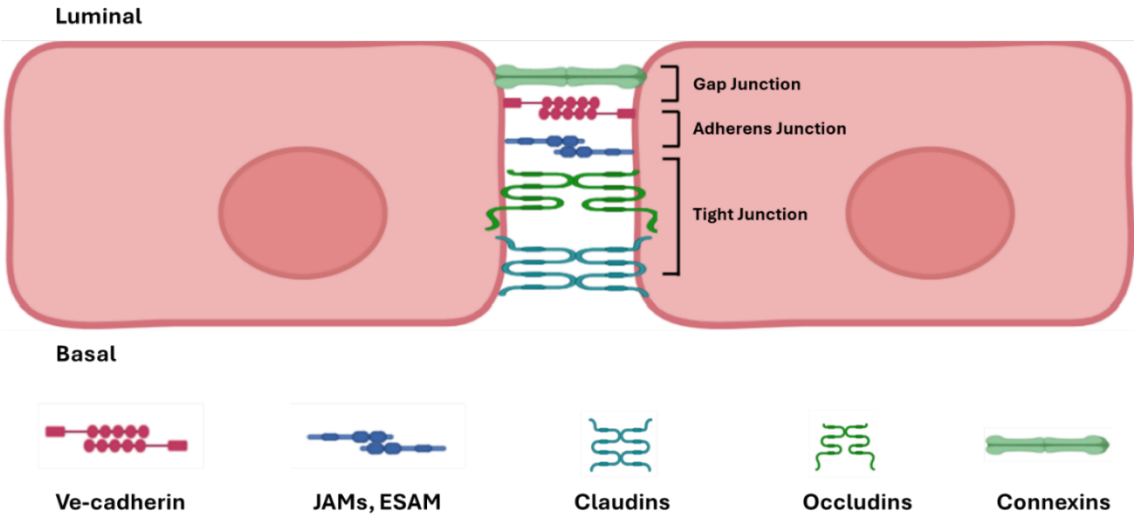

Figure 1.3: Endothelial cell gap junction, tight junction, and adherens junction proteins. Gap junction proteins include connexins, tight junction proteins include junctional adhesion molecules (JAMs) and Endothelial Cell Selective Adhesion Molecule (ESAM), claudins, and occludins, and adherens junctions include VE-Cadherin. Created in BioRender and PowerPoint.

1.2.3 Interaction with the basement membrane

ECs rest on a specialised basement membrane and interact with underlying components which make up the extracellular matrix (ECM). The ECM not only provides structural support but also acts as a signalling platform influencing survival, differentiation, and migration of ECs (Stratman and Davis, 2012). ECM synthesis and deposition surrounding the developing vasculature is critical for vessel remodelling and vessel tube maturation (Stratman and Davis, 2012). Pericytes, embedded within the basement membrane, interact closely with ECs to stabilise capillaries and regulate vascular maturation. EC-ECM interactions are key during dysfunction and damage to the endothelium, for example, during inflammation or tumour cell invasion (Kirkpatrick et al., 1989).

The ECM consists of a viscoelastic network of fibrillar proteins, surrounded by vascular proteoglycans (PGs), glycoproteins and various matricellular proteins. This network acts as a structural scaffold for cells and comprises two matrices. The pericellular matrix surrounds cells, and consists of laminin, perlecan, collagen IV and integrins, and the outermost interstitial matrix contains elastin, fibronectin, collagens, proteoglycans with associated glycosaminoglycans (GAGs), such as heparan sulfate (HS) and other ECM macromolecules (Figure 1.4) (Theocharis et al., 2016). Vascular proteoglycans are important regulators of the vascular wall, and support cell function by forming a glycocalyx around cells, mediating cell permeability and interaction with growth factors, cytokines and chemokines as well as crosstalk between vascular ECs and SMCs (Iozzo and Schaefer, 2015).

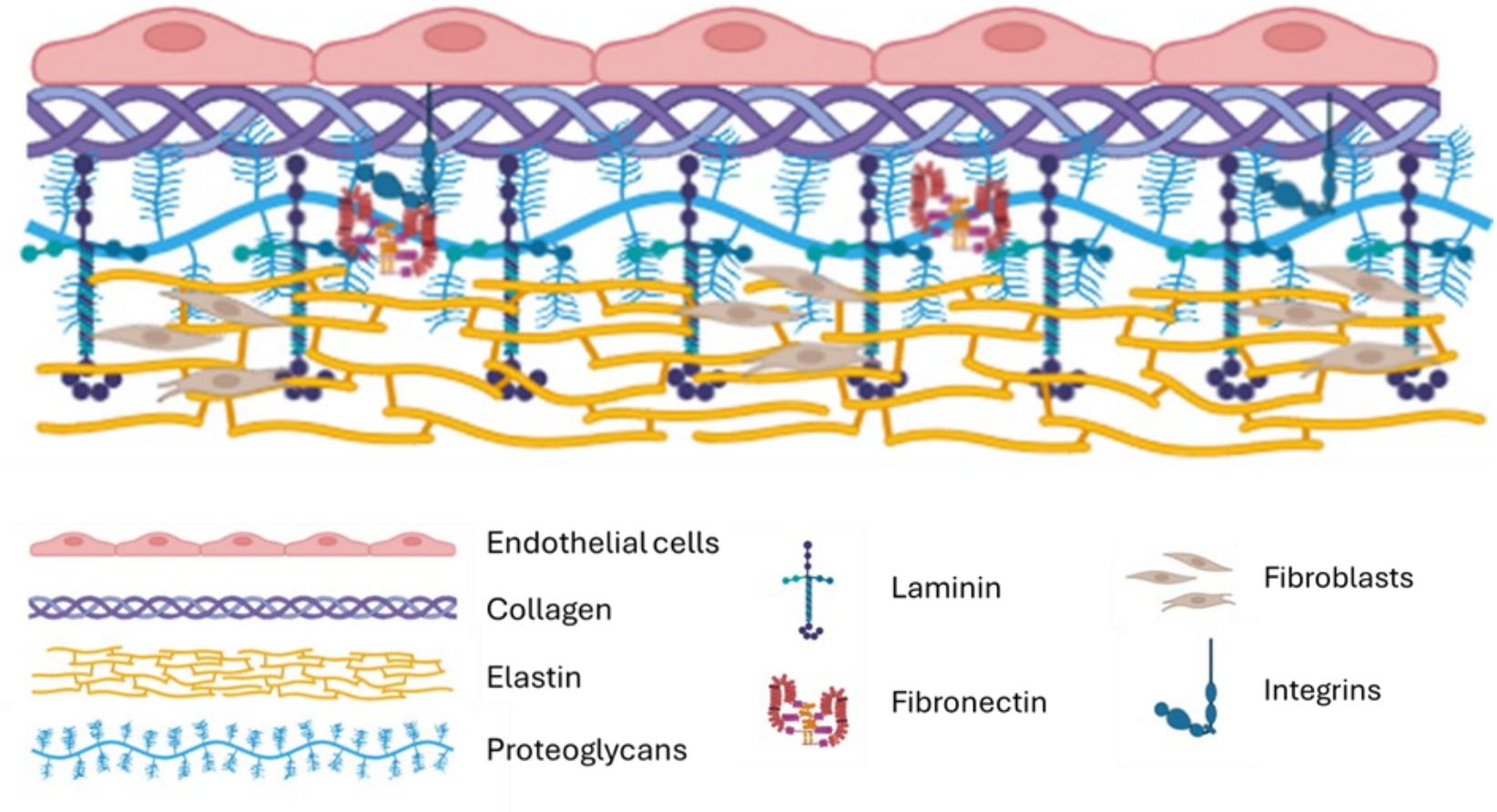

Figure 1.4: Endothelial structure and interaction with sub-endothelial basement membrane and its ECM components. ECM components include integrins, collagen, proteoglycans (including perlecan), elastin, fibroblasts, and glycoproteins- fibronectin and laminin). Created in BioRender and PowerPoint.

1.3 Endothelial Cell Functions

Although structurally the endothelium is a physical semi-permeable barrier separating the lumen from the vessel wall, it has a variety of functions, known to play an important role in cardiovascular physiology in health and pathophysiology during dysfunction. The endothelium is a major player in the control of blood fluidity, platelet function and aggregation, and wound healing, through the regulation of immunology, inflammatory response, coagulation, the exchange of fluids and solutes, and cell proliferation, migration and angiogenesis, among others (Baldwin and Thurston, 2001, Feletou, 2011).

Within each organ or tissue, ECs are highly specialised and transcriptionally distinct, even within single vessels (Dudley and Griffioen, 2023). The function of the endothelium varies depending on the blood vessel type and location within the vasculature (dela Paz and D'Amore, 2009), for example, the endothelium comprising the blood–brain barrier (BBB) has significantly different functional characteristics than the endothelium lining the aorta, as the BBB endothelium is a highly specialised, selective, and tightly regulated gatekeeper for maintaining the homeostasis of the central nervous system (CNS) (Xiang et al., 2025), whereas the aortic endothelium is optimised for systemic exchange, mechanotransduction, and vascular homeostasis (Mannion and Holmgren, 2023). However, all ECs share a common set of functions, including the regulation of haemostasis, maintenance of vascular permeability, mediation of acute and chronic immune responses to various types of injury, and control of vascular tone (Cyr et al., 2020).

ECs not only form a semi-permeable monolayer with anti-thrombotic properties, but also release angiocrine growth factors influencing organ development, regeneration, and metabolism (Rafii et al., 2016). EC-released factors act locally but also systemically when released into the bloodstream, allowing the endothelium to play crucial roles in the maintenance of body homeostasis, and pathologically in diseases such as cancer and CVD, contributing to their

pathogenesis through cell signalling (Preuss et al., 2023, Rafii et al., 2016, Alsina-Sanchis et al., 2021).

1.3.1 Barrier Function and the role of junctional proteins

During atherosclerotic processes, leukocyte infiltration into inflamed regions most frequently occurs through endothelial junctions composed of protein complexes of adherins, gap, and tight junctions (Figure 1.2) (Dejana, 2004). ECs function as gatekeepers that control the infiltration of leukocytes and plasma proteins into the walls of blood vessels. Junctions are not only sites of EC attachment but can also function as signalling structures having an important role in angiogenesis by modulating cell growth and apoptosis, whilst regulating vascular homeostasis (Dejana, 2004). The regulation of these junctions determines vascular permeability under physiological and pathological conditions.

1.3.2 Regulation of vascular permeability

As mentioned above, ECs function as a semi-permeable barrier between blood and tissues, regulating fluid exchange, nutrient delivery, and waste removal. The EC layer is a selective barrier that restricts the extravasation of macromolecules and cells while transporting gases and nutrients into tissues, therefore EC permeability is tightly regulated as it is essential for maintaining homeostasis and organ and tissue functions (Claesson-Welsh et al., 2021, Wakasugi et al., 2024).

Under quiescent conditions, the endothelial barrier suppresses the permeability of macromolecules and cells. When inflammation occurs, various mediators act on ECs to increase their permeability, allowing for the extravasation of large molecules and cells such as plasma proteins and leukocytes through two routes, the transcellular and paracellular pathways (Figure 1.5), which regulate the leakage of substances into the extravasation space. Leukocytes also extravasate through transcellular and paracellular routes during inflammation (Wakasugi et al., 2024, Wettschureck et al., 2019).

1.3.2.1 Paracellular permeability pathway

The majority of extravasation processes occur through the paracellular route which is controlled by the opening and closing of EC adhesions (tight and adherens junctions), which, under certain conditions, can move apart, open, or be internalised to make space between ECs to allow fluid or cells to pass into the extravascular space, and play essential roles in controlling endothelial paracellular permeability. Gap junctions are also involved in controlling endothelial permeability through the paracellular pathway (Wakasugi et al., 2024, Wettschureck et al., 2019).

1.3.2.2 Transcellular permeability pathway

Fluids and solutes are transported across the endothelial cytoplasm from the vessel lumen to the extravascular space at the basement membrane side through transcytosis (Wakasugi et al., 2024), where solutes or cells (particular macromolecules) are taken up by an EC and are transported by specialised structures including caveolae and vesiculo-vacuolar organelles (VVOs) (Wettschureck et al., 2019, Wakasugi et al., 2024).

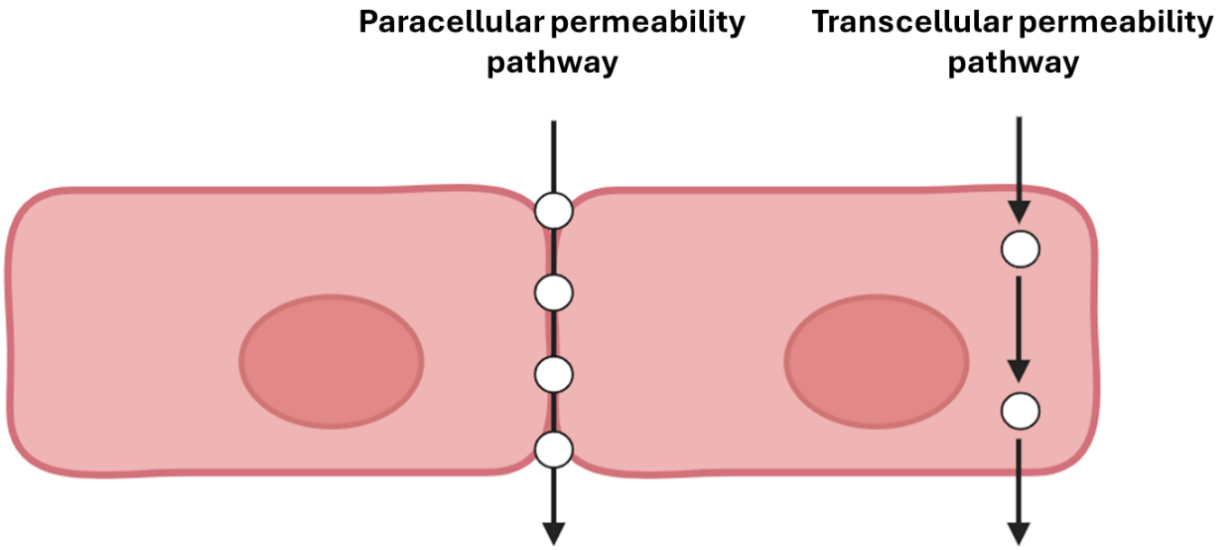

Figure 1.5: Endothelial paracellular and transcellular pathways. ECs form selective semi-permeable barriers which allow the transport of macromolecules from the blood through paracellular and transcellular pathways. Paracellular pathway allows the passage of macromolecules between endothelial cells by opening and closing endothelial junctions. Transcellular pathway involves transcytosis where molecules are transported across endothelial cells through caveolae and vesiculo-vacuolar organelles (VVOs). Created in BioRender and PowerPoint.

1.3.3 Vascular Tone Regulation

A healthy vascular endothelium controls vasodilation and vasoconstriction, which work in balance to maintain vascular homeostasis (Ross, 1999). ECs carry out these specific functions and actively regulate vascular tone and blood pressure and modulate various biological processes through signalling and the production and secretion of various mediators. These include vasodilators, such as nitric oxide (NO), prostacyclin (PGI_2), and smooth muscle cell vasorelaxants including C-type natriuretic peptide (CNP), an endothelium-derived hyperpolarising factor (EDHF) (Sandoo et al., 2010, Dickinson et al., 2024). Conversely, potent vasoconstrictors are produced by the endothelium, such as endothelin-1, and angiotensin II, which have directly opposing effects (Higashi, 2024).

ECs actively regulate vessel diameter by secreting vasoactive molecules including nitric oxide, endothelin-1, and prostacyclin.

1.3.3.1 Nitric oxide synthesis and function

Nitric oxide (NO) is produced and secreted by the endothelium following the stimulation of mechanosensors that sense shear stress (SS) caused by blood flow on vascular ECs (Palmer et al., 1987, Rees et al., 1989). NO is produced from L-arginine via activated eNOS and then released from ECs (Ignarro and Napoli, 2005). Endothelium-derived NO is a potent vasodilator in the vasculature. Endothelial dysfunction is strongly linked to decreased NO production reducing the amount of bioavailable endothelium-derived NO in the vasculature, generating an imbalance. This consequently results in an imbalance in vascular homeostasis and regulation of blood flow, generating a decreased vasodilatory response.

Additionally, reduced NO levels contribute to a pro-thrombotic, pro-inflammatory, and less compliant blood vessel wall as NO and its signalling functions, particularly in thrombosis and vascular tone, are central to the maintenance of vascular homeostasis (Medina-Leyte et al., 2021, Cyr et al.,

2020). This results in the suppression of platelet aggregation, leukocyte migration, and cellular adhesion to the endothelium (Vallance and Chan, 2001).

1.3.3.2 Endothelin and prostacyclin pathways

Prostacyclin (PGI_2) is an important protective hormone in the cardiovascular system produced by the vascular wall, whose synthesis is dependent on COX-1 and COX-2 enzymes (Figure 1.6). Prostacyclin is released from ECs and stimulates IP receptors on VSMCs, elevating intracellular cAMP levels, mediating relaxation and vasodilation (Majed and Khalil, 2012). The EC lining of the vascular system facilitates blood flow by prostacyclin-mediated vasodilation by causing a relaxation response in VSMCs as well as by prevention of excessive platelet aggregation (Suggs et al., 1986, Bunting et al., 1976). Angiotensin II, bradykinin, histamine, and thrombin can stimulate ECs to produce prostacyclin (Hong, 1980, Baenziger et al., 1980, Lonchampt et al., 2018).

Endothelin-1 (ET-1) has opposite actions depending on the receptor that it stimulates, ET_A or ET_B, influencing a range of homeostatic mechanisms throughout the body, and is instrumental in the control of vascular tone (Schneider et al., 2007, Banecki and Dora, 2023). ET_A is on SMCs and mediates constriction (classical response associated with ET-1), whilst ET_B is also present on ECs and stimulates the release of NO and PGI_2, mediating vasodilation (Schneider et al., 2007).

1.4 Haemostasis

1.4.1 Anti-coagulant and pro-coagulant properties

ECs play a key role in regulating haemostasis (Feletou, 2011), the physiological process that stops bleeding at the site of an injury, while maintaining normal blood flow elsewhere in the circulation (Gale, 2011). The pathway is a tightly regulated process that prevents significant blood loss following vascular injury. The normal haemostatic response depends on the closely linked interaction

between the blood vessel wall (mainly ECs), platelets, and blood coagulation factors (Zaidi and Green, 2025).

The luminal surface of quiescent ECs is non-thrombogenic, platelets and leukocytes do not adhere to it, and the haemostatic system remains inactivated (Kruger-Genge et al., 2019b). Healthy ECs release prostacyclin and NO to limit platelet activation and aggregation in the absence of vascular injury. The endothelium also produces ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1) or CD39, an ectonucleoside which hydrolyses circulating adenosine diphosphate (ADP) to adenosine monophosphate (AMP) (Hamilos et al., 2018), preventing unwarranted platelet activation.

By contrast, the components of the basal lamina, synthesised by and lying beneath ECs, are strongly thrombogenic, capable of recruiting and activating platelets and coagulation. The endothelium therefore provides an important barrier between the blood and the sub-endothelial matrix, which becomes exposed if the blood vessel is damaged (Figure 1.2, section 1.3). Several of the exposed components including collagen IV, fibronectin, and laminin lead to platelet recruitment and activation (primary haemostasis), and damage to the endothelium leads to the release of activators of coagulation (secondary haemostasis) which initiate the formation of a blood clot, composed primarily of platelets and fibrin (Gale, 2011).

1.4.2 Primary haemostasis (platelet activation)

Primary haemostasis refers to initial platelet aggregation and plug formation where platelets are activated and adhere to the site of injury and to each other (Gale, 2011).

Platelets circulate in a quiescent state and are kept inactive by endothelial derived NO and prostacyclin (Mitchell et al., 2008). When the continuity of the endothelium is disrupted, the sub-endothelial matrix and its constituent collagen fibres are exposed.

Von Willebrand factor (VWF), a multimeric protein, is predominantly synthesised by ECs and stored in Weibel–Palade bodies (WPBs), whilst also circulating in the

plasma (Jaffe et al., 1974). VWF strings released in the circulation stabilise FVIII and can bind to exposed collagen in the sub-endothelium. At sites of vascular injury, circulating VWF binds to the exposed collagen and facilitates platelet-EC interaction by recruiting platelets to sites of vascular injury by forming a bridge between the damaged vessel wall and platelets. The interaction of VWF with platelet GPIbα is crucial for initial platelet adhesion (Figure 1.5). VWF A1-GPIbα interaction allows firm adhesion of platelets to the exposed sub-endothelial matrix via the platelet collagen receptors GPVI and integrin α2β1 (Denorme et al., 2019). The GPIbα-VWF and GPVI/α2β1-collagen interactions induce downstream intracellular platelet signalling leading to activation of platelet αIIbβ3, which mediates further adhesion and aggregation via binding to fibrinogen and VWF (Denorme et al., 2019). Additionally, upon damage to the blood vessel wall, and endothelial activation, ECs release VWF from their WPBs to facilitate the development of inflammatory processes by recruiting leukocytes to inflamed tissues, leading to widespread thromboinflammation (Feletou, 2011, Denorme et al., 2019). These platelet–EC and leukocyte-EC interactions are at the interface between haemostasis and inflammation.

The secondary recruitment of platelets and their aggregation involve morphological changes and degranulation of the activated platelets (Sang et al., 2021). The release of ADP, ATP, and serotonin from the platelet dense granules, and VWF and fibrinogen from the platelet alpha granules, alongside synthesis and release of TXA2, further activates neighbouring platelets (Denorme et al., 2019). This then drives the recruitment of platelets to the platelet plug, amplifying the aggregation process via the activation of the integrin αIIbβ3 and fibrinogen binding (Denorme et al., 2019). TXA2 also induces VSM contraction, facilitating the formation of the haemostatic plug.

ECs can also contribute to the amplification of this process. In response to thrombin, following the activation of platelet protease-activated receptor (PAR) signalling, they release VWF, and the expression of TF is upregulated (Pearson, 1999). After platelet adhesion and activation, further recruitment, and

aggregation, the membrane phospholipid becomes exposed resulting in the activation of the clotting cascade (Zaidi and Green, 2025).

1.4.3 Secondary haemostasis (coagulation)

Secondary haemostasis refers to the synthesis of insoluble fibrin to stabilise the platelet plug. Fibrin is generated by the coagulation cascade that results in the cleavage of soluble fibrinogen by thrombin (Gale, 2011). Thrombin self-regulates its generation, and is responsible for further activating FV, FVIII, FXI and FXIII. Additionally, by interacting with its platelet receptor (PAR-1), it causes further platelet activation (Verhamme and Hoylaerts, 2006).

The initiation of the coagulation cascade is primarily mediated by tissue factor (TF) that is exposed following EC damage (Figure 1.6) (Park and Park, 2024). In healthy blood vessels, TF is located in the ECM underlying the ECs, in the adventitia, in a number of cells surrounding blood vessels, and in subcutaneous tissues (Park and Park, 2024). Exposure of TF to blood and it's subsequent binding to FVIIa initiates coagulation via the extrinsic pathway, forming an extrinsic tenase complex and activating FX to FXa (Park and Park, 2024). The intrinsic coagulation system is initiated by FXII. FXII is activated to FXIIa when in contact with negatively charged surfaces, such as sub-endothelial collagen (Park and Park, 2024). The initial step involves the conversion of plasma prekallikrein to kallikrein, which then activates additional FXII molecules. Intrinsic coagulation is driven by FXIIa-mediated FXI cleavage. FXIa activates FIX, which forms an intrinsic tenase complex with the cofactor FVIIIa, leading to FX activation (Park and Park, 2024). FXa catalyses the generation of thrombin (FIIa) through binding with its cofactor Va, and formation of the prothrombinase complex. Thrombin cleaves fibrinogen into fibrin, which then polymerises into a fibrin network, and is crosslinked by FXIIIa (Figure 1.7) (Gale, 2011).

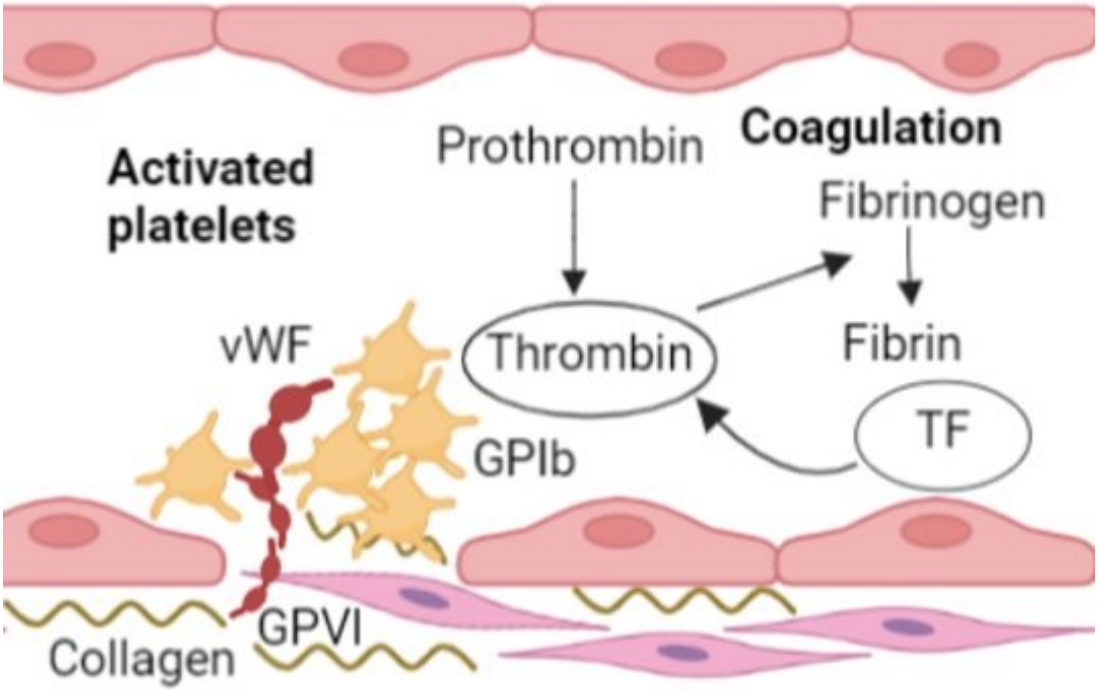

Figure 1.6: Pro-coagulant properties of ECs. At sites of vascular injury, VWF captures platelets from the circulation and mediates initial platelet adhesion and activation by GPIbα-VWF interactions, which enable the binding of GPVI to exposed sub-endothelial collagen. TF initiates the formation of thrombin from prothrombin which then acts to convert fibrinogen to fibrin, initiating the process of coagulation with platelets. Adapted from (Neubauer and Zieger, 2021). Created in BioRender and PowerPoint.

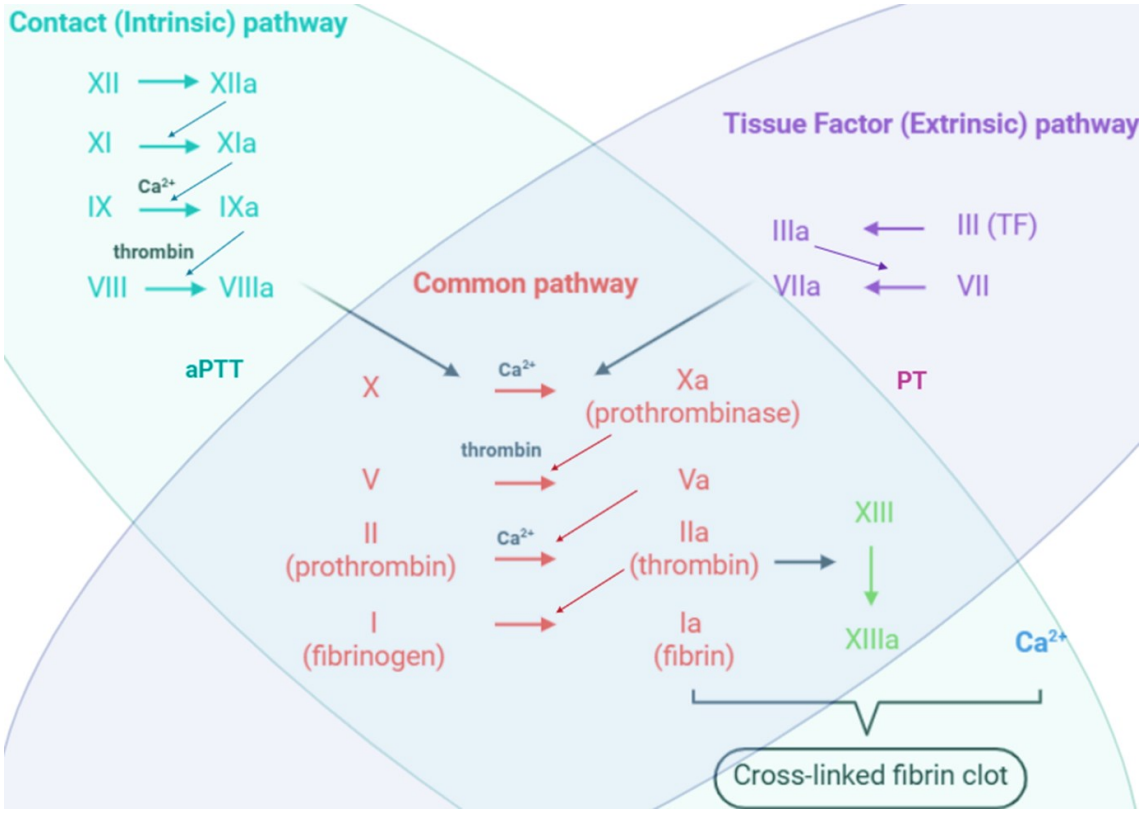

Figure 1.7: An overview of the coagulation cascade, demonstrating the intrinsic, extrinsic, and common pathways of coagulation. The coagulation cascade generates fibrin to stabilise a platelet plug after vascular injury. It is divided into the extrinsic and intrinsic pathways, which converge on a common pathway. The extrinsic pathway is initiated when TF exposed at the site of injury binds factor VIIa, activating factor X. The intrinsic pathway is triggered by contact activation, and with cofactor VIIIa, also activates factor X. In the common pathway, factor Xa with cofactor Va converts prothrombin (factor II) into thrombin, which cleaves fibrinogen into fibrin and activates factor XIII to crosslink fibrin into a stable clot. Thrombin also amplifies coagulation by activating cofactors (V, VIII, XI). Created in BioRender and PowerPoint.

The process of coagulation is controlled by negative regulators to prevent uncontrolled fibrin formation. Several inhibitors are derived from the endothelium including tissue factor pathway inhibitor (TFPI), HS and thrombomodulin.

TFPI is an alternatively spliced anti-coagulant protein that dampens the initiation phase of coagulation before thrombin is generated. TFPI's actions are localised to cells expressing TF and to sites of injury by altering cellular trafficking and signalling pathways driven by coagulation proteases of the TF pathway (Mast and Ruf, 2022). Essentially, TFPI blocks TF-FVIIa mediated generation of prothrombinase, FVa-FXa, in the common blood coagulation pathway (Mast and Ruf, 2022).

Two naturally occurring anti-coagulant pathways serve to regulate thrombin; antithrombin (AT), and the protein C pathway (Weitz, 2003).

HS achieves its anti-coagulant activity by interacting with AT, which then undergoes a conformation change, with the generation of the active form of AT that inhibits blood coagulation factors Xa and thrombin (Liu and Pedersen, 2007).

Thrombomodulin is a multidomain integral membrane glycoprotein constitutively and predominantly expressed on the luminal surface of vascular ECs of arteries, veins and capillaries (Maruyama et al., 1985). Thrombomodulin negatively regulates coagulation, converting thrombin from a pro-coagulant to an anti-coagulant enzyme, which then activates inhibitory mechanisms that act to restrain fibrin formation. Thrombomodulin-bound thrombin activates protein C, facilitated by the endothelial protein C receptor. Activated protein C (APC) with its cofactor protein S inactivates FVa and FVIIIa, resulting in decreased thrombin generation and reduced fibrin clot formation (Feletou, 2011).

Haemostasis relies on the tightly regulated balance between pro-coagulant systems (platelet activation and the fibrin formation/coagulation cascade) and anti-coagulant systems which exist to limit blood clot formation, including inhibitors of platelet activation, and anti-coagulant proteins which limit thrombin generation including APC/protein S, fibrinolysis, and serpins.

1.4.4 Thrombosis

Thrombosis, the pathological clotting of blood that affects arterial or venous circulation, is one of the leading causes of death worldwide, affecting men and women of all ethnicities globally (Ten Cate, 2021). Historically, three common factors predispose to thrombosis: 1) damage to the endothelial lining of a vessel wall, 2) hypercoagulable state, and 3) arterial or venous blood stasis.

Both arterial and venous thrombosis have similar risk factors, including age, obesity, smoking, chronic inflammation, hyperlipidaemia, hypertension, and metabolic syndrome (Previtali et al., 2011).

Damage to the vessel wall leads to reduced vascular integrity and exposure of the ECM, production of pro-inflammatory cytokines, increased expression of TF, proliferation of adhesion molecules, enhanced platelet activation through the production of thromboxane, and reduced expression of inhibitors of platelet activation (Mosevoll et al., 2018). The activation of leukocytes and ECs promotes the expression of adhesion molecules, which eventually initiate clot formation, alongside inflammation (Mosevoll et al., 2018). When an imbalance exists in the formation and lysis of clots, unwarranted thrombosis occurs (Ashorobi et al., 2021).

1.4.5 Fibrinolysis

Following successful blood clot formation and wound repair, for the vessel to be cleared of obstruction and blood flow to resume, the fibrinolytic system comprised of proteolytic enzymes dissolves the fibrin clot and restores a healthy blood vessel during the process of wound healing (Figure 1.8) (Feletou, 2011). Fibrinolysis is primarily composed of three serine proteases (plasmin, tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA)) that are present as zymogens in the blood and activated in the presence of fibrin(ogen). A healthy endothelium releases pro-fibrinolytic factors tPA and uPA (Figure 1.8) (Zaidi and Green, 2025).

tPA and plasma plasminogen come together on the surface of a fibrin clot, to which they both bind. tPA cleaves and activates plasminogen forming plasmin, which subsequently cleaves fibrin. uPA activates plasminogen in the presence of the uPA receptor (uPAR), which is found on various cell types including ECs (Collen and Lijnen, 1995). During haemostasis, production and release of tPA, and expression of uPAR is reduced, and damaged ECs also upregulate plasminogen activator inhibitor 1 (PAI-1) which inhibits tPA and uPA (Rau et al., 2007), AT, which inhibits thrombin, and Alpha-2-antiplasmin which directly inhibits plasmin, ultimately leading to a decrease in plasmin activation favouring fibrin formation (Figure 1.8). Once repaired, the vasculature returns to its pro-fibrinolytic state.

If haemostasis is out of balance, and the system is dysregulated due to a defect in one of these processes, then either thrombosis or pathological bleeding may result (Gale, 2011). While an efficient and rapid mechanism for stopping bleeding is essential for survival, it is equally important that this mechanism is tightly controlled, so that pathological thrombosis is prevented.

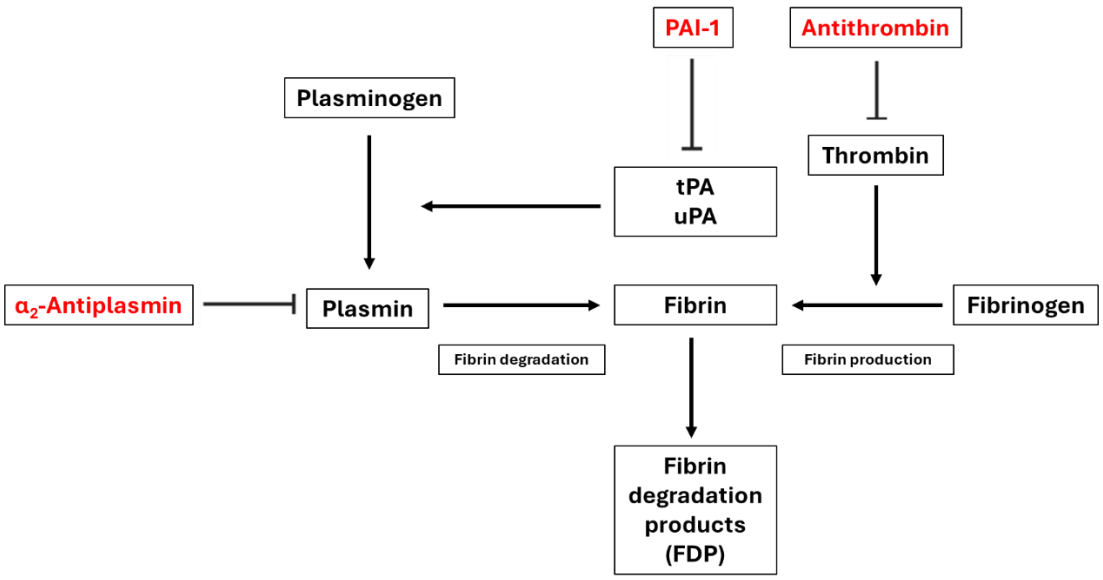

Figure 1.8: Fibrin production and degradation pathways. Normal haemostasis requires tight regulation between fibrin production (coagulation) and degradation (fibrinolysis). Thrombin drives fibrin formation from soluble fibrinogen. Conversely, fibrin degradation occurs through the conversion of plasminogen to plasmin, by tPA and uPA. Plasmin breaks down fibrin into fibrin degradation products (FDP). Serine protease inhibitors (SERPIN) exist to regulate these pathways. SERPINC1 (antithrombin) inhibits thrombin, preventing excessive fibrin formation, SERPINE1 (PAI1) limits plasmin generation by inhibiting tPA/uPA, and SERPINF2 (α2-antiplasmin) inhibits plasmin, preventing degradation of fibrin. Created in PowerPoint.

1.5 Immune and Inflammatory roles

Leukocytes play a key role in the regulation of inflammation, which initiates the expression of adhesion molecules such as ICAM-1, VCAM-1, and selectins, and production and release of cytokines TNFα, IL-6, and IL-8 from ECs. These endothelium-derived cytokines and receptors are involved in mediating inflammation and leukocyte recruitment in the vasculature. Pro-inflammatory cytokines produced and released by ECs activate neutrophils to form NETs and contribute to an inflammatory cascade, leading to increased inflammation and thrombotic events (Manoj et al., 2025, Hotchkiss and Karl, 2003).

1.5.1 Leukocyte adhesion and transmigration

Leukocyte interaction with the endothelium involves leukocyte tethering, rolling, activation, adhesion, and trans endothelial migration (TEM) or diapedesis (via paracellular and transcellular migration) through the basement membrane to the ECM (Ley et al., 2007). Most TEM takes place at endothelial borders via paracellular transmigration (Figure 1.4) (Muller, 2013).

1.5.2 Expression of adhesion molecules

ICAM-1

Intercellular adhesion molecule-1 (ICAM-1) is the only member of the ICAM family whose expression and function is inducible, and its surface expression is upregulated in ECs by pro-inflammatory cytokines like TNFα and IL-1β (Williams and Luscinskas, 2011, Singh et al., 2023). ICAM-1 mediates inflammation and promotes leukocyte migration and consequent atherosclerosis, playing a major role in regulating homeostasis and in pathological states including cancer, atrial fibrillation, myocardial infarction (MI), stroke, asthma, obesity, and kidney diseases (Singh et al., 2023).

VCAM-1

The role of vascular cell adhesion molecule-1 (VCAM-1) in endothelial dysfunction and leukocyte infiltration during vascular inflammation has established VCAM-1 as a critical contributor to the pathogenesis of

atherosclerosis. Disrupting VCAM-1 function blocks monocyte infiltration into the sub-endothelial space, which effectively prevents macrophage maturation and foam cell transformation necessary for atherosclerotic lesion formation (Ley and Huo, 2001, Pickett et al., 2023).

P-selectin

P-selectin is a constitutively synthesised adhesion molecule stored in WPBs in ECs, and is rapidly translocated to the cell membrane upon activation with inflammatory mediators like thrombin, histamine and platelet-activating factor (PAF), where it initiates endothelial-leukocyte interactions through binding P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, playing a significant role in leukocyte recruitment, tethering and rolling and contributing to the progression of atherosclerotic processes (Auvinen et al., 2014, Yang et al., 1999). Additionally, during platelet activation, P-selectin stored in platelet α-granules is exposed at the surface of the membrane and promotes platelet-leukocyte interaction, enabling direct leukocyte binding via PSGL-1, forming platelet–leukocyte aggregates promoting thromboinflammation (Dole et al., 2005).

1.5.3 Cytokine signalling and inflammatory response

There are many cytokines that have been extensively implicated in the process of inflammation, contributing to atherosclerosis, and consequent thrombosis. Pro-inflammatory cytokines produced and released from the endothelium such as TNFα, IL-6 and IL-8 are essential inflammatory mediators, aggravating the inflammatory response and inducing the expression of adhesion molecules in ECs (ICAM-1, VCAM-1) and p-selectin to recruit leukocytes, which worsen endothelial dysfunction (Medina-Leyte et al., 2021).

IL-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced by many cells, including ECs and is involved in many different immunological processes. It is the major regulator of acute phase response proteins and plays a crucial role in CAD (Bouzidi and Gamra, 2023), with IL-6 levels having shown to be associated with the development of cardiovascular events (Neumann et al., 1995). IL-6

plays a pro-inflammatory role in the contribution to CVD, involved in the activation of ECs, inducing aggregation and activation of platelets, promoting SMC proliferation and macrophage lipid accumulation, contributing to the development of atherosclerosis, and promoting clot formation and vessel occlusion in atherothrombosis (Zhang et al., 2018b, Reiss, 2017). IL-6 upregulates ICAM-1 expression in vascular ECs, promoting inflammatory and atherosclerotic processes, and is strongly involved in the maintenance and progression of inflammation by enhancing neutrophil function and migration (Higashi, 2024).

IL-8

IL-8 is produced and released by ECs and has pro-inflammatory properties, mainly activating neutrophils and enhancing their migratory potential playing roles in inflammatory diseases (Harada et al., 1994). Increased endothelial permeability along with high expression of IL-8 have been demonstrated at sites of injured endothelium and early-stage atherosclerotic plaque formation, suggesting that IL-8 participates in regulating endothelial permeability in the developing processes of vascular disease (Yu et al., 2013). Additionally, IL-8 increases endothelial permeability during early stages of angiogenesis, has been implicated in cancer development and progression (Waugh and Wilson, 2008).

TNFα

Tumour Necrosis Factor alpha (TNFα) plays an important role in endothelial dysfunction and inflammation (Idriss and Naismith, 2000). TNFα activates ECs and other cell types to express and release various inflammatory cytokines, chemokines and adhesion molecules. In clinical studies, increased TNFα circulating levels were found to be associated with the endothelial dysfunction in hypertensive patients (Naya et al., 2007), with carotid atherosclerosis thickness in patients with early atherosclerosis (Skoog et al., 2002), and increased CAD risk through increasing thrombosis (Kaptoge et al., 2014, Safranow et al., 2009).

1.6 Vascular Remodelling

1.6.1 EC proliferation, migration, and angiogenesis

Proliferation, migration and angiogenesis are crucial for processes such as tissue growth and wound healing and repair, ensuring the delivery of oxygen and nutrients in response to stimuli such as hypoxia and inflammation (Pena and Martin, 2024). These processes are highly regulated by the endothelium involving ECM (collagen) deposition, and tissue remodelling (Mao and Zhirou Lin, 2025).

Angiogenesis, sprouting of new vessels from pre-existing vessels, occurs when EC tube networks are formed through the recruitment of supporting cells including pericytes and vascular SMCs, primarily mediated by EC proliferation and migration (Mannell et al., 2021). During the early events in vascular morphogenesis, the vasculature is very dynamic and prone to remodelling in a flow environment (Stratman and Davis, 2012).

eNOS (endothelial nitric oxide synthase)-derived NO also plays a critical role in angiogenesis and vascular remodelling, where NO production is one of the final products of angiogenic signalling cascades (Cyr et al., 2020). Additionally, key angiogenic factors, such as vascular endothelial growth factor (VEGF) are crucial in regulating vascular tube formation through its contribution in angiogenesis and remodelling, mediating EC cell proliferation, migration, and survival, and increased permeability capabilities (Shi et al., 2023). VEGF signalling is a critical regulator of angiogenesis processes that are vital for the development of vascular systems, tissue repair, and the maintenance of homeostasis. Dysregulated VEGF signalling drives diverse pathological events, including CVD. Excessive VEGF activity promotes tumour growth, invasion, and metastasis, while insufficient signalling contributes to impaired wound healing and ischemic diseases, therefore an appropriate balance is required (Lee et al., 2025).

Angiogenesis is tightly regulated, and its dysregulation can lead to pathological conditions. Numerous diseases such as cancer and CVD are characterised or caused by abnormal or excessive angiogenesis or conversely by insufficient

angiogenesis or vessel regression (Fischer et al., 2005). Angiogenesis is regulated by various pro/anti-angiogenic molecules, and plays a crucial role in tumour growth, invasion, and metastasis (Liu et al., 2023). Additionally, there has been an association demonstrated between intralesional angiogenesis with atherosclerotic plaques that cause ACS, suggesting a link between neovascularisation and atherosclerosis (Tenaglia et al., 1998, Khurana et al., 2005).

1.7 Vascular (dys) function in CVD

Dysfunction of the vascular endothelium is a hallmark of various diseases under the umbrella of CVD. Vascular endothelial dysfunction plays an essential role in the onset, maintenance, and development/progression of atherosclerosis, and is strongly associated with known traditional cardiovascular and cardiometabolic risk factors (Sun et al., 2019), such as hypertension, diabetes, and dyslipidaemia which have been shown to impair vascular function, as well as coronary risk factors including age, smoking, obesity, menopause and lack of physical activity (Higashi and Yoshizumi, 2004, Higashi, 2024), ultimately triggering cardiovascular events (Vane et al., 1990, Higashi et al., 2009). Therefore, vascular dysfunction has been shown to be a predictor of cardiovascular events in various clinical trials and meta-analyses (Higashi, 2024, Tanaka et al., 2018) and can independently predict the development of CVD or cardiovascular events (Fischer et al., 2005, Lerman and Zeiher, 2005).

1.8 Atherosclerosis

Atherosclerosis is a chronic, pro-inflammatory, and lipid-driven mechanism that facilitates the build-up of lipid rich plaques in the blood vessel wall (Wang et al., 2021). Atherosclerotic plaque rupture or erosion activates circulating platelets, resulting in activation of the coagulation cascade and initiation of thrombus formation, the major cause of heart attacks and stroke (Braganza and Bennett, 2001).

EC dysfunction is a central initiator and contributor to the development of atherosclerosis, where during atherosclerotic processes, ECs can become

activated by inflammatory cytokines, and by other stresses such as hypoxia and metabolic stress (van Hinsbergh, 2012). Increased vascular permeability supports monocyte transmigration and differentiation into macrophages and foam cells, leading to the promotion of atherosclerotic plaque development (Friedl et al., 2002). The transport of lipoproteins through the endothelial barrier and into the sub-endothelial space plays a pivotal role in the pathogenesis of atherosclerosis, emphasising a key role for the endothelium and inflammation in all phases of atherosclerosis. Elevated circulating low-density lipoprotein (LDL) levels represent one of the best-characterised risk factors for CAD and the accumulation of LDL under the arterial endothelium represents the first step in the pathogenesis of atherosclerosis (Zhang et al., 2018a). Inflammatory processes have a key role not only in the initiation and progression of atherosclerosis but also in the stability of the established atherosclerotic plaques (Medina-Leyte et al., 2021). The endothelium is therefore a useful therapeutic target for atherosclerosis and consequent thrombus formation (Higashi, 2024).

Key stages of the development of atherosclerosis include:

1. Endothelial dysfunction- triggered by risk factors (LDL, hypertension, smoking), causing inflammation, increased oxidative stress, expression of adhesion receptors, production of inflammatory cytokines, increased permeability, and loss of endothelial barrier integrity (Wang and He, 2024).
2. Lipid retention and oxidation, and lesion initiation- LDL accumulates in the intima, becomes oxidised, and initiates inflammation (Jebari-Benslaiman et al., 2022).
3. Fatty streak formation- monocytes are recruited and extravasation occurs where monocytes enter the intima and differentiate to macrophages, ingest oxidised LDL and accumulate lipid, then form foam cells (Moore and Tabas, 2011).
4. Fibrous plaque formation and development- SMCs take up LDL, migrate and proliferate, and collagen deposition and fibrous cap formation

occurs over lipid core pools that have formed, promoting calcification and neovascularisation (Buja, 2023).

5. Plaque vulnerability- Inflammation degrades collagen, foam cells apoptose, fibrous cap thins, and plaque becomes prone to rupture (Buja, 2023, Jebari-Benslaiman et al., 2022).
6. Plaque rupture and thrombosis- Atherosclerotic plaques contain a lipid-rich core beneath the fibrous cap. When the fibrous cap ruptures, blood is exposed to the thrombogenic lipid core, triggering platelet activation which, along with the collagen in the plaque, triggers the coagulation cascade and thrombus formation (Libby, 2013).

1.9 Atherothrombosis

Atherothrombosis, a major cause of ACS and cardiovascular death, is characterised by atherosclerotic plaque disruption with superimposed thrombus formation (Viles-Gonzalez et al., 2004). Atherothrombosis is a multicellular process affecting various cell types and factors within the blood and vasculature and is initiated by EC dysfunction (Yau et al., 2015b, Da Silva et al., Asada et al., 2020). Atherosclerotic plaque disruption via rupture or erosion activates platelets. Subsequent platelet adhesion, aggregation, recruitment of leukocytes, and activation of the coagulation cascade, trigger the process of arterial thrombus formation with associated inflammation (thromboinflammation), resulting in atherothrombosis, all driven by a dysfunctional crosstalk between ECs, platelets and leukocytes (Viles-Gonzalez et al., 2004, Yamashita and Asada, 2012).

1.10 Challenges with current anti-thrombotic therapies

With the dominance of platelets in arterial thrombi, historically, anti-platelet therapy has been the cornerstone for prevention and treatment of atherothrombosis (Chan and Weitz, 2019), with Aspirin being administered as the most widely used anti-platelet agent, along with ADP P2Y12 receptor antagonists including the thienopyridines Clopidogrel and Prasugrel, Ticagrelor, a triazolopryrimidine, and Cangrelor, an ATP analogue (Arockiam et al., 2023). Anti-platelet therapy may also be used in combination with lipid lowering drugs, such as statins which are the gold standard for treating patients with thin-capped atheromatous plaques, slowing lipid accumulation and delaying plaque progression (Honda et al., 2018). Additionally anti-coagulant treatment exists (including direct oral anti-coagulants (DOACs), e.g. Dabigatran and Apixaban), and many patients rely on oral anti-coagulants to decrease the risk of ischemic stroke and other thromboembolic events, along with widespread use for the treatment of atherothrombosis/CAD (Amaraneni et al., 2025). However, despite their efficacy in preventing and treating thrombosis, bleeding remains a major side effect of anti-thrombotic therapeutics, demonstrated by one of many studies which identified significantly increased haemorrhage following Aspirin use (Wolfe et al., 2025), and efficacy is limited by variation across patient groups with co-morbidities, requiring careful monitoring of patients on anti-platelet therapy, presenting an unmet need for safer and more efficacious anti-thrombotic therapies. With current anti-thrombotic therapies, bleeding remains a concern and limits the ability to provide the most effective therapy to patients with or at risk of thrombosis (Chan and Weitz, 2019). Advances in the understanding of the contribution of ECs in thrombosis have prompted new treatment options, and since no current therapies target the endothelium, the endothelium presents potential as a novel targeting strategy.

1.11 Protein kinases

Over 500 protein kinases are known to be encoded in the human genome and are involved in diverse signalling pathways including the regulation of cardiac

function in health and disease states (Shahin et al., 2017), and modulating various cellular process in cardiovascular homeostasis (Bai et al., 2024).

Protein kinases have been demonstrated to play a role in vascular ECs, for example protein kinase C (PKC) has been implicated in pathways involving angiogenesis, endothelial permeability, vascular tone, and endothelial activation (Kant and Feng, 2025). Protein kinases catalyse the transfer of phosphate from ATP to a target protein substrate. Phosphorylation regulates protein function by inducing conformational changes, creating a network of hydrogen bonds among specific amino acids. Phosphorylation can also disrupt surfaces for protein-ligand interactions/create a ligand-binding surface, without inducing conformational changes. In turn, these post-translational modifications regulate the function of cellular proteins in cell metabolism, signal transduction, and various other biological processes (Cheng et al., 2011).

Protein kinases are regulated by protein-protein interactions, binding of ligands, and reversible/irreversible covalent modifications such as phosphorylation, including autophosphorylation and limited proteolysis (Cheng et al., 2011). Protein kinases that directly phosphorylate proteins are grouped into one of two types, tyrosine kinases, and serine/threonine kinases (Taylor et al., 1995). Protein phosphorylation on serine, threonine, or tyrosine residues, is one of the most important and widely understood post-translational modifications (Khoury et al., 2011). Reversible protein phosphorylation is catalysed by phosphatases which systematically remove phosphate groups maintaining the equilibrium of physiological protein phosphorylation (Simpson et al., 2023).

1.12 Pim kinases

Proviral Integration Site for MuLV (Murine Leukaemia virus) (Pim) kinases are a family of constitutively active serine/threonine kinases consisting of three highly homologous members: PIM1, PIM2, and PIM3 (Nawijn et al., 2011). They are referred to as isoforms due to being highly conserved with high amino acid

sequence homology, but are expressed by three independent genes, and located on different chromosomes in both the human and mouse genome (Nock et al., 2023). Alternative translation initiation sites have also been described for PIM1 and PIM2, giving rise to isoforms with different molecular weights (Warfel and Kraft, 2015).

All three Pim proteins can phosphorylate serine and threonine amino acids and can activate similar cellular pathways (Hoover et al., 1991). Individual Pim kinases are therefore considered compensatory, whereby the loss of one Pim kinase isoform can be alleviated by the expression of another Pim kinase isoform (Bellon and Nicot, 2023). Despite high sequence similarity however, Pim family isoforms show substrate specificity across the individual kinases.

1.12.1 Structure and regulation

1.12.1.1 Gene

Pim kinases are similar in human and mouse and share significant functional similarities, although chromosomal location differs. Features of Pim kinase isoforms are outlined in Table 1.1. Each gene has six codons surrounded by large untranslated regions (UTR) (Nock et al., 2023). Pim kinases lack regulatory domains within the protein and its structure, therefore they are considered to be constitutively active (Nock et al., 2023). Their kinase activity is tightly controlled through expression and stability via transcriptional, post-transcriptional, translational, and post-translational modifications and mechanisms (Nock et al., 2023). The activity of Pim kinases is mainly influenced by the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, nuclear factor kappa-B (NF-κB) (Warfel and Kraft, 2015), and heat shock protein 90 (HSP90) (Shay et al., 2005).

Transcriptional regulation of Pim kinases is usually in response to mitogenic stimulants such as platelet-derived growth factor (PDGF) in vascular SMC (Zhou et al., 2021), high glucose (Wang et al., 2017), hypoxia (Warfel et al., 2016), and inflammation such as in response to cytokines, IL-6 (Block et al., 2012), and TNFα treatment (Yang et al., 2011c), for example PIM1 has been shown to be

upregulated by signalling through granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor and its activation involves tyrosine kinase JAK2 signalling to activate PIM1 (Palaty et al., 1997a).

Post-transcriptional modifications are related to mRNA processing. Within the mRNA sequence of each isoform are five AUUA destabilising motifs in the untranslated region (UTR) at 3' which target the transcripts for rapid degradation (Wingett et al., 1991, Saris et al., 1991). Although this is a key mechanism for the regulation of the Pim kinases, this may not be the only mechanism of transcriptional regulation of Pim kinase isoform expression levels. In vascular SMCs there appear to be other transcriptional methods of control, such as delayed transcription and translation (Nock et al., 2023), for example, cell stimulation with PDGF-BB lead to a transient increase in PIM1 mRNA at 1hr, however the protein expression profile did not match this time course with protein levels peaking much later at 24hrs (Willert et al., 2010).

MicroRNAs (MiRNA) have also been identified to be important in controlling Pim kinase expression. MiRNAs interact with the UTR of their target mRNAs, and many have been identified as targets for Pim kinases within the cardiovascular system where one study has shown that the inhibition of miR-195 results in increased PIM1 (Zhang et al., 2020).

Table 1.1: Features of PIM1, PIM2, and PIM3 kinases. Adapted from (Nock et al., 2023).

Features	PIM1	PIM2	PIM3
Mouse chromosomal location	17	X	15
Human chromosomal location	6	X	22
Isoform molecular weights	34kDa 44kDa	34kDa 37kDa 40kDa	34kDa

1.12.1.2 Protein

Although they are classed as serine/threonine kinases, Pim kinases are constitutively active, uniquely lacking regulatory domains, with only kinase domains present (Asati et al., 2019). The kinases adopt the commonly observed bi-lobed kinase fold structure seen in other kinases, however, the Pim family members contain a novel two-stranded β-sheet with an αc helix, and a unique hinge region, distinguishing them from other kinases (Figure 1.9) (Kumar et al., 2005). All Pim kinases show spatial structural similarity in their hinge region which has two inserted proline residues, widening the ATP binding site. This widened ATP site does not allow for the typical hydrogen bonds to capture their ATP substrate, and results in high Michaelis constant (Km) values for ATP (Bullock et al., 2009). This unique feature provides potential to find selective inhibitors specifically for Pim kinases, making targeting the Pim kinase family attractive.

Post-translational modifications and regulation of Pim kinase include phosphorylation, dephosphorylation, ubiquitination, and Small Ubiquitin-like Modifier (SUMOylation) (Iyer et al., 2017). Additionally, Pim kinase autophosphorylation is also believed to be important for kinase stability, but not activity which is controlled by expression (Kreuz et al., 2015, Torres-Ayuso and Brognard, 2025).

Various proteins have been identified as possible mediators of Pim phosphorylation including Protein Kinase C α (PKCα) and endothelial/epithelial tyrosine kinase (Etk) (Kim et al., 2004).

Dephosphorylation and subsequent degradation of Pim kinase occur via protein phosphatase 2A (PP2A), with inhibition of PP2A activity increasing the half-life of Pim kinases (Losman et al., 2003). SOCS-1, involved in the degradation of proteins through ubiquitination, binds to Pim kinases, and is required for the PP2A-mediated degradation of Pim kinase (Chen et al., 2002, Liau et al., 2018, Losman et al., 2003).

PIM1 has been shown to be protected from ubiquitin-mediated proteasome degradation via a mechanism that involves HSP90 binding. During the treatment of cells with geldanamycin, a HSP90 inhibitor, rapid degradation and reduced kinase activity of PIM1 occurs (Mizuno et al., 2001, Shay et al., 2005). However, ubiquitin-mediated degradation may not extend to all Pim isoforms, as ubiquitination of PIM2 does not appear to target the kinase for degradation demonstrated by a study showing that inhibiting the formation of the cullin RING ligase-containing ubiquitin ligase complex via inhibition of NEDD8-Activating Enzyme did not lead to a modulation of PIM2 protein levels, despite control proteins being altered, and additionally, the E1-ubiquitin ligase inhibitor PYR-41 did not alter the stability of the PIM2 protein (Adam et al., 2015).

Degradation of Pim kinases may also occur via Small Ubiquitin-like Modifier (SUMOlyation). Mutation of the potential SUMO site E171 in PIM1 increases the protein half-life to almost double that of WT (Iyer et al., 2017). Whilst only PIM1 was studied, PIM2 and PIM3 also share similar consensus sequences for SUMOylation. Furthermore, silencing of the SUMO-targeted E3 ubiquitin ligase RNF4 also increases PIM1 levels (Iyer et al., 2017). This suggests ubiquitin-facilitated proteasomal regulation of Pim kinase levels.

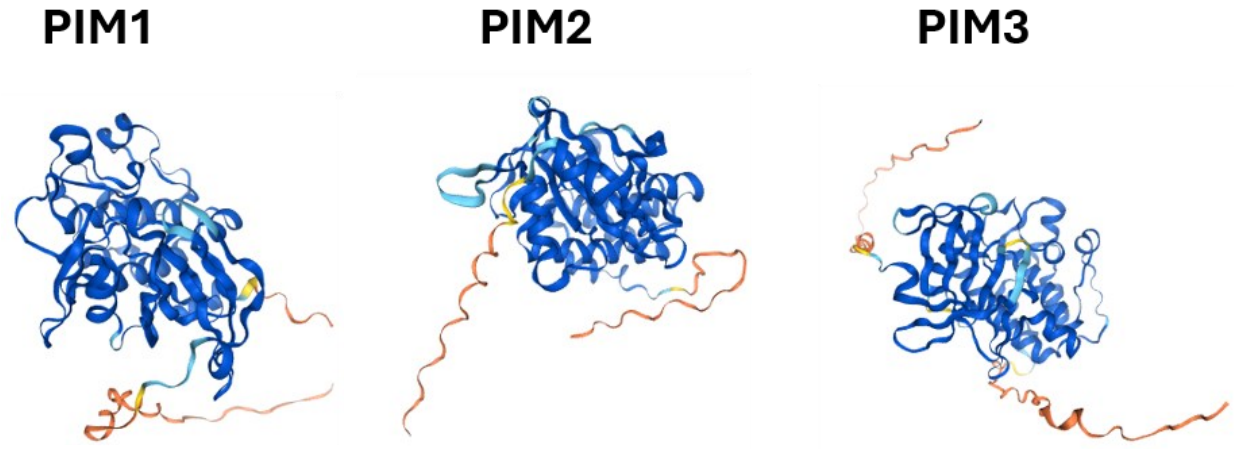

Figure 1.9: Predicted PIM1, PIM2, and PIM3 protein structures. Colours denote confidence for predicted structure where dark blue= very high, light blue= confident, yellow= low, and orange= very low confidence based on predicted local distance difference test (pLDDT). Taken from the Human Protein Atlas (ProteinAtlas.org, 2026).

1.12.2 Function

Pim kinases have been identified in various cell types in the body, contributing to many functions (Morishita et al., 2008). Pim kinases are downstream effectors of many cytokine and growth factor signalling pathways and are upregulated in various malignancies (PubChem, 2012b).

The majority of studies have investigated Pim kinase function in cancer metastasis and progression and established that Pim kinases are upregulated in various cancer types, predominantly haematological and solid tumour cancers (Shah et al., 2008), promoting cell survival, proliferation, and migration (Santio and Koskinen, 2017, Santio et al., 2015). Whilst most established for their role in cancer progression, there is increasing evidence for wider pathological roles of Pim kinases within the context of CVD, including a role for Pim kinases in ECs, platelets and thrombosis (Unsworth et al., 2021, Chen et al., 2016).

1.12.3 PIM1

PIM1, located on chromosome 6, encodes 34 and 44kDa isoforms that display comparable kinase activity. PIM-1S (34kDa) and PIM-1L (44kDa) can phosphorylate the androgen receptor at serine-213, whereas only the larger isoform could phosphorylate at threonine 850 *in vivo* suggesting some differential phosphorylation, which may be related to their localisation within the cell (Linn et al., 2012). The longer isoform of PIM1 is demonstrated to bind to the SH3 domain of the ETK/BMX tyrosine kinase, localising it to the plasma membrane, whereas the shorter isoform is primarily localised in the cytosol and nucleus (Xie et al., 2006). PIM1 key sites include K67, H68 (site mutation increases kinase activity), P81 (site mutation decreases kinase activity), P123 (proline region in hinge site which is unique to Pim family kinases), and Ser261 (phosphorylation site) (Kumar et al., 2005, Qian et al., 2005, Jacobs et al., 2005).

PIM1 inactivates pro-apoptotic protein Bcl-2-associated death promoter (Viles-Gonzalez et al.) by phosphorylating it on Ser112 (Aho et al., 2004). Furthermore, it promotes cell cycle progression through phosphorylation and activation of Cdc25 and phosphorylation and inhibition of Cdc25-associated kinase 1 (C-TAK1), an inhibitor of Cdc25C (Bachmann et al., 2004, Bachmann et al., 2006). The promotion of tumorigenesis through phosphorylation of c-Myc (Zhang et al., 2008) and regulation of mTORC (Rebello et al., 2018) is also mediated via PIM1. PIM1 has also been shown to promote cell migration and chemotaxis (metastasis) via the phosphorylation of CXCR4 (Santio et al., 2015).

1.12.4 PIM2

PIM2, located on the X chromosome, exists as three isoforms (34, 37, and 40kDa) that are all cytoplasmic (Warfel and Kraft, 2015). 37kDa and 34kDa isoforms of PIM2 have very short half-lives (less than 30 min), whereas the 40kDa isoform has a half-life approximately twice that of the shorter isoforms (Adam et al., 2015). PIM2 is transcriptionally regulated by cytokines, mitogens, and numerous growth factors (Cheney et al., 2007). PIM2 key sites include P119 (proline residue in hinge site, unique to Pim kinases), K121 (site mutation renders kinase inactive)

(Nock et al., 2023), and D128 and E171 (required for potent drug inhibition of PIM2 but not PIM1 and PIM3 (Ishchenko et al., 2015)).

PIM2 shares 66% amino acid sequence homology with PIM1, with the most substantial differences being within the C terminal (Nock et al., 2023). Within this sequence are six proline residues that are not predicted to form a helical structure; therefore, PIM2 lacks a C-terminal α helix compared to the other isoforms (Bullock et al., 2009). This lack of helical structure is thought to increase the flexibility of the terminus, resulting in structural changes to PIM2 compared with PIM1 and PIM3, which may offer an explanation as to why pan-Pim kinase inhibitors appear to be less potent on PIM2 compared to the other Pim family members (Dakin et al., 2012, Keeton et al., 2014). Despite these differences in structure, PIM2, like PIM1, can also phosphorylate c-Myc (Zhang et al., 2008) and maintain mTORC signalling by maintaining inhibitory phosphorylation on eukaryotic initiation factor 4E binding protein (4E-BP1) and BAD, allowing for cell growth and survival (Fox et al., 2003), however, they do have different levels of expression and protein stability. These differences in protein stability are likely due to the disordered N-terminal domains that can be recognised directly by the proteasome (Adam et al., 2015). Differences in the activity of the PIM2 isoforms have also been observed *in vitro*. The largest isoform has been shown to be less active when compared to the smaller isoforms which may be due to the larger 40kDa isoform containing an auto-inhibitory sequence (Yan et al., 2003).

1.12.5 PIM3

PIM3, located on chromosome 22, has only been described as a single isoform of 34kDa molecular weight (Atalay and Ozpolat, 2024). Similar to PIM2, PIM3 kinases are transcriptionally regulated by cytokines, mitogens, and numerous growth factors (Cheney et al., 2007). PIM3 key sites include the hinge region and Ser190 (predicted autophosphorylation site) (Palaty et al., 1997b).

PIM3 shares 77% sequence homology and similar protein structure with PIM1 (Nock et al., 2023). Similar to PIM1 and PIM2, PIM3 can phosphorylate BAD at Ser112, with PIM3 deletion shown to reduce phosphorylation at this site (Li et al., 2006, Popivanova et al., 2007). MYC activity can also be augmented by PIM3, along with control of protein synthesis (Beharry et al., 2011).

1.13 Pim kinases and cardiovascular disease

Whilst mostly well known for their role in cancer progression, there is increasing evidence for wider pathological roles of Pim kinases, including in CVD and thrombosis. The Pim kinase isoforms have widespread expression in cardiovascular tissues, including the heart, coronary artery, aorta, and blood (Figure 1.10), and have been demonstrated to be upregulated in several co-morbidities and risk factors for CVD (Nock et al., 2023), for example, PIM1 is increased 11-fold in CAD (Chittenden et al., 2006) and PIM2 is upregulated in atherosclerotic arteries in CAD (Archacki et al., 2003). Furthermore, Pim kinases have been linked to inflammation (Clements et al., 2025), particularly PIM1, which has been identified to contribute to inflammatory disease processes in various studies, contributing to the upregulation of pro-inflammatory cytokines (Baek et al., 2024, Ha et al., 2019).

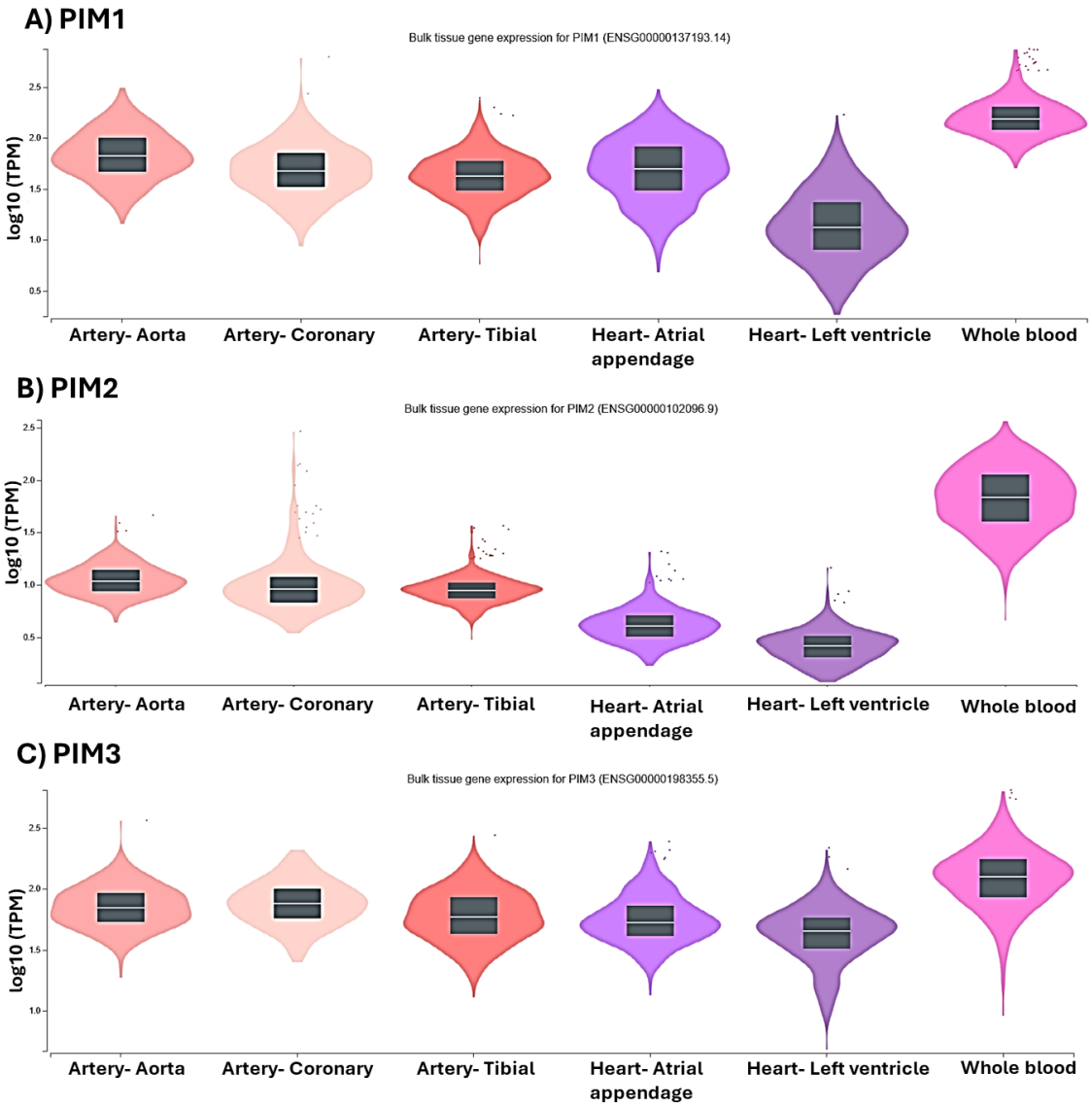

Figure 1.10: Bulk tissue gene expression of PIM1, PIM2, and PIM3 in tissues of the cardiovascular system. Pim kinase isoform genes A) PIM1, B) PIM2, and C) PIM3 are demonstrated to be present in the aorta, coronary artery, tibial artery, atrial appendage, left ventricle of the heart, and in whole blood. Created using the GTEX portal and PowerPoint.

1.13.1 Pim kinases, platelets and thrombosis

Pim kinases are highly expressed in haematopoietic cells where they are important for the differentiation and development of blood cells and precursors (An et al., 2013a), and play an important role in haematopoietic stem cell proliferation and survival (An et al., 2013a). It has previously been shown that PIM1 is expressed in human and mouse platelets and has shown to contribute to thrombosis (Unsworth et al., 2021), and a study has shown increased PIM1 expression in DVT (Chen et al., 2023). Additionally, it has more recently been shown that PIM1 contributed to an upregulation of TF, initiating an increased coagulation response in the development of sepsis (Wang et al., 2026).

A study investigating the role of Pim kinase in platelet function identified anti-platelet properties of pan-Pim kinase inhibitors where treatment of human platelets with AZD1208 caused a reduction in surface expression levels of the thromboxane A2 receptor (TPaR), leading to a reduction in signalling events downstream of TP-coupled G proteins (Gq and Ga13), and reduced platelet activation (Unsworth et al., 2021). In addition to regulation of TPaR-dependent platelet responses, Pim kinase inhibition also attenuated CXCR4-mediated platelet responses, with a reduction in CXCR4a ligand SDF-1a also observed following treatment with AZD1208 (Nock et al., 2024). These findings indicate that PIM1 inhibition could be a novel mechanism for anti-platelet therapies. This is supported by observations from thrombosis studies using PIM1 deficient mice, which show reduced thrombus formation using *in vitro* models and reduced thrombosis *in vivo* following acute treatment of mice with pan-Pim kinase inhibitor AZD1208 (Unsworth et al., 2021). Despite PIM1 mice demonstrating reduced thrombotic capacity, normal haemostatic functions were maintained, suggesting that PIM1 inhibition may be a suitable target for anti-platelet therapy, having no adverse effects on bleeding (Unsworth et al., 2021). The roles of PIM2 and PIM3 in platelet function and thrombosis have yet to be described.

1.13.2 Pim kinases and the endothelium

Various studies have identified roles for Pim kinases in the endothelium, particularly focused on HUVECs. Studies using HUVECs have identified a role for Pim kinase in the regulation of VEGF-dependent eNOS signalling downstream of the VEGF receptor Flk1, where siRNA-mediated knockdown of PIM1 in HUVECs reduced VEGF-induced eNOS phosphorylation at Ser-663 (Chen et al., 2016).

To date, little is known about the role of Pim kinase in arterial EC function and its contribution to CVD, however there are various studies that have explored Pim kinase and its effects on ECs within the context of cancer. All three isoforms of Pim kinase are expressed in ECs and have been shown to play regulatory roles in the adhesion, inflammation, and migration of ECs (Min et al., 2012, Walpen, 2012, Yang et al., 2011b, Zhang et al., 2017). PIM3 has demonstrated a role in regulating TNFα signalling, a key inflammatory cytokine involved in EC dysfunction (Yang et al., 2011c). Loss of PIM1 also increases actin polymerisation and EC adhesion, indicating that Pim kinase inhibitors have the potential to reduce atherothrombotic events linked to plaque erosion, which are responsible for a third of all heart attacks (Walpen et al., 2012).

1.14 Pim kinase inhibitors

Protein kinases have shown to be activated in heart disease, and their inhibition has been shown to improve cardiac function (Zheng et al., 2023). Therefore, with an increased understanding of the contribution of kinases to cardiovascular biology, protein kinases are increasingly a desirable target for CVD (Arrouchi et al., 2019, Shahin et al., 2017). The targeting of Pim kinases has great future potential for the repurposing of drugs against CVD using several inhibitors of Pim kinases that currently exist for the treatment of haematological and solid tumour malignancies (Arrouchi et al., 2019). As Pim kinases are constitutively active, they have become a potential target for cancer therapy, with numerous inhibitors being developed to treat various malignancies. Although these have

been developed and in clinical trials (Table 1.2), they demonstrate variable efficacies among different isoforms (Bellon and Nicot, 2023).

To date, most efforts to inhibit Pim kinases in cancer treatment generally use ATP-competitive drugs that target the kinase action of the protein (Luszczak et al., 2020), preventing it from phosphorylating its downstream effectors, either through quinones or other classes of small molecule inhibitors (Schroeder et al., 2016, Mohareb et al., 2019). The pharmacological profile and mechanism of action of Pim kinase inhibitors used in this study are ATP-competitive, blocking serine/threonine kinase activity of Pim kinases.

AZD1208, PIM447, and TP3654 can be achieved and tolerated in plasma between 0.1-10μM *in vivo*, as demonstrated in clinical studies (Keeton et al., 2014, Raab et al., 2019, Kunder et al., 2022, Lebedinsky et al., 2020).

1.14.1 AZD1208

AZD1208 is a potent, selective ATP-competitive pan-Pim inhibitor that inhibits all three Pim kinase isoforms at a low nanomolar range (inhibition constant (Ki) values of PIM1: 0.1nM, PIM2: 1.92nM, and PIM3: 0.4nM) (Keeton et al., 2014). The structure of AZD1208, a benzylidene-1,3-thiazolidine-2,4-dione, is shown in Figure 1.11. Cellular studies have demonstrated that AZD1208 reduces the expression and phosphorylation of downstream Pim kinase effectors such as STAT3 and mTOR (Yadav et al., 2019). AZD1208 has been used in various clinical trials to treat advanced solid malignancies and acute myeloid leukaemia (AML), as shown in Table 1.2.

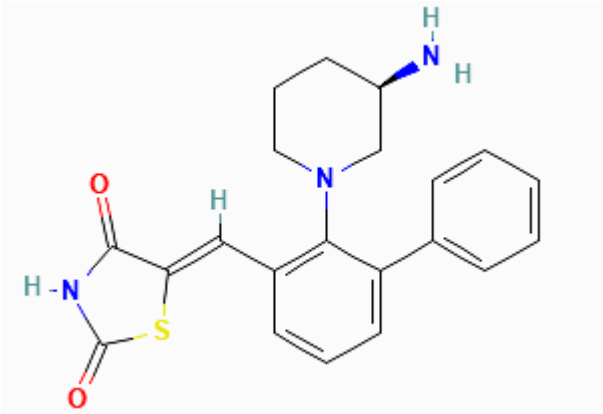

Figure 1.11: Chemical structure of AZD1208. Chemical formula of AZD1208; $C_{21}H_{21}N_3O_2S$, identified as benzylidene-1,3-thiazolidine-2,4-dione. Taken from (PubChem, 2012b).

1.14.2 PIM447 (LGH447)

PIM447 is a potent, highly selective, and orally bioavailable small molecule pan-Pim kinase inhibitor (Garcia et al., 2014). The structure of PIM447 is shown in Figure 1.12. PIM447 inhibits the Pim kinase isoforms at the following inhibition constant (Ki) values; PIM1: 6pM, PIM2: 18pM, and PIM3: 9pM (Burger, 2015). PIM447 has been used in various clinical trials to treat myelofibrosis, AML, and multiple myeloma, as shown in Table 2.1.

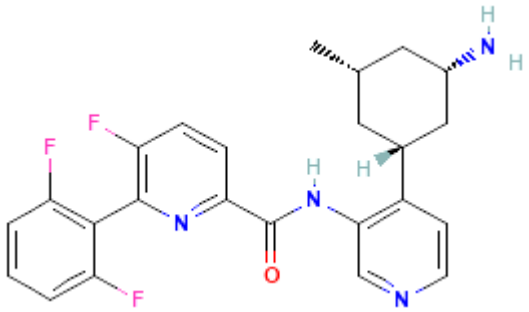

Figure 1.12: Chemical structure of PIM447. Chemical formula of PIM447; $C_{24}H_{25}Cl_2F_3N_4O$, identified as: trans-α,α-dimethyl-4-[[3-[3-(trifluoromethyl)phenyl]imidazo[1,2-b]pyridazin-6-yl]amino]cyclohexanemethanol. Taken from (PubChem, 2010).

1.14.3 TP3654

TP3654 (SGI-9481, Nuvisertib) is a second-generation small molecule pan-Pim inhibitor with improved potency and decreased cardiotoxicity compared to previous Pim kinase inhibitors. The structure of TP3654 is shown in Figure 1.13. TP3654 more selectively targets PIM1, with an inhibition constant (Ki) of 5nm for PIM1, 239nM for PIM2 and 42nM for PIM3 (Luszczak et al., 2020). This selectivity is often replicated by other Pim inhibitors, as many of them inhibit PIM2 much less efficiently than the other Pim kinase isoforms (Cervantes-Gomez et al., 2019). TP3654 has been used in various clinical trials to treat myelofibrosis and advanced solid tumours, as shown in Table 1.2.

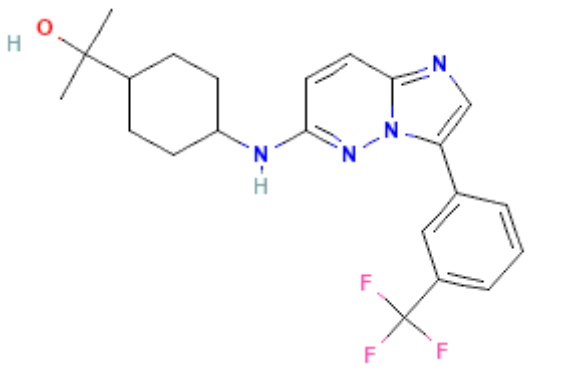

Figure 1.13: Chemical structure of TP3654. Chemical formula of TP3654; $C_{22}H_{25}F_3N_4O$, Identified as trans-α,α-dimethyl-4-[[3-[3-(trifluoromethyl)phenyl]imidazo[1,2-b]pyridazin-6-yl]amino]cyclohexanemethanol. Taken from (PubChem, 2012a).

Table 2.2: Pim kinase inhibitor clinical trial information. Taken from (ClinicalTrials.Gov, 2025).

Pim inhibitor	Target	Clinical trial number	Clinical trial phase	Status	Cardiovascular events/complications
AZD1208	Advanced solid malignancies/Malignant lymphoma	NCT01588548	Phase 1	Completed	-Thrombocytopenia -Atrial fibrillation (Cortes et al., 2018, Keeton et al., 2014)
	Acute myeloid leukaemia	NCT01489722	Phase 1	Terminated	-Hypotension (Cortes et al., 2018)
PIM447 (LGH447)	Myelofibrosis	NCT02370706	Phase 1	Completed	-No reported cardiovascular effects
	AML and high risk MDS	NCT02078609	Phase 1	Completed	-Blood pressure effects -Electrical conduction effects -Thrombocytopenia
	Multiple myeloma	NCT02160951	Phase 1	Completed	-Thrombocytopenia -QT prolongation
	Relapsed and refractory multiple myeloma	NCT02144038	Phase 1	Completed	-QT prolongation (Raab et al., 2019)
	Multiple myeloma	NCT01456689	Phase 1	Completed	-Cardiomyopathy -Rhythm alterations
TP3654 (Nuvisertib)	Advanced solid tumours	NCT03715504	Phase 1	Completed	-No reported cardiovascular effects
	Myelofibrosis	NCT04176198	Phase 1/2	Recruiting	-No reported cardiovascular effects (Mascarenhas, 2025, Lebedinsky et al., 2020)

Phase I clinical trials using Pim kinase inhibitors for the treatment of several malignancies have demonstrated drug efficacy and shown pan Pim kinase inhibitors, AZD1208, PIM447, and TP3654 to be safe and tolerable at therapeutic doses, without bleeding complications (Cortes et al., 2018, Raab et al., 2019). Additionally, genetic deletion of all three Pim kinase isoforms does not affect viability or fertility in mice, indicating that lack of Pim kinases is safe and well tolerated (An et al., 2013b, Mikkers et al., 2004). This indicates the potential of repurposing Pim kinase inhibitors for the treatment of CVDs and some of the associated risk factors in high-risk individuals. Repurposing pre-existing therapies may offer a more advantageous development strategy that quickens the drug development pipeline from synthesis to patient testing. Targeting the endothelium, based on the other anti-thrombotic effects of Pim kinase inhibitors, might mean they offer an alternative strategy.

1.15 Aims, objectives and hypothesis

1.15.1 Study aims

Current anti-thrombotic therapies are limited by variability between patients, having no effect in some patients while causing adverse bleeding in others. Therefore, developing novel, safer, and more efficient anti-thrombotic treatments is an unmet clinical need.

The various roles carried out by ECs such as regulation of barrier function, anti-thrombotic properties, and angiogenic capacity have broad impact in cardiovascular biology and therefore offer great potential for a CVD therapeutic targeting strategy (Afshar et al., 2023).

This project aims to investigate the influence of Pim kinase in regulating arterial EC control of thrombus formation and its contributing role in the pathways involved in atherothrombosis. This will allow Pim kinase to be explored as a novel endothelium targeting strategy for the prevention of cardiovascular-related events.

1.15.2 Hypothesis

Pim kinase inhibition will promote the anti-thrombotic capacity of the endothelium and reduce endothelial activation and dysfunction.

1.15.3 Objectives

The objectives in this project are:

- O1: Characterise the thrombotic profile of PIM1 deficient mice.
- O2: Investigate the effect of Pim kinase inhibition on arterial endothelial function.
- O3: Elucidate the therapeutic potential of Pim kinase inhibition on endothelium-blood crosstalk and thrombosis.

Chapter 2. Materials and methods

2.1 Ethics statement

This project requires the use of human whole blood, and primary cells (HCAECs and PECs). All health and safety, risk assessments and potential ethical issues were considered prior to the start of the project. Procedures and experiments involving human participants were conducted following appropriate approval obtained from The Science and Engineering Research Ethics and Governance Committee (REGC) at Manchester Metropolitan University (EthOS approval- 48062), in place from 16/11/2022 and the School of Medicine Research Ethics Committee at the University of Leeds (MREC 23-001) in place from 27/03/2024, conducted in accordance with the Declaration of Helsinki.

Materials

2.2 Primary Cells

Separate batches of primary HCAEC 500,000 human coronary artery endothelial cells (HCAECs) were used in this study at varying passages (3-6), purchased from PromoCell (Heidelberg, Germany). Mouse pulmonary endothelial cells (PECs) were isolated from wild-type (WT) and PIM1 global knockout ($PIM1^{-/-}$) mouse lungs, isolation methodology described in section 2.8.2. Cells were grown directly on plastic tissue culture flasks/plates.

2.3 $PIM1^{-/-}$ global knockout mice

$PIM1^{-/-}$ global knockout mice (C57/BL6/J background) were generated by MRC Harwell via electroporation of CRISPR/Cas9 reagents into 1-cell stage embryos. The CRISPR/Cas9 induced mutation deleted 3288 nucleotides from the Pim1 gene, across critical exons ENSMUSE00000235024 to ENSMUSE00000234945/ENSMUSE00001453577/ENSMUSE00001451542 to introduce a frameshift that results in a premature stop codon/null allele.

Heterozygous male and female mice were bred to yield homozygous PIM1 $^{-/-}$ and WT mice in a mendelian ratio. All mice generated were on a C57Bl/6J background and both male and female mice were used throughout this study. All pups were ear notched at weaning (~3 weeks of age) to allow for identification and isolation of genomic DNA (gDNA) for automated genotyping by Transnetyx.

2.3.1 PIM1 mouse genotyping

Genotyping was outsourced and performed by Transnetyx. To genotype the mice, the following primers and probes were used:

- Wildtype specific primer situated within the deleted region.

PIM1-DEL3267-WT1 assay (FAM labelled)

cccagagagctaccatatcatttcttcattttctgtctggaccagagaggcagagatggagagaaaaaaaaaatatggttgttttaa
agtcaaatctgcctgtctgcctcctgagagctgggattaaaggcatgtgccaccaaaccc**gagccaggcagtgtctttat**taaaaaa
tatttgaaaacct**agtggaggtggtggttcttgcttt**taatccagcccttggg**aggtagaggtaggcagatctc**cgagttcaaggcc
agcctggcctacagagtcacttccagagctacacagagaaactttggaggagaaaaaaaaaattaaaagaagcggctcagacta

Lower case letters denote the deleted sequence in the mutant allele.
Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo PIM1-DEL3288	5' label	Sequence 5' → 3'	3' label	Oligo Type
PIM1-DEL3288-WT_F	n/a	**GAGCCAGGCAGTGTCTTTAT**	n/a	WT Forward
PIM1-DEL3288-WT_PROBE	FAM	**AGTGGAGGTGGTGGTTCTTGCTTT**	ZEN-IBFQ	WT Probe
PIM1-DEL3288-WT_R	n/a	**GAGATCTGCCTACCTCTACCT**	n/a	WT Reverse

- Mutant specific primer that bridges the junction designed for the CRISPR mutant allele or is 3' of the junction.

PIM1-DEL3288-MUT1 assay (FAM labelled) (untested)

CCCCACCCTGGGCGCGCGGGTCGTTGGGACGCCGGGAGACCCCCGCGTGGGTCGAGCGCGCTGAG
TGTCCCGTGCCTC**TTTCCTAGGCAAAGAGAAGGAG**CCCCT**GGAGTCGGCCCTCCTTTGAAGAAA**TC
CGGAACCATCCATGGATGCAGGGTGACCTCCTGCCCCAGGCAG**CTTCTGAGATCCATCTGCACA**GTC
TGTCACCGGGGTCCAGCAAGTAGCAGCCTTTCTGCTGCTGTCTGTCAGACCCTCCAGGGAGGGAGA

Probe sequence is in bold and shaded grey
Primer sequences are in bold and underlined

Oligo PIM1-DEL3288	5' label	Sequence 5' → 3'	3' label	Oligo Type
PIM1-DEL3288-MUT _F	n/a	**TTTCCTAGGCAAAGAGAAGGAG**	n/a	MUT Forward
PIM1-DEL3288-MUT _PROBE	FAM	**TTTCTTCAAAGGAGGGCCGACTCC**	ZEN-IBFQ	MUT Probe
PIM1-DEL3288-MUT_R	n/a	**TGTGCAGATGGATCTCAGAAG**	n/a	MUT Reverse

2.3.2 Animal husbandry

All animal husbandry, housing and procedures were carried out in line with the guidelines and regulations of the University of Leeds Central Biological Services facility under the Animals (Scientific Procedures) Act 1986 and carried out under a United Kingdom Home Office approved project licence (PP0499799). Mice were housed in ventilated clear, plastic cages (GM500, Techniplast), containing standard bedding and maintained on a 12-hour light and dark cycle (21˚C and 50-70% humidity. relative humidity). Mice were fed standard rat and mouse no.1 maintenance diet (RM1, Special Diet Services) and given water through Hydropac pouches.

2.4 Media and supplements

Endothelial Cell Growth Medium MV2 (Promocell- [C-22022]) was supplemented with Endothelial Cell Growth Medium MV2 Supplement Pack (Promocell- [C-22121]) contents: 5ng/mL recombinant human (rH) epidermal growth factor, 10ng/mL rH basic fibroblast growth factor, 20ng/mL rH insulin-like

growth factor, 0.5ng/mL VEGF 165, 1µg/mL ascorbic acid and 0.2µg/mL hydrocortisone, supplemented with 5% (v/v) foetal calf serum (FCS) and 100U/ml (1%) penicillin-streptomycin (Fisher Scientific, Loughborough, UK).

2.5 Pharmacological Pim kinase inhibitor preparation

Pim kinase inhibitors AZD1208, PIM447 and TP3654 were purchased from Stratech Scientific (UK) and each dissolved in dimethyl sulfoxide (DMSO) (ThermoFisher Scientific, US), to make 10mM and 100mM stock solutions, which were stored at -20°C. Pim kinase inhibitors were used at a range of concentrations including 0.1, 0.3, 1, 3, 10, and 30µM.

Table 2.1: Pim kinase inhibitor cell-free kinase assay K_i values.

Pim inhibitor	**K_i values (inhibitory constant)**
AZD1208 [S7104-SEL, 10mg]	PIM1: 0.1nM PIM2: 1.92nM PIM3: 0.4nM (MedChemExpress)
PIM447 (LGH447) [S7985-SEL, 2mg]	PIM1: 6pM PIM2: 18pM PIM3: 9pM (MedChemExpress, 2025b, Selleckchem, 2025a)
TP3654 [S6774-SEL, 5mg]	PIM1: 5nM PIM2: 239nM PIM3: 42nM (MedChemExpress, 2025c, Selleckchem, 2025b)

0.1% DMSO was used as vehicle control in all experiments conducted.

2.6 Antibodies

Table 2.2: Antibodies used for Western blotting experiments (section 2.13) and supplier information.

Antibody	Dilution	Supplier
Primary		
Anti-PIM1 antibody [ZP003] (ab54503)	1:250	Abcam, Cambridge, UK
Recombinant Anti-PIM2 antibody [EPR6987] (ab129057)	1:1000 – 1:10000 (1:5000)	
Anti-PIM3 antibody - C-terminal (ab198842)	1:500	
Anti-beta Actin antibody - Loading Control (ab8227)	1:1000 – 1:5000 (1:2500)	
Secondary		
HRP-linked goat anti-rabbit IgG [7074]	1:1000–1:3000 (1:1000)	Cell Signalling Technology, Leiden, The Netherlands
HRP-linked horse Anti-mouse IgG [7076]	1:1000–1:3000 (1:1000)	

2.7 Fluorescence labels

Table 2.3: Fluorescence labels used for microscopy experiments (section 2.14) and supplier information.

Stain	Dilution/concentration	Supplier
Thermo Scientific™ DAPI	100 ng/mL	ThermoFisher Scientific
Invitrogen™ Rhodamine phalloidin	1:750	

2.8 Buffers

Table 2.4: Buffers used for Western Blotting experiments (section 2.13) and supplier information.

Buffer	Reagents	Supplier
10% Sodium Dodecyl Sulfate (SDS) solution	SDS RNAse-free water	Fisher Scientific
Laemmli sample buffer	62.5 mM Tris-HCl, pH 6.8 25% glycerol 2% SDS 0.01% Bromophenol Blue	Bio-Rad Laboratories Ltd
Tris-glycine running buffer	192mM glycine 25mM tris 1% SDS (pH 8.3)	-
Towbin buffer	25 mM Tris 192 mM glycine 20% (v/v) methanol (pH 8.3)	-
Tris-buffered saline (TBS)	10 mM Tris 150 mM NaCl	-
TBST	10 mM Tris 150 mM NaCl 0.1% Tween-20	-
Restore™ Western Blot Stripping Buffer	-	Sigma-Aldrich

2.9 Agonists/Mediators of endothelial cell dysfunction and platelet activation

Various agonists/mediators of endothelial dysfunction and platelet activation were used in experiments conducted for this project.

Table 2.5: Chemical mediators of endothelial dysfunction/platelet activation and supplier information.

Chemical mediator/agonist	**Supplier**
Human TNF-alpha Recombinant Protein, PeproTech®	ThermoFisher Scientific
Human IL-6 Recombinant Protein, PeproTech®	ThermoFisher Scientific
Phorbol 12-myristate 13-acetate **(Swystun et al.)**	Sigma-Aldrich
Thrombin (from human plasma)	Sigma-Aldrich
Tissue-type plasminogen activator (tPA)	Biosynth

Methods

PIM1 mice experiments

2.10 Murine blood analysis

2.10.1 Whole blood collection

Following schedule 1 euthanasia, murine blood was taken from the inferior vena cava into a sodium citrate (1:10v/v) containing syringe and used immediately for a full blood count (FBC) and coagulation analysis. Whole blood collection from mice was conducted by Dr Beth Webb and Dr Cedric Duval who held personal licences for animal work.

2.10.2 Full blood count

A FBC was carried out on the Sysmex haematology analyser. 20µL of citrated whole blood was added to measure various indices. RBC count, Hb and MCV, WBC count, lymphocyte, mixed (monocytes/eosinophils/basophils) and neutrophil counts and percentages, and platelet count, MPV, and PDW were measured.

2.10.3 ROTEM

Rotational thromboelastometry was performed using a ROTEM analyser (ROTEM Delta, Werfen) and ROTEM mini-cups according to the manufacturer's guidelines. For each assay, 105µL of citrated whole blood was pipetted into a pre-warmed mini-cup. For EXTEM, 7µL of EXTEM reagent (tissue-factor activator) was added, and for INTEM, 7µL of INTEM reagent (ellagic-acid activator) was used. Coagulation was initiated by addition of 7µL CaCl2 reagent, and measurements were recorded for 60 minutes. Standard parameters including clotting time (Burger), clot formation time (CFT), alpha angle (α), amplitude at 10 minutes (A10), and maximum clot firmness (MCF) were recorded.

2.10.4 Fibrin formation

30µL plasma mix (PPP from PIM1$^{-/-}$ mice and saline solution (1 in 6 diluted plasma)) was added to each well of a half volume 96-well plate. An activation mix was made containing thrombin (0.1U/mL), CaCl2 (10mM), and saline to investigate the common pathway of coagulation. To initiate fibrin formation, 20µL of activation mix was added to each well, giving a total volume of 50µL. The plate was then immediately read on the BioTek PowerWave HT microplate reader and absorbance (optical density) read at 340nm every 17 seconds for 1 hour at 37˚C to measure fibrin formation, using three parameters: lag time, time to 50% clotting, and time to MaxOD. Three technical repeats were run for each sample.

2.10.5 Fibrinolysis assay

20µL plasma mix (PPP from PIM1$^{-/-}$ mice and saline solution (1 in 6 diluted plasma)) were added to each well of a half volume 96-well plate. 30µL of activation mix containing thrombin (0.1U/mL), CaCl2 (10mM), tPA (20ng/mL), and saline was added to each well, giving a total of 50µL. Once activation mix was added, the plate was read immediately on a BioTek PowerWave HT microplate reader and absorbance (optical density) at 340nm determined every 17 seconds for 6 hours at 37°C. Three technical repeats were run for each sample measuring MaxOD to 50% lysis, and MaxOD to lysis Vmax.

2.11 Mouse pulmonary endothelial cell (Chen et al.) collection, isolation and culture

Following schedule 1 euthanasia, mouse lungs were removed and placed into a universal tube containing Hanks' Balanced Salt Solution (HBSS) (PLUS Ca^{2+}) Gibco 500ml (ThermoFisher Scientific). Lung collection from the mice was conducted by Dr Beth Webb and Dr Cedric Duval.

To carry out the EC isolation, firstly, tissue digestion was carried out where mouse lungs were minced in a petri dish using collagenase type 2 (Worthington Biochemical, US) in HBSS, then incubated in a falcon tube on a rotator for 45 minutes at 37°C. This was followed by cell straining of the digested tissue using

a 70µm Easystrainer (greiner- [542070]) into a 50mL falcon, and centrifugation for 5 minutes at 200*xg*. After the spin, the supernatant was aspirated, and 6.6% (v/v) BSA in PBS added to resuspend the cells before transfer to an eppendorf tube. Microbead labelling using 20µl Mouse CD146 Microbeads ((LSec) cat: 130-092-007, Miltenyi Biotec) were added to the suspension to isolate the PECs and the eppendorf placed on a rotator for 20 minutes at 4°C. After the incubation, cells were washed in 6.6% (v/v) BSA in PBS and centrifuged at 500*xg* for 5 minutes in a microfuge at RT. The supernatant was aspirated, and 6.6% (v/v) BSA in PBS added to resuspend the cells prior to magnetic isolation of the labelled PEC cells using a MACStand. Cells were added to a 30µm Pre-Separation Filter cup (Miltenyi Biotec- [130-041-407]) assembled with an MS column (Miltenyi Biotec- [130-042-201] and washed 3 times in PBS before elution. The column was removed from magnet and 6.6% (v/v) BSA in PBS added, then cells were retrieved using a plunger. Collected cells were centrifuged for 5 minutes at 400*xg* to pellet the recovered cells and then resuspended in supplemented EC Growth Medium MV2.

The PECs were then plated in the wells of 6-well plates coated in 0.2% gelatin (one set of lungs per 6-well plate). PECs were then cultured for 7-10 days with a media change every other day before being used in experiments.

2.12 Primary EC culture

Cell culture was performed in class II safety cabinets under sterile conditions. All primary cells were adherent and grown in T25 or T75 cell culture flasks, and experiments conducted in 6, 12, 24 or 96-well plates (NuncTM, ThermoFisher Scientific, US). Cells were cultured in a 5% CO2 incubator at 37° and passaged at 80-90% confluency with cell media changed every 48/72 hours.

2.12.1 HCAEC culture

Primary HCAECs [C-12221] were purchased from Promocell at passage 2 (P2), isolated from the coronary arteries (the right and left coronary artery) from 3 different donors. HCAECs were cultured up to passage 6 (P6) in supplemented

EC Growth Medium MV2 (Promocell- [C-22022]). Cell health was checked using trypan blue and viability was maintained above 90% used in experiments.

2.12.2 Cell passage and freezing

Once confluent, EC medium was aspirated and cells were washed with pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS; Lonza, Basel, Switzerland). Cells were then incubated at 37°C for 4 minutes with pre-warmed Trypsin-Ethylenediaminetetraacetic acid (EDTA; Lonza; 4mL per T75) until cells had fully detached from the base of the flask/plate. Trypsin was then neutralised with pre-warmed EC MV2 medium (8mL per T75) and cell suspension transferred to a 15mL falcon tube (Fisher Scientific, Loughborough, UK). ECs were centrifuged at 300*xg* for 5 minutes at RT, before resuspension of the cell pellet in 1mL pre-warmed EC MV2 medium. Number of cells were counted using the TC20 Automated Cell Counter (Bio-Rad Laboratories Ltd, Watford, UK). Cells were seeded in plates, flasks and ibidi VI 0.1 µ-slides (Thistle Scientific, Glasgow, UK).

For cryopreservation, cells were passaged as above, and the cell pellet resuspended in 90% (v/v) foetal bovine serum (FBS) (Lonza, Basel, Switzerland) supplemented with 10% (v/v) DMSO (Sigma Aldrich, Dorset, UK) at $1x10^6$ cells/mL. Cell suspension was then transferred to a 1.2mL cryopreservation vial (1mL/vial) and placed in a Mr Frosty freezing container (Sigma Aldrich) for 48 hours at -80°C, before being transferred to liquid nitrogen for longer term storage.

2.13 Culturing under orbital shear stress

The orbital shaker or 'swirling well' model allows for investigation of endothelial proliferation and function following exposure to different flow and shear environments (Warboys et al., 2019). Endothelial monolayers were grown to confluency on 6-well plates then subjected to shear stress (153 RPM) for 72 hours using a Stuart Mini Orbital Shaker (SSM1) (Richmond Scientific, Chorley, UK). Cells cultured in the outer periphery of the wells are exposed to the equivalent of atheroprotective laminar shear (HSS) (15 dyn/cm2), whilst cells in

the centre of the wells are exposed to atheroprone oscillatory shear (LSS) (0~ ± 5 dyn/cm2) (Warboys et al., 2019). Orbital shear stress (SS) was applied to HCAECs using an orbital shaker positioned inside an incubator set to 37°C with 5% CO2. Media was removed after 72 hours, and 3 washes performed with PBS for 5 minutes each prior to sample staining (section 2.14) and imaging. HCAECs in both regions (middle and outer) were imaged to investigate morphology changes at different shear rates.

2.14 HCAEC immunofluorescence

HCAECs were cultured until confluent and treated with vehicle control (0.1% DMSO) +/- AZD1208 (1μM), PIM447 (1μM), and TP3654 (0.1μM) for 24-72 hours at 37°C under static conditions or under orbital shaking (as described in section 2.9). After washing with PBS, HCAECs were fixed with 10% formalin for 10 minutes at RT. Following 3 PBS washes, fixed HCAECs were then permeabilised for 5 minutes with 0.1% Triton X-100-PBS, and then after 3 washes, blocked with 1% BSA-PBS for 1 hour at RT. To visualise the cells, F-actin was stained with Invitrogen™ Rhodamine phalloidin (ThermoFisher Scientific; 1:750 in 1% BSA-PBS), and DAPI (Sigma-Aldrich; 100 ng/mL) was used to stain the nuclei, incubating at 4°C overnight. HCAECs were then washed 3 times and imaged using an EVOS Auto 2 microscope. ImageJ (Fiji) thresholding was used to measure DAPI cell count (number of nuclei), quantify fluorescence intensity (FI) of Rhodamine phalloidin, and assess nuclear aspect ratio using shape descriptors to evaluate cell circularity.

2.15 Cell cytotoxicity assay (LDH)

HCAEC inhibitor cytotoxicity and survival was measured using an LDH Assay Kit (Abcam, UK- [ab65393]) after treatment with Pim kinase inhibitors. HCAECs cultured in 96-well plates were incubated for 24 and 48 hours with increasing concentrations of AZD1208, PIM447 and TP3654 (0.1, 0.3, 1, 3, 10, and 30μM), vehicle control (0.1% DMSO), or 1μg/mL phorbol myristate acetate (Swystun et al.). PMA was included as a positive control for cell lysis. A cell-free media control was also included. Cell lysis solution (10%) was added to untreated cells

as a total lysis control 30 minutes before the LDH Reaction Mix was added. 10µL media was removed from each well and added into a 96-well plate before 100uL LDH Reaction Mix was added, and the plate incubated for 30 minutes at RT. Absorbance was read at 450nm on the GloMax® Explorer Multimode Microplate Reader (Promega). Two replicate wells (technical duplicates) were included for each analysis, and four independent experiments were performed. Absorbance data collected was converted to percentage cytotoxicity, normalised to vehicle.

2.16 Cell growth inhibition assay (MTS)

HCAEC proliferation was measured using an MTS colorimetric assay (Abcam, UK) following treatment with Pim kinase inhibitors. HCAECs were plated in 96-well plates at $1x10^4$ cells/well and cultured overnight. The next day cells were treated for 24 and 48 hours with increasing concentrations of AZD1208, PIM447, and TP3654 (0.1, 0.3, 1, 3, 10, and 30µM). Vehicle control (0.1% DMSO) was used as a negative control, and 0.1% Sodium Dodecyl Sulfate (SDS) was included as a positive control for cell lysis. After 24-hour treatment, 100µL of MTS tetrazolium reagent (Abcam- [ab197010]) was added to each well and the plate incubated for 4 hours at 37°C before absorbance was read at 490nm on the GloMax® Explorer Multimode Microplate Reader (Promega). Two replicate wells (technical duplicates) were included for each analysis, and four independent experiments performed.

Absorbance (Abs) data collected was converted to percentage proliferation, normalised to vehicle (average absorbance of the vehicle wells was considered 100% of cell viability). The cell viability percentage of the other wells was calculated using the following equation:

Cell viability (%) = Abs treated HCAEC/Abs vehicle HCAEC × 100.

2.17 Holomonitor live cell imaging scratch assay (migration)

Cell migration following treatment with Pim kinase inhibitors AZD1208 (1, 10μM), PIM447 (1, 10μM), and TP3654 (0.1, 1μM) was investigated using a HoloMonitor M4 live cell imaging system. Ibidi 2-well cell culture silicone inserts (ibidi GmbH, Germany- [80209]) were placed into a 24-well plate (Sarstedt, Nümbrecht, Germany) using sterile forceps. 70μL of a $3x10^5$ cells/mL HCAEC suspension were seeded into each well and left overnight at 37°C. The following day, AZD1208, PIM447, and TP3654 were prepared in EC MV2 media, along with vehicle control (0.1% DMSO). Inserts were removed, media aspirated, and cells washed with PBS. Treated media was added to each well (reverse pipetted to avoid creating bubbles) and sterile HoloLids™ (Phase Holographic Imaging, Sweden) placed in each column of the plate. The plate was placed securely onto the PHI HoloMonitor® M4 System and set up to start. Images of the scratch were taken at 6 positions per well using the 10x objective, every 10 minutes up to 20 hours using the HoloMonitor® App Suite 3.5 Cell Imaging software (Wound Healing application). Cell migration, and scratch width (μm) data was obtained from 3 images per well and analysed using the HoloMonitor® App Suite 3.5 software to measure time to wound closure.

2.18 Angiogenesis assay (tube formation)

Using methods described previously, a Matrigel tube formation assay was carried out to analyse the angiogenic activity of HCAECs/PECs (Ponce, 2009). Wells of a 96-well plate were coated with 50μL/well of Matrigel® Basement Membrane Matrix (Corning, US) and then allowed to polymerise in an incubator for 30 minutes at 37°C. HCAECs were seeded on the coated plate at a density of 1 X 10^5 cells/well, treated with vehicle control (0.1% DMSO) +/- AZD1208 (1, 10μM), PIM447 (1, 10μM), and TP3654 (0.1, 1μM) and then incubated for 4 and 24hr at 37°C. Calcein was added after 24hr for visualisation of the tube network. WT and PIM1$^{-/-}$ PECs were seeded on the coated plate at a density of 1 X 10^5 cells/well and incubated for 4 hours at 37°C. HCAECs were imaged using an Invitrogen™ EVOS M5000 imaging system (ThermoFisher Scientific) at a 4x and

10x magnification, and PECs imaged using a GXM inverted microscope at a 4x and 10x magnification. 3 images were taken per condition and the number of branch points (cell sprouting), number of loops, and tube length (measuring the circumference of an intact loop) were calculated and averaged for HCAECs using an angiogenesis plugin in ImageJ.

2.19 Gene and protein expression

HCAECs were cultured under conditions described previously in section 2.12.1 and treated with Pim kinase inhibitors (0-10 µM) for 4- or 24-hours gene or protein expression or releasate experiments. HCAECs were stimulated with vehicle (0.1% DMSO), TNFα (5ng/mL) and IL-6 (100ng/mL) for experiments conducted in section 5.3.2. PECs were seeded from WT and PIM1$^{-/-}$ mice as described in section 2.11 for RT-qPCR and transcriptomics.

2.19.1 Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

2.19.1.1 RNA lysate preparation

RNA lysates were prepared from 24-well plates where following 3x washes with DPBS, 200µL buffer RL [P/N 90055] obtained from the Total RNA Purification Kit (Geneflow, Lichfield, UK- [48400]) was added to each well of cultured cells and the plate was immediately stored at -80°C. When required for use, the plate was thawed out, and a cell scraper was used to create lysates ready for RNA purification.

2.19.1.2 Total RNA extraction- RNA purification

RNA was extracted using the Total RNA Purification Kit (Geneflow, Lichfield, UK- [48400]) following the manufacturer's instructions. Genomic DNA was removed from the RNA lysate using a gDNA removal column and centrifuged at 14,000*xg* for 1 minute and the flowthrough stored on ice. The lysates were added to an RNA purification column with ethanol (60µl of 100% ethanol added to every 100µL of lysate), then centrifuged for 1 minute at 3,500*xg* (~6000 RPM). The column was washed three times with 400µL of wash solution A and centrifuged for 1 minute

at 3,500*xg*, flowthrough was discarded. RNA was eluted from the purification column using 50µL of elution solution A and centrifugation of the column for 2 minutes at 200*xg* (~2000 RPM) followed by 1 minute at 14,000*xg*. The purified RNA samples were aliquoted and stored at -20°C for immediate use or -80°C for long term storage.

2.19.1.3 Reverse transcription- RNA to cDNA

The concentration and purity of RNA were measured using the Nanodrop OneC Spectrophotometer (ThermoFisher Scientific) as per manufacturer's instructions. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1. An acceptable limit of purity was set at ~2.0, a purity of ~1.8 suggested DNA contamination (ThermoFisher Scientific, 2022). Purified RNA from samples was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (200) (QIAGEN, Germany- [205313]) according to the manufacturer's instructions and summarised below.

The following calculations were performed for each sample to determine the volume of sample required based on RNA concentration:

Volume of sample (µL) = 200 / sample concentration (ng/µL)

Total volume was then made up to 12µL by adding DNAse/RNAse free water to the sample. 2µL gDNA wipeout buffer was added to each sample, mixed, and kept on ice. Samples were then placed on a heat block for 2 minutes at 42°C to remove any residual gDNA, then immediately put back on ice. Per sample, 1µL RT, 1µL Primer mix, and 4µL RT buffer were added. The samples were placed in the TurboCycler 2 Thermal Cycler (Blue-Ray Biotech, Scientific Laboratory Supplies) for reverse transcription, conditions are listed in Table 2.6.

Table 2.6: Thermal Cycler setting for reverse transcription reaction.

Stage	Temperature	Cycles	Time
Primer annealing	25.0	1	3:00
DNA polymerisation	45.0	1	10:00
Enzyme inactivation	85.0	1	5:00

At the end of the run, 20µL DNAse/RNAse free water was added to each sample (to yield a final concentration of 5ng/µL). For each sample, cDNA was synthesised from 200ng total RNA in a final volume of 40µL and stored at -20°C until further use. cDNA was synthesised from up to six independent sets of EC samples.

2.19.1.4 quantitative PCR (qPCR)

NCBI Primer blast was used to design primers, ensuring exons were common to all transcripts. The PCR product size was specified to be 90-250, with a specified melting temperature of 65°C. The primers were intron spanning with an intron length range of 100-10,000. Primers were purchased from Sigma-Aldrich (primer sequences listed in Table 2.7). RT-qPCR reactions were performed in 96-well PCR plates (Scientific Laboratory Supplies) using the QuantiNova SYBR Green PCR kit (Qiagen, Germany- [208056]). Each 10µL reaction contained 5µL of SYBR Green, 3µL DNAse free water, 1µL primer master mix (made up of 392µL DNAse/RNAse free water, 4µL forward primer, 4µL reverse primer, making a 2µM final primer concentration) and 1µL of cDNA sample. cDNA (5ng) was used as template for RT-qPCR (PCR conditions are listed in Table 2.8). All RT-qPCR analyses were performed in duplicate (technical repeats) using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Ltd). Data was collected and analysed using the delta delta Ct (ΔΔCt) method (2–ΔΔCt; Livak and Schmittgen, 2001). Following successful amplification, gene of interest (GOI) cycle threshold (Burger) technical replicate values were averaged before being

normalised to the geometric means of averaged housekeeper (GAPDH/RPLPO) technical replicate values for each sample.

Delta Ct (ΔCt) was obtained using the following equation:

$$\Delta Ct = Ct\ (GOI) - Ct\ (geometric\ mean\ of\ housekeeping\ genes).$$

The delta-delta Ct value (ΔΔCt) was obtained by subtracting the control sample ΔCt value from the treated sample ΔCt value using the following equation:

$$\Delta\Delta Ct = \Delta Ct\ (treated\ sample) - \Delta Ct\ (control\ sample).$$

Finally, the mRNA fold change was calculated using $2^{-\Delta\Delta Ct}$.

Table 2.7: Primers and their sequences for RT-qPCR.

Primer name (gene)	Forward sequence (5' to 3'	Reverse sequence (5' to 3')
Housekeeping (reference) genes		
GAPDH	CGGATTTGGTCGTATTGGGCG	GTCTTCACCACCATGGAGAAGGC
RPLPO	GCAGCAGATCCGCATGTCCC	TCCCCCGGATATGAGGCAGCA
Human primers		
Pim kinases		
PIM1	TTCTGGCAGGTGCTGGAGGC	GCGGATCCACTCTGGAGGGC
PIM2	CTTCGCAGGACACCGCCTCA	AAAGGCCGCTCGAGGACCAG
PIM3	TTCGGGTGCGCTGCTCAAGG	GAGAAGCACGCCCAGCGACC
Thrombotic mediators		
VWF	TGCCATGGAACGTGGTCCCG	ACCACCGCCTTTGAGGCTCC
eNOS	TCGGCCGGAACAGCACAAGA	AAAGGCGCAGAAGTGGGGGT
PTGS-1 (COX-1)	ACGCACAGGAGCCTGCACTC	GGTCAAGGCCGAAGCGGACA
PTGS-2 (COX-2)	CTGGGCCATGGGGTGGACTT	CCTGCCCCACAGCAAACCGT
Mouse primers		
PIM1	CGCGACATCAAGGACGAGAACA	CGAATCCACTCTGGAGGACTGT
PIM2	CTGCTTCACGATGAGCCGTACA	GAGTAGGACACCTAGTGACCAG
PIM3	TGTGGTCTCTGGGTGTACTGCT	GACACCACTCAATAAGCTGCTGG

Table 2.8: qPCR reaction conditions.

Stage	Temperature	Cycles	Time
Incubation	95.0	1	300s
DNA denaturation	95.0	40	1200s
Primer annealing	62.0	1	20s
Primer extension	72.0	1	25s

2.19.2 RNA sequencing

RNA samples were prepared (lysed and extracted), as described previously, then concentration and purity were determined by NanoDrop, Qubit, 2.0, Agilent 2100. RNA sample analysis was then outsourced, and sequencing performed by BMKGene, using the following workflow (Figure 2.1). Library preparation was conducted using BMKGENE's standard directional mRNA sequencing protocol, which includes poly-A enrichment and strand-specific library construction. Sequencing was performed on the Illumina NovaSeq platform, generating paired-end reads with a minimum depth of 30 million reads per sample. Clean reads were aligned to the human reference genome (Homo_sapiens.GRCh38_release95.genome.fa.) using HISAT2, and gene-level quantification was performed. Differential expression analysis was conducted using DESeq2, with significance thresholds set at adjusted p-value <0.05 and |log2 fold change| >1.

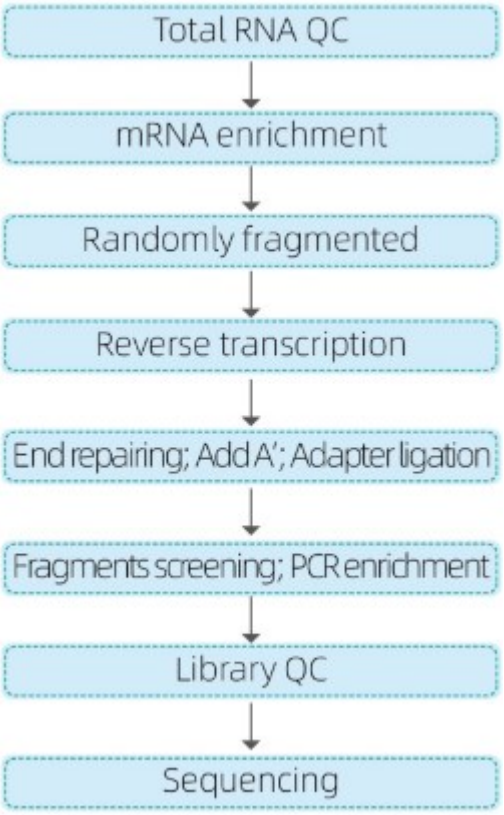

Figure 2.1: Workflow of mRNA sequencing. Includes sample preparation, library construction, library quality control and sequencing. Taken from BMKGene analysis report.

2.19.3 Western Blotting

2.19.3.1 Protein lysate preparation

To confirm protein expression of Pim kinase isoforms, HCAECs were cultured under conditions described previously in section 2.8.1. Protein lysates were prepared by extracting total protein from HCAECs using RIPA buffer (Abcam-[ab288006]), supplemented with protease and phosphatase inhibitors (Sigma-Aldrich), containing 2mM EDTA, 1mM PMSF, 10µg/mL aprotinin, 10µg/mL leupeptin, 0.7µg/mL pepstatin A, 2mM sodium orthovanadate, and 2% NP-40, pH 7.3 (10µL of each inhibitor was added per mL of RIPA). 6-well cell culture plates were put on ice and media removed from each well. PBS was added to each well of plate to wash, aspirated, then 200µL RIPA/protease/phosphatase inhibitor master mix added to each well. A cell scraper was used to create lysates which were then added to 0.5mL eppendorf tubes. Protein lysates were then immediately stored at -80°C until use.

2.19.3.2 Sample preparation

A Pierce BCA protein quantification assay (ThermoFisher Scientific) was performed to determine the protein concentration of each HCAEC lysate. The appropriate quantity (30µL) of protein lysates was then prepared by the addition of Laemmli sample buffer (Bio-Rad Laboratories Ltd). To reduce and denature the samples, each cell lysate in sample buffer was heated to 95°C in a Accublock Digital Dry bath incubator (Labnet International) for 5 minutes before use.

2.19.3.3 SDS-PAGE Gel Electrophoresis

Equal amounts of prepared lysates (30µL) at 10µg and 4µL of a molecular weight marker (GeneRuler 1kb DNA Ladder) (ThermoFisher Scientific) were loaded into the wells of a 10% Mini-PROTEAN TGX Stain-Free precast gel (Bio-Rad Laboratories Ltd) or hand-cast gels made with the TGX Stain-Free FastCast 10% Acrylamide Kit (Bio-Rad Laboratories Ltd). The gel was run with Tris-glycine running buffer in a Mini Trans-Blot® Cell (Bio-Rad Laboratories Ltd) at 60V for 30 minutes then increased to 120V for 60 minutes to ensure sufficient separation of the proteins.

2.19.3.4 Blotting

Transferring of the proteins from the gel to the membrane (blotting) was carried out using semi-dry transfer. A Polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich) was soaked in Towbin buffer before the transfer stack (gel and membrane embedded in filter paper) was prepared. Samples were then transferred from gel to membrane using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories Ltd), blotting at 10V for 35 minutes.

2.19.3.5 Blocking

Following transfer, the membrane was blocked with 5% BSA in TBST for 1 hour at room temperature (RT).

2.19.3.6 Antibody incubation

The membrane was then incubated with primary antibodies raised against PIM1, PIM2, and PIM3 (Table 2.4) overnight on a rocker at 4°C. Primary actin antibody (Abcam, UK) was used as a loading control to normalise the levels of protein detected following stripping of the membrane (as described in section 2.13.7 below).

The membrane was then washed 3 times with TBST for 5 minutes each. Membranes were then incubated with HRP-conjugated secondary IgG antibody (Sigma-Aldrich) (Table 2.4) for 1 hour at RT on a rocker.

2.19.3.7 Protein immunodetection and visualisation

After three washes of 5 minutes in TBST, the membrane was developed using the Immobilon® Western Chemiluminescent HRP Substrate (Millipore Ltd, Livingston, UK) and imaged with the Odyssey® Fc Imaging System (LI-COR Biosciences Ltd, Cambridge, UK) to detect antibody binding using an enhanced chemiluminescence (ECL) system. The membranes were washed again 3 times with TBST for 5 minutes each.

Stripping was performed by incubating the membrane in Restore™ Western Blot Stripping Buffer (Sigma-Aldrich) for 15 min at RT. The stripping buffer was then

discarded and the membrane was washed 3 times in TBST for 5 minutes each time.

2.20 Releasate preparation

To investigate changes in EC expression and release of thromboinflammatory mediators, conditioned cell media (containing cell releasate) was collected from experiments. Conditioned culture media was removed from each well without disturbing the adhered cells before aliquoting and storage at -80°C for future analysis. It was ensured that freeze/thaw cycles were minimised to preserve sample quality.

2.21 Nitrate assay

Nitrate was measured as a marker of NO production and eNOS activity using an Enzyme-linked immunosorbent assay (ELISA) Nitrate/Nitrite Fluorometric Assay kit (Cayman Chemical- [780051]). In summary, reagents, samples (supernatant collected from HCAECs after 24 hours of drug treatment), and standards were prepared ahead of use, following manufacturers' protocol. A standard curve was set up as per manufacturers' instructions. 10µL of sample conditioned media was added to wells, followed by 70µL of assay buffer, 10µL of Enzyme Cofactor mixture, and 10µL of the Nitrate Reductase mixture (total volume – 100µL) and samples left to incubate at RT for 1 hour. After the incubation, 10µL DAN reagent was added to each well and the plate was incubated for another 10 minutes. To stop the reaction, 20µL of NaOH was added to each well and fluorescence measured using the GloMax® Explorer Multimode Microplate Reader (Promega) at an excitation wavelength of 360-365nm and emission wavelength of 430nm. Data analysis was subsequently conducted using a standard curve constructed from a serial dilution of working standard samples to calculate nitrate production.

2.22 Human Luminex® Discovery Assay

2.22.1 Magnetic bead-based Multiplex Assay

The release of mediators of thrombosis and inflammation from ECs was measured using a bespoke 17-plex Human Luminex® Discovery Assay (R&D systems- [LXSAHM]) to detect the following analytes: VWF-A2, ADAMTS13, thrombomodulin, Serpin-C1, Serpin-E1, TF, TFPI, IL-1β, IL-6, IL-8, sICAM-1, sVCAM-1, sP-selectin and TNFα in conditioned cell culture media. The assay was performed as per manufacturers' instructions on a Luminex™ 200™ Instrument System (ThermoFisher Scientific), summarised below.

Reagents and standards were prepared, as instructed in the manufacturer's protocol. Prior to use, conditioned HCAEC culture media was centrifuged at 16,000*xg* for 4 minutes. 50µL of standard or sample was added to each well of the assay plate followed by an equal volume of Microparticle Cocktail and the samples were incubated for 2 hours at RT on a horizontal orbital microplate shaker (0.12" orbit) at 800 RPM. A magnet was applied to the bottom of the plate and 3 washes in 100µL wash buffer were performed to remove unbound substances (antibodies are immobilised by magnetic microparticles and magnetic platform). 50µL Biotin-Antibody Cocktail was then added to each well and the samples incubated for 1 hour at room temperature on the shaker at 800 RPM. Samples were washed 3 times to remove unbound biotinylated antibodies and 50µL of diluted Streptavidin-PE was added to each well, followed by a 30-minute incubation at RT on a shaker at 800 RPM. After this incubation, the plate was washed 3 times to remove unbound streptavidin-PE. Immediately prior to reading, the plate was shaken at 800 RPM for 2 minutes to resuspend the microparticles. The plate was read within 90 minutes of assay completion using the Luminex™ 200™ Instrument System (ThermoFisher Scientific). All measurements were within the standard curve and range of sensitivity.

2.23 Human blood sample preparation

Human blood/plasma samples were required for coagulation and *in vitro* thrombus formation experiments.

2.23.1 Ethics statement

Procedures and experiments involving human participants were conducted following appropriate approval obtained from The Faculty of Science and Engineering Research Ethics and Governance Committee (REGC) at Manchester Metropolitan University (EthOS approval- 48062), in place from 16/11/2022 and the School of Medicine Research Ethics Committee at the University of Leeds (MREC 23-001) in place from 27/03/2024.

2.23.2 Phlebotomy

All blood donors signed an informed participant consent form prior to donating blood (Appendix B). Peripheral blood was taken from healthy male and female volunteers aged 18-65 into 3.2% (v/v) sodium citrate 2.7, 4.5 or 9mL BD Vacutainer™ tubes (Scientific Laboratory Supplies Ltd, Nottingham, UK), before being gently inverted 3-4 times to mix with the anti-coagulant. Individuals with anaemia, blood-borne infections, blood clotting disorders, and those using anti-platelet or anti-coagulant therapy 10 days or less prior to donating were excluded from the study. Blood samples were kept at 37°C in an incubator and used within a maximum of 4 hours following venepuncture. Biological repeats were performed for each assay using blood from up to 5 different donors.

2.23.3 Preparation of platelet poor plasma

Whole blood collected in sodium citrate tubes was centrifuged at 1800*xg* for 10 minutes. Platelet poor plasma (PPP) was collected, and remaining blood discarded. PPP was stored at -20 ˚C for short term storage, or -80°C for long term storage.

2.24 aPTT assay

An aPTT assay was performed to measure the effects of Pim kinase inhibition on the intrinsic coagulation pathway using a Helena Biosciences C-4 coagulation analyser (Helena Biosciences). APTT si L minus reagent and 0.025M calcium chloride were warmed to 37˚C for 30 minutes. PPP was treated with Pim kinase inhibitors, or 1mM EDTA as a positive anti-coagulant control. A separate set of experiments was performed with HCAEC conditioned media (AZD1208/PIM447/TP3654 treated) in a 1:1 ratio with PPP to a final volume of 100 µL and left to incubate in cuvettes for 10 minutes. 100µL pre-warmed APTT reagent was added to the cuvette and incubated for 5 minutes at 37˚C followed by 100µL pre-warmed calcium chloride reagent and time to clot formation (seconds) was measured. Two technical replicates were performed for each condition.

2.25 Turbidity assay – fibrin formation

HCAEC conditioned media was added to PPP and saline solution (1 part media, 1 part PPP, and 4 parts saline) and 30µL plasma mix was added to each well of a half volume 96-well plate. An activation mix was made containing thrombin (0.1U/mL), CaCl2 (10mM), and saline to investigate the common pathway of coagulation. To initiate fibrin formation, 20µL of activation mix was added to each well, giving a total volume of 50µL. The plate was then immediately read on the BioTek PowerWave HT microplate reader and absorbance (optical density) read at 340nm every 17 seconds for 1 hour at 37˚C, giving a turbidity trace, allowing the measurement of fibrin formation using three parameters: lag time, time to 50% clotting, and time to MaxOD. Three technical repeats were run for each sample.

2.26 Fibrinolysis assay

HCAEC conditioned media was added to plasma and saline solution (1 part media, 1 part PPP, and 4 parts saline) and 20µL added to each well of a half volume 96-well plate. 30µL of activation mix containing thrombin (0.1U/mL),

CaCl2 (10mM), tPA (20ng/mL), and saline was added to each well, giving a total of 50µL. Once activation mix was added, the plate was read immediately on a BioTek PowerWave HT microplate reader and absorbance (optical density) at 340nm determined every 17 seconds for 6 hours at 37°C, giving a fibrinolysis trace. Three technical repeats were run for each sample measuring MaxOD to 50% lysis, and MaxOD to lysis Vmax.

2.27 In-vitro thrombus formation

Ibidi VI 0.1 µ-slides (Thistle Scientific) were used in a high throughput 6-syringe flow model setup to measure thrombus formation on HCAECs and collagen, enabling six chambers to be under flow at one time. A 1mL suspension of HCAECs was prepared at $4.5x10^6$ cells/mL and seeded in Ibidi 6-channel µ-slide chambers, then cultured for 24-72hr until cells were confluent. Another set of slide chambers were coated with 1.7µL 100µg/mL Type I collagen fibrils (Equine tendon-derived; Labmedics) and left for one hour at RT prior to performing experiments. The model was set up using a six-channel programmable syringe pump (ProSense, Chepstow, UK) with 5, 10 and 20mL syringes (ThermoFisher Scientific) connected to silicone tubing with an internal diameter of 0.8mm (RS Components, UK) and Luer adaptors. Whole blood was incubated with 1µM DIOC-6 for 15 minutes to label the platelet membrane at RT. Slide chambers were washed under flow with tyrodes-HEPES buffer (with added glucose) at an arterial flow rate of 46.7µL/min (15 dynes/cm2), before 330µL DIOC-6 fluorescently tagged whole blood was perfused through chambers for 8 minutes. Images were taken every 10 seconds with the 10x objective on the 488nm channel using a CELENA® S Logos Biosystems fluorescent microscope.

2.28 Statistical Analysis

Statistical analysis of data was performed and quantified using Microsoft Excel and GraphPad Prism Version 10 (GraphPad, California, USA). All experiments were performed with at least three biological repeats from blood donors or batches of HCAECs. Technical replicates in duplicate or triplicate were also performed where possible. Number of biological repeats are listed in

corresponding figure legends, and all data points included on graphs for visualisation of data. Depending on the number of groups per analysis, levels of significance of the differences between groups was analysed by a two-tailed Student's *t*-test, One-way analysis of variances (One-way ANOVA) or Two-way ANOVA followed by Tukey correction/Bonferroni correction (multiple comparisons). Statistical analysis was performed with the appropriate post-hoc test depending on whether data is parametric or non-parametric. Normal distribution of data was confirmed using a Shapiro-Wilk test. For data that are not normally distributed or when comparing percentages or arbitrary units, the appropriate non-parametric tests were used (Mann-Whitney U test). A confidence level of >95% ($P \leq 0.05$) was considered statistically significant. Results are expressed as mean ± standard error (Mangin et al.). Where data is presented as normalised, statistical analyses were performed prior to normalisation.

Chapter 3. Characterising the thrombotic profile of PIM1 deficient mice.

3.1 Introduction

Arterial thrombosis occurs when highly thrombogenic surfaces are exposed following plaque rupture or erosion, leading to the formation of platelet rich thrombi, which can partially or completely occlude vessels obstructing blood flow, and leading to acute cardiovascular events (Baaten et al., 2024). This process involves complex interactions between the blood vessel, platelets, and the coagulation cascade (Vilahur and Fuster, 2025).

3.1.1 PIM1 and thrombosis

Pim kinases have been identified to play roles in the contribution to thrombosis, and global PIM1$^{-/-}$ mouse models have previously been used to investigate the effects of PIM1 on platelet function. It has been shown by Unsworth *et al* that Pim kinases are expressed in human and mouse platelets and Pim kinase inhibitors or deletion of PIM1 cause a reduction in platelet function and thrombus formation (Unsworth et al., 2021). This attenuation appeared to be mediated via inhibition of platelet thromboxane receptors (Nock et al., 2025), and to some extent inhibition of platelet CXCR4 (Nock et al., 2024) signalling. Mechanistic studies further demonstrated that the effects on platelet thromboxane receptor signalling were driven by PIM1 specifically. Interestingly genetic deletion of PIM1 and pan-Pim kinase inhibitor treatment results in reduced thrombosis but are not associated with impaired haemostasis (Unsworth et al., 2021). Furthermore, this previous work from the lab has demonstrated that whilst ~50% reduction of thrombus formation on collagen is observed following Pim kinase inhibition *in vitro*, ~90% reduction is observed *in vivo* (Unsworth et al., 2021). This suggests that additional mechanisms may be involved, independent of the inhibitory effects on platelets. One possible option is that the endothelium may be contributing, and that PIM1 may play a role in the

endothelial contribution to thrombosis, therefore the effects of PIM1 deletion on ECs should be investigated.

In further support of a role for PIM1 in the promotion of thrombosis, another study has identified that PIM1 levels are elevated in the plasma of patients with lower limb deep vein thrombosis (DVT) (Chen et al., 2023), indicating that it may play a role in driving coagulation. There have been no investigations on the effects of PIM1 genetic deletion on coagulation, therefore studying coagulation profiles in PIM1$^{-/-}$ mice would provide deeper insight into the mechanisms of PIM1 action observed in previous thrombosis studies.

3.1.2 PIM1 and Endothelial cells

Endothelial cells (ECs) are crucial for maintaining vascular homeostasis and mediate several biological functions that are relevant to atherosclerosis and CAD, regulating vital processes including wound healing, inflammation, and thrombosis (Pepin and Gupta, 2024). Increased PIM1 expression has been demonstrated with the progression of atherosclerosis, with higher expression measured in unstable plaques (Xue et al., 2025). The same study also showed that endothelial cell-specific PIM1 genetic deletion regulates endothelial-to-mesenchymal transition (EndMT), where reduced EndMT markers (α-SMA, Slug, Snail) and preserved endothelial markers were measured after PIM1 deletion (Xue et al., 2025). The same study showed that PIM1 deletion attenuated atherosclerosis progression, demonstrated by less macrophage infiltration, and improved plaque stability (Xue et al., 2025), suggesting a role for endothelial PIM1 in atherosclerotic processes and the progression of CVD.

3.2 Chapter aim, objectives and hypothesis

In this chapter the role of PIM1 kinase in the regulation of coagulation and endothelial cell contributions to thrombosis was investigated using a PIM1-specific global KO mouse model and a combination of haematology and coagulation assays, pulmonary EC isolation and transcriptomics.

The aim of this chapter is to define how PIM1 activity influences coagulation to determine whether pharmacological inhibition of PIM1 kinase could offer a novel anti-coagulant targeting strategy.

To investigate this, whole blood was collected, platelet-poor plasma (PPP) obtained, and PECs isolated from WT and PIM1$^{-/-}$ mice.

The following objectives were addressed:

1. Investigate the impact of PIM1 deletion on whole blood and coagulation profiles *ex vivo*.
2. Assess the functional effects of PIM1 genetic deletion on PEC viability and angiogenesis.
3. Investigate the effect of PIM1 deletion on gene expression of regulators of coagulation, thromboinflammation, and endothelial activation.

3.2.1 Hypothesis: PIM1 is a positive regulator of coagulation, and its inhibition promotes the anti-thrombotic properties of endothelial cells.

3.2.2 Methods

To investigate this, whole blood, PPP, and PECs were isolated from PIM1$^{-/-}$ mice or WT controls and different functional assays performed. For analysis of blood cell profiles, full blood cell counts were performed using a haematology analyser. For the measurement of coagulation, ROTEM, clot formation, and fibrinolysis experiments were performed. EC experiments included measurement of gene expression, viability, angiogenesis, and transcriptomic

analysis, using standard assays described in Chapter 2 (Materials and Methods).

3.2.2.1 Visual methodology abstract

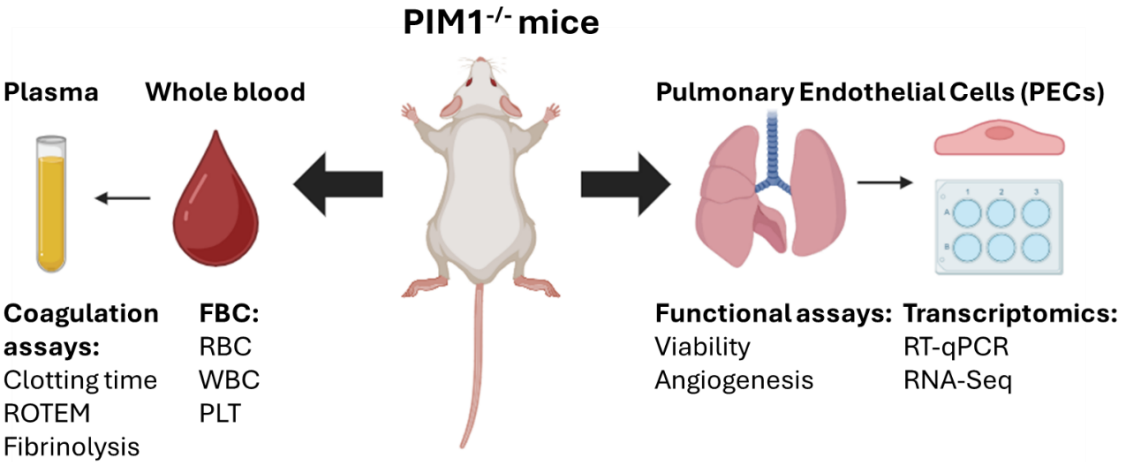

Figure 3.1: Methodological summary of experiments carried out in this Chapter. Experiments were carried out using whole blood, plasma (PPP), and pulmonary endothelial cells (PECs) from WT and PIM1$^{-/-}$ mice. Plasma experiments included coagulation assays including clotting time assays, ROTEM, and fibrinolysis. Whole blood measurements included a full blood count (FBC) measuring red blood cell (RBC), white blood cell (WBC), and platelet (PLT) parameters. PECs were used in functional assays, viability and angiogenesis, and transcriptomics, RT-qPCR and RNA-Seq experiments. Created in BioRender and PowerPoint.

3.3 Results

3.3.1 Gene expression of the three Pim kinase isoforms is decreased with PIM1 genetic deletion

To confirm that *PIM1* genetic deletion was successful in the mice, mRNA levels of *PIM1* were measured in WT and PIM1$^{-/-}$ PECs isolated from mice using real-time quantitative polymerase chain reaction (RT-qPCR). Additionally, the effects of genetic deletion of PIM1 were explored on gene expression of the other Pim isoforms, as it is well understood that the isoforms are complementary and functionally redundant (Nock et al., 2023). PIM1 was successfully deleted in PECs, demonstrated by a lack of *PIM1* gene expression (Figure 3.2A). Interestingly, deletion of *PIM1* also led to a significant decrease in *PIM2* and *PIM3* gene expression (Figure 3.2A and 3.2B) in PECs isolated from PIM1$^{-/-}$ mice compared to WT controls, indicating that the expression of Pim kinase isoforms is inter-regulated, and suggesting that deletion of one Pim isoforms can affect expression of the others.

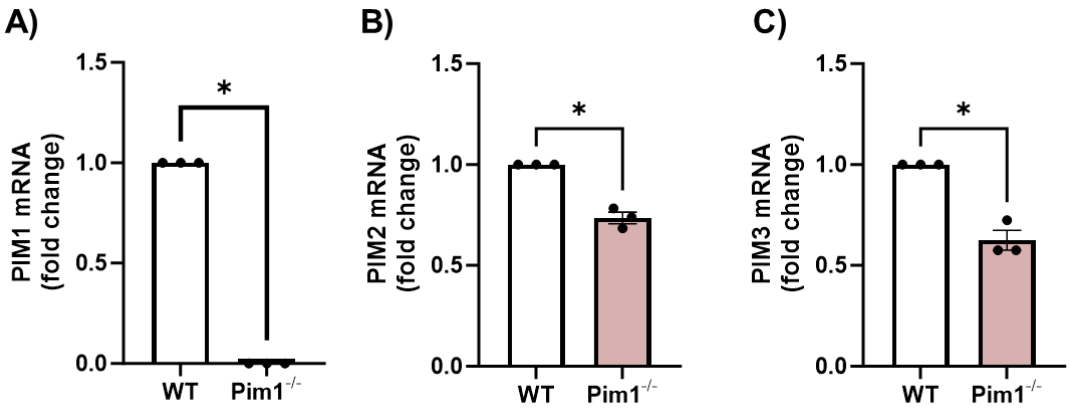

Figure 3.2: Individual Pim isoform gene expression is decreased following PIM1 deletion in PECs. WT and PIM1$^{-/-}$ mice PECs were lysed and RNA extracted. (A) *PIM1*, (B) *PIM2*, and (C) *PIM3* gene expression was analysed using RT-qPCR on WT and PIM1$^{-/-}$ PEC lysates. Technical repeats in each independent experiment were carried out in duplicate, and results were plotted as fold change in comparison with untreated cells. Data was analysed using an unpaired Mann-Whitney U nonparametric test, results are expressed as mean ± SEM for n=3. *P≤0.05. Where normalised data was shown, statistics were performed before normalisation.

3.3.2 Global knockout of PIM1 does not affect mice weight

Previous studies of PIM1 and triple PIM isoform deficient mice have observed reductions in mouse body weight (Mikkers et al., 2004, Liu et al., 2019). Mice were therefore weighed at 12-16 weeks old to determine any effects of PIM1 deletion on systemic health and metabolism compared to WT. No significant differences were observed between control and PIM1$^{-/-}$ mice, indicating the potential for using Pim kinase inhibitors clinically without the disruption of metabolic processes.

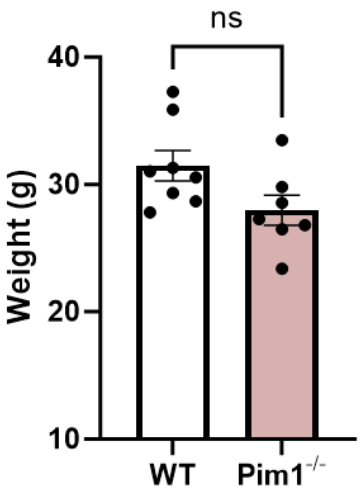

Figure 3.3: Genetic deletion of PIM1 does not significantly affect mice body weight compared to WT. Male WT and PIM1$^{-/-}$ mice were weighed at 12-16 weeks and age-matched for experiments. Results are expressed as mean ± SEM. Data was analysed using an unpaired t-test, n=8 (WT) and n=7 (PIM1$^{-/-}$).

3.3.3 Deletion of PIM1 alters blood cell indices

Having observed no effect of PIM1 deletion on mice body weight, the effects of PIM1 deletion on mice blood cell indices was subsequently determined using a full blood count (FBC). It has previously been shown in triple Pim kinase isoform deletion studies that the three Pim kinases are widely expressed in haematopoietic tissues and that all 3 Pim kinases are commonly overexpressed in haematological cancers (Alvarado et al., 2012), prompting us to investigate the effects of PIM1 deletion on the development of the hematopoietic lineages. A FBC was performed to compare blood cell indices between WT and PIM1$^{-/-}$ mice. Whilst red blood cell count (RBC) was unaltered (p=0.2796), both haemoglobin concentration (Hb) (p=0.0033) and mean cell volume (MCV) (p=<0.0001) of red cells were observed to be decreased in PIM1$^{-/-}$ mice compared to WT controls (Figure 3.4). These findings possibly indicating microcytic hypochromic RBC compared to WT mice, indicating a role for PIM1 in the regulation of erythropoiesis.

No alteration in the total white blood cell (WBC) count was observed between PIM1$^{-/-}$ and WT mice. However, differences in the proportion of the different WBC types were observed. The proportion (%) of lymphocytes were increased (p=0.0182), whilst the proportion (%) of mixed (monocytes/eosinophils/basophils) WBC were observed to be decreased in PIM1$^{-/-}$ mice compared to WT controls (Figure 3.5) Interestingly whilst the WBC differential and proportion of granulocyte (myeloid) vs lymphoid cells are affected, the individual lymphocyte or monocyte counts were not observed to be significantly altered in PIM1$^{-/-}$ mice versus WT controls. These differences in WBC differentials could suggest that Pim1 kinase plays a role in the regulation of lymphopoiesis or myelopoiesis which requires further investigation.

Finally, platelet indices were compared between WT and PIM1$^{-/-}$ mice. Platelet count was found to be significantly decreased in PIM1$^{-/-}$ compared to WT mice (p=0.0439). Despite the reduction in count (~20%) there were no significant differences observed in mean platelet volume (MPV) (p=0.7066) or Platelet

Distribution Width (PDW) (p=0.2046) (Figure 3.6), indicating no alteration in platelet size. A decreased platelet count indicates alterations in thrombopoiesis in PIM1$^{-/-}$ mice, which could have implications for haemostasis and future clinical use.

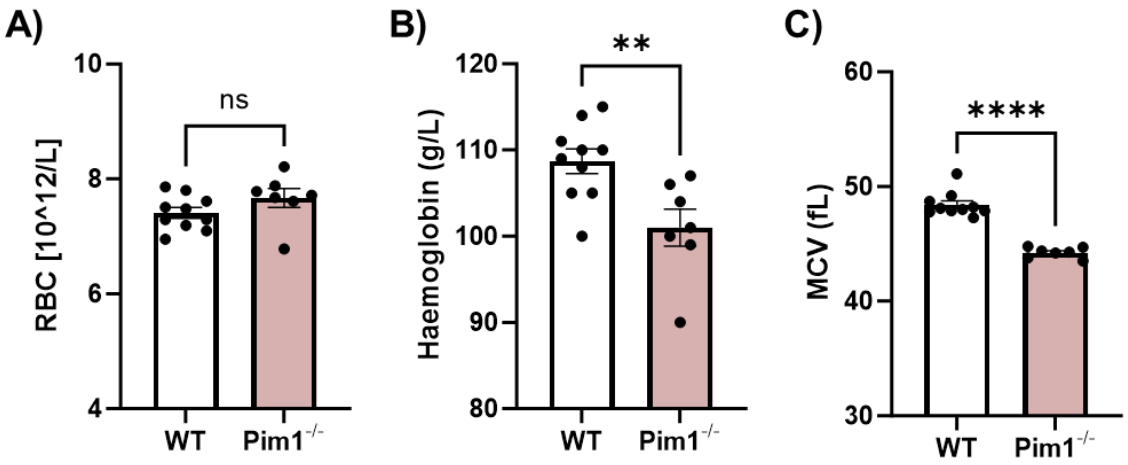

Figure 3.4: PIM1$^{-/-}$ mice have microcytic hypochromic red blood cells compared to WT controls. A full blood count was performed on whole blood collected from WT and PIM1$^{-/-}$ mice and RBC indices measured on the Sysmex Analyser including (A) RBC count, (B) Haemoglobin, and (C) Mean Cell Volume (MCV). Results are expressed as mean ± SEM. **P≤0.01, **** P≤0.0001. Data was analysed using unpaired t-tests, n=10 (WT) and n=7 (PIM1$^{-/-}$).

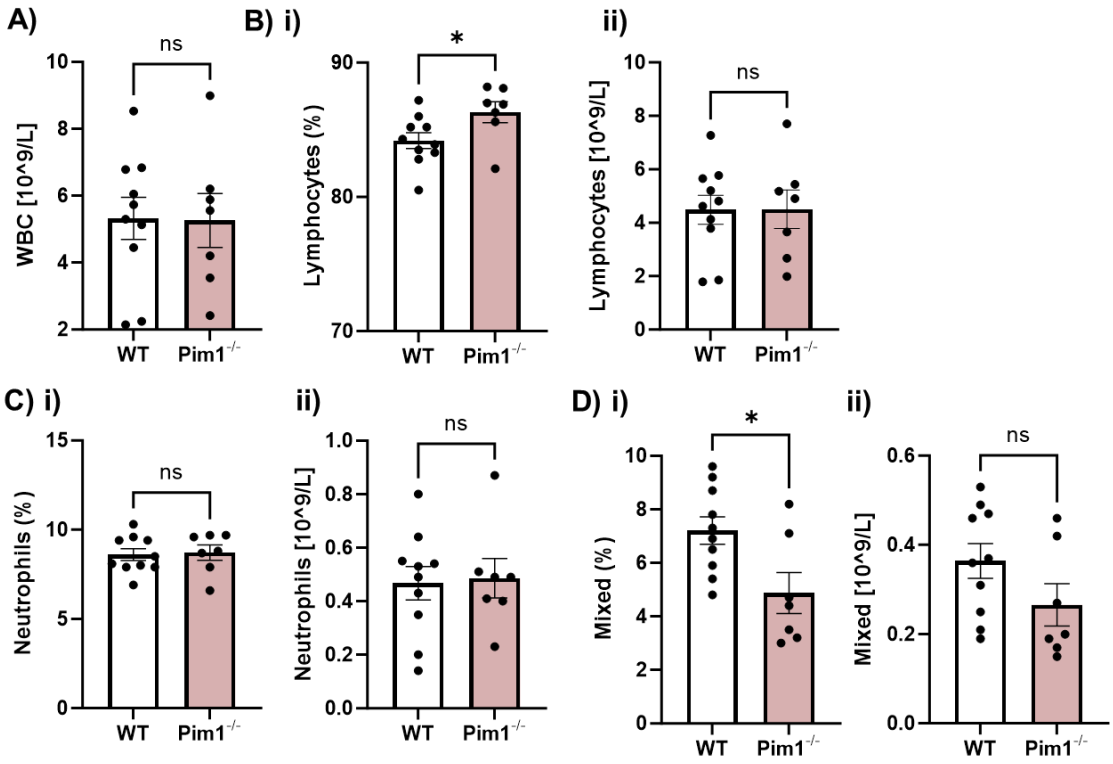

Figure 3.5: PIM1$^{-/-}$ mice have increased lymphocyte and decreased mixed WBC proportions compared to WT controls. A full blood count was performed on whole blood collected from WT and PIM1$^{-/-}$ mice measuring WBC indices on the Sysmex Analyser; (A) WBC count, (B) Lymphocyte, (C) Neutrophil, and (D) Mixed (Monocytes/Eosinophils/Basophils) WBC (i) %, and (ii) count. Results are expressed as mean ± SEM. *P≤0.05. Data was analysed using unpaired t-tests, n=10 (WT) and n=7 (PIM1$^{-/-}$).

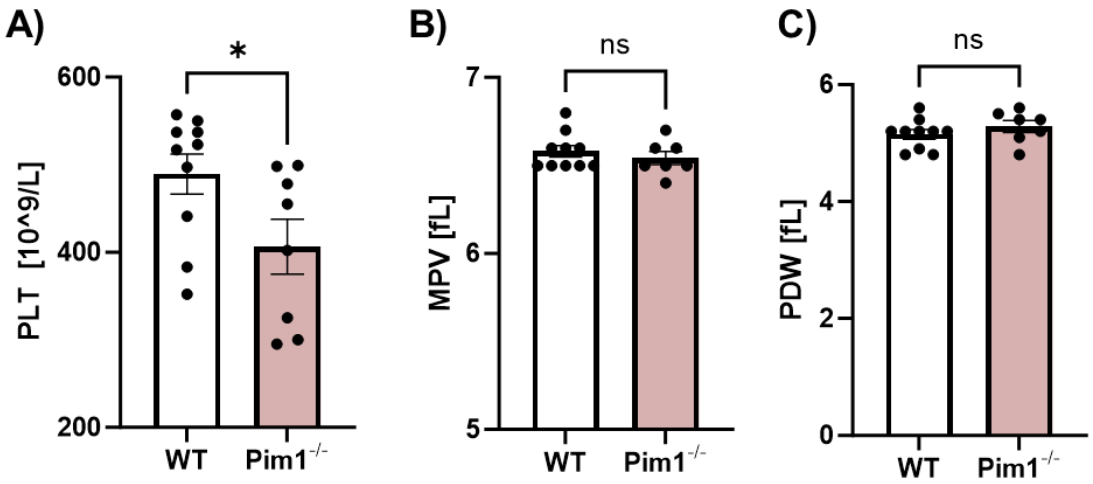

Figure 3.6: PIM1$^{-/-}$ mice have a decreased platelet count compared to WT controls. A full blood count was performed on whole blood collected from WT and PIM1$^{-/-}$ mice measuring platelet indices on the Sysmex Analyser; (A) Platelet count, (B) Mean Platelet Volume (MPV), and (C) Platelet Distribution Width (PDW). Results are expressed as mean ± SEM. Data was analysed using unpaired t-tests and graphs plotted using GraphPad Prism, n=10 (WT) and n=7 (PIM1$^{-/-}$).

The effects of PIM1 deletion on platelet production and function have been investigated and reported previously (Unsworth et al., 2021) and is the focus of a separate research project that is currently ongoing. Therefore, further investigations relating to platelet biology is not the focus of the work presented in this chapter and will therefore not be described further. To investigate the role of PIM1 in the regulation of coagulation and its contribution to thrombosis, the clotting properties of blood and plasma from $PIM1^{-/-}$ and WT mice were determined using whole blood and platelet poor plasma (PPP) coagulation assays.

3.3.4 PIM1 deletion delays clotting via the intrinsic pathway

To determine whether PIM1 deletion has any effects on whole-blood clot formation and stability *ex vivo*, rotational thromboelastometry (ROTEM) analysis was performed. Both extrinsic thromboelastometry (EXTEM) (tissue factor, Figure 3.7) and intrinsic thromboelastometry (INTEM) (contact activator, Figure 3.8) experiments were carried out to activate and investigate PIM1 contributions to the individual extrinsic and intrinsic coagulation pathways, respectively.

In EXTEM analysis, time to initiation of clotting (clotting time, CT) (Figure 3.7A), clot formation time (CFT) (Figure 3.7B) and maximum clot firmness (MCF) in whole blood was also found to be unaffected in blood from PIM1$^{-/-}$ mice compared to WT controls (Figure 3.7C). These findings indicate that PIM1 does not play a role in the regulation of the extrinsic pathway of coagulation.

Interestingly in contrast to EXTEM, time to initiation of clotting in INTEM was observed to be prolonged 1.5-fold in PIM1$^{-/-}$ mice compared to WT controls ($p=0.0177$) (Figure 3.8A). Indicating a role for PIM1 in the regulation of the initiation of the intrinsic, contact activated coagulation. Whilst a similar trend was observed with time to clot formation (Figure 3.8B) this was not statistically significant ($p=0.1389$). It initially appeared that there may be a slight decrease in maximum clot firmness in whole blood INTEM in PIM1$^{-/-}$ mice compared to WT controls (Figure 3.8C) but this was not found to be statistically significant following analysis ($p=0.1032$).

These findings indicate that PIM1 may play a role in the regulation of initiation of intrinsic coagulation where deletion or inhibition could prolong clotting time following intrinsic pathway activation.

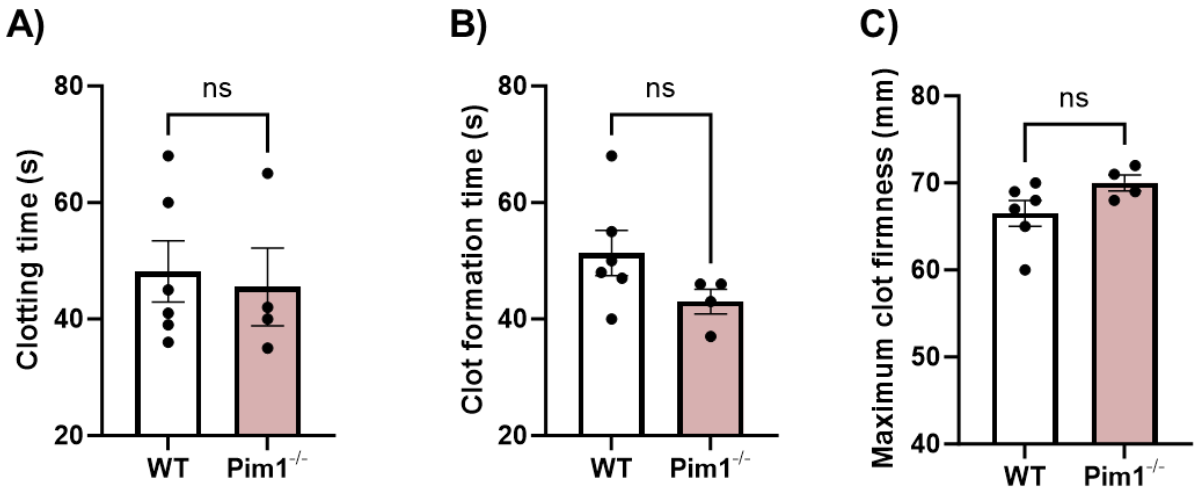

Figure 3.7: PIM1 deletion does not affect coagulation via the extrinsic pathway. Whole blood collected from WT and PIM1$^{-/-}$ mice was analysed by thromboelastography using a ROTEM and activation by EXTEM using tissue factor and phospholipids to activate the extrinsic pathway and time to (A) initiation of clotting (clotting time) CT, (B) clot formation (clot formation time) CFT, and (C) maximum clot firmness MCF measured. Results are expressed as mean ± SEM for n= 6 (WT) and n=4 (PIM1$^{-/-}$). Data was analysed using unpaired t-tests.

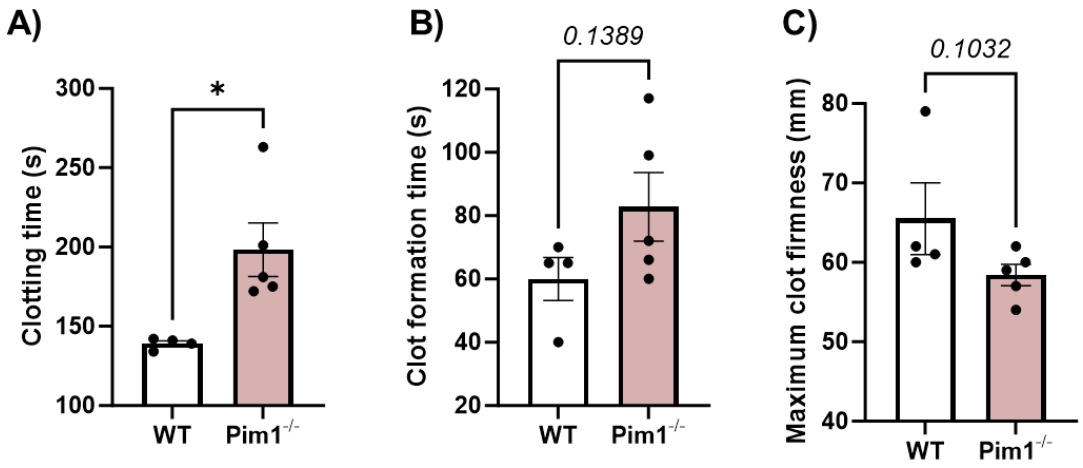

Figure 3.8: PIM1 deletion increases time to clot formation via the intrinsic pathway. Whole blood collected from WT and PIM1$^{-/-}$ mice was analysed by INTEM using partial thromboplastin and ellagic acid to activate the intrinsic pathway and time to (A) initiation of clotting (clotting time) CT, (B) clot formation (clot formation time) CFT, and (C) maximum clot firmness MCF measured using ROTEM. Results are expressed as mean ± SEM. *P≤0.05. Data was analysed using unpaired t-tests for n= 4 (WT) and n=5 (PIM1$^{-/-}$).

3.3.5 PIM1 does not directly regulate thrombin activity and fibrin formation

Next, to determine whether PIM1 deletion has any effects on clot formation without the interference of platelets, platelet poor plasma (PPP) was used. To investigate whether PIM1 deletion alters fibrin clot formation and structure, clots were made with plasma collected from both WT and PIM1$^{-/-}$ mice and analysed by turbidity measurements. Thrombin (0.1 U/mL) was added to PPP to initiate clotting, directly converting fibrinogen to fibrin, recapitulating the common pathway of the coagulation cascade. Clotting time was subsequently measured for the duration of 1 hour (Figure 3.9A).

Comparison of time to clot formation (lag time, Figure 3.9B), 50% clotting time (Figure 3.9C), time to complete clot formation (time to maximum optical density, Figure 3.9D), and maximum optical density (MaxOD, Figure 3.9E) found no significant differences between PIM1$^{-/-}$ mice and WT controls, indicating that PIM1 does not directly regulate thrombin activity, fibrinogen, or fibrin formation indicating that PIM1 kinase does not play a role in the direct regulation of the common pathway of coagulation.

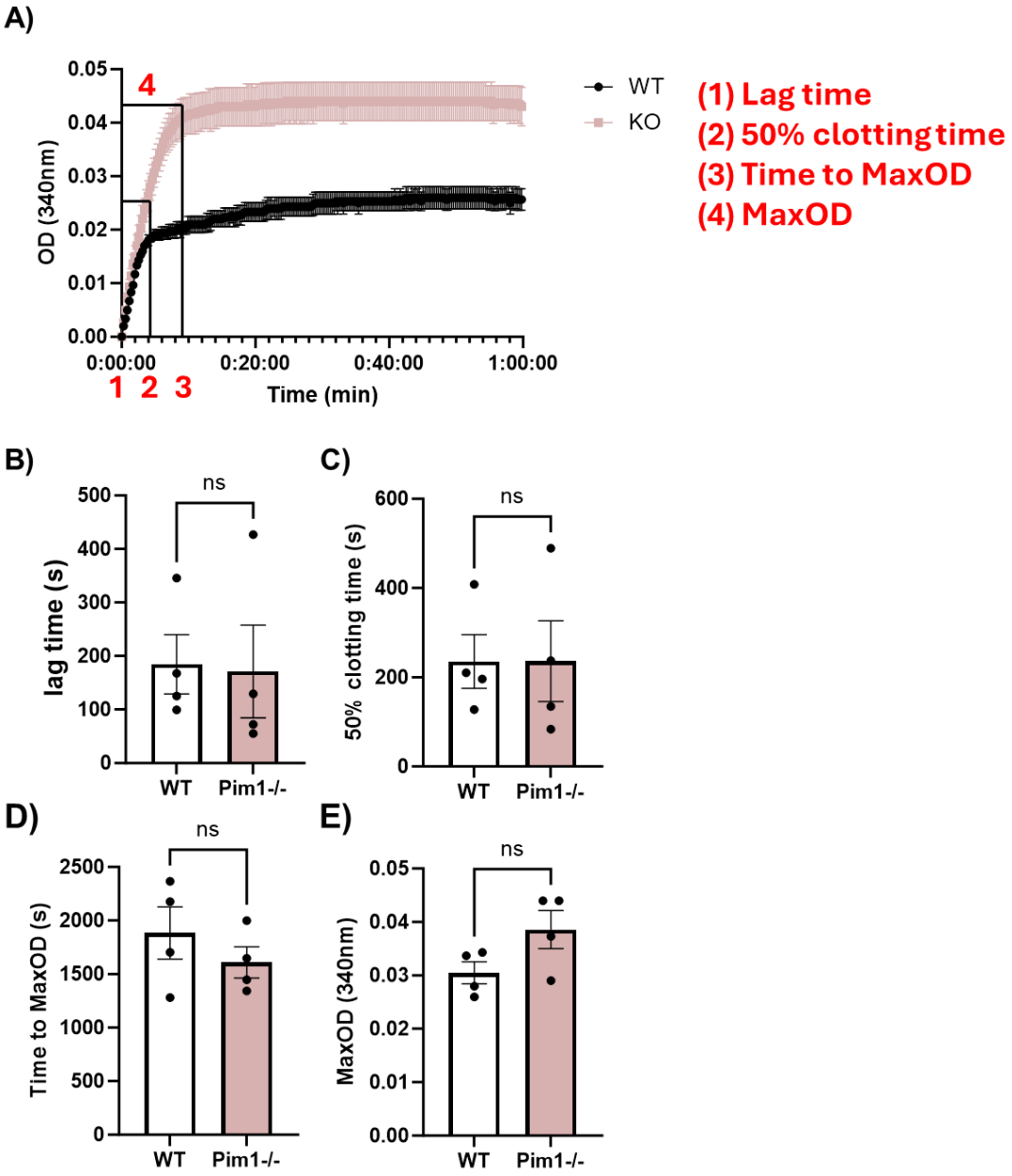

Figure 3.9: PIM1 does not regulate thrombin mediated fibrin clot formation. Platelet poor plasma (PPP) was isolated from whole blood collected from WT and PIM1$^{-/-}$ mice. (A) Turbidity analysis was performed to measure fibrin clot formation following initiation of clotting using thrombin (0.1 U/mL) (representative trace shown), and four parameters (B) lag time, (C) 50% clotting time, (D) Time to Maximum Optical Density (MaxOD), and (E) MaxOD determined. Turbidity was measured on a BioTek PowerWave HT microplate reader set at 340nm, taking absorbance readings every 17 seconds for 1 hour at 37°C. Results are expressed as mean ± SEM for n= 4. Data was analysed using unpaired t-tests.

3.3.6 Genetic deletion of PIM1 does not alter fibrinolysis

Fibrinolysis is a key physiological process following coagulation and clot formation, that breaks down fibrin in blood clots to prevent thrombosis. The effects of PIM1deletion on fibrinolysis was measured to investigate whether genetic deletion of PIM1 affects fibrin clot stability and clot breakdown. Turbidity assays using platelet poor plasma (PPP) isolated from PIM1$^{-/-}$ and WT mice were performed, where clots were formed with mouse plasma (PPP) initiated by thrombin, followed by the addition of tPA to lyse the clot.

There were no differences measured between WT control and PIM1$^{-/-}$ mice fibrinolysis measurements (Figure 3.10A), including time to MaxOD to 50% lysis (Figure 3.10B), or MaxOD to lysis Vmax (Figure 3.10C), suggesting that PIM1 deletion does not affect the fibrinolytic activity of plasma. Although this is the case, there seems to be a trend of increased time to lysis using PIM1$^{-/-}$ mice plasma, suggesting a possible inhibition or difference in clot structure making it less susceptible to lysis.

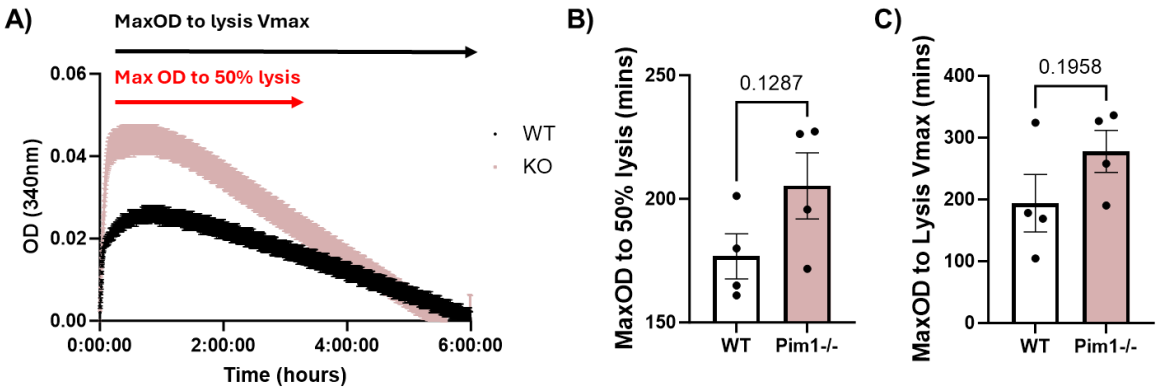

Figure 3.10: PIM1 deletion does not alter fibrinolysis. Fibrinolysis was measured following the formation of a clot with mouse plasma activated by thrombin, and the addition of tPA to lyse the clot. (A) The plate was read on a BioTek PowerWave HT microplate reader set at 340nm, taking absorbance readings every 17 seconds for up to 6 hours at 37°C producing a fibrinolysis trace (representative trace shown). (B) MaxOD to 50% lysis, and (C) MaxOD to lysis Vmax was measured. Results are expressed as mean ± SEM for n=4. Data was analysed using unpaired t-tests.

Taken together, these findings whereby PIM1 deletion prolongs clotting via the intrinsic coagulation pathway, together with data published previously, provide further evidence of a pro-thrombotic role for PIM1. Elucidating the cellular mechanisms that underlie these observations is required to allow the potential for targeting Pim kinases to be determined.

3.3.7 Deletion of PIM1 does not affect PEC survival or viability

Endothelial cells contribute to thrombosis and coagulation during dysfunction and infection/injury (Pepin and Gupta, 2024). The following experiments were focused on the contribution PIM1 to the regulation of endothelial biology and of the endothelial contribution to thrombosis and haemostasis. The following experiments used primary mouse Pulmonary Endothelial Cells (PECs) isolated from lungs collected from WT and PIM1$^{-/-}$ mice, as described in Section 2.11 (Materials and methods).

Pim kinases are well-established proto-oncogenes, contributing to cell survival, migration, and proliferation (Rathi et al., 2021). It was therefore important to determine the effects of PIM1 deletion on PEC survival and viability.

PECs were isolated and cultured from PIM1$^{-/-}$ and WT mice for 7-10 days and cell morphology, survival and viability determined (Figure 3.11). No significant differences were observed in cell morphology (Figure 3.11A), cell survival, measured by LDH assay (Figure 3.11B) or cell viability measured by MTS assay (Figure 3.11C) between WT and PIM1$^{-/-}$ PECs. These findings indicate that loss of PIM1 does not compromise PEC survival or viability.

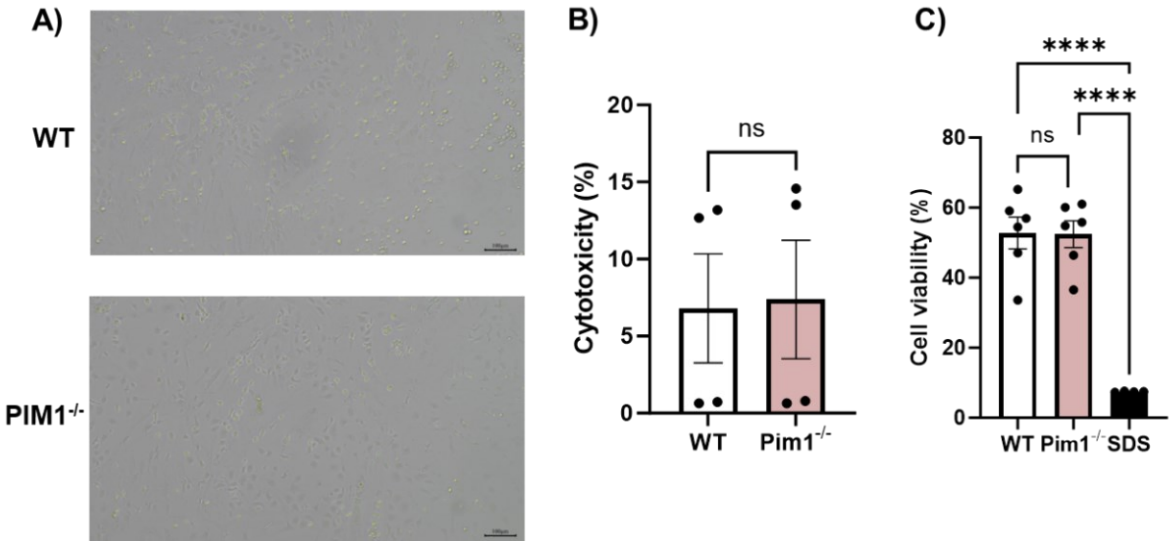

Figure 3.11: Loss of PIM1 does not compromise PEC survival and viability. Pulmonary endothelial cells were isolated from the lungs of WT and PIM1 $^{-/-}$ mice and cultured for 7-10 days before analysis for morphology, survival, and viability determined by analysis of (A) Images taken on a GXM inverted microscope at 4x magnification 24 hours after seeding, (B) culture media by LDH assay (Abcam- [ab65393]) and (C) MTS assay (Abcam- [ab197010]). SDS (sodium dodecyl sulfate) was used as a positive control for cell lysis. Representative images shown in (A). Results are expressed as mean ± SEM for n=4 for LDH and n=6 for MTS assay. **** P≤0.0001. Data was analysed using an unpaired t-test for LDH assay, and One-Way ANOVA for MTS assay.

3.3.8 Genetic deletion of PIM1 appears to reduce PEC tube formation at 4hr

It has previously been demonstrated that PIM3 silencing in HUVECs prevents the activation of EC sprouting compared to control cells, confirming that PIM3 exerts pro-angiogenic characteristics *ex vivo* (Yang et al., 2011a). An angiogenesis assay was therefore carried out using PECs isolated from PIM1$^{-/-}$ and WT mice to investigate whether this effect is specific to PIM3, or a function shared by PIM1.

PECs were seeded on Matrigel, and tube formation observed. Genetic deletion of PIM1 appeared to reduce PEC tube formation when imaged after 4 hours (Figure 3.12), however due to technical limitations, and lack of consistency between experiments, images are only available for n=2 and PEC tube formation using traditional measures (number of branch points, number of loops formed, and tube length) was not quantifiable. Therefore, further repeats are needed to confirm this initial observation.

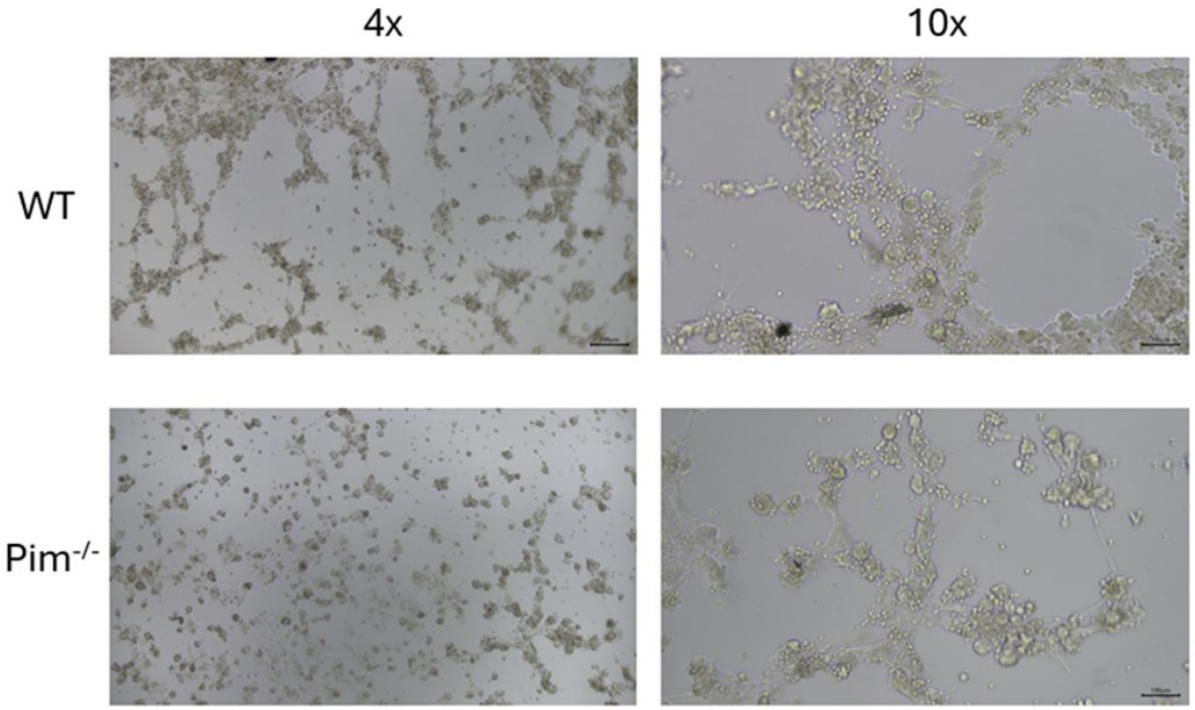

Figure 3.12: Loss of PIM1 appears to reduce PEC tube formation. WT and PIM1$^{-/-}$ PECs were seeded on Matrigel and incubated for 4 hours. Cells were imaged using a GXM inverted microscope at a 4x and 10x magnification and analysed using ImageJ software. Images are representative for n=2.

3.3.9 PIM1 deletion alters the PEC transcriptome

The identification of genes and pathways contributing to vascular endothelial function is important for the development of new pharmacological approaches to target the endothelium for the prevention and treatment of cardiovascular events (Medina-Leyte et al., 2021).

The aim of this section was to determine the impact on gene expression following genetic deletion of PIM1 in ECs. PEC lysates from PIM1$^{-/-}$ and WT mice were prepared, RNA extracted and outsourced to BMKGene for sequencing.

Due to low sample quality and following independent assessment of the RNA-Seq data and principal component analysis, only 2 experimental data sets were considered acceptable for comparison. Therefore, statistical analyses and subsequent volcano plots could not be carried out on this dataset. A preliminary analysis of gene expression levels of various, endothelial and thrombotic regulators was grouped across four functional categories and is described below (Figure 3.13).

3.3.9.1 (A) Upstream PIM kinase signalling pathway components

JAK/STAT are upstream of Pim kinase and are involved in their regulation. Exploring the endothelial transcriptomic profile of PIM1 deletion on JAK and STAT expression is key to determine the upstream effects on Pim signalling pathway components. While JAK1 was relatively unchanged, JAK2 looked to be decreased, indicating variable responses. STAT1 was increased, along with STAT5B which was only slightly increased, while STAT3 and STAT5A were very slightly decreased following PIM1 deletion.

3.3.9.2 (B) thromboinflammatory molecules

Gene expression of thromboinflammatory molecules was compared between WT and PIM1$^{-/-}$ PECs to elucidate the role of PIM1 kinase in PEC function.

Regulators of platelet function

Whilst no statistical comparisons can be made, comparison of negative and positive regulators of platelet adhesion and activation identified that *NOS3*

(eNOS) appears to be increased in PIM1$^{-/-}$ PECs compared to WT (9-fold), along with ENTPD1 (CD39) and flow regulatory genes KLF2 and KLF4. Although these observations point towards an anti-thrombotic endothelial phenotype, more repeats are required to confirm these observations. Conversely, *VWF* (4-fold), *TBXAS1 and TBXA2R* (thromboxane receptor) were also upregulated in PIM1$^{-/-}$ PECs compared to WT.

Inflammatory mediators

IL6, IL6R, and *IL1β* looked to be decreased following genetic deletion of PIM1 compared to WT, suggesting a role for PIM1 in inflammation, and highlighting its contribution to the increased expression of inflammatory markers in the endothelium.

3.3.9.3 (C) Endothelial adhesion molecules

Genes associated with adhesion to the endothelium appeared to be increased in PIM1$^{-/-}$ PECs compared to WT, including *SELE* (E-selectin), *SELP* (P-selectin), *PECAM1* (7-fold) and possibly *ICAM1*. In addition, gene expression of proteins involved in the maintenance of endothelial junctions including *CDH5* (VE-Cadherin) (8-fold), and *F11R* (JAM-A) were also observed to be increased in PIM1$^{-/-}$ PECs compared to WT. An increase in endothelial adhesion molecules following PIM1 deletion suggests that PIM1 plays a role in maintaining the intact endothelial barrier and may facilitate leukocyte recruitment to drive wound healing. This mechanism needs to be further elucidated.

3.3.9.4 (D) Coagulation and fibrinolysis regulators

Activators of fibrinolysis, *PLAT* (tPA) and *PLAU* (uPA) are increased, possibly indicating a protective effect of PIM1 deletion in fibrin breakdown following thrombus formation. *F5* (coagulation factor V) is also decreased, indicating that PIM1 may play a role in the formation of the prothrombinase complex and coagulation. Additionally, *SERPING1* (C1 inhibitor), an inhibitor of the contact pathway of coagulation, was also increased, together demonstrating an anti-coagulant response following PIM1 deletion. Conversely, *SERPIND1* (Heparin

cofactor II), which promotes thrombin inactivation was also decreased, indicating a variable coagulation regulator response.

These preliminary observations in the transcriptomic profile of PECs indicate a variable endothelial thromboinflammatory phenotype following PIM1 deletion. Further investigation and more experimental repeats are needed to conclude any effects with statistical significance and confirm these observations.

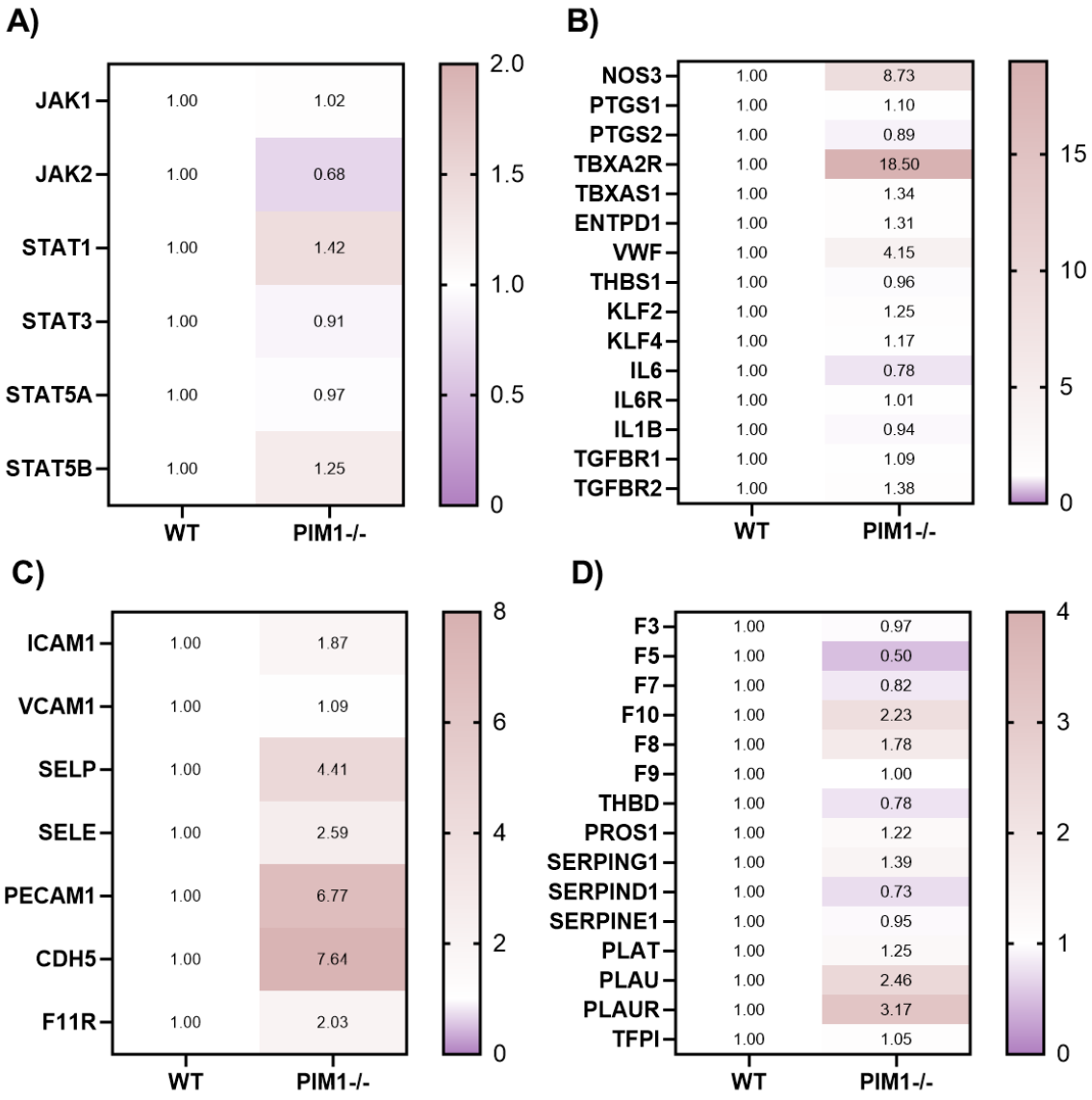

Figure 3.13: PIM1 genetic deletion differentially modulates endothelial regulators of thrombosis and inflammation. WT and PIM1$^{-/-}$ PECs were lysed and RNA extracted. RNA-Seq was performed on the Illumina NovaSeq platform, generating paired-end reads with a minimum depth of 30 million reads per sample. Heat maps display fold change gene expression levels compared to vehicle control for various regulators across four functional categories: (A) upstream PIM kinase signalling pathway components, (B) thromboinflammatory molecules, (C) endothelial adhesion molecules, and (D) coagulation and fibrinolysis regulators. Each row represents an individual gene, and colour intensity reflects relative expression magnitude. Data represents mean ± S.E.M for n=2. Heat maps were plotted using GraphPad Prism.

3.4 Discussion

The aim of this chapter was to determine the effects of global PIM1 deletion on whole blood parameters and pathways of coagulation, along with functional and transcriptomic effects on the endothelium to elucidate the role played by PIM1 in various cell types that contribute to thrombosis.

Key Findings:

- PIM1 deletion downregulates gene expression of PIM2 and PIM3.
- PIM1 deletion impacts normal myelopoiesis, decreasing red blood cell size, the proportion of monocytes, and platelet count.
- PIM1 deletion delays clotting time via the intrinsic pathway.
- PEC transcriptome demonstrates a variable thromboinflammatory response following PIM1 deletion.

These findings identify PIM1 as a potential regulator of vascular homeostasis that positively regulates intrinsic pathway coagulation and endothelial function.

3.4.1 The effect of global PIM1 deletion on mice viability

Ahead of investigating the functional effects of PIM1 deletion, mice were weighed (ages varying between 12-16 weeks), as weight is a key biological indicator of health and metabolic processes. Whilst mice body weight, on inspection, looked to be decreased following $PIM1^{-/-}$ compared to WT control, this was not significantly different, indicating that normal metabolic processes are not altered supporting that PIM1 deletion is safe. These observations of a lack of an effect on body weight is in contrast to previous studies where mice lacking expression of PIM1, PIM2, and PIM3 (triple knockout) showed a reduction in body size at birth (neonates) and throughout postnatal life, weighed again at 12 weeks, however they were still viable and fertile (Mikkers et al., 2004). Another study using $PIM1^{-/-}$ mice demonstrated that in a 12-week follow-up, the body weight of $PIM1^{-/-}$ mice was significantly lower than that of control littermates, showing overall growth retardation (Liu et al., 2019). It should be noted however, that mice weighed in this chapter were male, therefore this is not representative

of female, and effects may differ due to hormonal and sex-specific differences, alongwith differences in physiology and metabolism. Additionally, mice weighed in our study were between 12-16 weeks which could be impacting the results, as a decreased trend is observed but the differences between different ages of mice make the data more variable, and therefore less significant. It should also be acknowledged that this study is underpowered and required further repeats. However, a PIM1$^{-/-}$ mice study has shown that body weights of mice deficient in PIM1 were in the range of their heterozygous and WT littermates (Laird et al., 1993), therefore indicating that PIM1 deletion does not affect metabolic processes and consequently body weight.

3.4.2 PIM1 deletion affects normal myelopoiesis

Bone marrow cells from PIM1$^{-/-}$ mice have demonstrated decreased hematopoietic progenitor colony-forming ability (An et al., 2013b), indicating that PIM1 plays a role in haematopoiesis. A full blood count (FBC) using PIM1$^{-/-}$ mice was performed in our study to investigate if this further affected the production of mature blood cells. FBC analysis identified a reduction in platelet count but no alterations in RBC or WBC counts. However, alterations in RBC size, and proportion of monocytes, basophils and eosinophils were observed in PIM1$^{-/-}$ mice compared to WT controls. Our observations and the literature suggest a shift in immune balance following PIM1 deletion which may be due to lineage-specific effects or altered cytokine or growth factor signalling due to transcriptional changes induced by PIM1 deletion, affecting haematopoiesis.

A study by Dutta *et al*, investigating the effects of PIM1 in myelofibrosis, noted a decrease in the colony forming units (CFU) of the myeloid lineage in the bone marrow of PIM1$^{-/-}$ mice compared to WT, but interestingly less of an effect in the later haematopoietic progenitors, granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) (Dutta et al., 2022). In the same study, a significant decrease in WBC and platelet counts were observed following genetic deletion of PIM1 whilst RBC count was not affected, supporting our observations of altered platelet count, and alterations in the proportion of

monocytes. This highlights a role for PIM1 in haematopoiesis, and specifically in the development of myeloid cells. In a pathological context, this presents PIM1 as an appropriate target for thrombotic and inflammatory diseases, and the development of atherosclerotic processes. The lack of effect on the MEPs supports our observations of lack of effect on the RBC count, which was also confirmed by this study.

3.4.2.1 Red blood cells

Observed decreases in red blood cell (RBC) mean cell volume (MCV) and haemoglobin are supported by previous studies in PIM1 and triple PIM kinase isoform deficient mice. A study by Zhang *et al* describes a role for PIM1 in normal erythropoiesis, demonstrated by PIM1 deletion in human erythroid cells inhibiting erythroblast enucleation, a key process in erythropoiesis (Zhang et al., 2025). Similar to our observations, an earlier specific PIM1$^{-/-}$ mice study demonstrated that the MCV of RBC is significantly smaller in PIM1 deficient mice compared to their wild-type littermates (Laird et al., 1993). Laird *et al* described that the peripheral blood concentration of RBC was not elevated in PIM1 deficient mice to compensate for the microcytosis, which meant that haemoglobin levels were also reduced (Laird et al., 1993) in further support of our observations. Given that RBCs are linked to the early myeloid progenitors, and RBC parameters were altered, it is more likely a reduction in this shared early progenitor, which then alters the proportions.

Interestingly studies using mice deficient in all three PIM kinase isoforms also demonstrate that triple PIM$^{-/-}$ mice have a decreased erythrocyte MCV which supports our observations, however they also reported a slight increase in the number of RBCs (Mikkers et al., 2004). Additionally, An *et al* also observed erythrocyte hypochromic microcytosis, consistent with our observations indicating that PIM1 is the main driver of normal erythropoiesis.

Taken together, these findings indicate that PIM1 deletion affects RBC indices, making them smaller with a reduced amount of haemoglobin. This could affect clotting and fibrinolysis processes, as indicated by a study which has shown that

RBCs support fibrin film formation, and fibrin film coverage is enhanced with higher haematocrit (Alkarithi et al., 2024). Alkarithi *et al* observed that reductions in fibrin film under thromboinflammatory conditions support continued clot growth whilst fibrin film formation impairs fibrinolysis (Alkarithi et al., 2024). An alteration of RBC indices could therefore suggest an alteration in fibrin film formation, affecting clot growth and fibrinolysis processes.

3.4.2.2 White blood cells

Whilst an increase in the proportion of lymphocytes was observed in PIM1$^{-/-}$ mice compared to WT controls, this was associated with a reduction in the proportion of monocytes, eosinophils and basophils. As cell counts were unaffected in our analysis, it is unclear whether these alterations in proportions are due to an increase in lymphocytes or a reduction in granulocytes (monocytes, basophils, eosinophils), however given that evidence suggests that PIM1 is involved in haematopoiesis at the early progenitor stage, it is very likely a reduction in myeloid cells (Dutta et al., 2022). Other studies using PIM1-specific and triple Pim knockout mice confirm our observations. Another study has identified that PIM1 plays a significant role in lymphopoiesis, particularly in B-cell development and function (Cui et al., 2024, Xu et al., 2016), and a study by An *et al* demonstrated that compared to WT controls, Gr-1^{+} granulocytes were reduced in triple Pim knockout mice (An et al., 2013b).

A reduction in monocytes, specifically during times of inflammation, could be beneficial by attenuating atherosclerosis and thromboinflammation. TF expression in monocytes within atherosclerotic plaques is a primary trigger for thrombosis after plaque rupture (Owens and Mackman, 2012). Therefore, decreased monocytes could lower atherosclerosis and consequent thrombosis due to less recruitment to the vessel wall, and less TF expression.

3.4.2.3 Platelets

In our study, we observed a reduction in platelet count in PIM1$^{-/-}$ compared to WT controls. This however, contrasts with observations by Laird et al, which states no difference in the platelet counts of PIM1$^{-/-}$ mice compared to their WT

littermates (Laird et al., 1993). Studies using triple PIM deficient mice have also demonstrated lower peripheral blood platelet count (An et al., 2013b), which aligns with the observations in our study, where platelet count was reduced while other parameters (MPV/PDW) were unaffected in PIM1$^{-/-}$ mice, indicating that Pim kinases may regulate thrombopoiesis. To support this hypothesis, PIM1 deficient PECs also showed reduced PIM2 and PIM3 mRNA expression, and therefore this suggests that it could also be the reduction in these that are contributing, along with PIM1.

It remains to be determined whether the observed alterations in Pim-deficient mice would translate to pathological effects following administration of PIM1-specific inhibitors in patients e.g. the development of anaemia.

3.4.3 Coagulation and fibrinolysis

It has been shown previously that PIM1 is elevated in the plasma of deep vein thrombosis (DVT) patients, which is a coagulation driven pathology, however its role and contribution to coagulation processes remains to be explored (Chen et al., 2023). It was therefore important to determine the effects of PIM1 deletion on clotting ability and time using whole blood and platelet poor plasma (PPP) to evaluate the effects of PIM1 on coagulation.

Time to fibrin clot formation was not affected when clotting was initiated with the addition of thrombin to PPP and measured using a turbidity assay, however PIM1 deletion delayed clot formation via the intrinsic pathway measured using INTEM but did not appear to affect the extrinsic pathway as EXTEM was not altered. Interestingly, *SERPING1* (C1 inhibitor), a serine protease inhibitor that functions as a major negative regulator of several biological pathways, including the contact pathway of coagulation (Grover et al., 2023), was increased following PIM1 deletion. This indicates that PIM1 may play a negative role in the regulation of coagulation, affecting clotting factors and mechanisms specifically linked to the intrinsic coagulation pathway, which include coagulation factors FXII, XI, IX, and VIII, or negative regulators e.g. thrombomodulin/protein C/ protein S (Cadroy et al., 1997). PIM1 is regulated via JAK/STAT, and JAK/STAT pathways have shown

to broadly regulate hepatocyte gene expression, with implications for downstream physiology including potential effects on production and regulation of liver-derived factors (Kaltenecker et al., 2019). As our PIM1$^{-/-}$ mouse model is a global knockout, it is possible that PIM1 deletion in hepatocytes could be affecting the liver and therefore production of coagulation factors (fibrinogen (I), prothrombin (II), V, VII, IX, X, XI, XII, XIII).

Coagulation cofactors (FVIII and FV) are key contributors to clotting, majorly influencing thrombin generation, and are commonly defected when clotting time is selectively prolonged (Bos and Camire, 2010), producing delayed but eventual clot formation due to reduced efficiency of the intrinsic tenase (FIXa and FVIIIa activating FX) and prothrombinase (FXa and FVa activating FII) complexes (Chaudhry et al., 2025). Interestingly, *FV* gene expression appeared to be decreased in PIM1$^{-/-}$ mice PECs, taken from our RNA-Seq transcriptomic data (Figure 3.10D), which may contribute to prolonged coagulation time. However, the prothrombinase complex is also required for extrinsic pathway-initiated coagulation and EXTEM was not impacted, indicating that the common pathway was not affected.

ECs, particularly in the liver sinusoids, are the primary source of FVIII (Cohen et al., 2020) which is a cofactor in the intrinsic clotting pathway. Additionally, ECs regulate FVIII stability (via VWF production) (Swystun et al., 2018). FVIII is highly sensitive to cellular signalling, and together with FIXa, activates FX (Konkle, 2025), initiating the common pathway to form a stable fibrin clot. Deficiencies in FVIII and FIX defects cause prolonged clotting, as they are required for the conversion of FX to FXa (Mehta et al., 2023). Additionally, VWF is needed to maintain FVIII levels, and transcriptomic data suggested increased VWF expression in PIM1$^{-/-}$ mice, which may rule out a PIM1 effect on FVIII as it is well-established that VWF is a key partner for FVIII, playing significant roles in FVIII function, and its production and its stabilization (Federici, 2003). Alternatively, an increase in VWF could indicate an attempt to overcome lower protein levels of FVIII.

Using plasma from PIM1$^{-/-}$ mice in FV, FVIII, FIX, and FX activity assays, or ELISA (for levels of the coagulation factors) and assays to measure levels or activity of inhibitors of the intrinsic pathway (e.g. Protein C Chromogenic Assay) may therefore be appropriate to deduce PIM1 effects on the specific factors that contribute to the intrinsic pathway of coagulation which may explain the delayed clotting time that has been observed.

The deficiency in coagulation factors associated with intrinsic coagulation such as FVIII and FIX specifically affect clot formation and stability, not the mechanisms of clot dissolution, therefore fibrinolysis itself remains normal. The resulting clots are, however shown to be frequently porous and fragile, making them more susceptible to the normal fibrinolytic process (Chapin and Hajjar, 2015). Although maximum clot firmness (MCF) was not altered between WT and PIM1$^{-/-}$ mice, and clot breakdown not affected, it would be useful to investigate the effect of PIM1$^{-/-}$ on physical clot properties such as clot structure and stability to better understand its contribution to clot formation and fibrinolytic processes.

Additionally, EXTEM and INTEM assays performed use whole blood therefore still include platelet activity and so the observations could be explained by platelet function (Kjellberg et al., 2020). Although this could be the case, no effect on EXTEM supports that the INTEM result is coagulation rather than platelet driven. EXTEM and INTEM experiments would need to be performed again (FIBTEM- which prevents platelet-mediated clot retraction using potent platelet inhibitor cytochalasin D and is therefore a reflection of fibrinogen activity). Clot formation (turbidity) assays should also be repeated with PPP and either TF or contact activators to be sure the effects observed are not platelet driven.

Thrombosis is a balance between clot formation and clot breakdown, where alterations in fibrinolysis can increase or decrease thrombotic potential. Whilst deletion of PIM1 significantly prolonged intrinsic-pathway–mediated clot formation, the fibrinolytic system operates separately from the initial coagulation cascade factors, and fibrinolysis was shown to be unaffected by

PIM1 deletion, indicating that PIM1 does not contribute to the regulation of fibrinolysis mediators, or alter clot structure making it more or less resistant to dissolution. Normal fibrinolysis after PIM1 genetic deletion indicates that PIM1 is not involved in the regulation of plasminogen activation, tPA/uPA activity, PAI-1, or α2-Antiplasmin function. Although this is the case, there does appear to be trend of increased time to lysis, suggesting a possible inhibition, which could suggest reductions in fibrinolytic factors or alterations in clot structure. Interestingly however, increased gene expression of fibrinolytic enzymes *PLAT* (tPA) and *PLAU* (uPA) gene was identified in PECs by our RNA-Seq analysis. The effects of this would need to be investigated further using ELISAs to measure levels of tPA and uPA (a PAI-1 complexed tPA/uPA ELISA may be appropriate to assess plasminogen activator release, as the proteases are rapidly inhibited by PAI-1 following secretion), plasmin generation assays to measure their activity, and qPCR/western blotting to determine mRNA and protein levels. A lower concentration of thrombin may be required to determine whether fibrinolysis would be altered, as the concentration of thrombin used to drive clotting could be too high so it's not sensitive enough to detect small changes in fibrinolytic enzymes.

3.4.4 Pulmonary Endothelial Cells

The endothelium is a key regulator of thrombosis and haemostasis (Yau et al., 2015a). Previous studies using both mouse models and human EC lines have indicated that PIM1 deletion or inhibition can be protective in vascular pathology (Xue et al., 2025). This section of the chapter therefore aimed to investigate the effects of PIM1 genetic deletion on endothelial function and transcriptional mechanisms. The impact of PIM1 deletion on pulmonary endothelial cells (PECs) isolated from PIM1$^{-/-}$ and WT control mice was therefore determined. Due to limited time and mouse numbers, only preliminary experiments were performed using PECs, which require further investigation and characterisation.

3.4.4.1 PEC function

Initial characterisation of PECs deficient in PIM1 compared to WT controls showed no apparent differences in EC survival or viability. PIM1 is a well-established proto-oncogene which is overexpressed and has known roles contributing to the inhibition of apoptosis (PIM1 inactivates pro-apoptotic protein BAD by phosphorylating it on Ser112) (Aho et al., 2004), promotion of cell cycle progression (through phosphorylation and activation of Cdc25) (Bachmann et al., 2006), and phosphorylation of regulatory proteins and modulation of cell cycle/apoptotic pathways (phosphorylation and inhibition of Cdc25-associated kinase 1 (C-TAK1), an inhibitor of Cdc25C) (Magnuson et al., 2010, Bachmann et al., 2004). As cells remained viable and death was not induced following PIM1 deletion, this suggests that the PIM1 role in cell survival and proliferation may be a cell specific mechanism, as cancer cells require PIM1 for increased pathological proliferation that is uncontrolled, but it may not be needed for normal proliferation in ECs. Alternatively, it could indicate that ECs adapt to a lack of PIM1, and therefore proliferation and survival is not affected. Following these observations, functional assays were carried out.

Angiogenesis is an important function of ECs, dependent on EC proliferation, survival, migration and tube formation, enabling them to form new blood vessels. Although this is a physiological function, it can also contribute to pathological processes and enhance atherosclerosis. Previous studies have shown that PIM1 promotes angiogenesis through phosphorylation of eNOS at Ser-633 in COS-7 cells co-transfected with eNOS and PIM1 expression plasmids (Chen et al., 2016). Following PIM1 deletion, qualitative analysis showed PEC tube formation appeared reduced after 4 hours in Matrigel, indicating that PIM1 is required for normal endothelial pro-angiogenic function and supports previous observations in HUVECs that PIM1 positively regulates endothelial angiogenic capacity (Chen et al., 2016). These findings potentially highlight PIM1 as a key regulator of pulmonary endothelial angiogenesis, supporting previously published observations. However, only 2 repeats of this experiment were carried out, and more time points are required to confirm this observation. Additionally,

the PIM1$^{-/-}$ mice were global knockouts so it is not known for certain whether these effects are EC driven.

Interestingly, FVIII a key regulator of the intrinsic coagulation pathway has also been demonstrated to drive angiogenesis in the literature where it was observed that factor VIII promotes angiogenesis and vessel stability (Olgasi et al., 2024). Having observed an attenuation of both intrinsic pathway clotting time and angiogenesis, this could indicate FVIII expression as a possible downstream target of PIM1 that requires further investigation.

3.4.4.2 PEC gene expression and thromboinflammatory profile

Gene expression of the Pim kinase isoforms was measured following PIM1 deletion to investigate whether compensatory effects between the 3 Pim kinase isoforms (PIM1, PIM2 and PIM3) exist. Interestingly, PIM2 and PIM3 mRNA was decreased in PIM1 deficient PECs, indicating positive autoregulation, where all 3 Pim isoform gene expression is decreased with the deletion of one isoform, which is possible as they are on different chromosomes (see Table 1.2), demonstrating coordinated gene family regulation.

Further investigation of transcriptional effects of PIM1 deletion demonstrated an increase in platelet mediators *NOS3* (eNOS) and *ENTPD1* (CD39) gene expression and flow regulatory genes *KLF2* and *KLF4*. Interestingly, an increase in TBXAS1 and TBXA2R (the receptor for TXA2 which is converted from PGH2 through TBXAS1), which play a significant role in platelet aggregation, were also increased, indicating a variable PIM1 endothelial-platelet mediator response.

An increase in *VWF,* which recruits platelets to sites of vascular injury, following PIM1 deletion is in contrast to the literature where a study has shown that JAK inhibitor Ruxolitinib reduced surface and secreted VWF when added to activated (TNFα)–stimulated ECs (Beckman et al., 2023), indicating that JAK/STAT signalling contributes to endothelial pro-thrombotic activation. As PIM1 is downstream of JAK/STAT signalling, this indicates that PIM1 may contribute to a pro-thrombotic endothelial phenotype, therefore a decrease in VWF would be expected following PIM1 deletion. Although this is the case, the study measured

protein secretion and surface expression, whereas we measured gene expression. VWF assays (activity assays/ELISA) would be appropriate to investigate this further. Further repeats are required to elucidate the effect as n=2 is not sufficient and does not provide statistical significance.

The downregulation of *IL-6, IL-6R*, and *IL1β* demonstrates an-anti-inflammatory response following PIM1 deletion, indicating that PIM1 plays a role in inflammatory processes. These observations are supported by a study that demonstrated that PIM1 deletion attenuated the LPS-mediated upregulation of *IL1B*, *TNFα*, and *IL6* in macrophage-like THP-1 cells (Baek et al., 2024), confirming a role for PIM1 in inflammation.

Endothelial adhesion and signalling molecules *E-selectin* and *P-selectin,* which play roles in leukocyte rolling and the inflammatory response of the endothelium*,* and *PE-CAM1* and *F11R* (JAM-A), which are junctional proteins involved in regulating trans-endothelial migration of leukocytes were increased in PIM1$^{-/-}$ mice. This indicates increased endothelial activation and potential for increased leukocyte recruitment following PIM1$^{-/-}$ genetic deletion. While this response is beneficial during acute inflammation and wound healing, sustained upregulation promotes endothelial dysfunction, excessive leukocyte infiltration, and inflammation, contributing to proatherogenic and thromboinflammatory processes. Further investigation is required to deduce specific effects including measurement of cytokine levels, barrier integrity assays, and leukocyte adhesion and migration assays.

Interestingly, *SERPIND1* (Heparin cofactor II), which inhibits thrombin was decreased in PIM1$^{-/-}$ mice (Figure 3.13D) although thrombin induced clotting time was not altered (Figure 3.9). Heparin cofactor potency in inhibiting thrombin is drastically enhanced in the presence of heparan sulphate, which is expressed on the surface of ECs (Shirk et al., 1996), however this was missing from the clot formation assays performed. Additionally, it should be noted, a high concentration of thrombin (0.1 U/mL) was used to initiate clotting in clot formation assays.

Statistics were not carried out on the transcriptomic dataset as n numbers were too low. Six samples of mice PECs were prepared for RNA-Seq, 3 WT and 3 KO, see Appendix C. RNA-Seq analysis reported by BMKGene suggested that two sets of the RNA-Seq samples were highly correlated overall, with tight clustering, however one set of samples (one WT, one KO) formed a distinct subgroup and demonstrated low correlations with their biological repeat samples, suggesting sample-specific variation (Appendix C). Consequently, these samples were excluded from analysis leaving only n=2, therefore further repeats are required to confirm the changes in gene expression. Additional experiments are also required to elucidate the thromboinflammatory mechanisms and effects following changes in gene expression from PIM1$^{-/-}$ PECs.

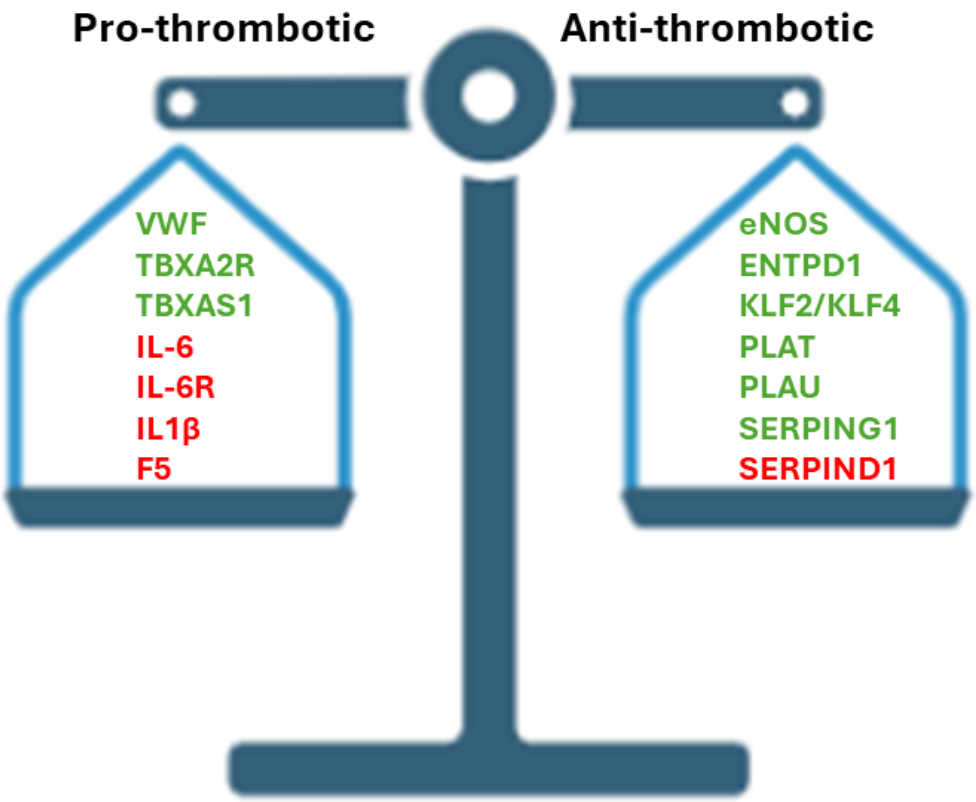

Figure 3.14: 'thrombotic' scale of genes identified by transcriptomics. Pro-thrombotic vs anti-thrombotic upregulated (green) and downregulated (red) genes in PIM1$^{-/-}$ mice compared to WT identified by transcriptomic analysis. Created in PowerPoint.

3.4.5 Limitations

Limitations for PEC experiments conducted in this chapter include little yield of material and a low cell retrieval so therefore low n numbers, and lack of power for statistical analyses. Although this is the case for the experiments conducted for this chapter, this provides pilot data for future work. Findings from this chapter however demonstrate the importance of *ex vivo* work in investigating systemic cardiovascular effects, enabling the investigation of the effects of genetic PIM1 deletion on several cardiovascular cell types and processes.

1. Analysis of coagulation and EC function is incomplete. Further assays could be performed to confirm our observations and elucidate cell specific effects. Clot formation assays could be carried out with the addition of C1 inhibitor to confirm observations from ROTEM assays and further elucidate the role of PIM1 in coagulation processes. Additionally, further clot formation assays could use different activators e.g. TF to initiate clot formation to investigate the effects of PIM1 deletion on the extrinsic pathway of coagulation further.
2. Endothelial experiments conducted in this chapter were using a global $PIM1^{-/-}$ mouse model which complicates the investigation of the EC contribution to thrombosis alone, however it provides a comparison to therapeutic intervention which is systemic. An endothelial-specific $PIM1^{-/-}$ mouse model may be more appropriate in this context to study EC specific effects.
3. Although not completely representative of the arterial endothelium, pulmonary ECs (PECs) were used for the experiments in this chapter as PEC isolation is a well-established and optimised method within LICAMM and has been successfully demonstrated to yield ECs from mouse lungs. PECs are representative only of the lung vasculature, and not representative enough of the arterial endothelium in the context of atherothrombosis, therefore an alternative cell type would have more disease relevance in this context. The most relevant blood vessels in human arterial thrombosis are the coronary arteries therefore the next

chapter will focus on experiments conducted using human coronary artery endothelial cells (HCAECs) *in vitro* as a more representative model of the arterial endothelium.

3.4.6 Chapter Conclusions

Findings from this chapter demonstrate the importance of *ex vivo* work in investigating systemic cardiovascular effects, enabling the investigation of the effects of genetic PIM1 deletion on several cardiovascular cell types and processes. PIM1 deletion was demonstrated to impact haematopoiesis/myelopoiesis, specifically altering RBCs, platelets, and monocytes. Prolonged clotting time was observed via the intrinsic pathway, and transcriptomic data demonstrated that *SERPIND1* was increased, suggesting that PIM1 plays a role in intrinsic coagulation. Altered expression of anti- and pro-thrombotic genes in PECs, including platelet mediators, and leukocyte trans endothelial migration (TEM) regulators, as well as endothelial barrier and junctional regulators requires further investigation. Together the data from this chapter demonstrates that PIM1 promotes endothelial-driven coagulation effects and angiogenesis, whilst having no effect on fibrinolytic processes, highlighting a functional role for PIM1 in the endothelium. PIM1 offers a potential anti-thrombotic target, however a variable coagulation profile and endothelial response following PIM1 deletion suggests that the other Pim isoforms may play a role. This is further supported by the decrease in *PIM2* and *PIM3* gene expression following PIM1 deletion, suggesting that the expression of Pim kinase isoforms is co-ordinated and inter-regulated. The effects of Pim kinase inhibitors that target all 3 Pim isoforms are required to further elucidate Pim kinase effects on the functions of the endothelium and coagulation. The next chapter will investigate the role of all three Pim kinase isoforms on HCAECs using pan-Pim kinase inhibition.

3.5 Chapter 3 summary abstract

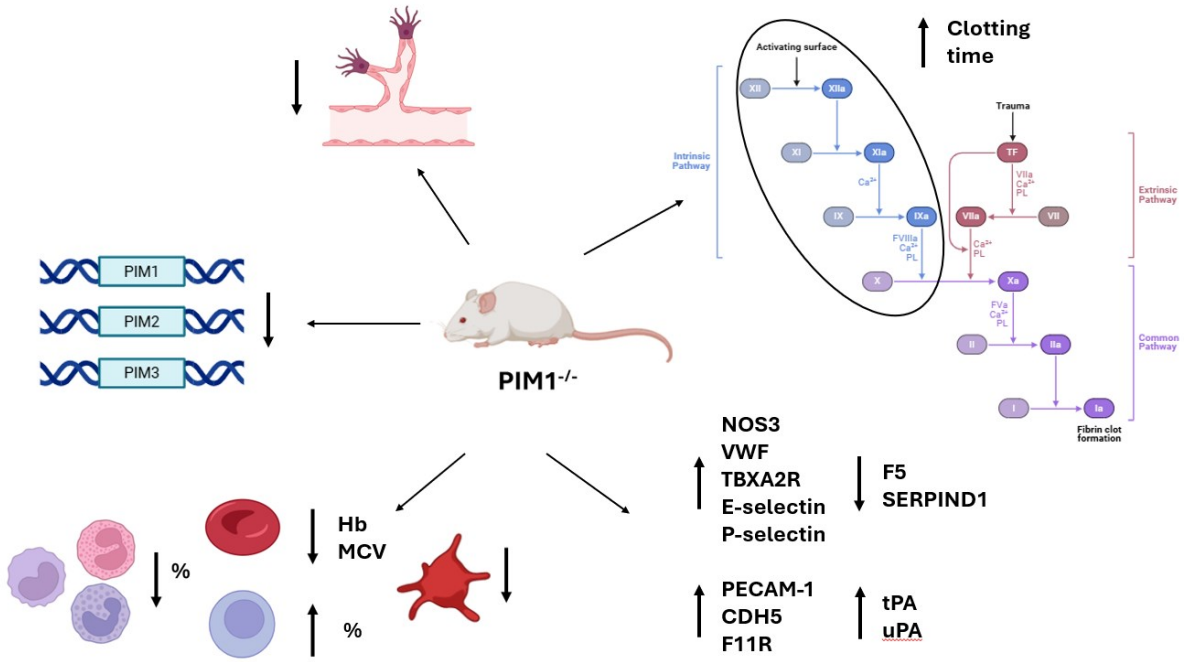

Figure 3.15: Summary abstract of PIM1$^{-/-}$ mice experiment results in this chapter. Created in BioRender and PowerPoint.

Chapter 4. Investigating the effect of Pim kinase inhibition on arterial endothelial function.

4.1 Introduction

4.1.1 Repurposing potential of Pim kinase inhibitors

Alterations in thrombotic potential following PIM1 deletion have been identified in Chapter 3 and published previously (Unsworth et al., 2021), identifying Pim kinase inhibitors as possible novel anti-thrombotic therapies.

The progression of Pim kinase inhibitors into Phase 1 and 2 clinical trials as cancer therapies demonstrates that Pim kinase targeting therapies can be safely administered (Mascarenhas, 2025). This provides an opportunity for drug repurposing, for the prevention and treatment of cardiovascular events. Pan-Pim kinase inhibitors AZD1208, PIM447, and TP3654 are currently in Phase 1 and 2 clinical trials for cancer treatment and have been shown to target various functions which contribute to cancer-related pathologies such as cancer-associated thrombosis and inflammation (Bellon and Nicot, 2023). Although these inhibitors are considered pan inhibitors, and target all 3 Pim isoforms, they have different specificities for each isoform and TP3654 is a highly selective PIM1 favourable inhibitor.

ECs have demonstrated to play key roles in the regulation of atherosclerosis and thrombosis, and the role of the Pim kinase isoforms in ECs is relatively unknown. Investigation of the effects of Pim kinase inhibition on EC function requires further investigation so that the potential repurposing of Pim inhibitors as a cardiovascular targeting therapy can be determined.

4.1.2 Pim kinase and its role in ECs

ECs line blood vessels and carry out many functions essential for maintaining vascular integrity, yet their dysfunction can drive vascular pathologies, contributing to thrombotic processes that drive cardiovascular

events. There is increasing evidence that Pim kinases play a role in thrombosis, including the endothelial contribution to thrombosis (Wang et al., 2026). In studies of cancer biology, Pim kinases have been identified to play roles in the regulation of cell survival, migration, and proliferation (Fox et al., 2003, Szydlowski et al., 2021), which are central to endothelial function and vascular biology, however endothelial-specific roles of Pim kinase remain unclear.

There are limited studies where the effects of Pim kinase inhibition or isoform deletion have been investigated on EC function, particularly in arterial ECs. It has been previously demonstrated that Pim kinases are expressed in different EC subtypes across different vascular beds including HUVECs (Zippo et al., 2004, Wang et al., 2026), dermal blood microvascular ECs (Santio et al., 2024), and mouse aortic ECs (MAECs) (Walpen et al., 2012). Studies have found that the three Pim isoforms play various roles in the regulation of the endothelium, particularly in the context of angiogenesis (Chen et al., 2023, Garbicz et al., 2020). The current understanding of the role of Pim kinase isoforms in the endothelium is that PIM1 and PIM3 play an essential role in EC spreading and migration, promoting angiogenesis (Chen et al., 2016, Yang et al., 2011a). PIM3 has shown to promote HUVEC angiogenesis by stimulating cell sprouting, migration, and vascular tube formation, often through interaction with factors like TNFα and VEGF, helping to form new blood vessels. Furthermore, PIM1 has been shown to mediate vascular endothelial growth factor (VEGF) responses in mice endothelial precursor cells, and in HUVECs, RNAi knockdown showed that PIM1 is required for VEGF-A-dependent EC viability and migration (Zippo et al., 2004).

Additionally, studies have demonstrated that PIM1 expression in HUVECs increased with the progression of atherosclerosis, and that PIM1$^{-/-}$ attenuated ox-LDL-induced EndMT (Xue et al., 2025). There is very limited investigation of the role of PIM2 and specific roles for PIM2 in the regulation of the endothelium has not yet been identified.

4.1.3 Arterial endothelial cell biology

Previous studies of endothelial Pim kinase isoforms, including the findings presented in Chapter 3, have used HUVECs or mouse derived ECs (pulmonary or endothelial precursor cells) to investigate Pim kinase biology which are not representative of the human arterial circulation. ECs from different vascular beds differ in their responses to biological processes and stimuli. This has been demonstrated previously where it has been shown that HUVECs and HCAECs show differential responses in gene expression of thrombotic regulators and fibrinolytic markers in response to flow rate and inflammation (Humphreys et al., 2025). In this chapter, it was important to determine whether Pim kinases impact general arterial EC biology and function before investigating their thrombotic targeting potential to determine clinical impact. Arterial ECs, such as coronary artery derived HCAECs, would therefore be a more representative model of atherothrombosis, a primary contributor to heart attacks and stroke. Therefore, in this chapter, the effect of Pim kinase inhibitors on human coronary arterial endothelial cells (HCAECs) was investigated as a model of the arterial endothelium, and the contribution of the Pim kinase family to endothelial cellular functions and processes investigated.

4.2 Chapter aim, objectives and hypothesis

The aim of this chapter is to elucidate the pharmacological effects of Pim kinase inhibition on the function of HCAECs to ascertain that inhibition of Pim kinases does not affect healthy endothelial functions. To investigate this, pan isoform-Pim kinase inhibitors, AZD1208, PIM447, and TP3654 (an inhibitor which has greater efficacy for PIM1 over PIM2 and PIM3) were used.

The following objectives were addressed:

1. Determine the effects of Pim kinase inhibitors on Pim kinase isoform expression and activity in HCAECs.
2. Assess the cytotoxicity of Pim kinase inhibitors on HCAECs viability and proliferation.
3. Characterise the effects of Pim kinase inhibition on EC functions, including migration, angiogenesis, and morphology.
4. Investigate the impact of Pim kinase inhibitor treatment on the HCAEC transcriptome.

4.2.1 Hypothesis: Pim kinase inhibition maintains a healthy endothelial phenotype *in vitro*.

4.2.2 Methods

Human coronary artery endothelial cells (HCAECs) were treated with or without pharmacological Pim kinase inhibitors, AZD1208 (1-10μM), PIM447 (1-10μM), and TP3654 (0.1-1μM) and cell survival, viability, morphology, adhesion, migration and angiogenesis determined using standard assays described in Chapter 2 (Materials and Methods).

4.2.2.1 Visual methodology abstract

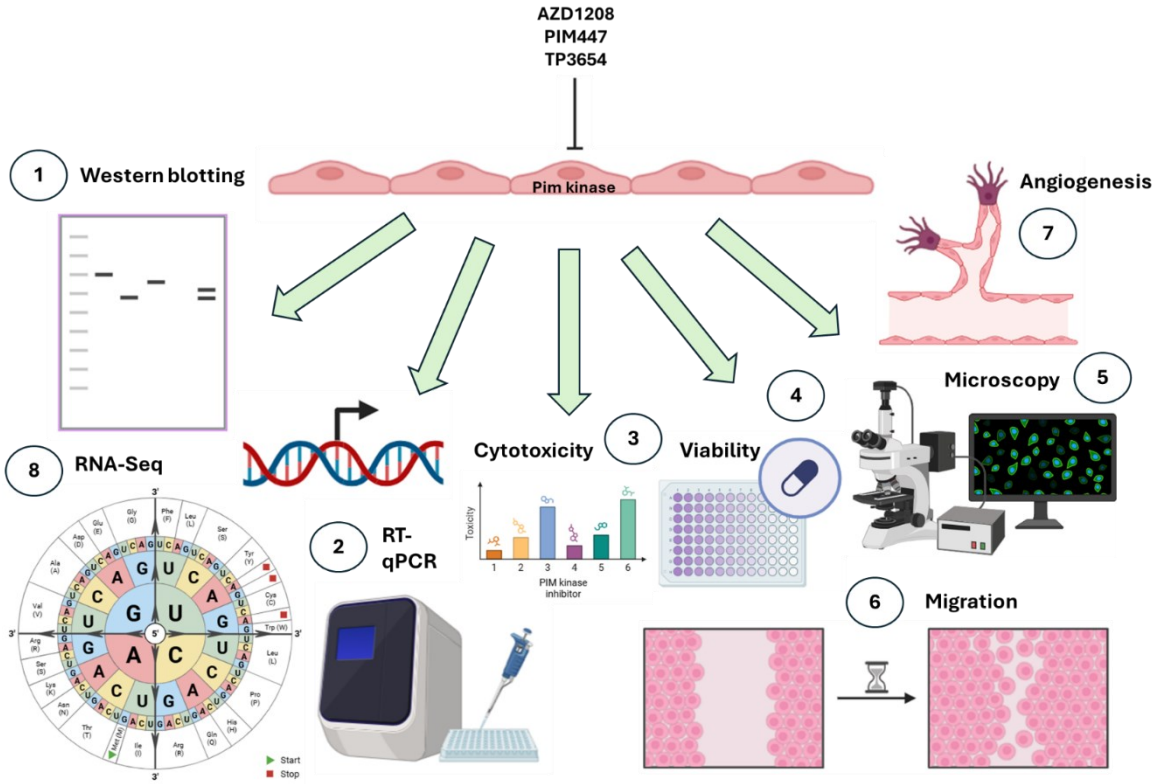

Figure 4.1: Functional EC experiments carried out in this chapter. (1) Western blotting, (2) RT-qPCR, (Owens and Mackman) LDH drug cytotoxicity assay, (Nelson et al.) MTS cell viability assay (5) microscopy for morphology assessment, (6) scratch wound migration assay, (7) angiogenesis assay, and (8) RNA-Seq. Created in BioRender and PowerPoint.

4.3 Results

4.3.1 Pim kinase isoforms are expressed in HCAECs

Pim kinases are constitutively active, and Pim kinase isoform activity is controlled via its expression. Proteomic and transcriptomic expression of the Pim kinase isoforms has been previously described in HUVECs (Chen et al., 2016), although PIM2 has been shown to be expressed at low levels compared to PIM1 and PIM3 (Santio et al., 2024). Bulk tissue gene expression of Pim kinases in tissues relating to the cardiovascular system has shown that Pim kinases are expressed in cardiovascular tissues, including the heart, coronary artery, aorta, and blood (Figure 1.8) (Nock et al., 2023), providing evidence that all three Pim kinase isoforms are expressed and potentially functionally relevant in ECs.

For this study, the effects of Pim kinase inhibition on arterial EC function using HCAECs was to be investigated as a representative model for atherothrombosis. It was therefore necessary to first confirm individual Pim kinase isoform protein expression in HCAECs. To do this, western blotting was performed on HCAEC lysates between passages 4-6 from three male donors aged between 50-65. PIM1, PIM2 and PIM3 western blot analysis identified protein bands at the expected 34kDa molecular weights, identifying all three short form variants of the Pim kinase isoforms were expressed, along with the longer 37kDa PIM2 isoform, supporting their expression in HCAECs (Figure 4.2). The longer form variants of PIM1 (44kDa) and PIM2 (40kDa) proteins were not visible in these experiments.

The existence of all three Pim kinase isoforms in HCAECs indicates that the short form variants of these enzymes may play a role in the regulation of HCAEC function.

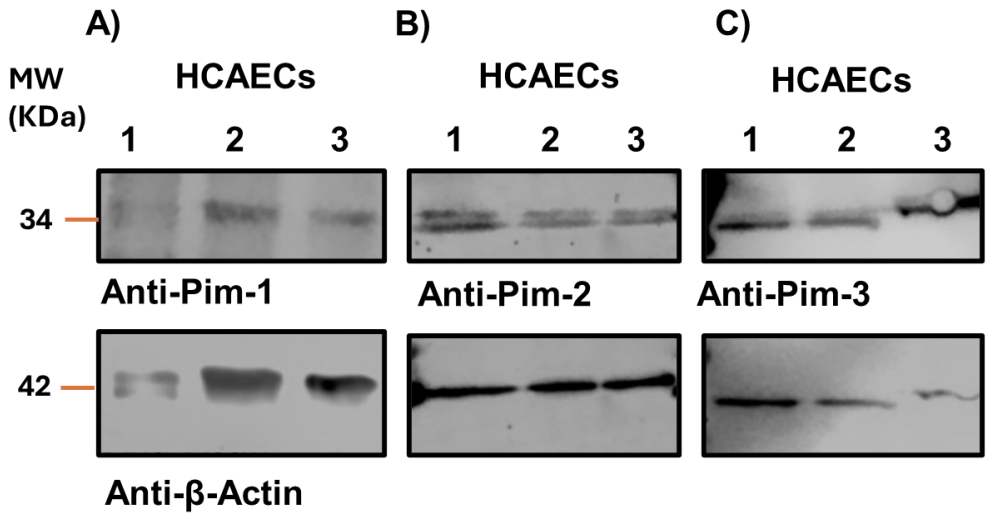

Figure 4.2: Protein expression of PIM1, PIM2, and PIM3 short (34kDa) forms in HCAECs. Healthy HCAEC lysates from three separate HCAEC donors were prepared (indicated by 1, 2, 3 figure labels) with the addition of Laemmli sample buffer, separated on SDS-PAGE gels, and transferred to membranes before immunoblotting with (A) PIM1, (B) PIM2, and (C) PIM3 antibodies for 1 hour. Protein detection was performed using a HRP-labelled antibody and chemiluminescence, identifying 34kDa molecular weight bands for all three Pim kinase isoforms, and the longer 37kDa PIM2 isoform. β-Actin was included as a loading control. Representative blots are shown for N=3. MW=molecular weight.

4.3.2 Gene expression of the three Pim kinase isoforms is increased with inhibitor treatment

Based on molecular modelling data, it has been established that the kinase domains of Pim proteins share unique structural features, which make them constitutively active (Qian et al., 2005). In contrast to most other kinases, cellular Pim activity is not dependent on post-translational modifications, but tightly controlled at both transcriptional and translational levels (Santio and Koskinen, 2017), regulated by expression and degradation, and consequently the amount of Pim isoform in the cell. It is therefore important to investigate how HCAECs respond to a reduction in kinase activity following treatment with Pim kinase inhibitors. The effects of Pim kinase inhibition on PIM1, PIM2, and PIM3 gene expression in HCAECs was therefore explored.

HCAECs were treated with AZD1208 (1μM), PIM447 (1μM), TP3654 (0.1μM) or vehicle control (0.1% DMSO) for 24 hours before individual isoform gene expression determined using RT-qPCR. Following treatment with Pim kinase inhibitors an increase in PIM1 ($P=0.0314$) mRNA expression was observed following AZD1208 treatment. PIM2 ($P=0.0349$) and PIM3 ($P=0.0480$) were upregulated with PIM447 treatment, and PIM1 ($P=0.0485$) and PIM2 ($P=0.0379$) were increased following TP3654 treatment, indicating a regulatory feedback loop where pan-Pim kinase inhibition leads to an upregulation of each of the three Pim kinase isoforms.

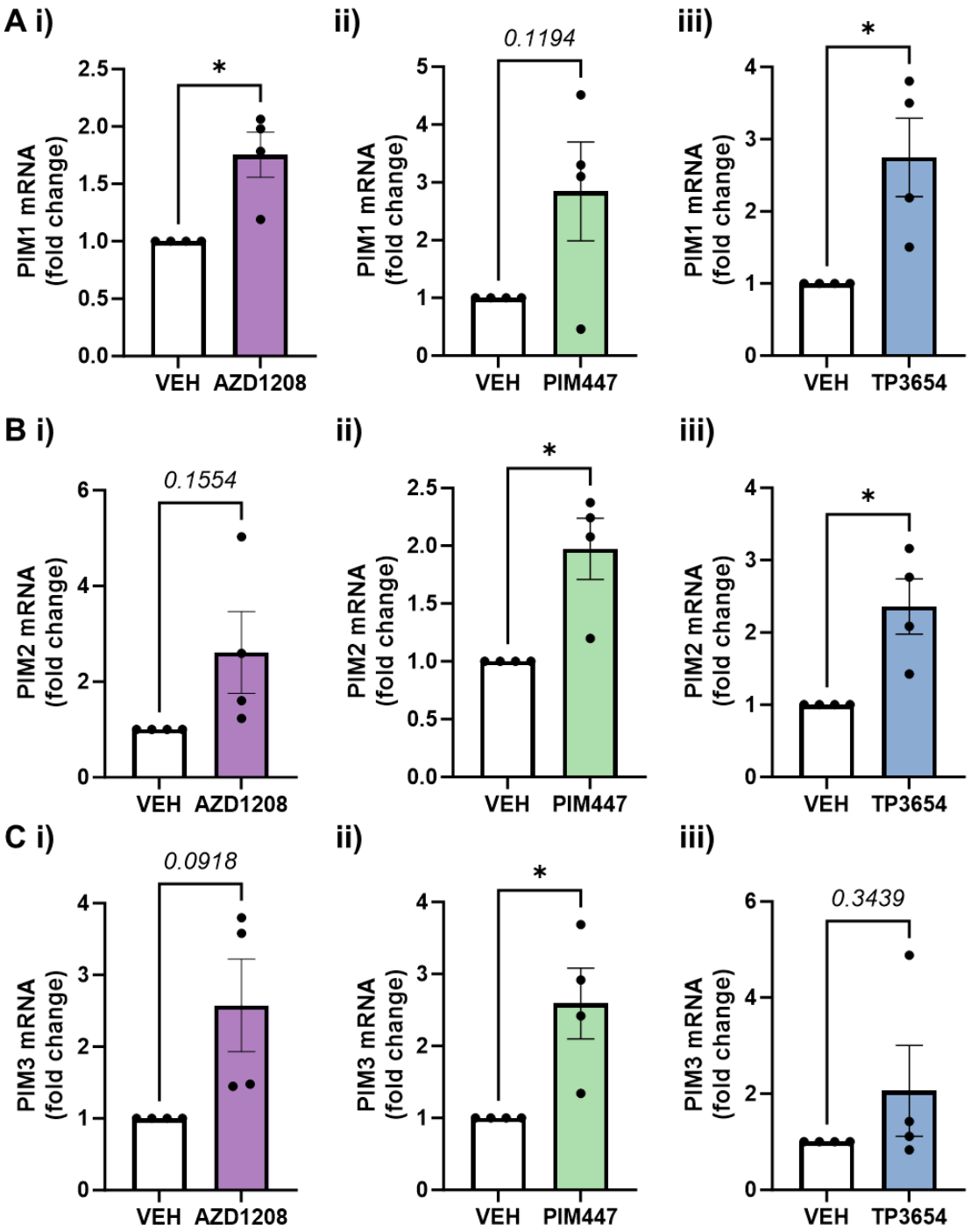
A i)
PIM1 mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
VEH
AZD1208
ii)
0.1194
5
4
3
2
1
0
PIM1 mRNA (fold change)
VEH
PIM447
iii)
*
4
3
2
1
0
PIM1 mRNA (fold change)
VEH
TP3654
B i)
0.1554
6
4
2
0
PIM2 mRNA (fold change)
VEH
AZD1208
ii)
*
2.5
2.0
1.5
1.0
0.5
0.0
PIM2 mRNA (fold change)
VEH
PIM447
iii)
*
4
3
2
1
0
PIM2 mRNA (fold change)
VEH
TP3654
C i)
0.0918
4
3
2
1
0
PIM3 mRNA (fold change)
VEH
AZD1208
ii)
*
4
3
2
1
0
PIM3 mRNA (fold change)
VEH
PIM447
iii)
0.3439
6
4
2
0
PIM3 mRNA (fold change)
VEH
TP3654

Figure 4.3: HCAECs compensate Pim kinase inhibitor treatment by increasing Pim kinase isoform expression. HCAECs were treated with or without Pim kinase inhibitors AZD1208 (1μM), PIM447 (1μM), TP3654 (0.1μM) or vehicle control (0.1% DMSO) for 24 hours before individual isoform gene expression determined using RT-qPCR. (A) PIM1 (B) PIM2 and (C) PIM3 gene expression was analysed in HCAECs ± (i) AZD1208, (ii) PIM447, and (iii) TP3654 treatment. RT-qPCR experiments were carried out in duplicate, and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean ± SEM for n=4. *P≤0.05. Data was analysed using paired t-tests. Where normalised data was shown, statistics were performed before normalisation.

4.3.3 Pim kinase inhibitors are not cytotoxic to HCAECs at physiologically achievable concentrations

Following confirmation of gene and protein expression in HCAECs, it was next important to confirm that pharmacological Pim kinase inhibition is not toxic to HCAECs. Pim kinases have previously been shown to play important roles in cell survival promoting cell evasion of apoptosis signals (Chen et al., 2016), and it has been shown that the inhibition of Pim kinase enables the activation of pro-apoptotic BAD leading to activation of BAX/BAK and inhibition of anti-apoptotic proteins BCL-2 and BCL-xL, initiating apoptosis (Macdonald et al., 2006). Although this is effective for targeting uncontrolled cell growth in cancer, we wanted to ensure that Pim kinase inhibitors do not induce apoptosis in healthy HCAECs.

The effects of Pim kinase inhibitor treatment on HCAEC survival were therefore determined using; concentrations of AZD1208, PIM447, and TP3654 (0.1-10μM), which can be achieved and tolerated in plasma *in vivo*, as shown in clinical studies (Keeton et al., 2014, Raab et al., 2019, Kunder et al., 2022, Lebedinsky et al., 2020), as well as an increased 30μM concentration. HCAECs were treated with either 0.1% DMSO (vehicle control), 1μg/mL PMA (positive control), or increasing drug concentrations (0.1, 0.3, 1, 3, 10, and 30μM) for 24 and 48 hours, and cytotoxicity determined using an LDH assay.

As expected, treatment of cells with PMA caused a significant decrease in HCAEC survival after 24 (P=0.0243) and 48 hours (P=0.0342) (Figure 4.4). Treatment with increasing concentrations of AZD1208 over 24 hours up to 3μM did not affect HCAEC survival however 10μM AZD1208 significantly increased HCAEC cytotoxicity (P=0.0479) (Figure 4.4Ai). 0.1, 1 and 10μM AZD1208 did not affect HCAEC survival at 48 hours of treatment, but HCAEC survival was reduced following treatment with concentrations 0.3 (P=0.0428), 3 (P=0.0084) and 30 μM (P=0.0036) (Figure 4.4Aii). In contrast, PIM447 did not affect HCAEC survival at any concentration up to 30μM at both 24 and 48 hours (Figure 4.4B), however 30μM PIM447 significantly increased HCAEC cytotoxicity (P=0.0005) at 24 hours

(Figure 4.4Bi), indicating that Pim kinase inhibition with PIM447 does not initiate EC apoptosis at physiologically achievable concentrations *in vivo*. Treatment with all concentrations of TP3654 tested was not observed to affect HCAEC survival after 24 hours of treatment (Figure 4.4Ci), however interestingly the lower concentrations tested 0.1 (P=0.0078), 0.3 (P=0.0391), and 1 µM (P=0.0368) of TP3654 induced a decrease in cell viability in HCAECs after 48 hours (Figure 4.4Cii). This looks to be increasing with dose however no significant differences compared to control were observed at higher concentrations.

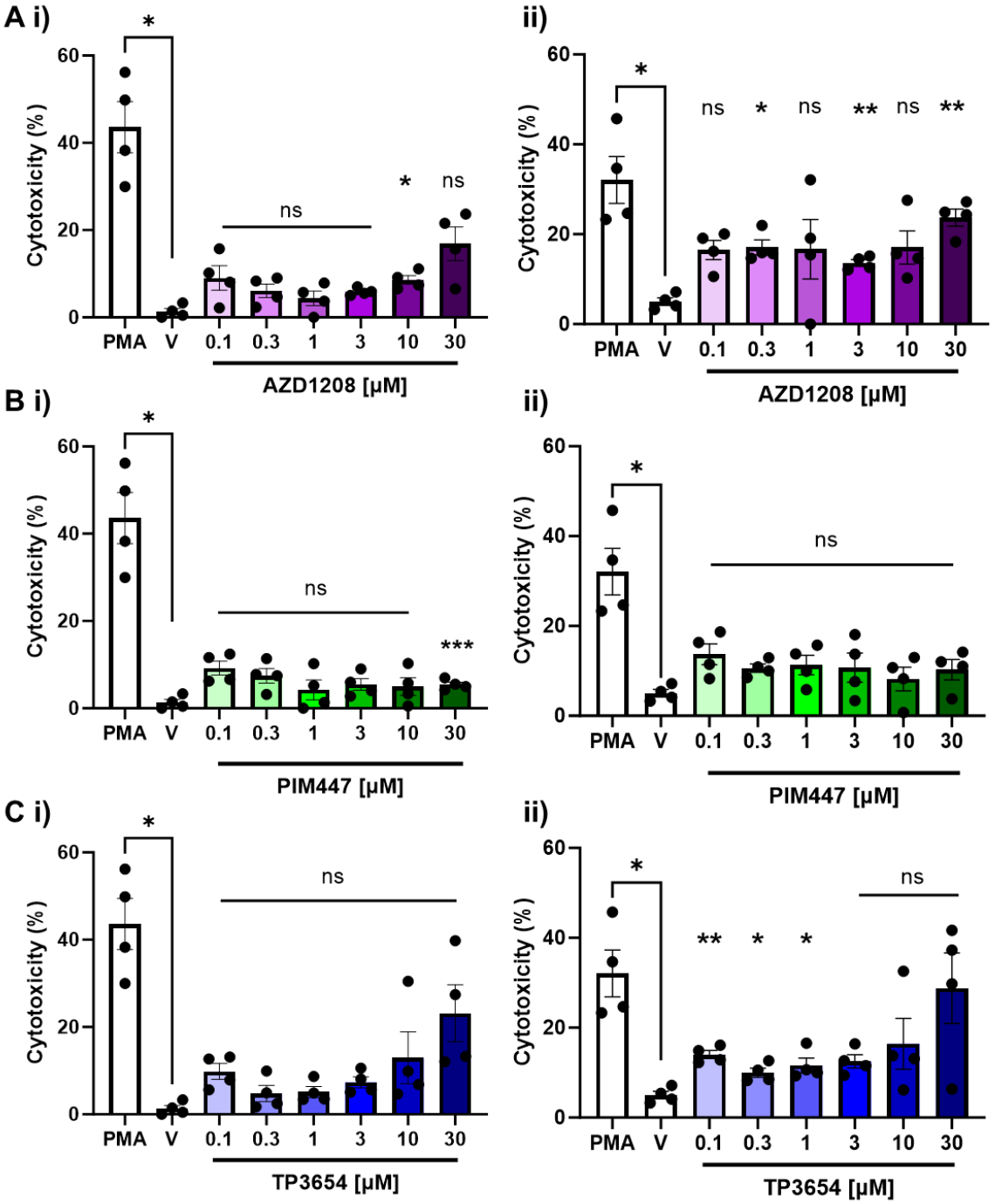

Figure 4.4: Pim kinase inhibitors demonstrate variable cytotoxicity to HCAECs after 24 and 48-hour treatment. HCAECs were treated with increasing concentrations of pan-Pim kinase inhibitors (A) AZD1208, (B) PIM447, and (C) TP3654 (0.1, 0.3, 1, 3, 10, and 30μM) for (i) 24 and (ii) 48 hours and drug cytotoxicity determined using LDH assay. LDH reaction mix (Abcam-[ab65393]) was added, as per manufacturers' instructions. Absorbance was read at 450nm on a GloMax® Explorer Multimode Microplate Reader (Promega). Results are expressed as mean + SEM for N=4. *P≤0.05, **P≤0.01, ***P≤0.001. Data was analysed using One-Way ANOVA, comparing concentration conditions to vehicle.

4.3.4 Pim kinase inhibitors do not affect HCAEC viability and proliferation at concentrations reached *in vivo*

Having observed no consistent patterns of cytotoxicity following treatment of HCAECs with Pim kinase inhibitors at concentrations achievable *in vivo* (1-10μM), the effect of Pim kinase inhibitors AZD1208, PIM447 and TP3654 on HCAEC proliferation and viability was subsequently determined using an MTS assay.

HCAECs were treated with either 0.1% DMSO (vehicle control), 0.1% SDS (positive control), or increasing drug concentrations (0.1, 0.3, 1, 3, 10, and 30 μM) for 24 and 48 hours before the addition of MTS reagent.

As expected, treatment of HCAECs with 0.1% SDS positive control caused a significant reduction in HCAEC viability after 24 (P=0.0010) and 48 hours (P=0.0003) (Figure 4.5). Following treatment with increasing concentrations of AZD1208 and PIM447 even at the highest (supraphysiological) concentration (30μM) tested, no significant effect on EC viability or proliferation was observed at both 24 and 48 hours, (Figure 4.5), indicating that Pim kinase inhibition with AZD1208 and PIM447 does not alter cell viability (Cortes et al., 2018). Interestingly, treatment of HCAECs with TP3654 appeared to cause a dose dependent decrease in HCAEC proliferation and viability with the highest concentrations of TP3654 causing a significant decrease in HCAEC viability at 10μM (P=0.0061) and 30μM (P=0.0077) at 24 hours (Figure 4.5Ci). Only treatment with 30μM TP3654 was significantly reduced (P=0.0252) after 48 hours (Figure 4.5Cii). These concentrations of TP3654 10μM and 30μM TP3654 are supraphysiological concentrations, which are not reached *in vivo,* based on available preclinical and clinical data (El Chaer et al., 2022, Foulks et al., 2014). As physiologically achievable concentrations of Pim kinase inhibitors did not affect HCAEC survival and viability, future experiments were restricted to these concentrations (1μM and 10μM for AZD1208 and PIM447, and 0.1μM and 1μM for TP3654). These results are promising for the potential repurposing of Pim kinase inhibitors as anti-thrombotic therapies; however, further characterisation

and experiments will be performed to validate these findings using physiological concentrations in future experiments.

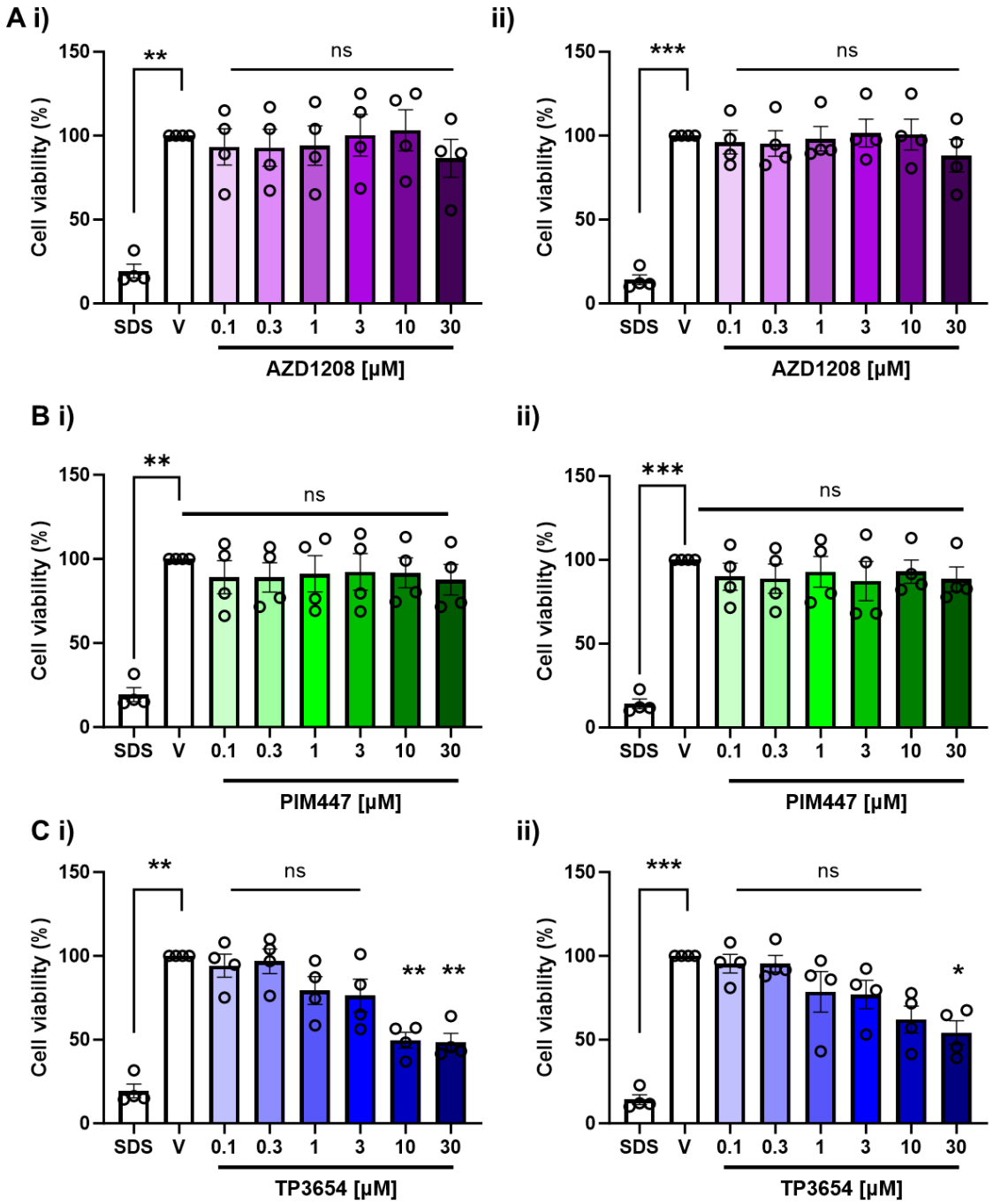

Figure 4.5: Physiologically achievable concentrations of Pim kinase inhibitors do not alter HCAEC viability. HCAECs were pre-treated with a range of concentrations of pan-Pim kinase inhibitors (A) AZD1208, (B) PIM447, and (C) TP3654 (0.1, 0.3, 1, 3, 10, and 30µM) for (i) 24 and (ii) 48 hours and cell viability determined using an MTS assay. MTS reagent (Abcam- [ab197010]) was added, as per manufacturers' instructions, 4 hours before absorbance was read at 490nm on a GloMax® Explorer Multimode Microplate Reader (Promega). Results are expressed as mean + SEM for N=4. *P≤0.05, **P≤0.01, ***P≤0.001. Data was analysed using One-Way ANOVA, comparing concentration conditions to vehicle (considered 100% of cell viability).

4.3.5 AZD1208 decreases HCAEC actin fluorescence under oscillatory shear

ECs in culture are different from those *in vivo*, lacking hormonal and biomechanical stimuli (Li et al., 2005). While the hormonal environment can be compensated for by adding supplementary growth factors into the culture medium, the lack of biomechanical stimulation makes ECs in culture phenotypically different from those *in vivo* (Galbraith et al., 1998). To address the impact of biomechanical stimulation, shear stress was applied to HCAECs to compare the effects of Pim kinase inhibition on EC cell morphology under flow and static conditions.

HCAECs were treated with Pim kinase inhibitors (AZD1208, PIM447, and TP3654) or vehicle control (0.1% DMSO) and cultured under static, laminar and oscillatory (atheroprone) shear conditions to determine the effect of Pim kinase inhibition on HCAEC morphology; including cell attachment and actin cytoskeleton structure and organisation, and nuclear aspect ratio, defined as the ratio between the long and short axes of the nucleus (Humphreys et al., 2025). Shear stress (153 RPM) was applied to cell cultures using the orbital shaker method (Warboys et al., 2019). Cells cultured in the outer periphery of the wells are exposed to the equivalent of atheroprotective laminar shear (HSS) (15 dyn/cm2), whilst cells in the centre of the wells are exposed to atheroprone oscillatory shear (LSS) (0~ ± 5 dyn/cm2).

Similar to observations made previously (Dardik et al., 2005, Galbraith et al., 1998) exposure to laminar shear stress caused HCAECs to adopt an elongated morphology with the cells aligned to the direction of flow, compared to the cobblestone morphology etc observed in HCAECs cultured under static conditions (Figure 4.6, 4.7 and 4.8). In contrast, HCAECs exposed to oscillatory shear demonstrated a morphology and pattern of adhesion similar to static conditions, appearing randomly oriented and uniformly polygonal.

4.3.5.1 AZD1208

Under both static culture and laminar shear stress conditions, treatment with AZD1208 (1μM) was not observed to alter cell attachment, measured by counting the number of cells per field of view, or affect actin polymerisation, measured using phalloidin fluorescence intensity, compared to vehicle treated control (Figure 4.6). In contrast, under oscillatory shear conditions, treatment with AZD1208 was observed to cause a 10% decrease in phalloidin fluorescence intensity and actin polymerisation compared to vehicle treated control. Cell adhesion (cell count) and morphology was unaffected. This decrease in polymerised f-actin possibly indicates a disruption in the HCAEC actin cytoskeleton reorganisation following inhibition of Pim kinase under oscillatory shear conditions. An increase in nuclear aspect ratio was observed in static AZD1208-treated HCAECs compared to control, indicating more elongated and less circular nuclei, however this was not observed under laminar and oscillatory shear stress conditions.

4.3.5.2 PIM447

Treatment with PIM447 (1μM) did not demonstrate any changes in the number of cells adhered, phalloidin fluorescence intensity, or nuclear aspect ratio compared to vehicle, under static and both laminar and oscillatory flow conditions (Figure 4.7). This indicates PIM447 treatment does not alter HCAEC morphology, cytoskeletal structure, or adhesive properties.

4.3.5.3 TP3654

Similar to PIM447, treatment with TP3654 (0.1μM) did not demonstrate any changes in cell morphology, the number of nuclei (cell count), f-actin polymerisation, or nuclear aspect ratio, under static or both laminar and oscillatory flow conditions (Figure 4.8). This suggests that treatment with TP3654 does not affect EC morphology, adhesive properties, or cytoskeletal structure.

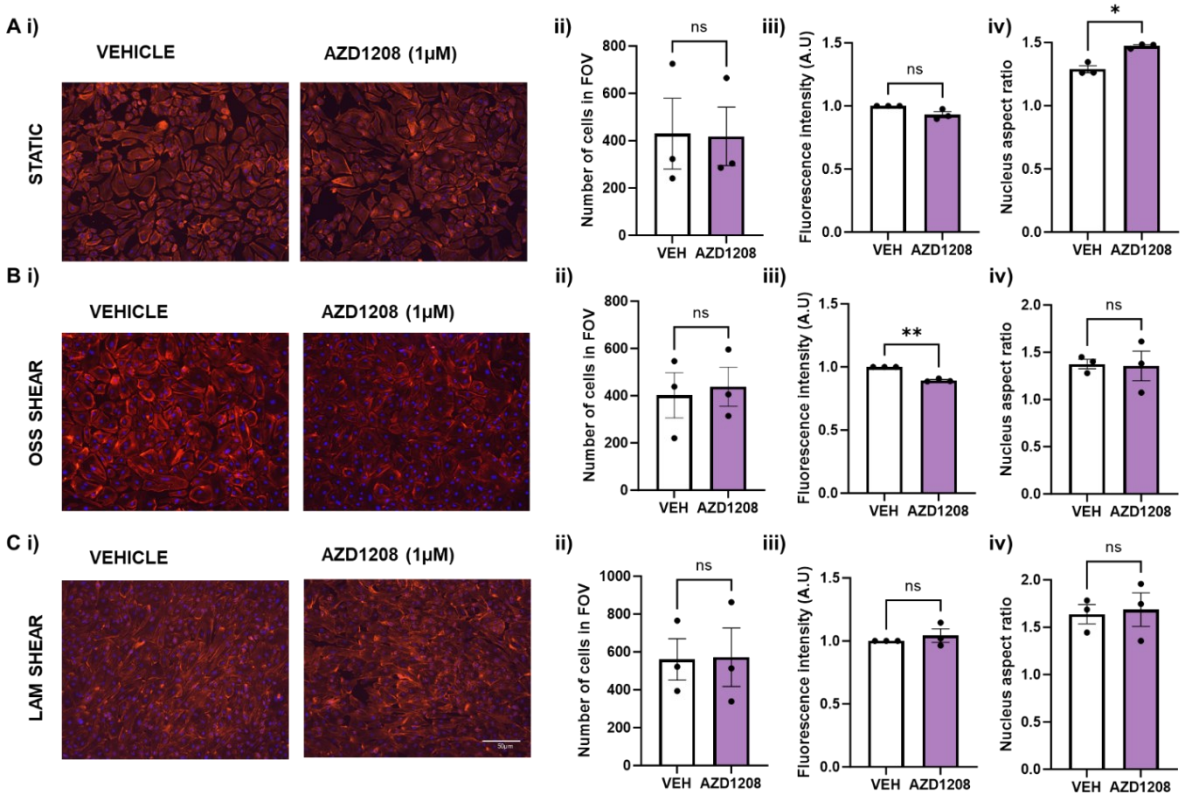

Figure 4.6: Treatment with AZD1208 increases nuclear aspect ratio in static HCAECs and decreases actin fluorescence intensity under oscillatory shear but does not alter cell adhesion or count. HCAECs treated with AZD1208 were seeded in 24-well plates and incubated for 24 hours under (A) static, (B) oscillatory, and (C) laminar flow conditions. Cells were fixed and stained with DAPI (100ng/mL) and Invitrogen™ Rhodamine phalloidin (1:750 in 1% BSA-PBS) for visualisation of the actin cytoskeleton prior to imaging using an EVOS Auto 2 microscope at a 10x magnification. Scale bar represents 50μm. (i) images taken were analysed to determine (ii) cell count and (iii) actin fluorescence using ImageJ software. Data analysis is from multiple images per well. Data represent mean + SEM for n=3. **p<0.01. Where normalised data was shown, statistics were performed before normalisation.

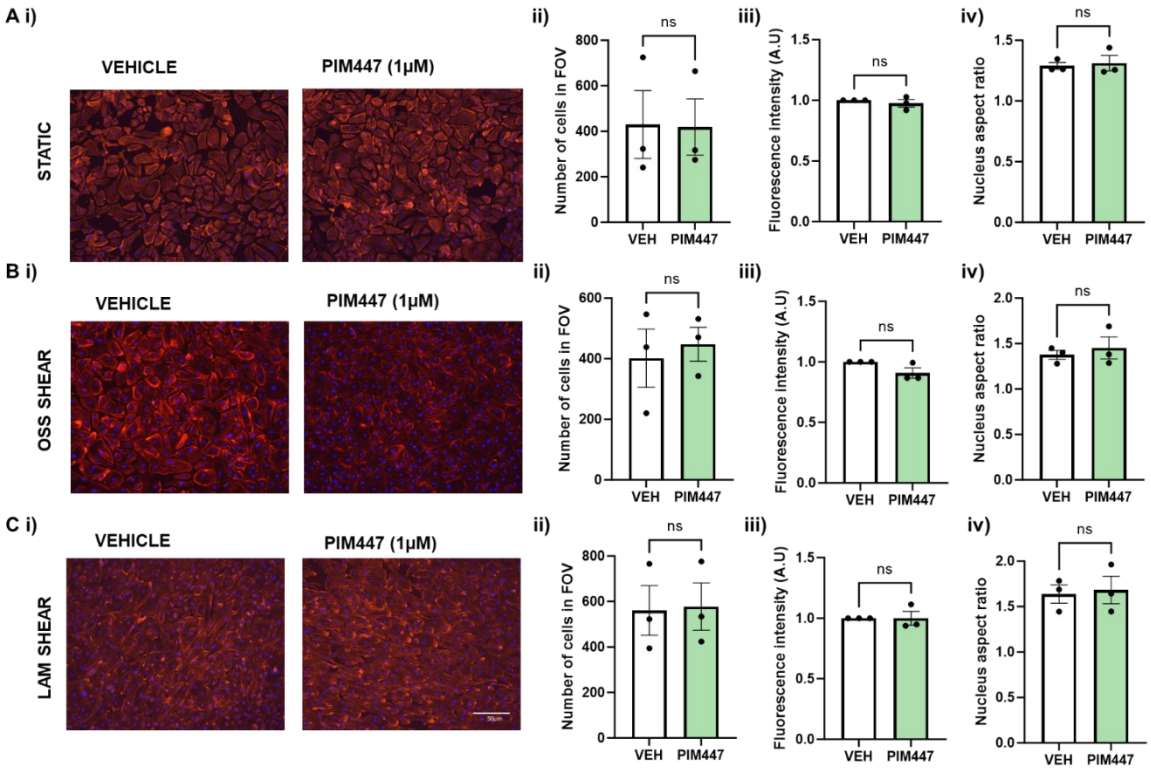

Figure 4.7: Treatment with PIM447 does not alter cell count or adhesion, actin fluorescence, or nuclear aspect ratio under static, laminar or oscillatory flow conditions. HCAECs treated with PIM447 were seeded in 24-well plates and incubated for 24 hours under (A) static, (B) oscillatory, and (C) laminar flow conditions. Cells were fixed and stained with DAPI (100ng/mL) and Invitrogen™ Rhodamine phalloidin (1:750 in 1% BSA-PBS) for visualisation of the actin cytoskeleton prior to imaging using an EVOS Auto 2 microscope at a 10x magnification. Scale bar represents 50μm. (i) images taken were analysed to determine (ii) cell count and (iii) actin fluorescence using ImageJ software. Data analysis is from multiple images per well. Data represent mean + SEM for n=3. Where normalised data was shown, statistics were performed before normalisation.

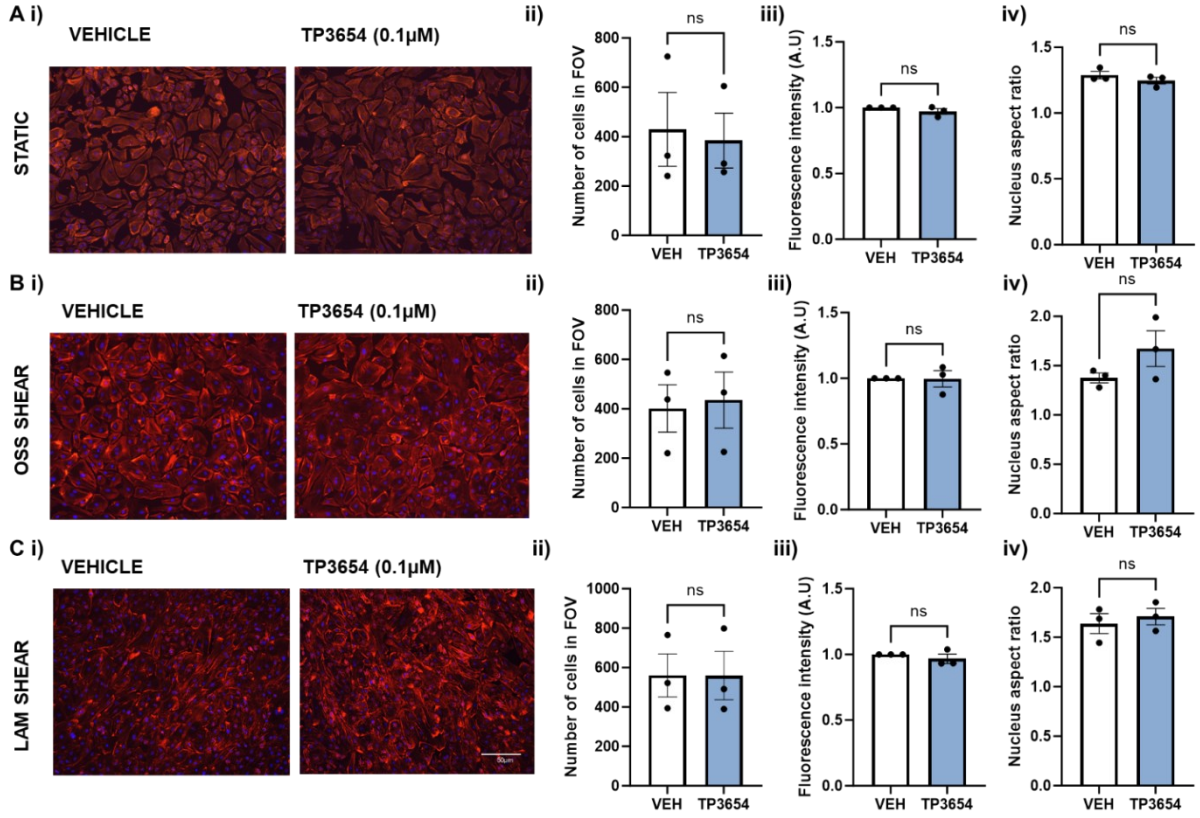

Figure 4.8: Treatment with TP3654 does not alter cell count or adhesion, actin fluorescence, or nuclear aspect ratio under static, laminar or oscillatory flow conditions. HCAECs treated with TP3654 were seeded in 24-well plates and incubated for 24 hours under (A) static, (B) oscillatory, and (C) laminar flow conditions. Cells were fixed and stained with DAPI (100ng/mL) and Invitrogen™ Rhodamine phalloidin (1:750 in 1% BSA-PBS) for visualisation of the actin cytoskeleton prior to imaging using an EVOS Auto 2 microscope at a 10x magnification. Scale bar represents 50μm. (i) images taken were analysed to determine (ii) cell count and (iii) actin fluorescence using ImageJ software. Data analysis is from multiple images per well. Data represent mean + SEM for n=3. Where normalised data was shown, statistics were performed before normalisation.

4.3.6 Pim kinase inhibition does not affect HCAEC migration

The next set of experiments explored the effects of Pim kinase inhibition on HCAEC migration. Migration of ECs is vital during various physiological processes, including angiogenesis and vascular repair where EC migration is a crucial early step in tissue growth and repair and neovascularisation (Du et al., 2024). As migration is a normal function of ECs, it was important to ensure that it wasn't compromised with inhibition.

HCAECs were treated with the three Pim kinase inhibitors at 1 and 10μM (AZD1208 and PIM447), 0.1 and 1μM (TP3654) or vehicle control (0.1% DMSO) and cell migration monitored over 20 hours using a scratch assay and live cell imaging. Scratch width was determined as a measure of cell migration (Figure 4.9, 4.10, and 4.11). In vehicle treated HCAECs scratch width significantly decreased over time with gap closure achieved by 20 hours. Treatment with 1 and 10μM AZD1208 (Figure 4.9) or PIM447 (Figure 4.10) was not observed to significantly alter scratch width over time compared to vehicle treated control, indicating that these pan-Pim kinase inhibitors did affect HCAEC migration. Interestingly treatment with the highest concentration (1μM) of TP3654 (Figure 4.11) did appear to reduce scratch width at 20 hours compared to vehicle treated control, possibly indicating increased migration, although lack of effect at earlier timepoints indicates this is not due to increased migration kinetics.

Overall, these results indicate that pan-Pim kinase inhibition does not have a notable effect on EC migration, suggesting Pim kinase inhibition would not impact HCAECs contribution to vascular repair.

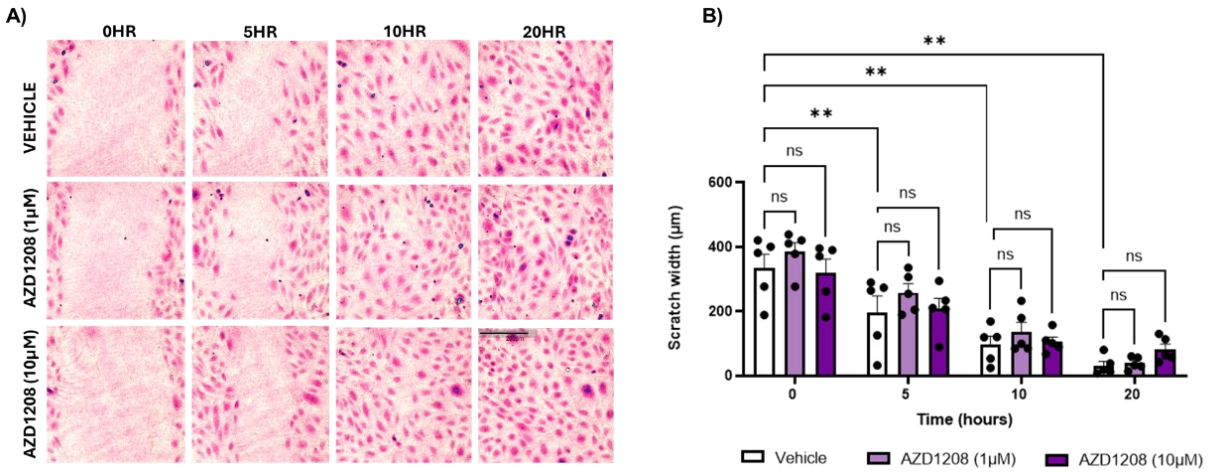

Figure 4.9: AZD1208 does not affect HCAEC migration. A) Representative images of the gap at 0, 5, 10 and 20 hours are shown, B) quantified data for scratch width. Cells were cultured in Ibidi 2-well inserts in a clear-bottomed 24-well plate to confluency. The inserts were removed to create a 'scratch'/gap for migration to occur, and cells treated with AZD1208 (1, 10μM or vehicle (0.1% DMSO). Cell migration was monitored for 20 hours using the 20x objective of the HoloMonitor Live Cell Imaging System. Images were analysed using the HoloMonitor App Suite cell imaging software. Scale bar represents 200μm. Data analysis is from multiple images per well. Data represents mean + SEM for n=4. **p<0.01

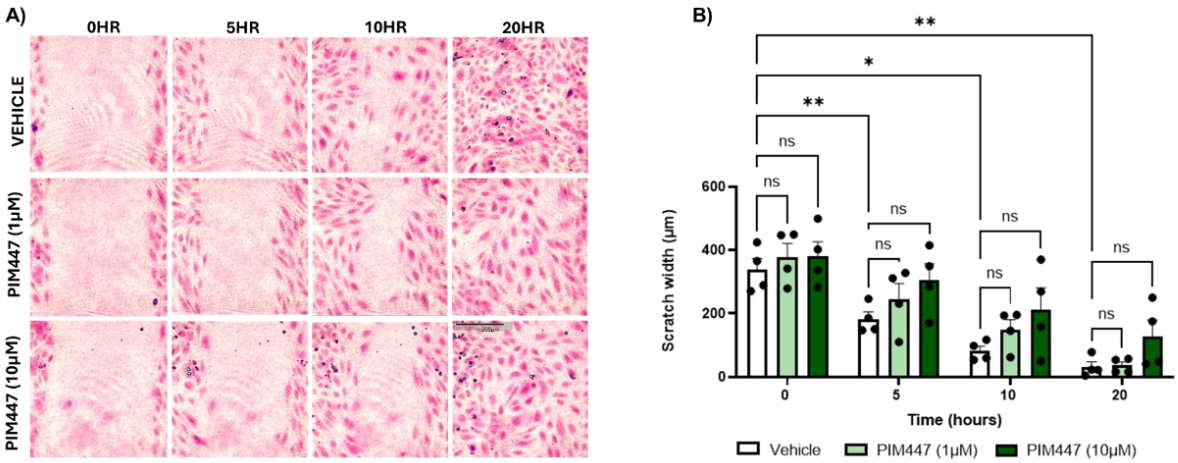

Figure 4.10: PIM447 does not affect HCAEC migration. A) Representative images of the gap at 0, 5, 10 and 20 hours are shown, B) quantified data for scratch width. Cells were cultured in Ibidi 2-well inserts in a clear-bottomed 24-well plate to confluency. The inserts were removed to create a 'scratch'/gap for migration to occur, and cells treated with PIM447 (1, 10μM) or vehicle (0.1% DMSO). Cell migration was monitored for 20 hours using the 20x objective of the HoloMonitor Live Cell Imaging System. Images were analysed using the HoloMonitor App Suite cell imaging software and graphs plotted using GraphPad Prism. Scale bar represents 200μm. Data analysis is from multiple images per well. Data represents mean + SEM for n=4. *p<0.05, **p<0.01

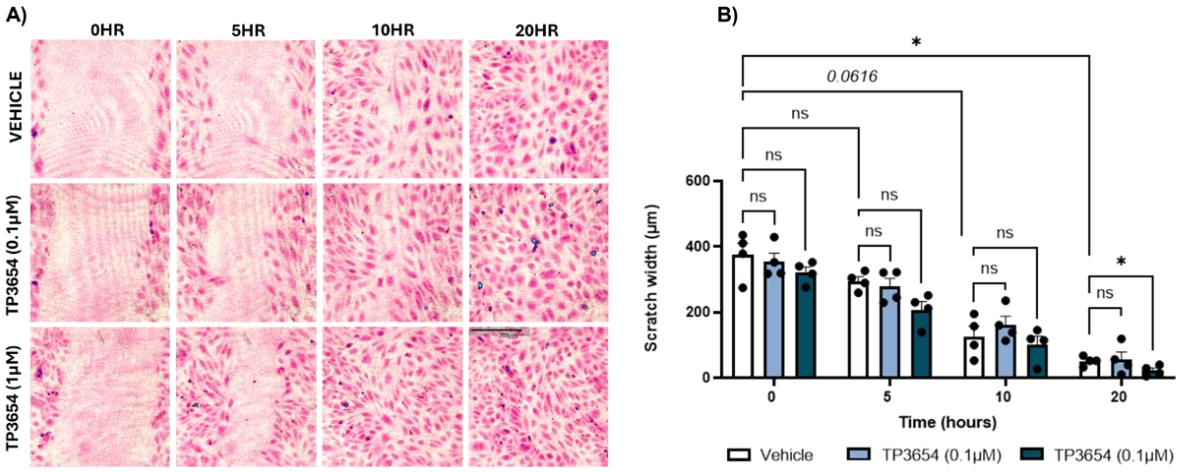

Figure 4.11: TP3654 does not affect HCAEC migration. A) Representative images of the gap at 0, 5, 10 and 20 hours are shown, B) quantified data for scratch width. Cells were cultured in Ibidi 2-well inserts in a clear-bottomed 24-well plate to confluency. The inserts were removed to create a 'scratch'/gap for migration to occur, and cells treated with TP3654 (0.1, 1μM) or vehicle (0.1% DMSO). Cell migration was monitored for 20 hours using the 20x objective of the HoloMonitor Live Cell Imaging System. Images were analysed using the HoloMonitor App Suite cell imaging software and graphs plotted using GraphPad Prism. Scale bar represents 200μm. Data analysis is from multiple images per well. Data represents mean + SEM for n=4. *p<0.05.

4.3.7 HCAEC angiogenesis is delayed by Pim kinase inhibitors

It was next important to investigate whether Pim kinase inhibition impacts EC contributions to angiogenesis where ECs migrate and proliferate to ischemic tissues, promoting blood vessel development.

PIM1 has previously been shown to drive angiogenic processes and preliminary observations in mouse PECs (Section 3.3.8, Figure 3.12) support this. However, there is currently a lack of studies which consider the angiogenic roles of Pim kinases in HCAECs, and there is limited information on the contribution of the PIM2 and PIM3 isoforms. We therefore investigated the effect of pan-Pim kinase inhibitor treatment on HCAEC angiogenesis. A Matrigel tube formation assay was performed following treatment of HCAECs with vehicle control (0.1% DMSO) or Pim kinase inhibitors AZD1208 (1, 10μM), PIM447 (1, 10μM), and TP3654 (0.1, 1μM), and the number of branch points, number of loops, and tube length were measured as indicators of angiogenic activity, at 4 and 24 hours.

Whilst the number of branch points and number of loops were unaffected by treatment with either concentration of AZD1208 (Figure 4.12), treatment with PIM447 (Figure 4.13) and TP3654 (Figure 4.14) were observed to significantly decrease the number of branch points at both 1 and 10μM at 4 hours, and the number of branch points and number of loops were observed to also be inhibited by TP3654 at 24 hours. All three of the Pim kinase inhibitors were observed to reduce tube length at 4-hours (Figure 4.12, 4.13 and 4.14) with inhibition maintained by TP3654 at 24 hours compared to vehicle treated control.

Taken together these observations demonstrate that pharmacological inhibition of the Pim kinase family delays angiogenesis, indicating a positive role for Pim kinase isoforms in the regulation of blood vessel formation.

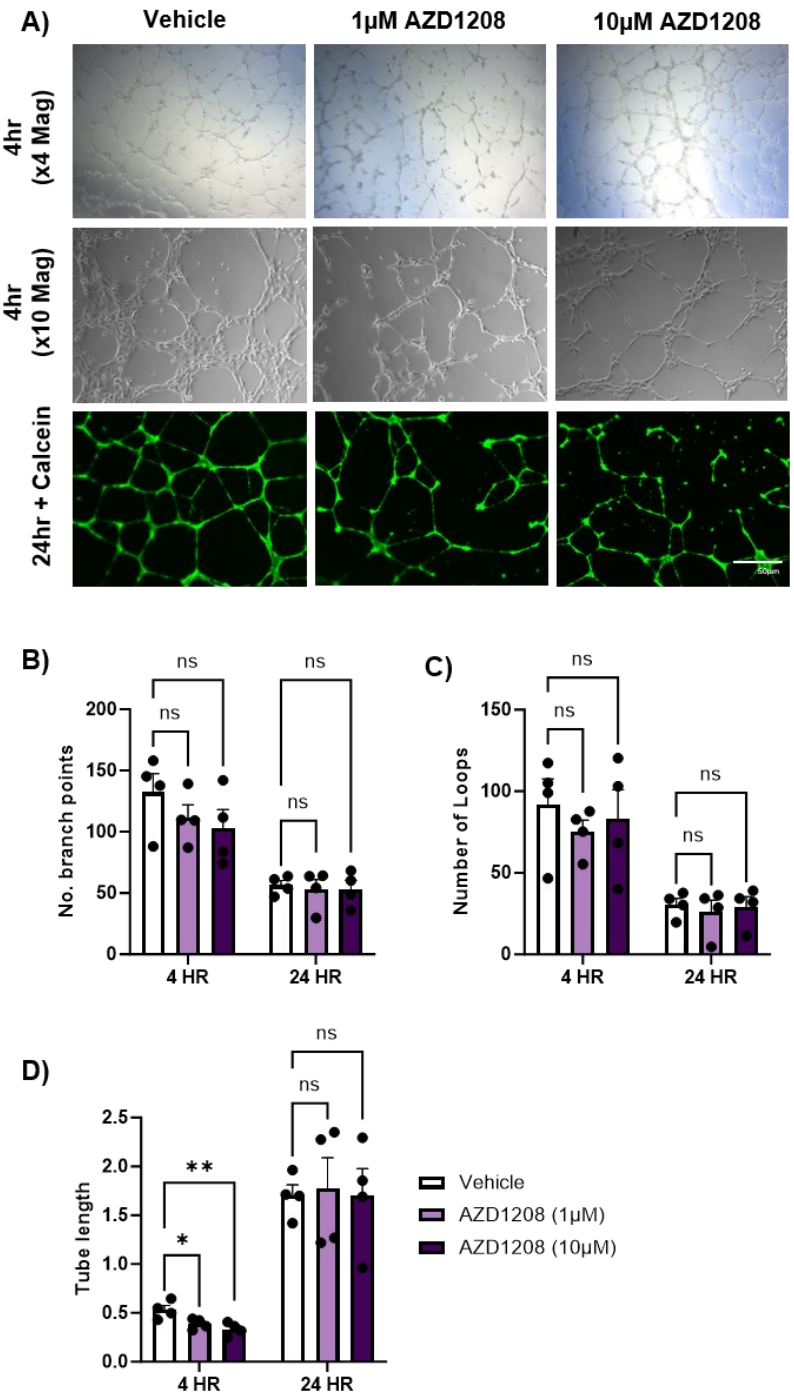

Figure 4.12: Treatment with AZD1208 decreases tube length at 4 hours. A) 4hr and 24hr calcein stained images taken on an EVOS microscope, representative images shown, B) Number of branch points, C) Number of loops and D) Tube length (measuring the circumference of an intact loop). HCAECs treated with AZD1208 were seeded on Matrigel and incubated for 24 hours. Cells were imaged at 4 and 24 hours using a Leica DMRX-A light microscope at a 4x and 10x magnification and analysed using ImageJ software. Scale bar represents 50µm. Calcein was used for visualisation of the tube network. Data analysis is from multiple images per well. Data represent mean + SEM for N=4. *p<0.05, **p<0.01.

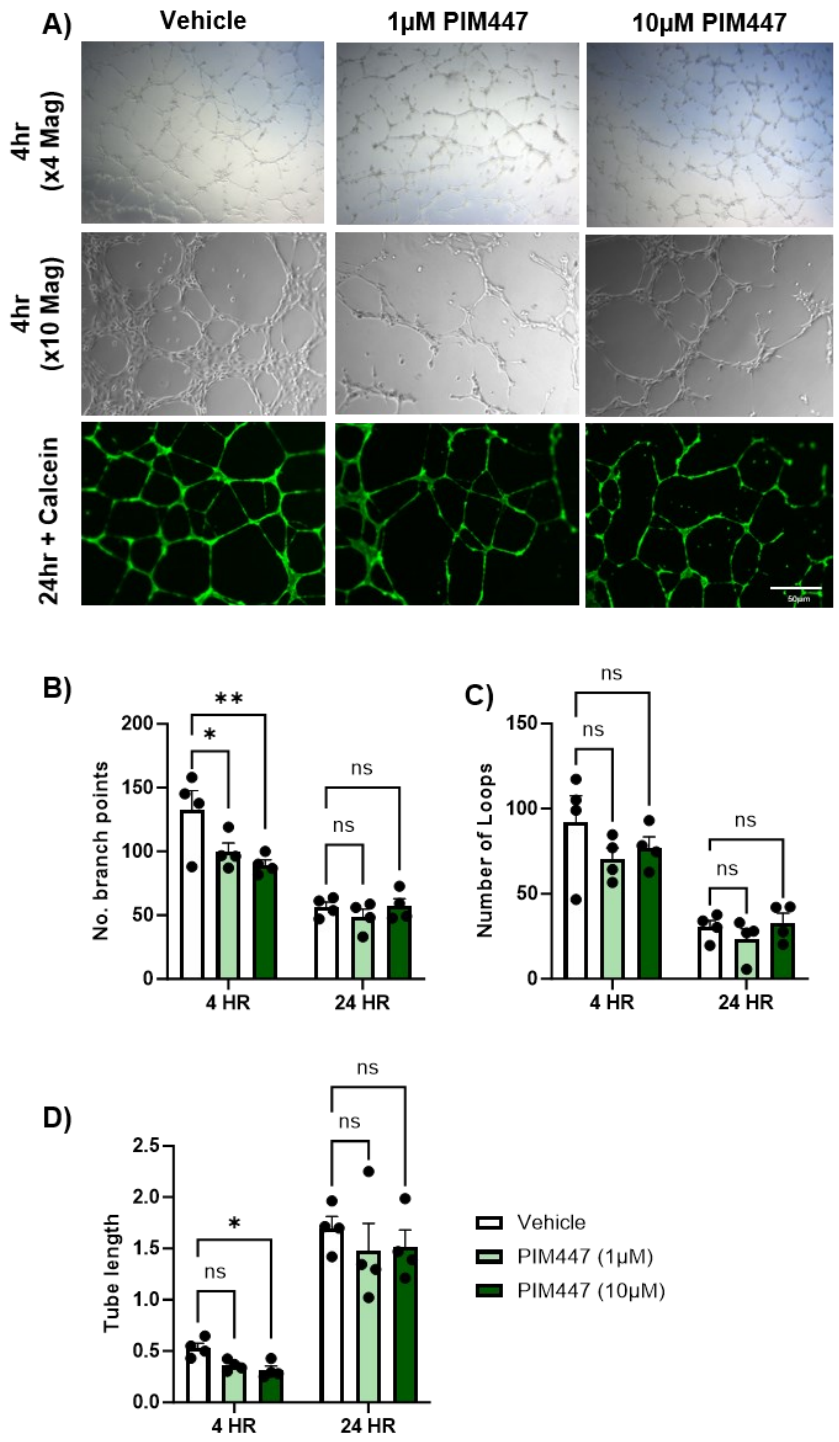

Figure 4.13: Treatment with PIM447 inhibits HCAEC angiogenesis at 4 hours. A) 4hr and 24hr calcein stained images taken on an EVOS microscope, representative images shown, B) Number of branch points, C) Number of loops and D) Tube length (measuring the circumference of an intact loop). HCAECs treated with PIM447 were seeded on Matrigel and incubated for 4 and 24 hours. Cells were imaged using a Leica DMRX-A light microscope at a 4x and 10x magnification and analysed using ImageJ software. Scale bar represents 50μm. Calcein was used for visualisation of the tube network. Data analysis is from multiple images per well. Data represent mean + SEM for N=4. *p<0.05, **p<0.01.

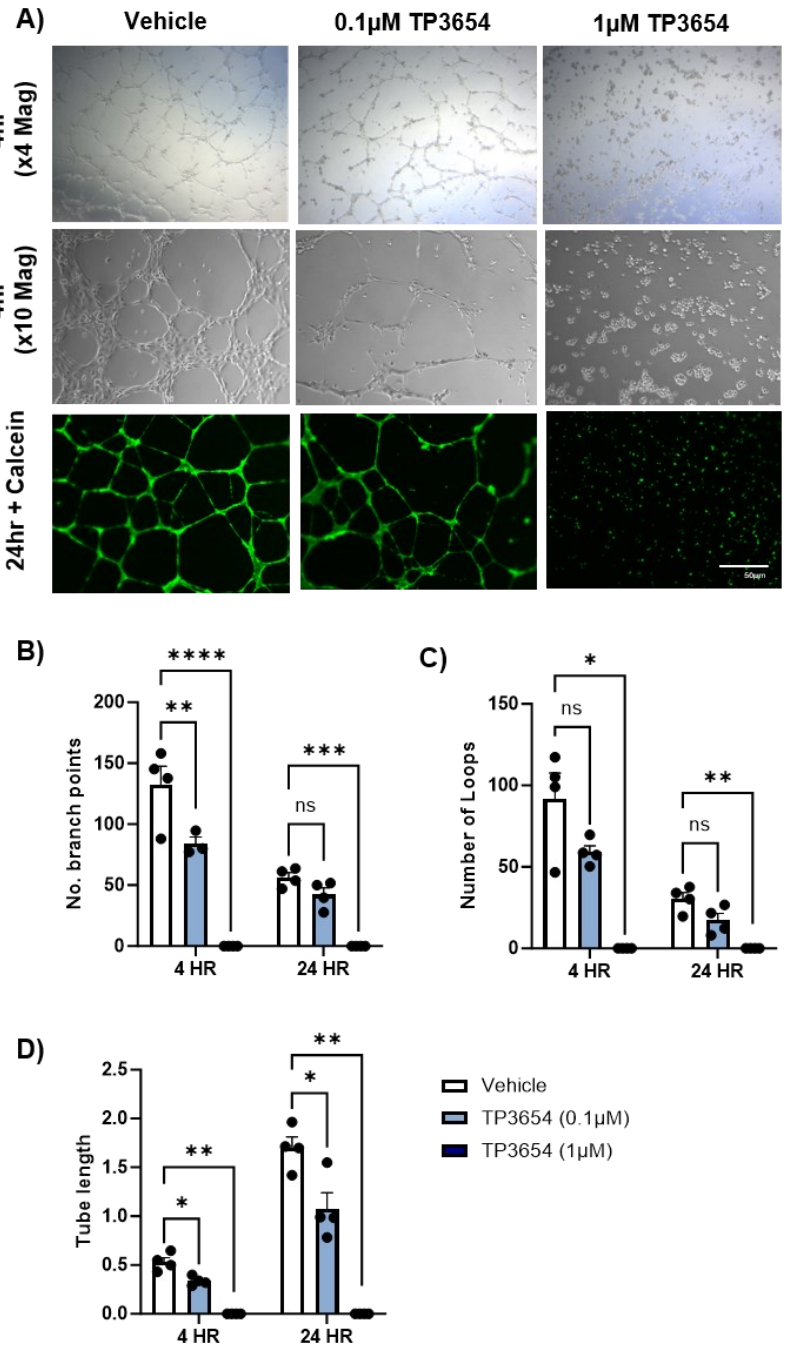

Figure 4.14: Treatment with TP3654 inhibits HCAEC angiogenesis at 4 and 24 hours. A) 4hr and 24hr calcein stained images taken on an EVOS microscope, representative images shown, B) Number of branch points, C) Number of loops and D) Tube length (measuring the circumference of an intact loop). HCAECs treated with TP3654 were seeded on Matrigel and incubated for 4 and 24 hours. Cells were imaged after 4 and 24 hours using a Leica DMRX-A light microscope at a 4x and 10x magnification and analysed using ImageJ software. Scale bar represents 50μm. Calcein was used for visualisation of the tube network. Data analysis is from multiple images per well. Data represent mean + SEM for N=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3.8 Pim kinase inhibition alters HCAEC transcriptome

To investigate the molecular and transcriptional effects of Pim kinase inhibition on endothelial function, RNA sequencing was outsourced to BMKGene and performed on HCAECs treated with pan-Pim kinase inhibitor PIM447 (1μM) for 24 hours. Similarity in experimental repeats of conditions (Vehicle vs PIM447) is demonstrated by Figure 4.15.

As shown in Figure 4.16, 447 genes were found to be significantly differentially expressed in HCAECs following treatment with PIM447 compared to vehicle treated control, 86 genes were found to be upregulated, and 361 genes downregulated. Differential expression analysis revealed significant changes in genes involved in endothelial regulation of signalling, adhesion, inflammation, atherosclerosis and thrombosis (Figure 4.17). Downregulated genes following PIM447 treatment include; CD34, which has previously been shown to play roles in promoting HUVEC angiogenesis and migration (Siemerink et al., 2012), F2R, a receptor for thrombin that plays an important role in regulating endothelial function and thrombotic responses coordinating hematopoietic and vascular development (Yue et al., 2012), and TNFSF18, which contributes to the positive regulation and activation of NF-κB transcription factor activity, which is crucial for a wide range of functions including cell survival which has been linked to Pim kinases, specifically PIM1 and PIM2 (Liu et al., 2020).

Upregulated genes include OSGIN1, which is crucial for cellular stress response, and acts as a tumour suppressor by regulating apoptosis and influencing cell growth, KLF4, a flow regulatory gene which has anti-atherosclerotic properties in the endothelium, AFT3 which is known to play crucial roles in the pulmonary endothelium, in the development of multiple inflammatory pulmonary diseases (Li et al., 2023) and ICAM4 which mediates cell adhesion to the endothelium.

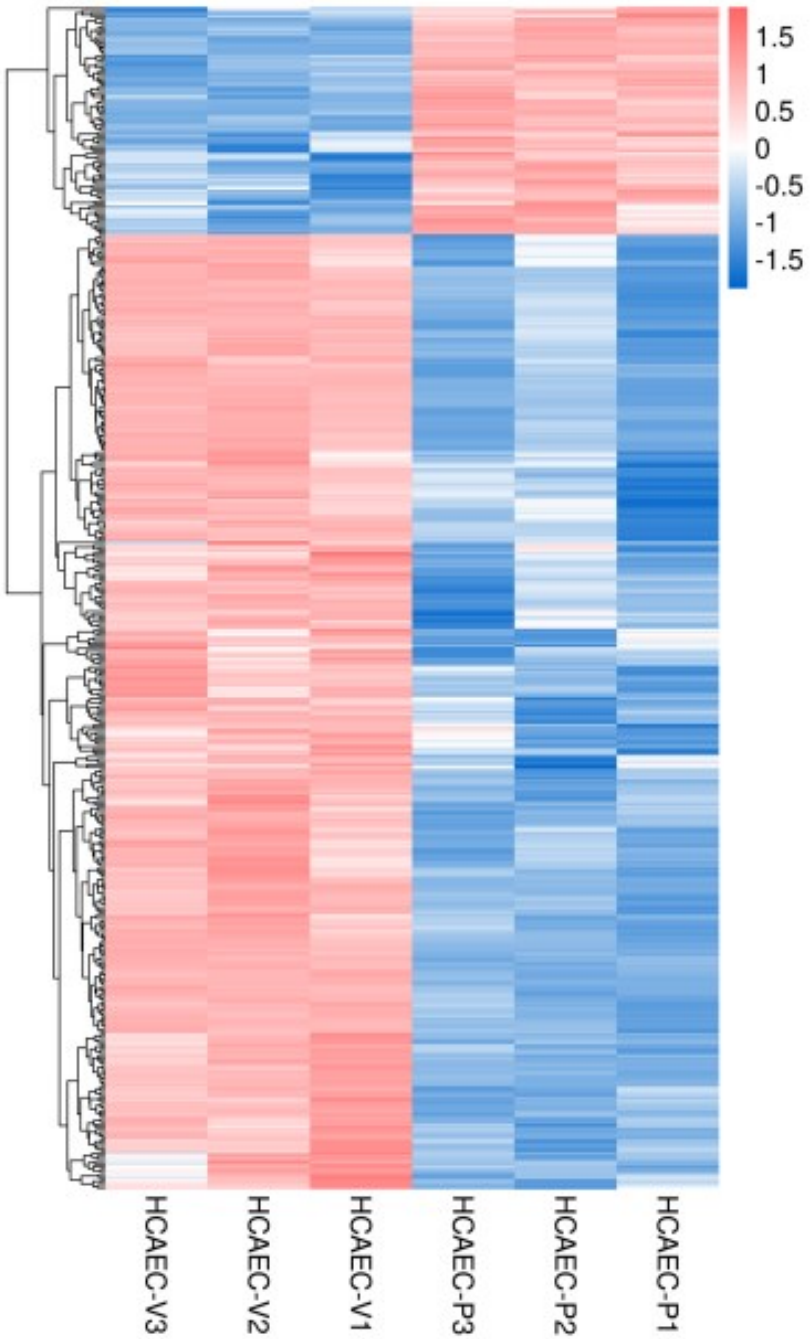

Figure 4.15: BMKGene analysis demonstrating similarity in experimental repeats of conditions. HCAECs were treated with PIM447 (1μM) for 24 hours and lysed for RNA isolation and analysis by RNA seq. Taken from BMKGene analysis report.

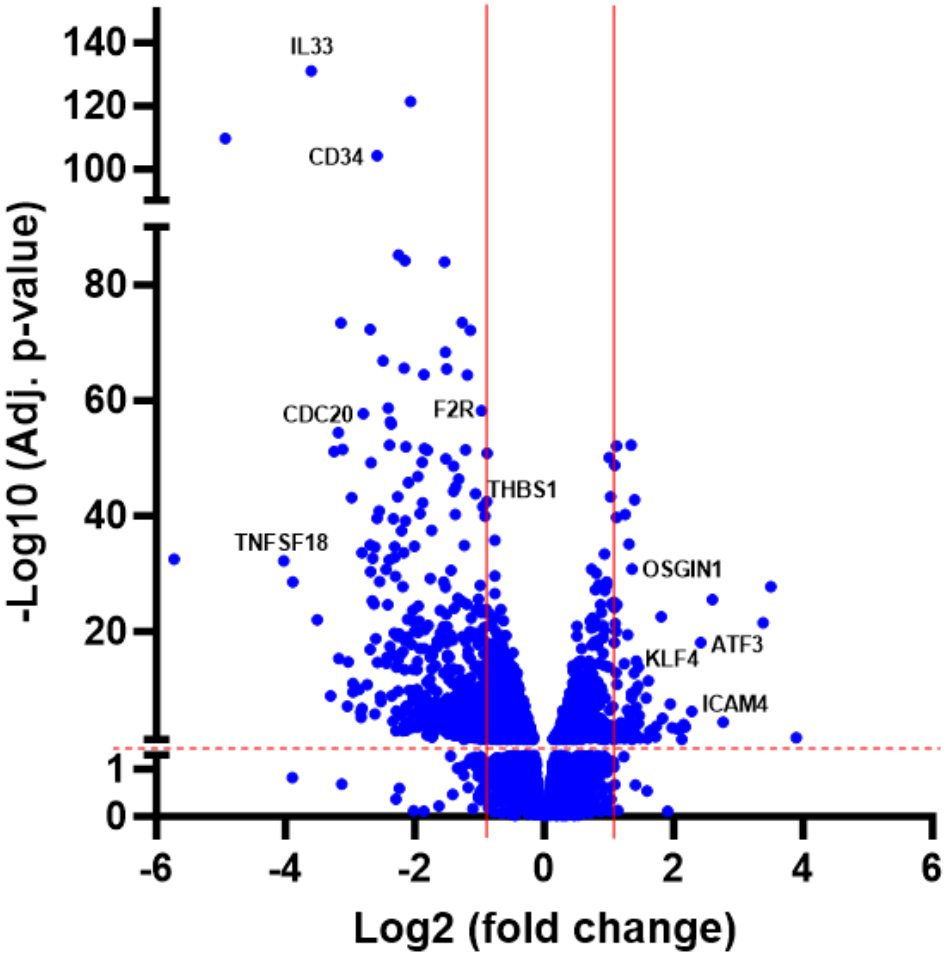

Figure 4.16: PIM447 treatment leads to changes in the HCAEC transcriptome. HCAECs were treated with PIM447 (1μM) for 24 hours and lysed for RNA isolation and analysis by RNA seq. Volcano plot analysis indicating changes in gene expression following HCAEC treatment with PIM447. Data retrieved from BMKGENE was transferred to Excel and P-values and fold changes were log transformed. Genes relevant in thrombosis and haemostasis, and vascular functions were annotated on the plot. Fold change is relative to control. Data is presented for n=3.

Analysis of differential expression also revealed significant changes in genes involved in endothelial regulation of inflammation and platelet and leukocyte adhesion and activation. Analysis of gene expression levels of various, endothelial and thrombotic regulators was therefore grouped and compared across five functional categories to determine the impact of Pim kinase inhibition on these processes.

4.3.8.1 (A) upstream PIM kinase signalling pathway components

First the impact of Pim kinase inhibition on the expression of known upstream regulators of Pim kinase activity was investigated. In confirmation of our earlier observations, PIM1 and PIM2 gene expression were increased following 24hr PIM447 treatment compared to vehicle, along with JAK1, JAK2 and JAK3, upstream regulators of Pim kinase expression potentially indicating a compensatory effect when Pim kinase is pharmacologically inhibited (Figure 4.9cA). In contrast to our earlier observations, PIM3 was significantly decreased, along with STAT1, and STAT5B, possibly indicating PIM3 inhibition via regulation of STAT upstream of Pim kinases.

4.3.8.2 (B) endothelial adhesion molecules

Next, the impact of Pim kinase inhibition on the gene expression of endothelial adhesion molecules was investigated. *ICAM-1* was significantly increased following inhibition with PIM447 compared to control, whilst *VCAM-1, P-selectin, PECAM-1*, and *F11R* were all observed to be significantly decreased, indicating that Pim kinase inhibition reduces gene expression of molecules that contribute to adhesion to the ECM, and of other cells to the endothelium.

4.3.8.3 (C) thromboinflammatory molecules

During endothelial dysfunction, the release of thromboinflammatory molecules that recruit platelets and leukocytes to the endothelium contributes to increased immunothrombosis and the progression of atherosclerosis. Gene expression of thromboinflammatory molecules was compared following HCAEC treatment with PIM447 to elucidate the role of Pim kinase in HCAEC function.

Regulators of platelet function

ENTPD1 (CD39), a regulator of platelet activation, was significantly decreased, along with VWF, which contributes to and promotes platelet adhesion and aggregation at sites of injury, indicating a variable endothelial response in gene expression of platelet mediators.

Inflammatory mediators

Thrombospondin1 a modulator of immune functions, and regulator of leukocyte recruitment and activation, and inflammatory molecules *IL6*, and *TGF beta receptor 2* (TGFBR2) were significantly decreased following PIM447 treatment compared to vehicle, demonstrating a variable HCAEC inflammation response to Pim kinase inhibition. *KLF2* and *KLF4*, which have shown to suppress vascular inflammation by inducing and upregulating eNOS (Mastej et al., 2022) were increased, indicating atheroprotective effects of Pim kinase inhibition on EC function and vascular integrity.

4.3.8.4 (D) coagulation and fibrinolysis regulators

Next the expression of regulators of coagulation and fibrinolysis were compared to determine HCAEC responses to Pim inhibition in the context of clot resolution. *PROS1* (protein S), a cofactor to protein C in the inactivation of FVa and FVIIIa, and *TFPI,* an inhibitor of TF, were significantly decreased, indicating that Pim kinase inhibition with PIM447 results in downregulation of negative regulators of coagulation and activators of fibrinolysis. Conversely, *SERPINE1* (PAI1), *PLAT* (tPA), and *PLAUR* (uPA receptor) increased, suggesting that an increase in tPA, and uPA receptor, activators of fibrinolysis, subsequently results in an increase in PAI-1, a plasminogen activator inhibitor, indicating that Pim kinase affects regulators of coagulation and fibrinolysis.

4.3.8.5 (E) endothelial growth factors

Finally, endothelial growth factors and drivers of angiogenesis were investigated. There were no effects measured on EC growth factors, VEGFA, or VEGFB.

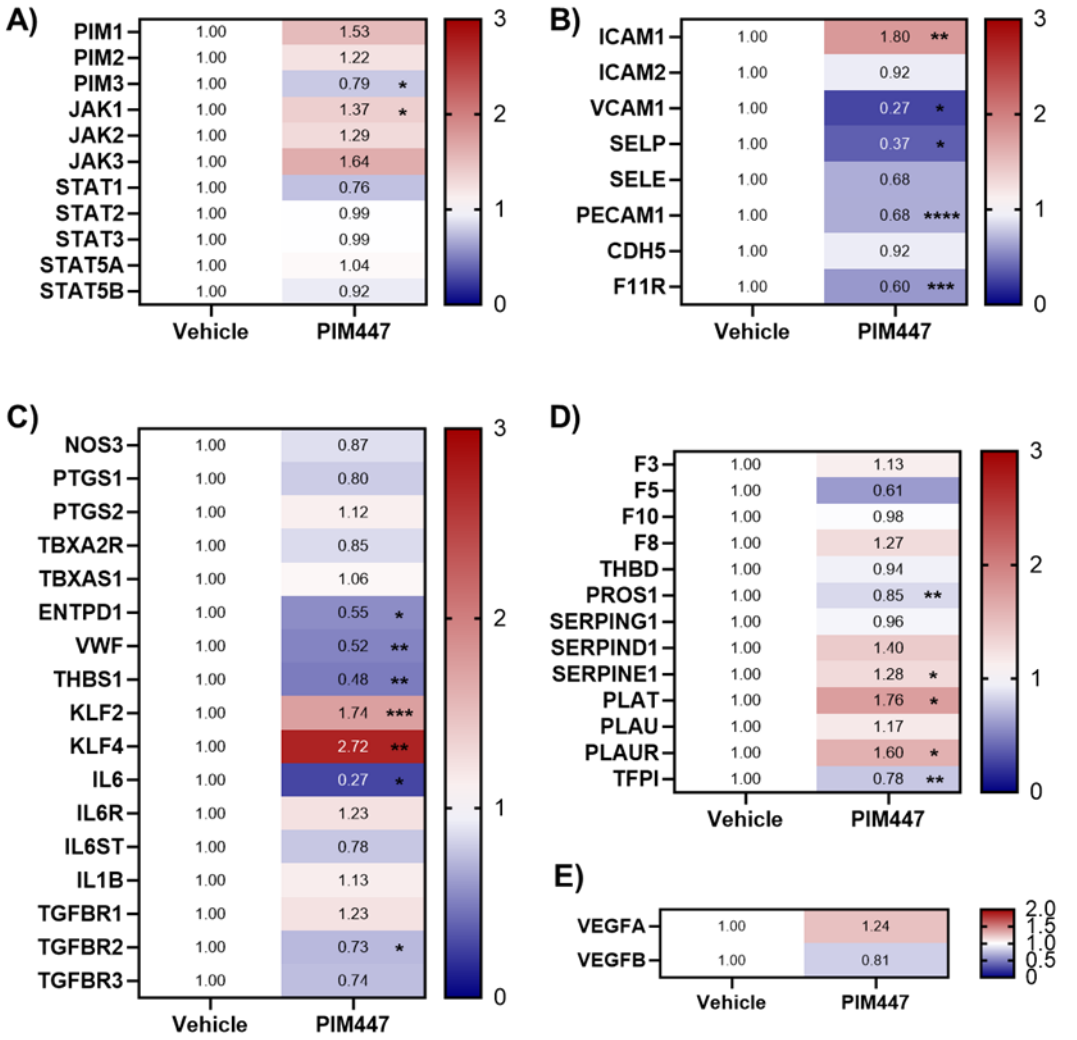

Figure 4.17: Pim kinase inhibition differentially modulates endothelial regulators of atherothrombosis and signalling. HCAECs were treated with vehicle (0.1% DMSO) or PIM447 (1μM) for 24 hours before lysis and RNA extraction. RNA-Seq was performed on the Illumina NovaSeq platform, generating paired-end reads with a minimum depth of 30 million reads per sample. Heat maps display fold change gene expression levels compared to vehicle control. Key regulators were highlighted and grouped into five functional categories: (A) upstream PIM kinase signalling pathway components, (B) endothelial adhesion molecules, (C) thromboinflammatory molecules, (D) coagulation and fibrinolysis regulators and (E) endothelial growth factors. Each row represents individual gene expression, and colour intensity reflects relative expression magnitude. Quantified data represents mean ± S.E.M for n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Heat maps were plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

4.4 Discussion

In this chapter, experiments were performed to investigate the effects of Pim kinase inhibition on functions of the arterial endothelium. Protein and gene expression of the three Pim kinase isoforms, PIM1, PIM2 and PIM3 was confirmed in HCAECs and treatment with pan-Pim kinase inhibitors were observed to not have cytotoxic effects on HCAECs when used at concentrations that are physiologically achievable *in vivo*. This supports the notion that Pim kinase inhibitors could be used to target the endothelium as they appear to be safe and tolerable.

Key Findings:

- PIM1, PIM2, and PIM3 are present in HCAECs.
- Pim isoform gene expression is increased in HCAECs following pharmacological Pim inhibition.
- Pim kinase inhibition does not have any detrimental effects on HCAEC viability, proliferation or migration.
- Pim kinase inhibition in HCAECs delays angiogenesis.
- HCAEC transcriptome demonstrates a variable thromboinflammatory response to PIM447 treatment.

4.4.1 Pim kinase expression and activity

Endothelial protein expression of all 3 short forms (34kDa) of Pim kinase isoforms was confirmed, along with the longer 37kDa PIM2 isoform, while longer forms of PIM1 (44kDa) and PIM2 (40kDa) were not shown to be expressed in western blotting experiments carried out in this chapter (Section 4.3.1). This could be due to the short isoform being expressed at much higher levels than the longer isoform in HCAECs. Additionally, the longer isoform may be stimulus dependent and only be induced under stress or in response to pathological mediators.

Interestingly, following pharmacological Pim kinase inhibition, PIM1, PIM2, and PIM3 gene expression was increased, possibly indicating a regulatory

compensatory feedback mechanism, whereby inhibition of Pim kinases leads to an increase in their own expression to increase kinase activity. This supports previous findings, in megakaryocyte-like (MEG-01) cells where 24-hour treatment with AZD1208 resulted in increased PIM1 expression (Nock et al., 2024). Importantly, Pim kinase activity is regulated by their expression (and degradation) as they are constitutively active and are not turned on or off by phosphorylation or cofactor binding like other kinases (Torres-Ayuso and Brognard, 2025). Considering this mechanism, cells may have the potential to adapt over time by increasing their expression to overcome pharmacological inhibition. This could have clinical implications with Pim kinases presenting resistance to Pim kinase inhibitors, which is frequently observed with cancer therapy (Torres-Ayuso et al., 2024).

RNA-Seq transcriptomics data confirmed these observations of increased PIM1 and PIM2 gene expression following 24hr PIM447 treatment demonstrated by an increase in fold change, although not significant (Figure 4.17). Furthermore, it has recently been shown that treatment of PC3, LNCaP, and C4-2 prostate cancer cells with different pan-PIM kinase inhibitors (SGI-1776, AZD1208, and PIM447) increased the protein levels of all three Pim kinase isoforms (Torres-Ayuso et al., 2024), stabilising levels of Pim, supporting our observations. JAK1 gene expression was also significantly increased, indicating a compensatory effect upstream of Pim kinase which could underlie the observed increased in PIM isoform expression, as JAK/STAT translocation to the nucleus and binding to the Pim promoter controls Pim isoform expression, particularly PIM1 (Warfel and Kraft, 2015, Liang and Li, 2014). This indicates that pharmacological Pim inhibition results in JAK upregulation to promote increased Pim production as a regulatory negative feedback mechanism. PIM3 expression however showed assay-dependent regulation following pharmacological inhibition, with RT-qPCR indicating increased expression while RNA-Seq analysis suggested overall downregulation, potentially reflecting isoform-specific or transient transcriptional effects. A downregulation of PIM2 and PIM3 isoform expression was also observed in PIM1$^{-/-}$ mice demonstrated in Chapter 3 (Figure 3.2).

PIM3 is translated from a single translation start site, but PIM1 and PIM2 both have multiple start sites, leading to variants of different molecular weights (Toth and Warfel, 2021). The shorter form of PIM1 (PIM-1S) (~34kDa) localises primarily to the nucleus, whereas the longer form (PIM-1L) (~44kDa) is cytoplasmic and localises at the plasma membrane (Saris et al., 1991), anchored by the additional N-terminal sequence (Egli et al., 2015). These observations suggest that differences between PIM-1S and PIM-1L might have some substrate specificity and therefore perform different functions. The identification of only the shorter forms of the PIM1 and PIM2 variants from western blotting analysis indicates that the different variants of each Pim isoform may play different roles in HCAECs. Less is known about the different PIM2 variants, although it has been suggested that the shorter 34kDa variant is more associated with anti-apoptotic activity than the medium (37kDa) and longer (40kDa) forms (Fox et al., 2003).

4.4.2 Cell viability and proliferation

Pim kinase inhibitors were originally identified to have anti-cancer properties and developed to induce cancer cytotoxicity by disrupting cell survival pathways and promoting apoptosis. Various Pim kinase inhibitors have been shown in several cell line studies to reduce cell viability and prevent cellular proliferation. In a study using pan isoform Pim kinase inhibitor SGI-1776 to target acute myeloid leukaemia (AML) using AML cell lines MV-4-11, MOLM-13 and OCI-AML-3 overexpressing Pim kinases (Chen et al., 2011), a concentration-dependent induction of apoptosis was observed, correlated with the inhibition of global RNA and protein synthesis and anti-apoptotic protein MCL-1 transcript decline after SGI-1776 treatment. Additionally, in a study using Pan-Pim inhibitor AZD1208 to target chronic lymphocytic leukaemia (CLL) using primary human CLL lymphocytes, which showed higher levels of PIM2 at both transcript and protein levels (Cervantes-Gomez et al., 2019), treatment of CLL lymphocytes with AZD1208 resulted in modest cell death, whereas practically no cytotoxicity was observed in healthy lymphocytes. These drugs are effective in targeting AML and CLL, and cells overexpressing Pim kinases, showing selective toxicity for

cancer cells over healthy ones as Pim kinase isoform expression is elevated under disease conditions, as demonstrated in the study by Cervantes-Gomez *et al*. However, it is important to investigate the effects of Pim kinase inhibitors on HCAEC survival and viability to ensure that cytotoxicity and apoptosis was not being induced in HCAECs.

Concentrations of Pim kinase inhibitors that can be reached in plasma in patients (Keeton et al., 2014, Raab et al., 2019, Kunder et al., 2022, Lebedinsky et al., 2020) were not observed to be cytotoxic to HCAECs and did not affect cell survival or viability after 24-hour treatment *in vitro* (Figure 4.4 and 4.5). These observations indicate that Pim kinase inhibitors would be a suitable and safe treatment option for targeting ECs.

4.4.3 Cell morphology and adhesion

Experiments were conducted to investigate HCAEC morphology, adhesion, and cytoskeleton dynamics following Pim kinase inhibition. It has previously been shown that pan-Pim inhibitor SEL24/MEN1703-treated HUVECs cultured under static conditions were depleted of higher-order actin structures necessary for efficient angiogenesis. These included reductions in actin stress fibres, membrane ruffles and lamellipodia (Garbicz et al., 2020), indicating that Pim kinase activity is required for the cytoskeletal organisation that underpins endothelial migration and angiogenesis, and that pan-PIM inhibition disrupts actin polymerisation and cytoskeletal remodelling.

In the experiments conducted, ECs were cultured under static conditions, as a simplified and controlled model to investigate the effects of Pim kinase inhibition on HCAEC function, without the additional complexity experienced by introducing flow dynamics. *In vivo*, mechanosensitive ECs in the coronary artery are constantly subject to blood flow shear stress, specifically to a laminar arterial shear rate of around 15dynes/cm2, with cells lined up in the direction of flow, as observed in a study by (Kroon et al., 2017). Under static culture conditions or following exposure to oscillatory/atheroprone shear, however, ECs have a cobblestone appearance, and are devoid of the atheroprotective

mechanisms exerted by laminar flow. This is akin to the areas of arteries such as bifurcations and bends, which are exposed to complex blood flow patterns with low or oscillatory shear stress (OSS), where atherosclerotic plaques are most likely to occur (Dong et al., 2024, Souilhol et al., 2020).

Shear stress was applied to HCAECs to investigate morphology parameters; cell adhesion, phalloidin fluorescence intensity (as an indicator of f-actin polymerisation and actin filament organisation of the cytoskeleton), and nuclear aspect ratio following treatment with Pim kinase inhibitors. A study by Walpen *et al* has shown that loss of PIM1 imposes a hyper adhesive phenotype on mouse aortic endothelial cells (MAECs) under static conditions demonstrated by decreased migration, slowed cell detachment and increased electrical resistance across the endothelial monolayer, and an upregulation of junctional molecules promoting endothelial barrier integrity (Walpen et al., 2012). Cell count was measured as an indicator of adhesion and was in the work outlined in this chapter observed to be relatively unchanged between vehicle and Pim kinase inhibitor-treated cells for all 3 treatments under both laminar and oscillatory flow conditions (Figure 4.6, 4.7 and 4.8), indicating that Pim kinase does not affect HCAEC adhesion, as opposed to the study described previously. Walpen *et al* used a PIM1-specific knockout mouse model, and experiments conducted were under static conditions, therefore the effects seen may be PIM1 isoform specific under these conditions. Although this is the case, TP3654 is favourable to PIM1 inhibition, and therefore this should be investigated further.

Actin filaments, the fundamental structure of the cytoskeleton, play an important role in cytoskeleton arrangement and endothelial integrity. Slee and Lowe-Krentz observed that laminar shear stress of 15 dyne/cm2 stimulates the disruption of actin realignment in ECs (Slee and Lowe-Krentz, 2013). The contribution of Pim kinases to actin cytoskeleton rearrangement has been investigated in various studies in the context of cancer. It has previously been shown that PIM1 enhances prostate cancer cell motility by phosphorylating actin capping proteins, modifying actin dynamics by destabilising actin filaments and consequently increasing migration (Santio et al., 2020).

Under static culture conditions, no alterations in F-actin polymerisation was observed following treatment with either of the 3 Pim kinase inhibitors, contrasting with results in other studies which show destabilisation or reductions in actin filaments in Pim inhibitor-treated HUVECs (Garbicz et al., 2020).

Interestingly under conditions of oscillatory shear, phalloidin fluorescence intensity, and therefore F-actin polymerisation was found to be decreased by ~5% in HCAECs treated with AZD1208 compared to control treated cells, although this effect was not seen with PIM447 or TP3654. It is well documented that cytoskeleton arrangement is disordered, and endothelial permeability is increased when ECs are exposed to low or OSS, versus high (laminar) shear (Chen et al., 2024). An observed decrease in fluorescence intensity with AZD1208 treatment suggests that Pim kinases may positively contribute to f-actin polymerisation and cytoskeletal rearrangement in pathological, disturbed flow conditions, contributing to pathological processes such as the initiation and progression of atherosclerosis (Chen et al., 2024).

Nuclear aspect ratio was measured as an indicator of cell circularity and was shown to be increased in AZD1208-treated HCAECs compared to vehicle control under static conditions, indicating more elongated and less circular nuclei, however this was not observed under laminar and oscillatory shear stress conditions.

Further appropriate measures of morphology would include cell surface area indicative of cellular organisation, along with the measurement of actin stress fibres and lamellipodia, however these would require additional analysis of existing data. A recent study has shown that PIM3 inhibition in mice following treatment with AZD1208 leads to increased pulmonary vascular barrier leakage and gaps in alveolar EC junctions. Impairment of the EC barrier was also observed following PIM inhibition with AZD1208 and PIM3 gene silencing, indicated by a decrease in junctional proteins (Santio et al., 2024), suggesting that PIM3 is protective in maintaining endothelial barrier functions in the

pulmonary endothelium. There were no alterations observed in cell contacts, or visible gapping, indicating that they still maintained cellular contacts under all conditions tested.

Analysis of the gene expression of molecules associated with endothelial barrier function in the transcriptomic data set collected following treatment of HCAECs with PIM447, identified that both *PECAM-1* and *JAM-A* were downregulated in HCAECs following Pim inhibition with PIM447, supporting previous observations and indicating that Pim kinases may play protective roles in maintaining endothelial barrier function by supporting junctional protein expression. The effects of Pim kinase inhibition on other tight and adherins endothelial junctional proteins (including VE-Cadherin and catenins) in HCAECs should be investigated as an indicator of vascular barrier function and to investigate the effects of Pim kinase on cellular contacts between ECs. Functional assays such as trans endothelial electrical resistance (TEER) or FITC-Dextran permeability assays would provide further quantitative insight into the integrity of the vascular endothelial barrier.

4.4.4 HCAEC migration and angiogenesis

Previously, studies have shown that PIM1$^{-/-}$ mouse aortic ECs (MAECs) have altered adhesion and migration, demonstrated by decreased migration and slowed cell detachment in PIM1 deficient ECs (Walpen et al., 2012). Additionally, it has been shown that treatment with Pim kinase inhibitor AZD1208 attenuated the ability of MEG-01s (megakaryocytes) to migrate towards CXCL12 (which facilitates platelet production and thrombopoiesis (Avecilla et al., 2004)), represented by a significant reduction in average cell motility and migration over a 24-hour period, along with the attenuation of CXCR4-/CXCL12-mediated megakaryocyte migration across a CXCL12 transwell gradient (Nock et al., 2024). Interestingly CXCL12 also promotes EC migration and has shown to promote the integration of ECFC-derived cells into developing vascular networks (Newey et al., 2014). PIM3 also promotes the spreading and migration of ECs (Zhang et al., 2009b).

Scratch wound healing experiments were carried out to determine the effect of Pim kinase inhibitor treatment on HCAEC migration which was observed to not be affected by Pim kinase inhibitor treatment, indicating that normal endothelial migration functions can still be carried out effectively. These differences in cellular effects when compared to the literature could be explained by sub-optimal inhibition by the pharmacological inhibitors, which typically have variable potency across isoforms, and it has been shown that cells try to overcome the inhibition by increasing gene expression (Figure 4.3). EC migration is influenced by key molecules including chemokines, growth factors (VEGF) and cell adhesion molecules (ECM and endothelial surface proteins/Ve-Cadherin/ICAM-1/VCAM-1/selectins), and is required for the development of new and existing vascular networks (Huveneers and Phng, 2024). Analysis of the gene expression patterns of key regulators of cellular migration in the transcriptomic data following HCAEC treatment with PIM447, demonstrated no alterations in gene expression of key EC growth factors, VEGFA, or VEGFB following Pim kinase inhibition with PIM447 compared to control. The expression of VEGF receptors which stimulate ECs to migrate should be measured to further investigate the effects.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a complex and dynamic process, involving endothelial activation, basement membrane degradation, tip cell formation, migration and proliferation, and finally vessel maturation, essential for growth and development (Dudley and Griffioen, 2023). Conversely, angiogenesis has been described in a pathological context where there has been an association demonstrated between intralesional angiogenesis with atherosclerotic plaques that cause ACS (Tenaglia et al., 1998). Inflammatory cytokines derived from the plaque and other vascular cells increase permeability via the formation of actin stress fibres, filopodia, and membrane ruffles in ECs, influencing cell migration and angiogenesis (Yang et al., 2011a, Gao et al., 2002). This indicates a link between neovascularization and atherosclerosis (Khurana et al., 2005), and an increased

atherothrombotic risk, therefore treatments capable of preventing this process could lower the risk of atherothrombotic events.

Further studies investigating Pim kinase inhibition and silencing has identified decreased viability, migration and formation of tube structures in Matrigel by ECs (Garbicz et al., 2020), and that Pim kinases promote new vessel formation via phosphorylation of TNFα and eNOS, demonstrating decreased angiogenic properties following the lack of Pim kinase in HUVECs. In support of previously published data using PIM isoform deficient HUVECs and different Pim kinase inhibitors (Zhang et al., 2009b, Chen et al., 2016), HCAEC angiogenesis was decreased by all 3 Pim inhibitors AZD1208, PIM447 and TP3654 used in this study at 4 hours, and after 24 hours. This indicates that whilst migration remains unaffected, other processes involved in HCAEC angiogenesis, including endothelial sprouting may be disrupted, indicated by decreases in tube length (AZD1208, PIM447 and TP), number of branch points by (PIM447 and TP), and the number of loops (TP3654).

Interestingly however, despite Pim kinase inhibitor treatment causing an attenuation of angiogenesis, this does not appear to be mediated via gene expression regulation of key angiogenic signalling pathways including VEGF and eNOS as there appeared to be no effects on *VEGFA* and *VEGFB* gene expression, or *NOS3* (eNOS) gene expression following Pim inhibition with PIM447 (Figure 4.17). This is in contrast to a study that has identified a role for Pim kinase in angiogenesis, where a link between Pim kinase and regulation of eNOS has been highlighted, showing the PIM1 isoform to directly phosphorylate and activate eNOS at Ser-633 in vascular ECs and promote NO production in HUVECs, enhancing angiogenic effects (Chen et al., 2016). Additionally, this attenuation also appears to be independent of genetic regulation of thrombospondin-1, a potent angiogenesis inhibitor that has shown to inhibit EC migration and proliferation and induce apoptosis in cancer cells, regulating tumour progression (Ren et al., 2006), as this was observed to be downregulated in HCAECs following treatment with PIM447 (Figure 4.17).

PECAM-1 (CD31) is highly expressed at EC intercellular junctions, where it functions as a regulator of leukocyte trafficking and in the maintenance of EC junctional integrity (Privratsky and Newman, 2014), and has shown to be implicated in angiogenesis, playing a major role in cell adhesion, migration, and sprouting angiogenesis (Kondo et al., 2007). *PECAM-1* gene expression was decreased following Pim kinase inhibition with PIM447 as shown in the RNA-Seq data (Figure 4.17), supporting the previous observations of the role of Pim kinase in angiogenesis processes.

These observed reductions in angiogenesis can result from impaired endothelial migration, proliferation, or survival or defective growth factor signalling amongst various other things. Growth factors (e.g. VEGF) should be investigated further to elucidate this effect using an ELISA or commercially available VEGF bioassay. A reduction in angiogenesis indicates a disruption in normal EC processes, however this could be beneficial in the context of disease and atherosclerosis where neovascularisation occurs and could be beneficial in increasing plaque stability.

4.4.5 Regulation of thromboinflammatory mediators

Following Pim kinase inhibition with PIM447, RNA-Seq transcriptomic analysis identified a complex regulatory pattern in the context of endothelial integrity and thromboinflammation.

4.4.5.1 Endothelium integrity and inflammation

Gene expression of KLF2 and KLF4, transcription factors that regulate endothelial function and maintain vascular integrity (Sangwung et al., 2017), were significantly increased following Pim kinase inhibition with PIM447, indicating that Pim kinases may play roles in endothelial dysfunction. Overexpression of both KLF2 and KLF4 in ECs has been shown to present an anti-inflammatory and vasoprotective cellular phenotype (Fan et al., 2017) through inhibiting pro-thrombotic factors PAI-1 and TF, and upregulating anti-thrombotic factors thrombomodulin and eNOS expression under inflammatory conditions (Lin et al., 2005), and KLF4 also inhibits NF-κB activation (Hamik et al., 2007,

Zhou et al., 2012). This suggests that Pim kinase inhibitors offer protective properties that would enhance the anti-inflammatory properties of KLF2 and KLF4 in ECs, and along with reduced IL-6 expression, which is regulated by JAK/STAT and NF-κB pathways, indicates a shift toward an anti-inflammatory endothelial state, confirming observations from Chapter 3 (Figure 3.13B) where KLF2 and KLF4 were increased, while IL-6 was decreased following PIM1 deletion. Additionally, KLF2 and KLF4 inhibit angiogenesis, suppress capillary-like tube formation and strongly inhibit the proliferation of ECs through multiple signalling pathways including inhibition of the transcription of VEGF receptor 2 (VEGFR2) (Bhattacharya et al., 2005, Zheng et al., 2013). Pim kinases therefore present as an appropriate target in proliferative vascular disorders and pathological angiogenesis in the context of inflammation.

4.4.5.2 Adhesion molecules and leukocyte mediators

Endothelial cell adhesion molecules ICAM-1 and VCAM1-1, P-selectin and JAM-A promote leukocyte transmigration across the endothelial barrier during inflammation (Singh et al., 2023). *VCAM-1* gene expression was significantly decreased by PIM447 treatment, along with *JAM-A* and *SELP (P-selectin)* in the RNA-Seq analysis, whilst *ICAM-1* was increased following inhibition with PIM447, demonstrating that leukocyte adhesion molecule expression was not suppressed for all genes. JAM-A, VCAM-1, ICAM-1 and P-selectin play roles in monocyte recruitment, infiltration and thromboinflammatory processes, and their inhibition is protective against atherosclerosis (Yago et al., 1999). This suggests that whilst Pim kinase inhibition does not suppress endothelial activation completely, with differences in gene expression of endothelial adhesion molecules demonstrated, it may selectively modulate leukocyte recruitment pathways particularly those associated with atherosclerosis and thromboinflammation.

4.4.5.3 Platelet mediators and coagulation regulators

A variable endothelial Pim kinase inhibition response was demonstrated in the alteration of gene expression of platelet mediators and coagulation regulators, indicating that the inhibition of Pim kinase does not uniformly affect haemostatic

and thrombotic processes. A decrease in *ENTPD1* (CD39) was observed in PIM447-treated HCAECs, along with a decrease in *PROS1* (protein S), suggesting that the inhibition of Pim kinases dampens the anti-thrombotic profile of the endothelium.

Contrary to this, a decrease in *VWF* was observed following PIM447 treatment of HCAECs, which is at odds with transcriptomic data in the previous chapter where an increase in *VWF* was observed in PIM1$^{-/-}$ PECs (Figure 3.13). Interestingly, this suggests that pan-Pim kinase inhibition may be required for the regulation of pro-thrombotic VWF in the endothelium.

Western blotting to measure levels of protein expression would be appropriate to confirm phenotypic responses. An increase in *PLAT* (tPA), and *PLAUR* (uPA receptor), along with *SERPINE1* (PAI1) suggests that expression of plasminogen activators is downregulated by Pim kinase, along with their inhibitors, possibly demonstrating an autoregulatory mechanism. Further investigation is required on thromboinflammatory effects following Pim kinase inhibition in HCAECs.

4.4.6 Limitations

1. Static HCAECs resembled an appearance similar to cells exposed to oscillatory shear, uniformly polygonal, whilst laminar shear aligned cells to the direction of flow. While static shear models are widely used in endothelial research, they do not fully recapitulate the features of laminar flow *in vitro*. Importantly, the conclusions drawn in this chapter from the majority of the functional experiments and transcriptomics are therefore restricted to endothelial responses under static shear conditions which may represent more pathological conditions (Qiao et al., 2016).
2. Gene expression is not necessarily a marker of protein activity, therefore functional experiments to measure protein levels e.g. western blotting should be carried out to confirm RNA-Seq data.
3. Damage could be induced in HCAECs to determine Pim kinase inhibition effects of thromboinflammatory (e.g. interleukins, VWF etc) and endothelial adhesion markers (e.g. ICAM-1, VCAM-1, p-selectin) following

endothelial dysfunction, which would better resemble an atherosclerosis disease environment, as this is where Pim kinases have been previously demonstrated to be upregulated (Xue et al., 2025).

4. HCAECs used in experiments in this chapter were from male donors due to a lack of commercially available female HCAECs. These therefore aren't representative of female biology and doesn't consider sex-specific differences. In future, HCAECs from female donors should be used.
5. The analysis for f-actin fluorescence intensity was not blinded but was largely automated in ImageJ due to the dynamic nature of the actin cytoskeleton, using software-defined limits for thresholding of images. Each image, therefore, went through an automated process of thresholding to determine f-actin fluorescence intensity, enabling standardisation of results. Morphological classification of ECs (cell number and angiogenesis parameters) however, was performed manually and was not blinded, therefore the analysis was subject to some bias, despite parameters set for identifying and measuring number of branch points, tube length, and number of loops for angiogenesis analysis. To avoid unblinded analysis bias, the analysis could be adapted and automated using specialist software. However, it is important to consider that specialised software will require training and testing to ensure accurate and replicable results.

4.4.7 Chapter conclusions

In summary physiologically achievable concentrations of Pim kinase inhibitors did not alter HCAEC survival and viability, indicating that their role in the induction of apoptosis and cell death is cancer-cell specific, enabling further exploration of their effects on endothelial function. The upregulation of gene expression all 3 Pim kinases following treatment with Pim kinase inhibitors demonstrates a compensatory feedback mechanism that requires further investigation. In this chapter we identify anti-angiogenic properties of pan-Pim kinase inhibitors on ECs, indicating that Pim kinase inhibitors have targeting potential in neovascularisation in the context of atherosclerosis. The observed upregulation of KLF2, KLF4, further indicates a protective anti-inflammatory response following inhibition, reinforcing Pim kinases as promising therapeutic targets in an atherosclerotic context through shared transcriptional signalling pathways. Variable gene expression of mediators of thrombosis, haemostasis, and endothelial function was observed, identifying a need to investigate these further. The next Chapter will therefore focus on how Pim kinase inhibition influences the anti-platelet, anti-coagulant, and anti-inflammatory properties of the arterial endothelium.

4.5 Chapter 4 summary abstract

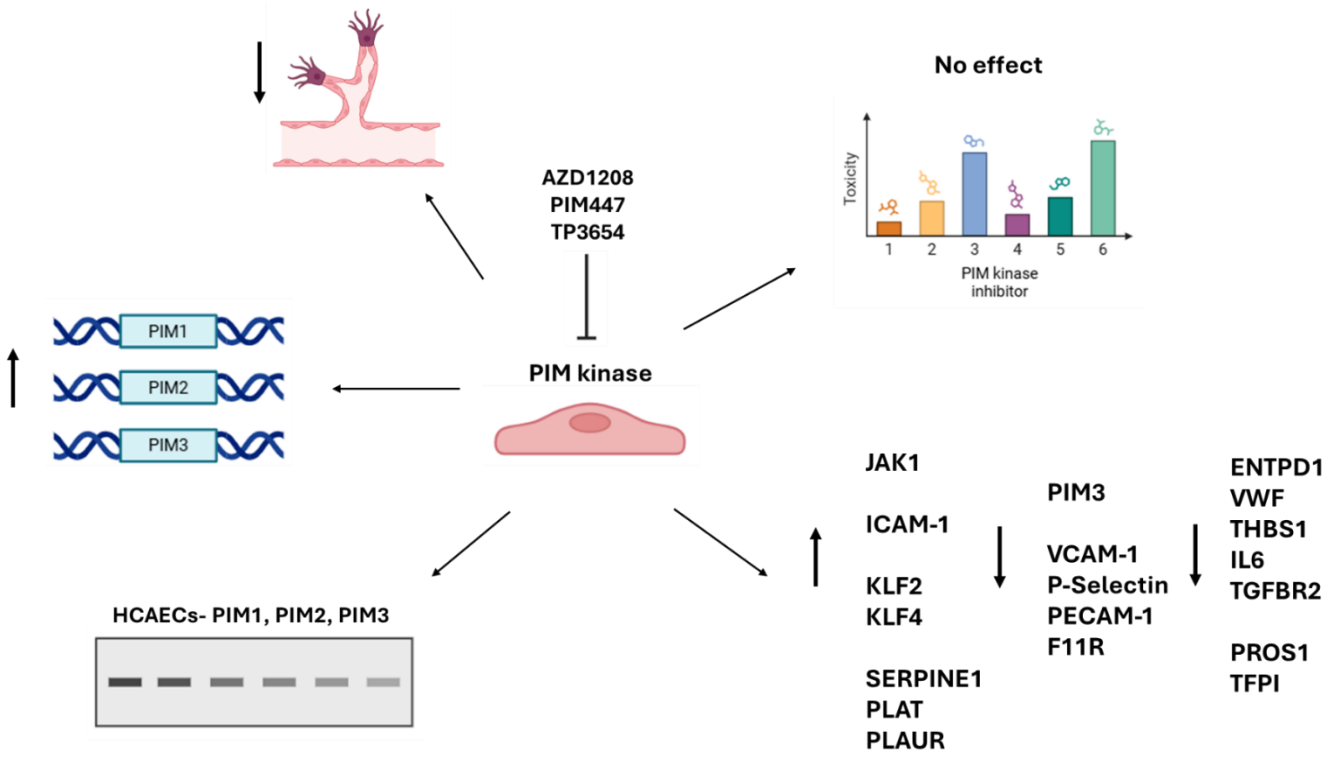

Figure 4.18: Summary abstract of the effects of Pim kinase inhibition in HCAECs experiment results in this chapter. Created in BioRender and PowerPoint.

Chapter 5. Investigating the therapeutic potential of Pim kinase inhibition on endothelium-blood crosstalk and thrombosis.

5.1 Introduction

5.1.1 Endothelial regulation of thrombosis

The endothelium is a key regulator of haemostasis and thrombosis, providing anti-coagulant protection in the healthy state, and conversely, turning pro-coagulant and pro-adhesive following damage or dysfunction (Neubauer and Zieger, 2022).

In ECs, during inflammation, PIM3 has been shown to mediate inflammatory TNFα signalling in ECs, with TNFα treatment associated with increased PIM3 mRNA expression in HUVECs *in vitro* and Pim-3 silencing attenuating EC sprouting *in vivo* (Yang et al., 2011a). These findings support a positive role for PIM3 in TNFα inflammation-mediated cytoskeletal rearrangements, vascular permeability (cell detachment), and angiogenesis, demonstrating the potential of targeting Pim kinases to protect against pathological EC activation to reduce vascular permeability and neovascularisation. To support this, in the previous chapter roles for Pim kinase in HCAEC angiogenesis were demonstrated where treatment with Pim inhibitors attenuated angiogenesis (Section 4.3.7), further highlighting the role of Pim in EC angiogenesis, and the potential for targeting Pim kinases in a pathological disease setting.

5.1.2 Pim kinase and thromboinflammation

Pim kinase expression and activity are not limited to cancer, with findings in several studies that have demonstrated Pim kinase upregulation in inflammatory diseases including the overexpression of PIM1 in idiopathic pulmonary fibrosis (Gao et al., 2022) and rheumatoid arthritis (Ha et al., 2019). Inflammation is an

established driver of thrombotic events and increased cardiovascular risk (Stark and Massberg, 2021).

In support of a role for Pim kinase in CVD and related pathologies, Pim kinase isoforms demonstrate widespread expression in cardiovascular tissues (Figure 1.8) and have demonstrated an upregulation in patients with a high risk of CVD such as smoking and hyperglycaemia (Wang et al., 2017, de Vries et al., 2014).

Roles for PIM1 and PIM3 isoforms have been identified in the published literature in thrombotic and inflammatory diseases. An epigenetic study identified that DNA methylation at the *PIM3* locus was altered in individuals with ischemic stroke compared with controls, suggesting an epigenetic association between *PIM3* and stroke risk (Soriano-Tarraga et al., 2020). Furthermore, an additional study demonstrated PIM1 expression to be upregulated in patients with lower extremity deep venous thrombosis (LEDVT) (Chen et al., 2023), indicating a role for Pim kinase in thrombosis. In studies of inflammatory conditions, it has been demonstrated that PIM1 and PIM3 are both upregulated by TNFα and IL-6 in rheumatoid arthritis synoviocytes (Ha et al., 2019), indicating that Pim kinases are actively induced by inflammatory cytokines in rheumatoid arthritis, and should be investigated further with different cardiovascular cell types to determine their contributions to thromboinflammatory mechanisms. In contrast to PIM1 and PIM3, a role for PIM2 has not yet been described in inflammation and thrombosis. Transcriptomic data comparing the impact of Pim kinase inhibitor treatment on HCAECs collected and described in Chapter 4 (Section 4.3.8) also identified alterations in gene expression of key endothelial modulators of thrombosis and inflammation, demonstrating possible roles for Pim kinase in thromboinflammation. This has prompted the investigation into the effects of pan-Pim kinase inhibitors on HCAEC regulation of thromboinflammation in this chapter.

5.1.3 Pim kinase inhibitors and thrombosis

It has previously been shown that platelet mediated thrombus formation is significantly reduced with AZD1208 treatment *in vitro* with similar effects

observed *in vivo* using the FeCl3 model of thrombosis. Interestingly *in vivo* studies demonstrate more pronounced attenuation compared to *in vitro* studies (Unsworth et al., 2021), indicating that other cell types may be involved in the anti-thrombotic properties of Pim kinase inhibitors, which needs to be investigated further.

The role of the Pim kinase isoforms on the anti-thrombotic and anti-inflammatory properties of the arterial endothelium has not yet been determined and will be investigated in this chapter. Various changes in thromboinflammatory mediators were identified from RNA-Seq data in Chapter 4, highlighting the need for follow up work to understand how Pim kinase inhibitors could functionally impact thrombosis. Exploring Pim kinase inhibition in the arterial endothelium in the context of thrombosis would allow the identification of the contribution of Pim kinase isoforms and provide potential targeting strategies for novel anti-thrombotic therapies.

5.2 Chapter aim, objectives and hypothesis

The aim of this chapter is to investigate the effects of Pim kinase inhibitors on the inflammatory and thrombotic potential of HCAECs.

The following objectives will be addressed:

1. To elucidate the effect of Pim kinase inhibition of the anti-inflammatory properties of the arterial endothelium.
2. To investigate the effect of Pim kinase inhibitors on the anti-platelet properties of the arterial endothelium.
3. To characterise the effect of Pim inhibition on the anti-coagulant properties of the arterial endothelium.

5.2.1 Hypothesis: Pim kinase inhibition protects HCAECs from converting to an inflammatory and thrombotic phenotype.

5.2.2 Methods

Human coronary artery endothelial cells (HCAECs) were treated with or without pharmacological Pim kinase inhibitors, AZD1208 (1-10μM), PIM447 (1-10μM), and TP3654 (0.1-1μM) and expression and release of modulators of inflammation, platelet activation and adhesion and coagulation and fibrinolysis determined using RT-qPCR, ELISA based assays, clot formation and breakdown assays and an *in vitro* endothelialised model of thrombus formation using standard assays described in Chapter 2 (Materials and Methods). HCAECs were treated with TNFα (5ng/mL) or IL-6 (100ng/mL) for some experiments conducted (Section 5.3.1 and 5.3.2).

5.2.2.1 Visual methodology abstract

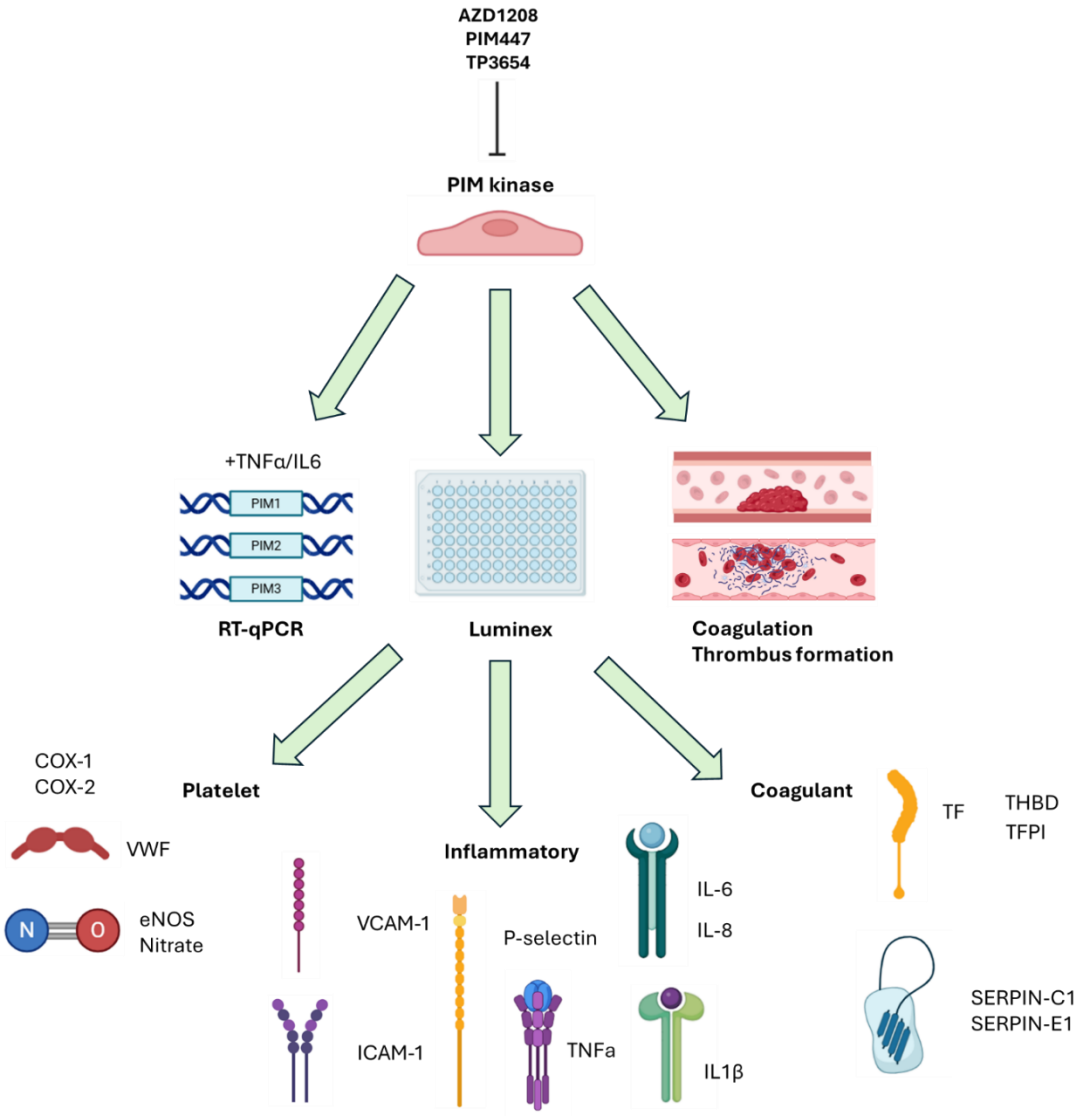

Figure 5.1: Visual representation of the methodology used to address the objectives in this chapter. Investigating the anti-platelet, anti-inflammatory, and anti-coagulant properties of the arterial endothelium following PIM kinase inhibition. Created in BioRender and PowerPoint.

5.3 Results

5.3.1 PIM kinase isoform gene expression is increased in HCAECs in response to inflammatory mediators

Pim kinases have been previously shown in various cell types to be upregulated in response to mediators of inflammation (Ha et al., 2019, Liong et al., 2017), and it has previously been described that altered protein kinase activity may be regulated by an increase in cell stimulation and/or exposure to pathological mediators in CVD (Shahin et al., 2017). Endothelial activation/dysfunction associated with atherosclerosis and thromboinflammation is triggered by various stimuli including inflammatory cytokines TNFα and IL-6 (Theofilis et al., 2021). Therefore, Pim kinase gene expression in healthy HCAECs treated with inflammatory mediators TNFα and IL-6 was determined using RT-qPCR.

Significant differences were observed in Pim kinase gene expression in HCAECs treated with TNFα (5ng/mL) or IL-6 (100ng/mL) for 4 and 24 hours compared to vehicle control. PIM1 gene expression was significantly upregulated following HCAEC stimulation with TNFα after 4 ($P=0.0164$) and 24 ($P=0.0019$) hours, whilst PIM3 gene expression was increased after 24 hours of TNFα treatment ($P=0.0357$). IL-6 treatment of HCAECs significantly upregulated gene expression of all 3 Pim isoforms at 24 hours ($P=0.0308$, 0.0139, 0.0084 for PIM1, 2, and 3 respectively) (Figure 5.2). These results strongly indicate that inflammatory mediators induce Pim kinase gene expression in HCAECs and suggest that Pim kinase may play a role in inflammation-mediated endothelial activation, however this needs to be investigated further.

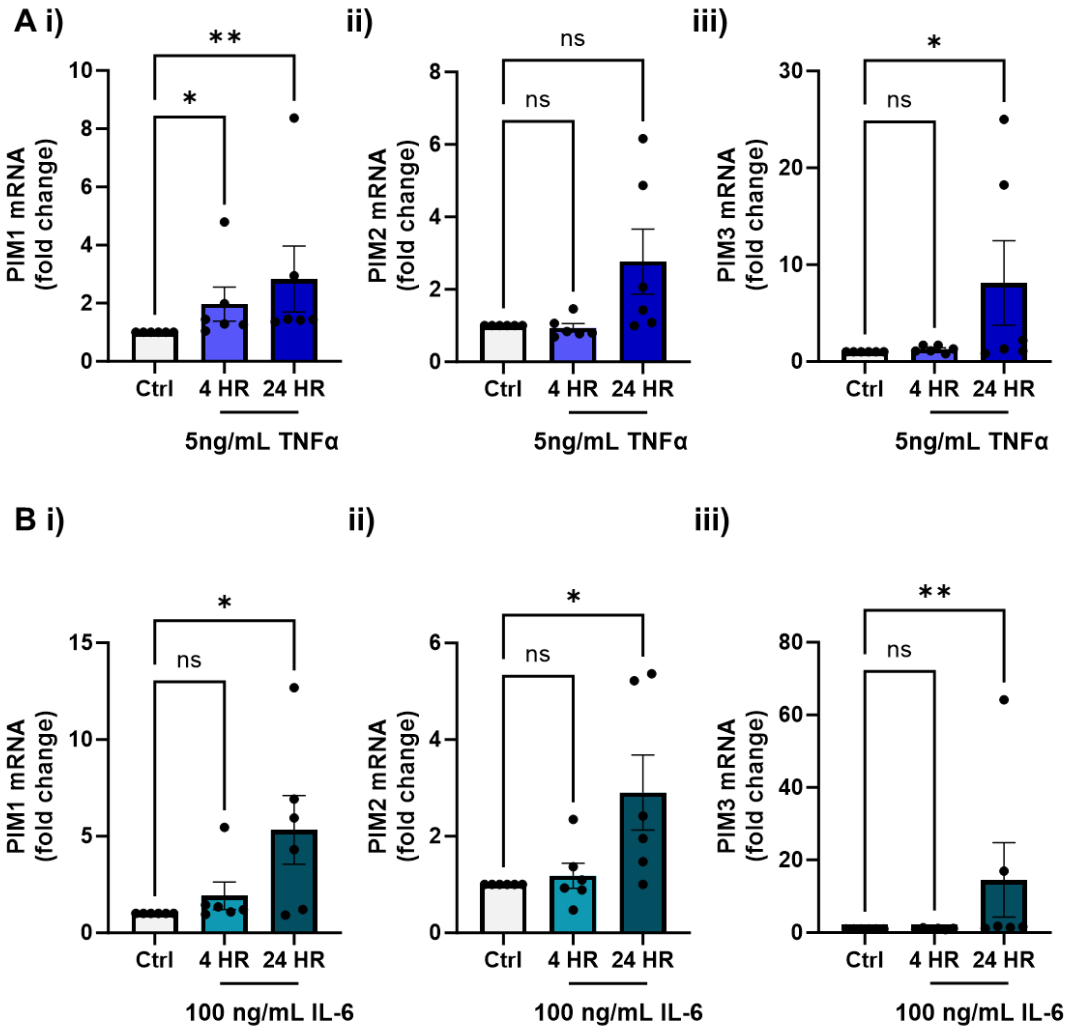

Figure 5.2: Mediators of inflammation upregulate Pim kinase isoform expression in HCAECs. *PIM1, PIM2,* and *PIM3* gene expression was analysed in HCAECs treated with (A) TNFα (5ng/mL) and (B) IL-6 (100ng/mL) for 4 and 24 hours. Gene of interest (GOI) Ct values were normalised to housekeepers GAPDH and RPLP0. RT-qPCR experiments were carried out in duplicate, and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean ± S.E.M for n=6. *P≤0.05, **P≤0.01. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.2 TNFα and IL-6 do not induce inflammation or thrombotic effects in static-cultured HCAECs

Prior to investigation of the effects of Pim kinase inhibition on EC activation, the impact of inflammatory mediators TNFα (5ng/mL) and IL-6 (100ng/mL) on gene expression of various known mediators of thrombosis and inflammation in HCAECs was determined.

Unexpectedly, under the conditions used, TNFα and IL-6 were not observed to affect gene expression of thrombotic and inflammatory mediators such as *VWF, VCAM-1*, and *PTGS1* (COX-1) after 24 hours of inhibitor treatment (Figure 5.3). Similar observations were also made at 4 hours of treatment (data not shown). Additionally, they did not have any effects on other endothelial mediators including *PTGS2* (COX-2), *eNOS,* and *THBD (thrombomodulin)*, indicating that TNFα and IL-6 did not cause activation of HCAECs and endothelial dysfunction under the conditions used.

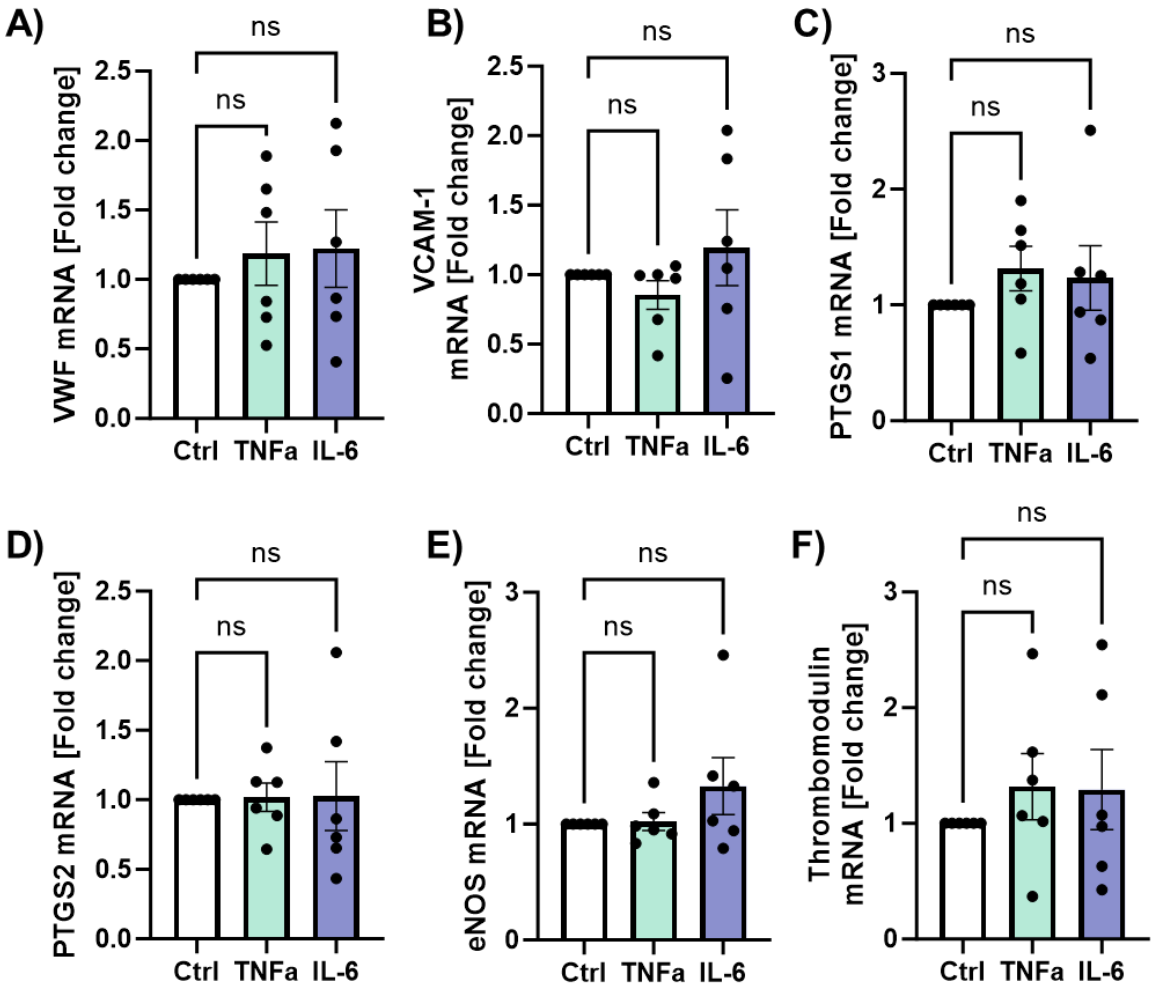

Figure 5.3: TNFα and IL-6 do not induce a thromboinflammatory phenotype in static-cultured HCAECs. Gene expression of various thromboinflammatory mediators A) VWF, B) VCAM-1, C) PTGS1, D) PTGS2, E) eNOS, and F) thrombomodulin was analysed in HCAECs treated with TNFα (5ng/mL) (green) and IL-6 (100ng/mL) (blue) for 24 hours. Gene of interest (GOI) Ct values were normalised to housekeepers GAPDH and RPLP0. RT-qPCR experiments were carried out in duplicate, and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean ± S.E.M for n=6. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.3 Static conditions differ to laminar shear

To establish the best model that would reflect a dysfunctional endothelium, using data made publicly available by (Afshar et al., 2023), the differences in endothelial phenotype following static adhesion vs shear were determined by comparing the effect of exposure of HUVECs to laminar shear (15 dynes/cm2) for 24 hours to static culture conditions. Compared to static culture, flow-responsive genes; KLF4 increased 9-fold, KLF2 increased 10-fold, and eNOS increased 6-fold following exposure to laminar shear (Table 5.1). As expected, gene expression of thromboinflammatory mediators *ICAM-1, VCAM-1, VWF*, and *IL-6* was decreased in response to laminar shear (Table 5.1), indicating that laminar flow is protective compared to static-culture of HUVECs. In further support of Pim kinases being involved in endothelial activation, expression of all 3 Pim kinases was reduced in response to laminar shear compared to static culture (Table 5.1).

Table 5.1: Gene expression fold changes in flow (laminar) vs static cultured HUVECs after 24 hours. Data taken from (Afshar et al., 2023).

Gene	Protein	Fold change (flow (laminar) vs static)
Pim kinases		
PIM1	PIM1	0.49689236
PIM2	PIM2	0.037324155
PIM3	PIM3	-7.770532032
Platelet mediators/coagulation		
VWF	VWF	-1.7344905
F3	Tissue factor	-0.653335648
TFPI	TFPI	-0.470746734
PTGS1	COX-1	3.365225536
NOS3	eNOS	6.217699093
THBD	Thrombomodulin	0.046270647
Flow sensing		
KLF2	KLF2	10.15291976
KLF4	KLF4	9.893612455
Adhesion		
VCAM-1	VCAM-1	-1.10505845
PECAM-1	PECAM-1	-1.971916577
ICAM-1	ICAM-1	-0.367987424
Inflammation		
IL-6	Interleukin-6	-0.302110938

5.3.4 Static conditions appear similar to oscillatory flow

As demonstrated in Chapter 4 (Figure 4.6, 4.7 and 4.8) and in the literature (Qiao et al., 2016), HCAEC morphology during static culture appears similar to that of oscillatory 'atheroprone' flow, possibly indicating a 'dysfunctional/activated' phenotype.

Having identified in Table 5.1 that static ECs appear dysfunctional and thromboinflammatory compared to ECs exposed to laminar shear, endothelial phenotype was compared between ECs exposed to static culture and atheroprone oscillatory shear, using publicly available data from (Qiao et al., 2016). The expression of genes involved in the regulation of thrombosis were compared in static versus oscillatory conditions. There was no change in gene expression of thrombotic mediators, including *VWF, TFPI*, and *NOS3* (eNOS) (Figure 5.4), indicating that static and oscillatory flow conditions are similar.

Furthermore, comparison of Pim kinase isoform gene expression demonstrated that *PIM1* and *PIM2* gene expression is similar in static and oscillatory flow conditions, whilst *PIM3* is more highly expressed in static conditions compared to oscillatory shear stress (Figure 5.5).

As these data indicated that gene expression profiles of HCAECs under static culture is similar to or potentially more pathological compared to oscillatory shear, further experiments were carried out using static conditions (with the addition of Pim kinase inhibitors).

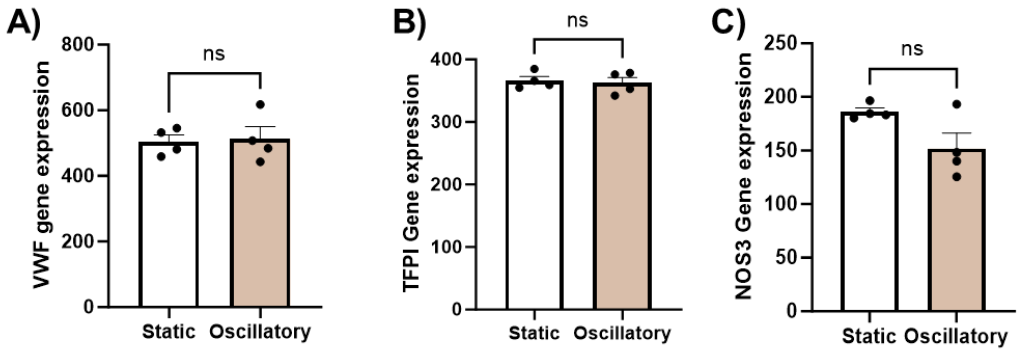

Figure 5.4: RNA-Seq transcriptomic data demonstrating thrombotic mediator gene expression of HCAECs in static and oscillatory conditions. Data was taken from (Qiao et al., 2016). Results are expressed as mean + S.E.M for n=4. Data was analysed using t-tests and graphs plotted using GraphPad Prism.

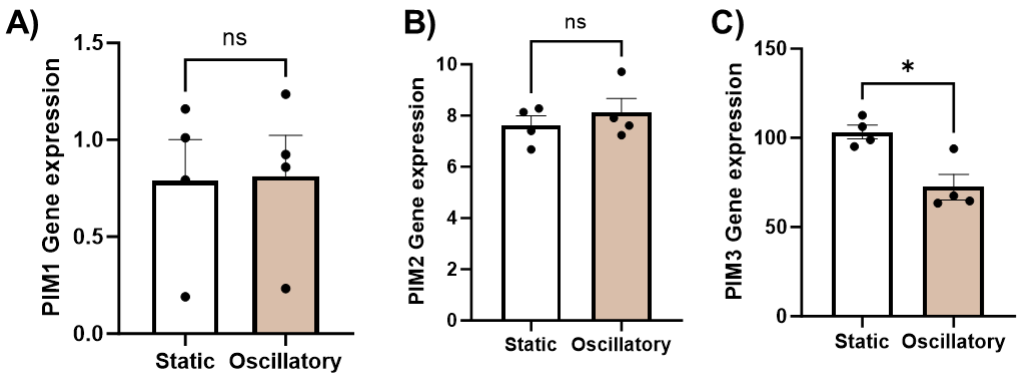

Figure 5.5: RNA-Seq transcriptomic data demonstrating Pim isoform gene expression of HCAECs in static and oscillatory conditions. Data was taken from (Qiao et al., 2016). Results are expressed as mean ± S.E.M for n=4. *P≤0.05. Data was analysed using t-tests and graphs plotted using GraphPad Prism.

5.3.5 Pim kinase inhibitors reduce HCAEC contributions to thrombus formation

Models of arterial thrombus formation allow the investigation of platelet function and testing of novel anti-thrombotic drugs (Drysdale et al., 2024). The *in vitro* flow model used in this experiment enables the early steps of arterial thrombus formation to be assessed including tethering of platelets to collagen, platelet activation and recruitment, and platelet aggregation and thrombus growth under the influence of ECs (Drysdale et al., 2024).

In vitro thrombus formation experiments were carried out to determine the effects of Pim kinase inhibitor treatment on the endothelial contribution to platelet activation and thrombus formation by investigating platelet adhesion on both HCAECs and collagen following HCAEC stimulation with TNFα (5ng/mL) to induce endothelial dysfunction.

Untreated whole blood (stained with DiOC6) was flowed at an arterial shear rate (15 dynes/cm2), which translates into a flow of 46.70µL/min, over healthy and TNFα-stimulated HCAECs treated with or without AZD1208 (1µM) and PIM447 (1µM) and then over collagen coated channels to elucidate endothelial effects of Pim kinase inhibitors on thrombosis.

5.3.5.1 Platelet adhesion/thrombus formation on HCAECs

Platelet (and leukocyte) adhesion appeared to be decreased on unstimulated (untreated) HCAECs following treatment with either of the Pim kinase inhibitors AZD1208 and PIM447 compared to vehicle treated controls, with complete lack of adhesion evident in PIM447 (1µM) treated HCAECs (Figure 5.6). Interestingly despite previous observations of a lack of changes in gene expression of thromboinflammatory mediators in response to TNFα treatment, increased adhesion of platelets and leukocytes (the larger green circles) was observed following HCAEC treatment with TNFα (5ng/mL) compared to unstimulated control, and this also appeared to be decreased with Pim inhibitor treatment, particularly following treatment with PIM447. However, due to technical and experimental limitations, statistical analysis of the data wasn't possible.

Therefore, thrombus formation using traditional measures (average thrombi number, size, area coverage and volume) was not quantifiable. Further repeats are needed to confirm this preliminary observation.

5.3.5.2 Platelet adhesion/thrombus formation on collagen

Type I collagen is the most thrombogenic ECM protein found in the blood vessel wall (Mangin et al., 2021), and is enhanced in atherosclerotic lesions (Shekhonin et al., 1987). ECs usually function to prevent platelet activation and adhesion. To investigate the anti- and pro-thrombotic profile of HCAECs following treatment with Pim kinase inhibitors, platelet adhesion to collagen was determined following exposure to HCAECs. To achieve this, whole blood was passed through HCAEC chambers and then through collagen coated chambers at an arterial shear rate (15 dynes/cm2), which translates into a flow of 46.70µL/min, in the presence and absence of TNFα.

Blood flowed over TNFα-treated HCAECs appeared to increase platelet adhesion and thrombus formation on collagen, compared to unstimulated conditions (Figure 5.7). This looked to be decreased for Pim inhibitor conditions; however, this should be confirmed qualitatively following additional repeats.

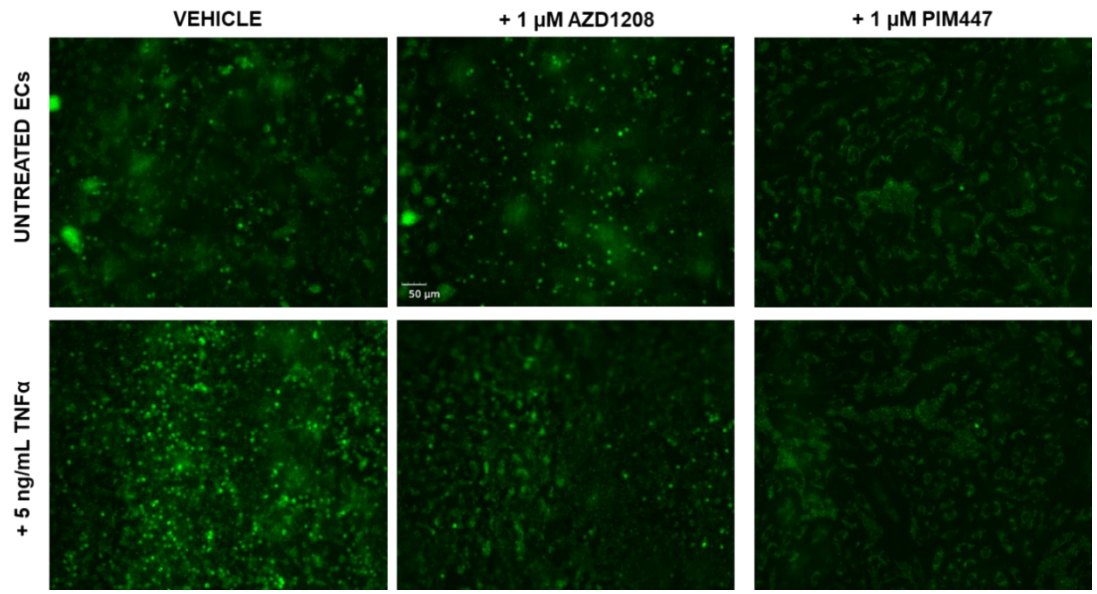

Figure 5.6: Pim kinase inhibitors reduce HCAEC thrombus formation. DiOC6 labelled untreated whole blood was perfused over untreated HCAECs and HCAECs treated with TNFα (5ng/mL) or vehicle (0.1% DMSO) in the presence or absence of Pim kinase inhibitors AZD1208 (1µM) or PIM447 (1µM) at an arterial shear rate (15 dynes/cm2) for 8 minutes. Images of DIOC-6-labelled platelets (and leukocytes) were taken using the CELENA® S Logos Biosystems fluorescent microscope. Representative images shown for n=2.

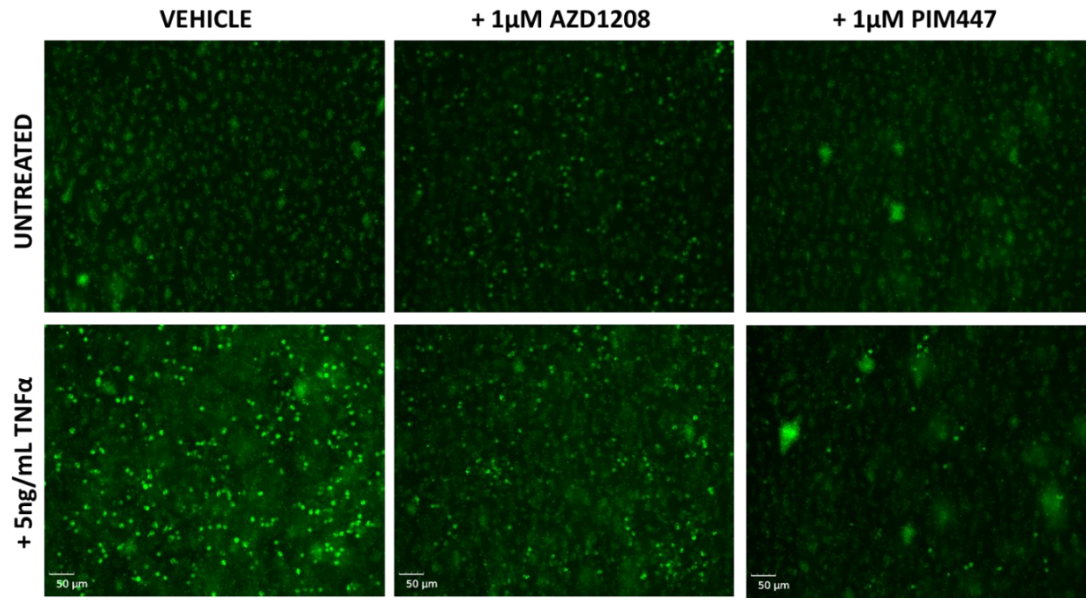

Figure 5.7: TNFα-treated HCAECs increased thrombus formation on collagen, which was then decreased following Pim kinase inhibitor conditions. Whole blood was perfused over healthy HCAECs and HCAECs treated with TNFα (5ng/mL) or vehicle (0.1% DMSO) in the presence or absence of Pim kinase inhibitors AZD1208 (1µM) or PIM447 (1µM), then through collagen coated channels at an arterial shear rate (15 dynes/cm2) for 8 minutes. Images of DIOC-6-labelled platelets were taken using the CELENA® S Logos Biosystems fluorescent microscope. Representative images shown for n=2.

5.3.6 Pim kinase inhibition does not alter endothelial regulation of platelet mediators

It has been previously described that Pim kinase inhibitor AZD1208 inhibits platelet driven thrombosis *in vivo* (Unsworth et al., 2021). The endothelium prevents platelet activation through the expression and release of anti-platelet mediators, including CD39 and eNOS, and when activated, expresses VWF to facilitate platelet adhesion; therefore, it was important to determine the effects of Pim kinase inhibition on the mediators involved in the endothelial control of platelet activity and adhesion.

5.3.6.1 eNOS gene expression and nitrate release are unchanged in damaged HCAECs following Pim kinase inhibition

eNOS function is critical for the maintenance of vascular homeostasis through the synthesis of NO (Cyr et al., 2020) which inhibits platelet activation and adhesion (Radziwon-Balicka et al., 2017). In Chapter 4, transcriptomic analysis indicated no significant alteration in eNOS expression following Pim kinase inhibition (Figure 4.17).

HCAEC eNOS mRNA expression was measured using RT-qPCR, and NO release determined by measuring nitrate levels in HCAEC conditioned media/releasate following 24-hour treatment with Pim inhibitors AZD1208 (1μM), PIM447 (1μM), and TP3654 (0.1μM) and confirmed the observations made in the transcriptomics. Treatment with any of the three Pim kinase inhibitors did not demonstrate any notable effect on eNOS gene expression or nitrate release (Figure 5.8), indicating no alteration in eNOS expression, activity, or NO production.

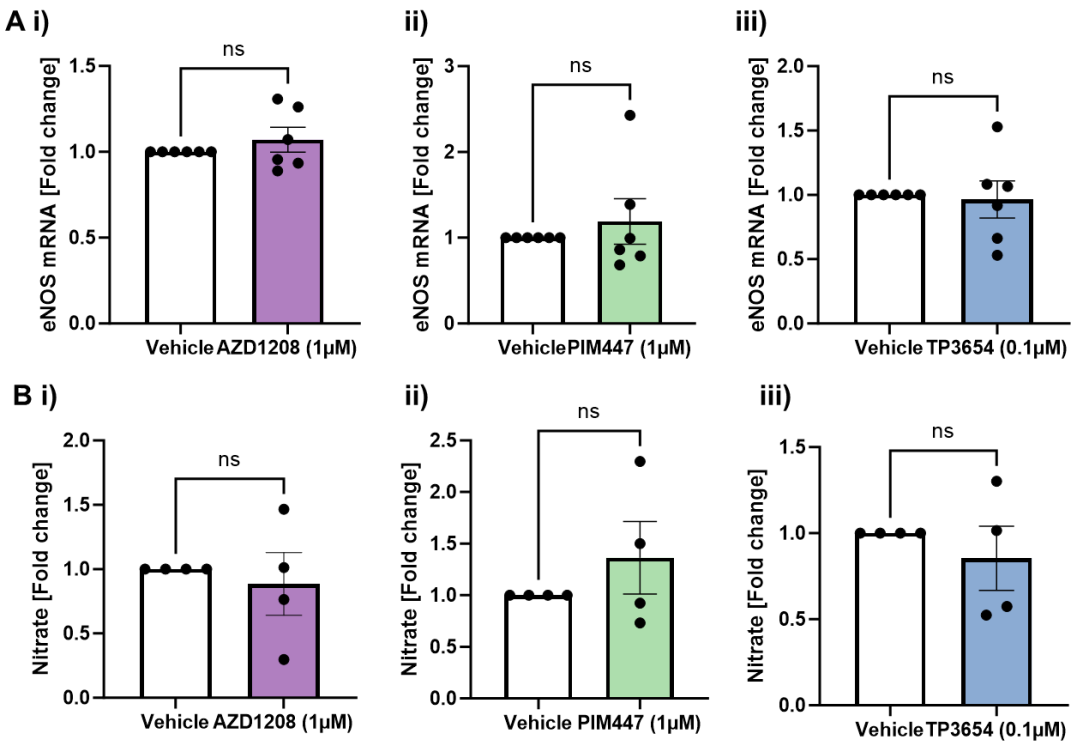

Figure 5.8: Pim kinase inhibitor treatment does not affect eNOS gene expression or nitrate release. HCAECs were treated with (i) AZD1208 (ii) PIM447 (iii) TP3654 treatment for 24 hours and conditioned media collected for releasate analysis, and cell lysates made for RNA extraction. (A) eNOS gene expression and B) Nitrate release was analysed. Nitrate release was determined as a measure of NO production using a Luminex assay. All measurements were within the standard curve, and range of sensitivity. Experiments were carried out in duplicate, and qPCR results were plotted as fold change in comparison with untreated cells. Gene of interest (GOI) Ct values were normalised to housekeepers GAPDH and RPLP0. Results are expressed as mean + SEM, n=6 for qPCR experiments, and n=4 for Luminex. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.6.2 Pim kinase inhibition does not affect VWF expression or ADAMTS13 release

Increasing evidence indicates that inflammation can cause thrombosis by driving VWF secretion, assembly and buildup of large hyper adhesive VWF multimers, strings and fibers through the inhibition of its cleavage by its regulator, ADAMTS13 contributing to adhesion and deposition of VWF-platelet thrombi in the vasculature (Chen and Chung, 2018).

The transcriptomics performed in the previous chapter indicated decreased VWF gene expression following PIM447 treatment (Figure 4.17). This observation was therefore validated and the effects of Pim kinase inhibition on VWF gene expression, VWF release and ADAMTS13 was measured to give an indication of thrombotic potential.

In contrast to the transcriptomics, no changes in *VWF* gene expression were observed following inhibition of Pim kinase in HCAECs with any of the 3 Pim kinase inhibitors used, including PIM447 (Figure 5.9). Interestingly, an increase in VWF release was observed following inhibition with PIM447 (1µM) (Figure 5.9Bii), although this was only a slight increase, and this effect was not seen with AZD1208 or TP3654. ADAMTS13 release appeared to be unaffected in HCAECs following Pim kinase inhibition. Interestingly, despite the increase in VWF following PIM447 treatment, as with the other Pim kinase inhibitors the VWF:ADAMTS13 ratio, an indicator of the balance between VWF and ADAMTS13 was unaffected by any of the Pim kinase inhibitors.

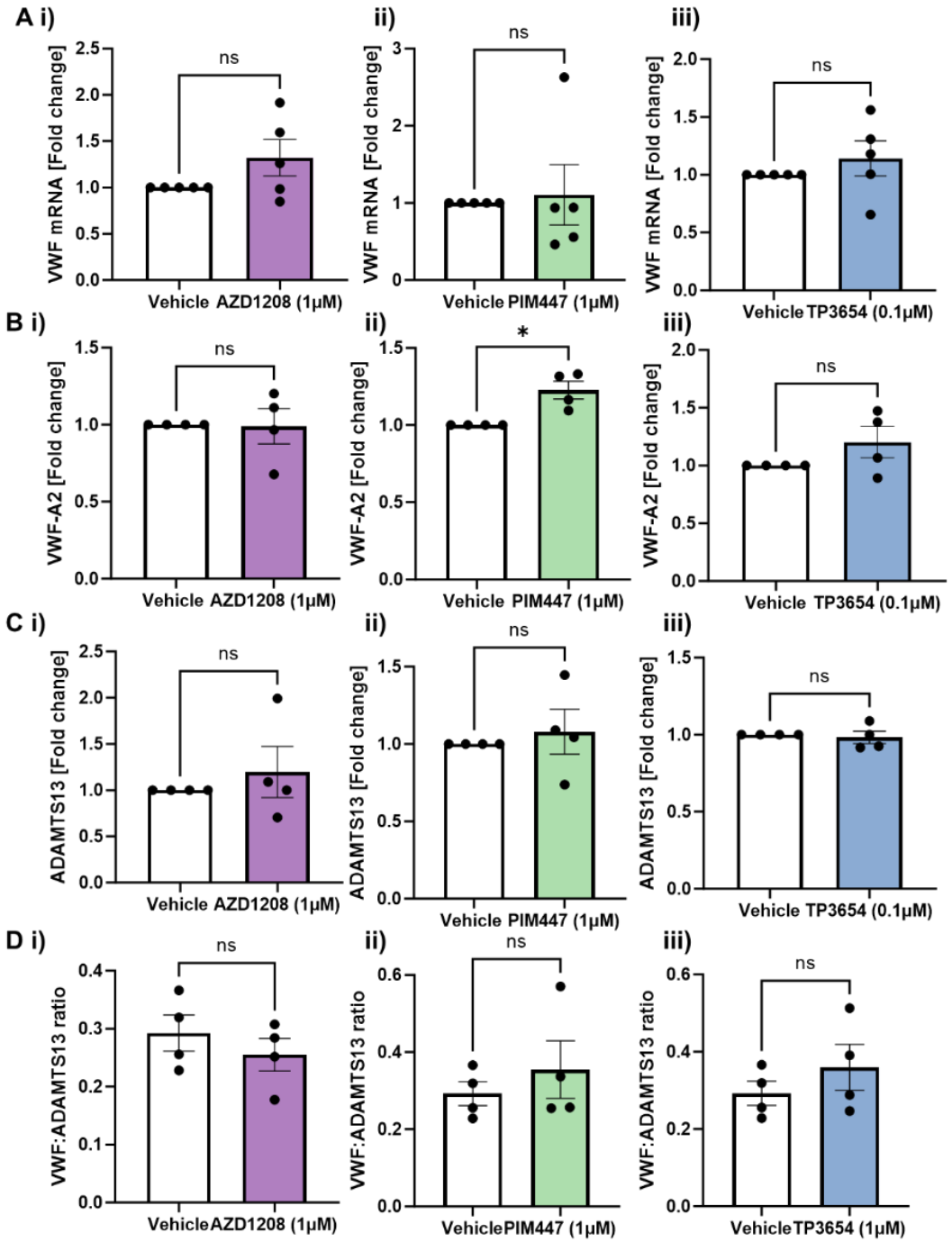

Figure 5.9: Pim kinase inhibition does not alter the VWF:ADAMTS13 axis. HCAECs were treated with Pim kinase inhibitors for 24 hours, conditioned media collected for releasate analysis, and cell lysates made for RNA extraction. (A) VWF gene expression was analysed in HCAECs treated with (i) AZD1208, (ii) PIM447, or (iii) TP3654 treatment for 24 hours. (B) VWF concentration and (C) ADAMTS13 concentration was measured in conditioned media from ECs treated with (i) AZD1208 (ii) PIM447, or (iii) TP3654 treatment for 24 hours, carried out using a Luminex assay. (D) VWF:ADAMTS13 determined from concentration values. Technical repeats were done in duplicate, and qPCR results plotted as fold change in comparison with untreated cells. Gene of interest (GOI) Ct values

were normalised to housekeepers GAPDH and RPLP0. Results are expressed as mean ± SEM, n=5 for qPCR experiments, and n=4 for Luminex. All measurements were within the standard curve, and range of sensitivity. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.6.3 Pim kinase inhibition does not affect COX-1 or COX-2 expression

Endothelial cells produce prostaglandins such as prostacyclin (PGI_2) that suppress platelet activation and aggregation; however, during inflammation, ECs can also generate thromboxane A_2, a potent driver of platelet activation, through COX-1– and COX-2–dependent pathways. It has been previously shown that PIM1 deletion in macrophage-like THP-1 cells suppressed LPS-induced upregulation of COX-2 (Baek et al., 2024), indicating that PIM1 is a positive regulator of COX-2 expression and may play a role in COX regulation in HCAECs.

The effect of PIM kinase inhibition on gene expression of both *PTSG1* (COX-1) and *PTSG2* (COX-2) enzymes was therefore determined in HCAECs to elucidate whether there are any vascular effects. Both COX-1 and COX-2 gene expression was unaffected by Pim kinase inhibition in HCAECs (Figure 5.10).

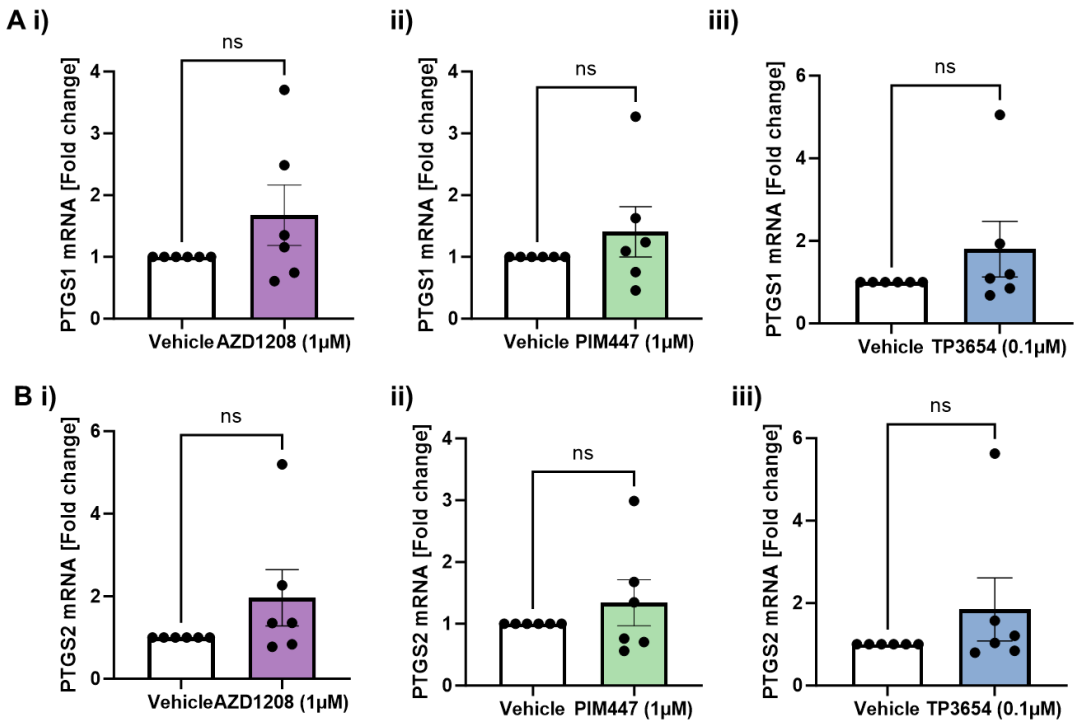

Figure 5.10: Pim kinase inhibitor treatment does not affect COX-1 or COX-2 gene expression. A) COX-1 and B) COX-2 gene expression was analysed in HCAECs treated with (i) AZD1208 (1μM) (ii) PIM447 (1μM) or (iii) TP3654 (0.1μM) treatment for 24 hours. Technical repeats were done in duplicate, and qPCR results were plotted as fold change in comparison with untreated cells. Gene of interest (GOI) Ct values were normalised to housekeepers GAPDH and RPLP0. Results are expressed as mean ± SEM, n=6 for qPCR experiments. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.7 Pim kinase inhibition does not alter endothelial regulation of leukocyte recruitment and adhesion

5.3.7.1 Pim kinase inhibition does not reduce endothelial release of Inflammatory cytokines

The damaged activated endothelium releases cytokines to facilitate leukocyte recruitment, including TNFα, IL-6 and IL-8, with increasing evidence of a crosstalk between leukocytes and ECs in the regulation of immune cell differentiation and activation (Carbone and Failla, 2021). Endothelial production of inflammatory cytokines was therefore measured including IL1β, IL-6, IL-8, and TNFα.

24-hour treatment with Pim kinase inhibitors, AZD1208 (1μM), PIM447 (1μM) and TP3654 (0.1μM) did not statistically alter HCAEC release of IL1β, IL-6, IL-8, or TNFα (Figure 5.11). However, although not statistically significant, AZD1208 and PIM447 did appear to slightly decrease IL-6 release, while AZD1208 also looked to reduce IL-8 release.

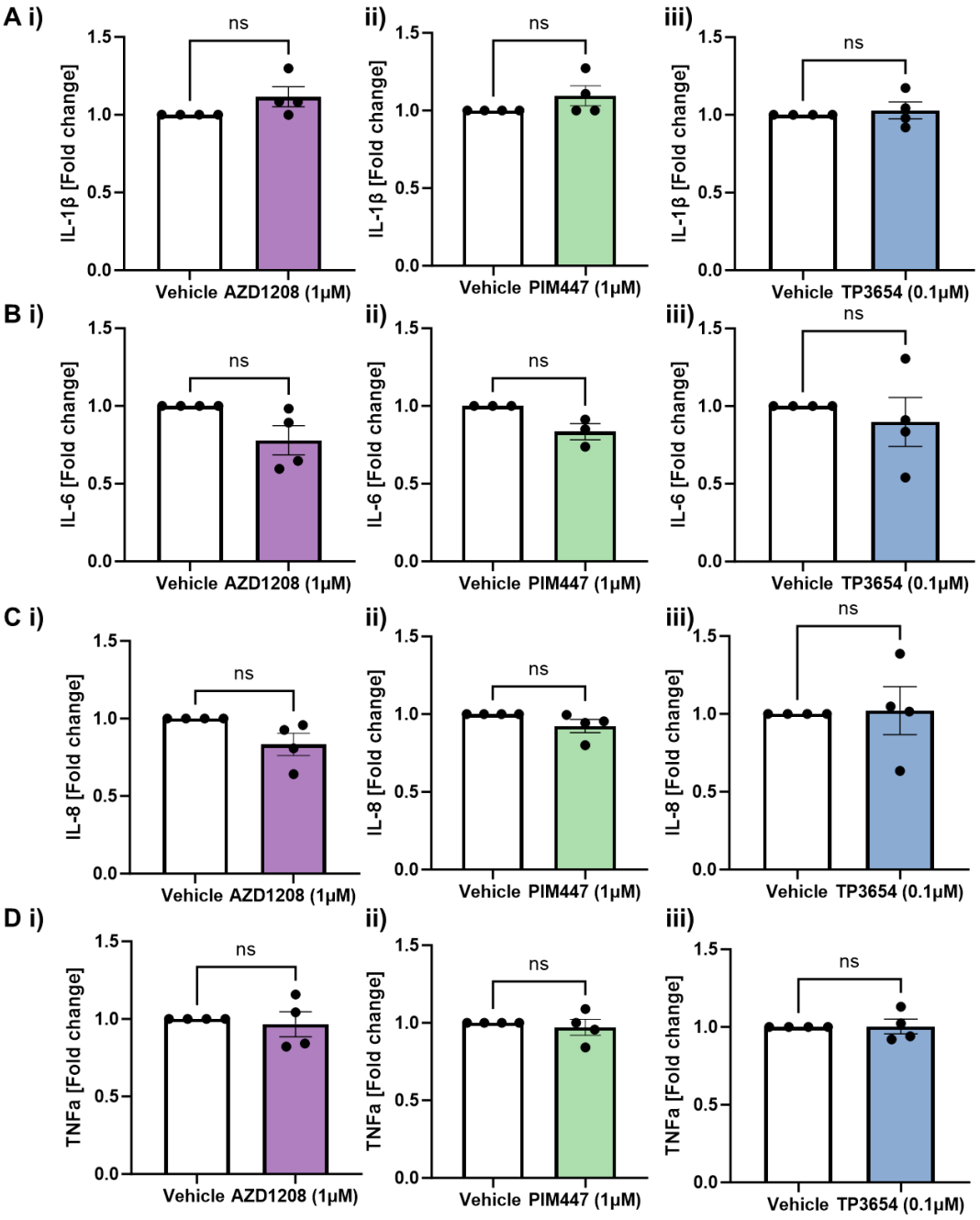

Figure 5.11: Pim kinase inhibitor treatment does not affect IL1β, IL-6, IL-8 or TNFα release in HCAECs. A) IL1β, B) IL-6, C) IL-8, and D) TNFα release was analysed in HCAECs were treated with Pim kinase inhibitors (i) AZD1208 (1μM) (ii) PIM447 (1μM) or (iii) TP3654 (0.1μM) for 24 hours and conditioned media collected. Released levels of inflammatory cytokines were measured using a Luminex assay. Technical repeats were done in duplicate. All measurements were within the standard curve, and range of sensitivity. Results are expressed as mean ± SEM, n=4. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were always performed before normalisation.

5.3.7.2 Pim kinase inhibition does not reduce endothelial release of soluble adhesion molecules

Leukocytes adhere to the activated endothelium via the surface expression of adhesion receptors that are upregulated during inflammation including ICAM-1, VCAM-1 and P-selectin (Granger and Senchenkova, 2010). All three, ICAM-1, VCAM-1, and P-selectin are shed following proteolytic cleavage from activated ECs generating soluble forms that serve as markers of endothelial activation and dysfunction (Pigott et al., 1992). Transcriptomic analysis described in Chapter 4 (Figure 4.9c) demonstrated increased *ICAM1* but decreased *VCAM1* and *SELP* (P-selectin) gene expression in HCAECs following PIM447 treatment.

Therefore, the effects of PIM kinase inhibitor treatment AZD1208 (1μM), PIM447 (1μM) and TP3654 (0.1μM) on endothelial shedding of leukocyte adhesion receptors was measured to determine the inflammatory role of the Pim kinase family in HCAECs. sICAM-1, sVCAM-1, and sP-selectin levels in conditioned HCAEC media were measured using a Luminex assay kit (described in Chapter 2.21).

Interestingly despite alterations in gene expression of ICAM, VCAM, and SELP (P-selectin), treatment with either AZD1208 (1μM), PIM447 (1μM) or TP364 (0.1μM) for 24 hours was not observed to alter endothelial shedding of any of the receptors from HCAECs (Figure 5.12).

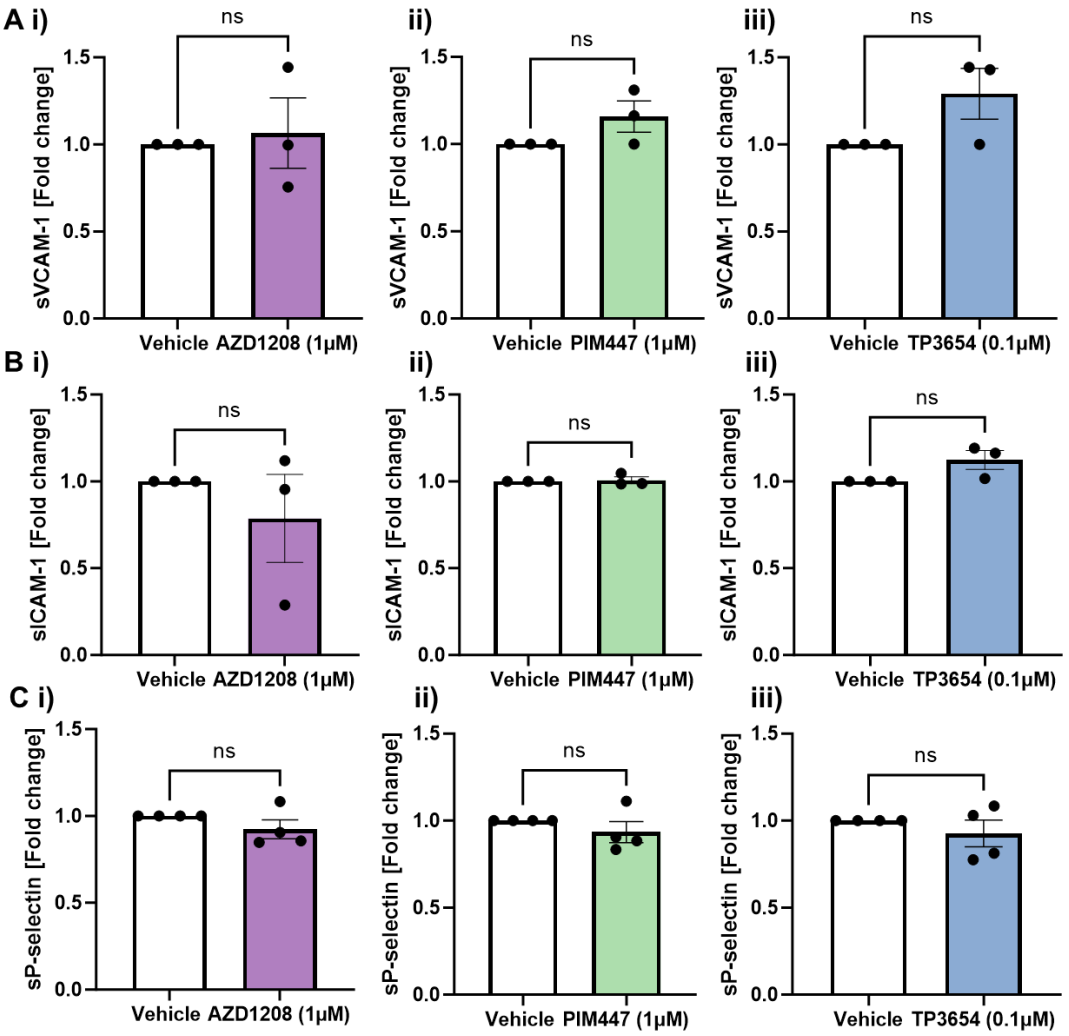

Figure 5.12: Pim kinase inhibitor treatment does not affect sVCAM-1, sICAM-1 or sP-selectin shedding. (A) sVCAM-1, (B) sICAM-1, and (C) sP-selectin concentration was measured in conditioned media from ECs treated with (i) AZD1208 (ii) PIM447, or (iii) TP3654 treatment for 24 hours, carried out using a Luminex assay. All measurements were within the standard curve, and range of sensitivity. Results are expressed as mean ± SEM for n=3. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were always performed before normalisation.

5.3.8 Pim kinase inhibition alters endothelial contributions to coagulation

5.3.8.1 Pim kinase inhibitors delay time to clot formation via the intrinsic pathway

It was demonstrated previously in Chapter 3 that blood from *PIM1*$^{-/-}$ mice delayed clotting time via the intrinsic pathway of coagulation using ROTEM (Section 3.2.4). It was therefore important to investigate whether this observation was mediated via the endothelium, and whether this effect can be replicated using pharmacological inhibitors.

An aPTT assay was carried out to investigate the effects of endothelial Pim kinase inhibition on the intrinsic pathway of coagulation using conditioned media collected from HCAECs treated with Pim kinase inhibitors AZD1208 (1μM), PIM447 (1μM) or TP3654 (0.1μM). Similar to the observations made in INTEM experiments comparing whole blood collected from WT and *PIM1*$^{-/-}$ mice, fibrin clot formation time was significantly delayed in PPP following the addition of releasate from Pim kinase inhibitor treated HCAECs compared to vehicle treated releasate (0.1% DMSO treated cells) ($p=<0.0001$) (Figure 5.13). This effect was true for all 3 Pim kinase inhibitor treatments, demonstrating that Pim kinase inhibition delays clot formation via the intrinsic pathway of coagulation.

Adding releasate from Pim inhibitor treated ECs to PPP confirms that there is an endothelial contribution to observed delayed intrinsic pathway coagulation processes, as data from PPP treated directly with Pim kinase inhibitors did not delay intrinsic pathway coagulation time, indicated by no changes observed in aPTT measurements (see Appendix C, Figure 1).

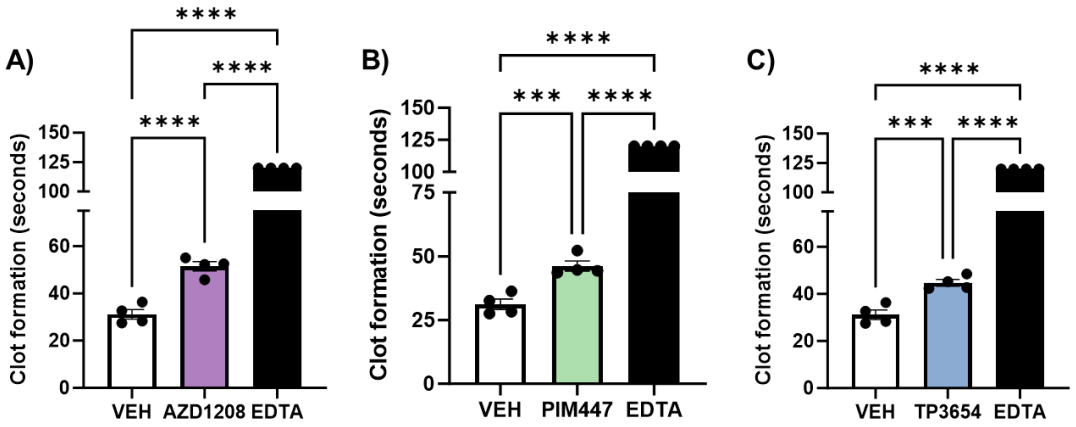

Figure 5.13: Pim kinase inhibitor treatment significantly increases clot formation time via the intrinsic pathway. Releasate from HCAECs treated with (A) AZD1208 (1μM), (B) PIM447 (1μM), and (C) TP3654 (0.1μM) treatment for 24 hours was added to PPP before the addition of aPTT si L minus reagent and calcium chloride (0.025M), and clot formation time in seconds measured on a Helena C-4 Coagulation Analyser. EDTA was used as a positive anti-coagulant control. Technical repeats were done in duplicate. Results are expressed as mean + SEM for n=4. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism.

5.3.8.2 Thrombin initiated clotting time is unaltered by Pim kinase inhibition

It was shown previously in Chapter 3 that whilst the intrinsic coagulation cascade was delayed, thrombin initiated clotting time was unaffected by PIM1 $^{-/-}$ (Section 3.3.5). Having identified that this delay in intrinsic coagulation appears to be driven by the endothelium (Figure 5.13), to investigate whether thrombin initiated fibrin clot formation is maintained following inhibition of all 3 Pim kinase isoforms, conditioned media containing releasate from HCAECs following 24 hour treatment with Pim kinase inhibitors was added to PPP and turbidity assays performed following stimulation by thrombin (0.1U/mL) (Figure 5.14A), and time to initial clotting (lag time) (Figure 5.14B), time to 50% clot formation (Figure 5.14C), time to clot formation (time to maximum optical density) (Figure 5.14D), and maximum optical density (MaxOD) (Figure 5.14E) measured.

All four parameters of clotting time were unaltered by pharmacological Pim inhibition by AZD1208 (1μM), PIM447 (1μM) or TP3654 (0.1μM) in HCAECs (Figure 5.14), indicating that prolonged clotting observed via the intrinsic pathway is not due to a thrombin defect. This suggests that Pim inhibitors don't impact thrombin function, or the conversion of fibrinogen to fibrin, demonstrating that normal clotting can be maintained, which is essential for haemostasis.

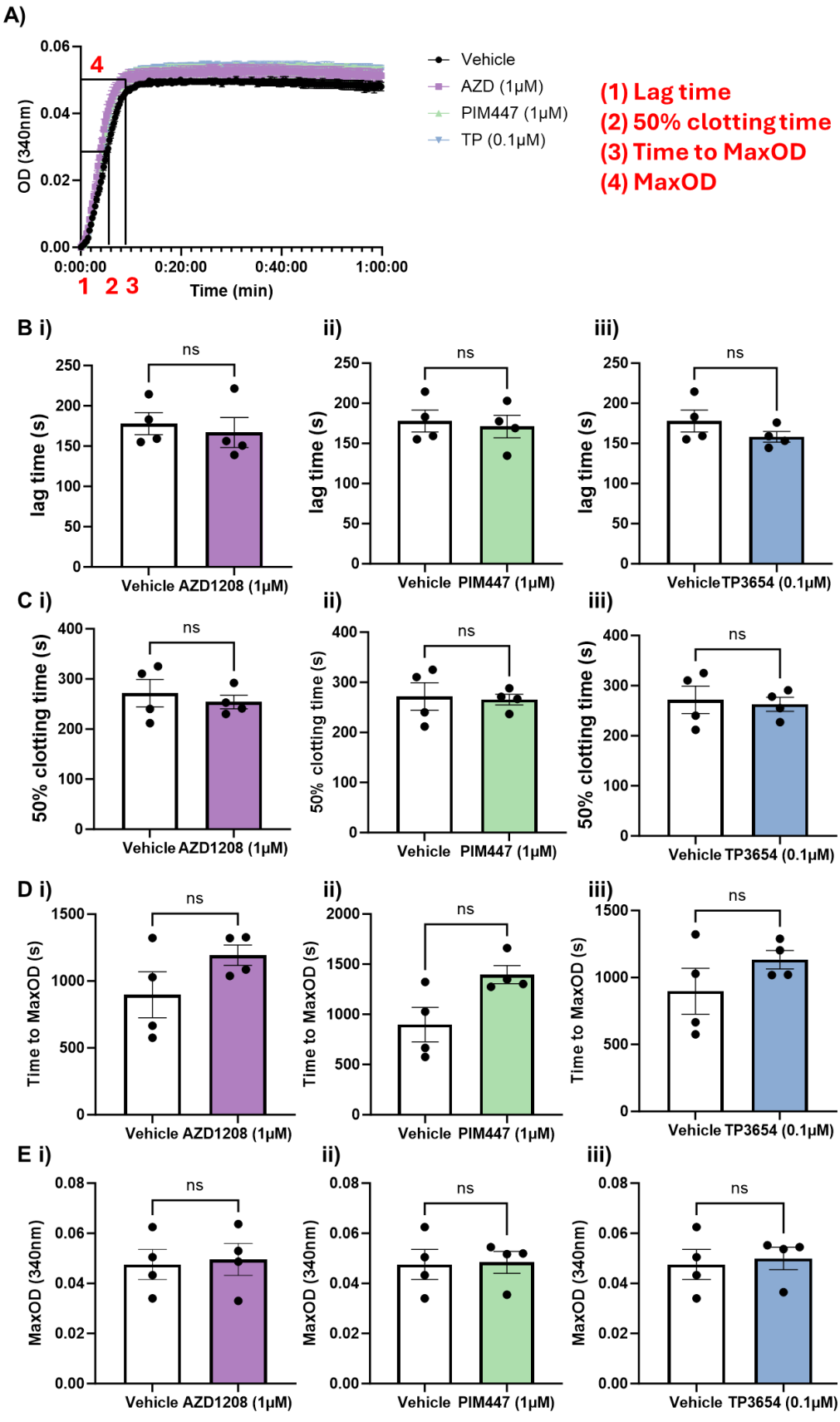
A)
OD (340nm)
Time (min)
1 2 3
4
Vehicle
AZD (1μM)
PIM447 (1μM)
TP (0.1μM)
(1) Lag time
(2) 50% clotting time
(3) Time to MaxOD
(4) MaxOD
B i)
ii)
iii)
lag time (s)
C i)
ii)
iii)
50% clotting time (s)
D i)
ii)
iii)
Time to MaxOD (s)
E i)
ii)
iii)
MaxOD (340nm)
ns
Vehicle AZD1208 (1μM)
Vehicle PIM447 (1μM)
Vehicle TP3654 (0.1μM)

Figure 5.14: Pim kinase inhibition does not affect thrombin-induced fibrin formation. (A) Turbidity assays were performed to measure Thrombin (0.1U/mL) stimulated fibrin clot formation using plasma supplemented with (i) AZD1208, (ii) PIM447, and (iii) TP3654 or vehicle (0.1% DMSO)-conditioned media, producing a turbidity trace (representative trace shown). (B) time to initiation of clot formation (lag time), (C) time to 50% clot formation (50% clotting time), (D) time to fibrin clot formation (time to maximum optical density), and (E) maximum optical density (MaxOD) were recorded and compared. Measurements were taken on a BioTek PowerWave HT microplate reader set at 340nm, taking absorbance readings every 17 seconds for 1 hour at 37°C. Results are expressed as mean ± SEM for n=4. Data was analysed using paired t-tests and graphs plotted using GraphPad Prism.

5.3.8.3 Fibrinolysis

Having identified that Pim kinase inhibition enhances the anti-coagulant properties of the endothelium, delaying clot formation initiated by contact activators (Figure 5.13), but not altering the properties of fibrin formation (Figure 5.14 – thrombin initiated), the effect of Pim kinase inhibition on the endothelial contribution to fibrinolysis was determined next.

Fibrinolysis assays were performed, in the presence of tPA (20ng/mL) and stimulation by Thrombin (0.1U/mL) using plasma treated with conditioned media collected from HCAECs treated with AZD1208 (1μM), PIM447 (1μM) or TP3654 (0.1μM) and vehicle treated control (0.1% DMSO) (Figure 5.15A). Comparison of time to lysis demonstrated that treatment with any of the 3 Pim kinase inhibitors did not alter HCAEC fibrinolytic potential, with no alteration in fibrinolysis measurements, time to 50% lysis (Figure 5.15B), or time to complete clot lysis (Figure 5.15C), compared to vehicle control observed. Interestingly, although no differences were observed in time to 50% lysis, and not statistically significant, a trend towards increased time to full clot lysis was observed following inhibitor treatment compared to vehicle treated control, possibly indicating alterations in clot structure or availability of fibrinolytic factors that requires further investigation.

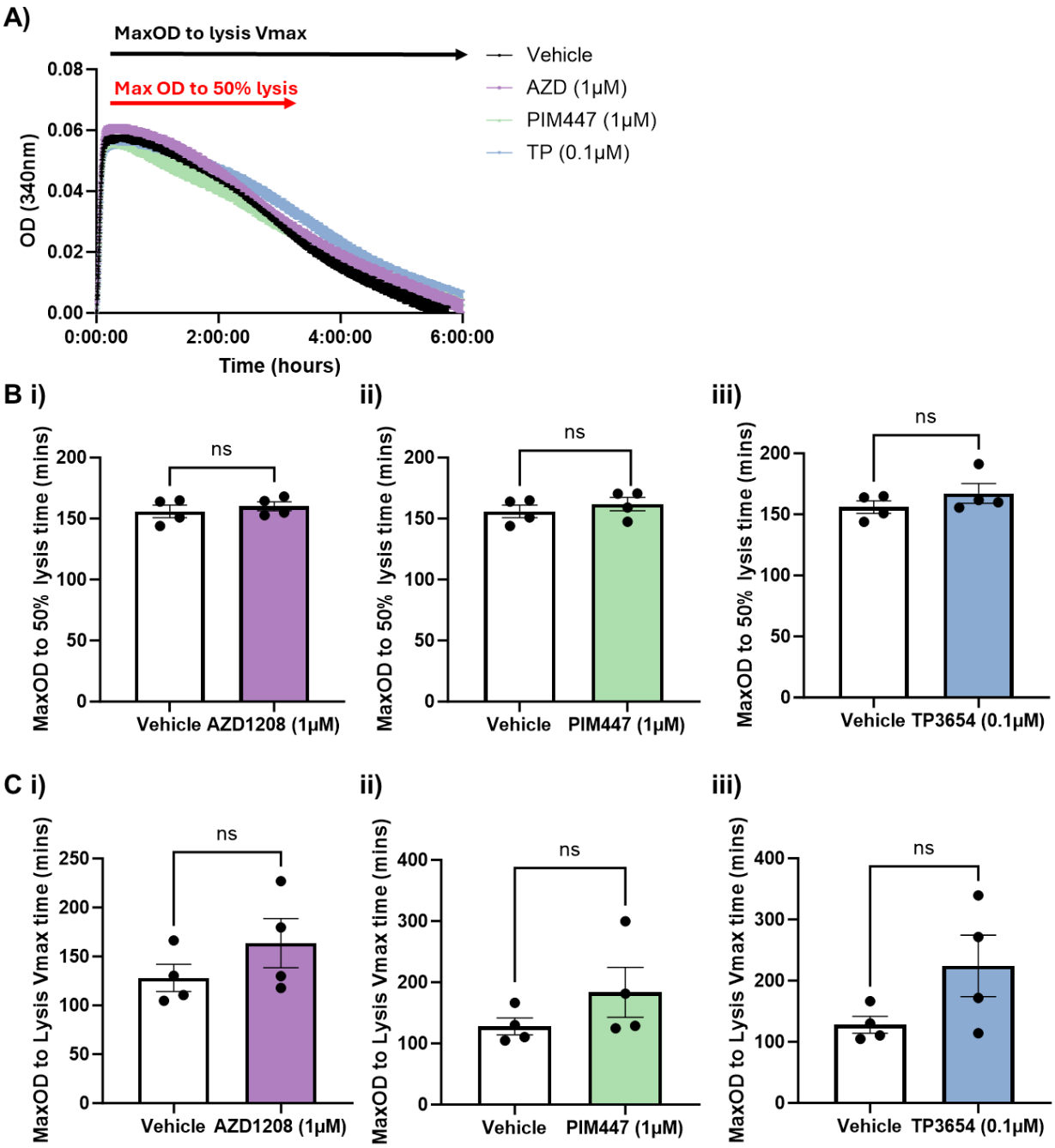
A)
MaxOD to lysis Vmax
Max OD to 50% lysis
Vehicle
AZD (1μM)
PIM447 (1μM)
TP (0.1μM)
OD (340nm)
0.08
0.06
0.04
0.02
0.00
0:00:00
2:00:00
4:00:00
6:00:00
Time (hours)
B i)
ii)
iii)
MaxOD to 50% lysis time (mins)
ns
Vehicle AZD1208 (1μM)
Vehicle PIM447 (1μM)
Vehicle TP3654 (0.1μM)
C i)
ii)
iii)
MaxOD to Lysis Vmax time (mins)
Vehicle AZD1208 (1μM)
Vehicle PIM447 (1μM)
Vehicle TP3654 (0.1μM)

Figure 5.15: Pim kinase inhibition does not affect the endothelial contribution to fibrinolysis. (A) Fibrinolysis was measured following the formation of a clot with plasma activated by thrombin and $CaCl_2$ with the addition of (i) AZD1208, (ii) PIM447, and (iii) TP3654 conditioned media, and the addition of tPA to lyse the clot (representative trace shown), allowing the following measurements, (B) MaxOD to 50% lysis time, and (C) MaxOD to lysis Vmax time. The plate was read on a BioTek PowerWave HT microplate reader set at 340nm, taking absorbance readings every 17 seconds for up to 6 hours at 37°C. Results are expressed as mean ± SEM for n=4. Data was analysed using a paired t-test and graphs plotted using GraphPad Prism.

5.3.9 Endothelial mediators of Coagulation

Having identified Pim kinase inhibited mediated effects on the endothelial contribution of intrinsic coagulation, the effect of Pim kinase inhibition on endothelial gene expression of regulators of coagulation were determined.

5.3.9.1 Mediators of coagulation

In addition to the intrinsic, contact activation pathway, during endothelial damage coagulation is initiated by TF, which activates FVII and the extrinsic coagulation pathway. Previous observations in Chapter 3 show no alteration in EXTEM in $PIM1^{-/-}$ mice (Section 3.3.4), and transcriptomic analysis of PIM447 treated HCAECs did not show any alteration in F3 (TF) gene expression (Section 4.3.8). To further validate these findings, endothelial release of TF was compared in Pim kinase inhibitor treated HCAEC conditioned media compared to vehicle treated controls.

No significant changes in release of TF were observed following Pim kinase inhibition in HCAECs with all 3 inhibitors (Figure 5.16).

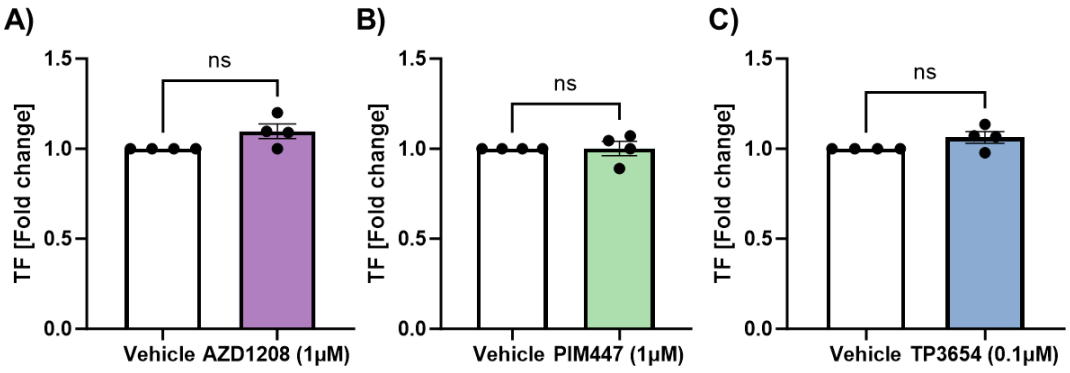

Figure 5.16: Pim kinase inhibitor treatment does not affect TF release. TF release was analysed in HCAECs treated with (A) AZD1208 (1μM), (B) PIM447 (1μM), or (C) TP3654 (0.1μM) treatment for 24 hours, carried out using a Luminex assay. Technical repeats were done in duplicate. Results are expressed as mean ± SEM, n=4. All measurements were within the standard curve, and range of sensitivity. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.9.2 Inhibitors of coagulation

Although TF expression was unchanged, TF activity is critically regulated by TFPI. As there were no alterations observed in TF release, TFPI release was measured to indicate potential regulatory effects of TFPI secretion. Interestingly, TFPI release was decreased by AZD1208 (1μM), PIM447 (1μM), and TP3654 (0.1μM) treatment of HCAECs (Figure 5.17A), reflecting a shift toward a pro-coagulant or dysfunctional endothelial state.

Further inhibitors of coagulation were therefore measured to elucidate the demonstrated pro-coagulant effects of Pim kinase inhibition in HCAECs. Serpin-C1 (antithrombin) release was unaltered following Pim kinase inhibition in HCAECs (Figure 5.17B).

Furthermore, to investigate the effects of Pim kinases on mediators of thrombosis and haemostasis in the arterial vasculature, the effect of Pim kinase on endothelial release of thrombomodulin (THBD), a negative regulator of coagulation (that impacts the intrinsic pathway because it can inhibit FVIII) was measured in conditioned media following 24-hour treatment with Pim kinase inhibitors. There were no notable differences measured between control and treated cells, demonstrating that 24-hour AZD1208, PIM447, or TP3654 treatment did not alter THBD release (Figure 5.17C). This indicates that Pim kinase does not affect THBD levels in HCAECs following 24 hours of treatment.

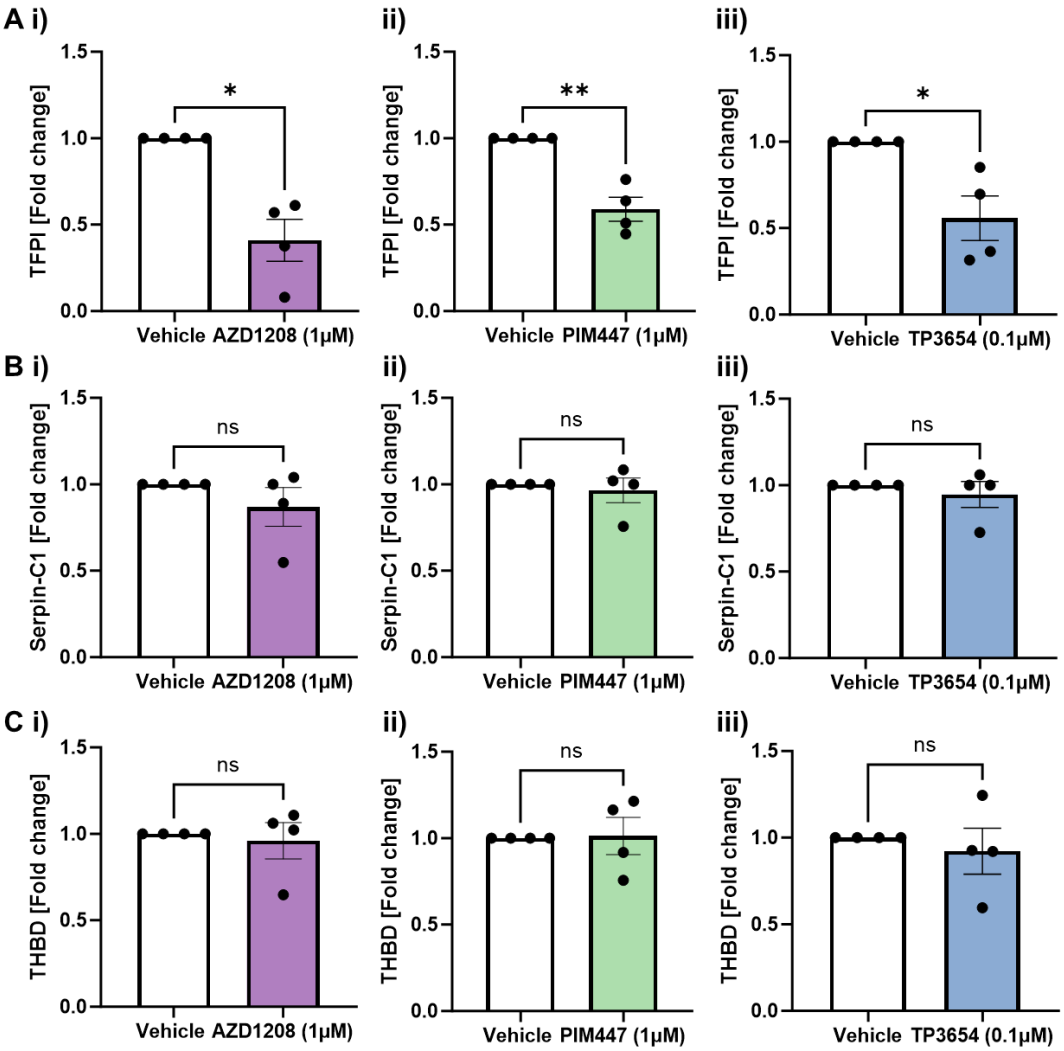

Figure 5.17: Pim kinase inhibitor treatment decreases TFPI release but does not affect Serpin-C1 or thrombomodulin levels. TFPI release was analysed in HCAECs treated with (A) AZD1208 (1μM), (B) PIM447, (1μM) or (C) TP3654 (0.1μM) treatment for 24 hours, carried out using a Luminex assay. All measurements were within the standard curve, and range of sensitivity. Technical repeats were done in duplicate. Results are expressed as mean ± SEM, n=4. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.3.9.3 Regulators of fibrinolysis

Transcriptomics data in Chapter 4 (Figure 4.17) demonstrated increased *Serpin-E1* (PAI-1) gene expression following PIM447 treatment of HCAECs, along with *PLAT* (tPa) which was also increased, indicating that Pim kinase inhibitors regulate mediators of fibrinolysis. Serpin-E1 release was measured to determine functional effects and to confirm that the transcriptional increase led to increased protein production and release. Treatment with AZD1208 (1µM), PIM447 (1µM), or TP3654 (0.1µM) did not result in alterations in Serpin-E1 release (Figure 5.18), indicating that despite the observed increases in gene expression, HCAEC PAI-1 levels were not increased.

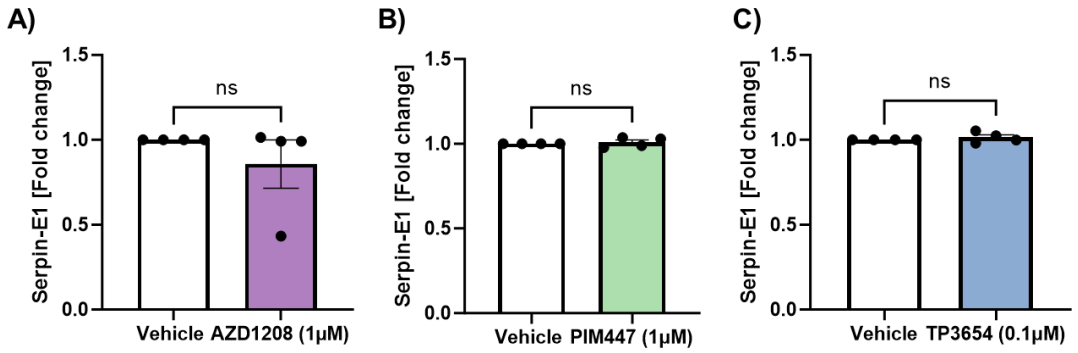

Figure 5.18: Pim kinase inhibitor treatment does not affect SERPIN-E1 release. SERPIN-E1 release was analysed in HCAECs treated with (A) AZD1208 (1µM), (B) PIM447 (1µM), or (C) TP3654 (0.1µM) treatment for 24 hours, carried out using a Luminex assay. All measurements were within the standard curve, and range of sensitivity. Technical repeats were done in duplicate. Results are expressed as mean ± SEM, n=4. Data was analysed using t-tests and graphs plotted using GraphPad Prism. Where normalised data was shown, statistics were performed before normalisation.

5.4 Discussion

The experiments in this chapter focused on investigating the effects of Pim kinase inhibition on the anti-platelet, anti-inflammatory, and anti-coagulant properties of the endothelium, and its interaction with other cells and coagulation factors of the cardiovascular system.

Key Findings:

- TNFα and IL-6 increase Pim isoform gene expression in HCAECs.
- Pim kinase inhibitors delay time to clot formation via the intrinsic pathway.
- TFPI release is decreased following Pim kinase inhibition in HCAECs.
- The role of Pim kinase in endothelial contributions to thrombosis require further investigation.

Gene expression of all 3 Pim kinase isoforms was observed to be increased in HCAECs following exposure to inflammatory mediators TNFα or IL-6. This indicates that Pim kinases are upregulated in inflammation and therefore potentially play a role in EC inflammatory processes. Pim kinases have been previously shown in various cell types to be upregulated in response to mediators of inflammation (Ha et al., 2019, Liong et al., 2017). PIM1 has shown to be involved in LPS-mediated inflammatory responses in macrophage-like cells (Baek et al., 2024), whilst PIM3 mRNA has shown to be increased by TNFα in HUVECs (Yang et al., 2011c) Additionally, a recent study has shown that PIM1 is directly involved in endothelial inflammatory activation during sepsis, demonstrating upregulation in an inflammatory disease context, through promoting TF activity (Wang et al., 2026). Taken together these observations and findings from the literature, Pim kinases are likely key modulators of cellular inflammation. To follow this up, western blotting would be appropriate to measure protein expression to confirm observations from gene expression measurements and provide better insight into phenotypic and functional effects.

Transcriptomic data comparing the impact of Pim kinase inhibitor treatment on HCAECs collected and described in Chapter 4 (Section 4.3.8) identified alterations in gene expression of key endothelial modulators of thrombosis and inflammation, demonstrating possible roles for Pim kinase in thromboinflammation. Gene expression and endothelial-mediated release of various markers and cytokines involved in thrombosis, haemostasis, and endothelial activation were measured in the following categories: platelet regulators (eNOS, VWF, ADAMTS13 and COX-1/COX-2) (Section 5.3.6), leukocyte inflammatory mediators (IL1β, IL-6, IL-8, and TNFα) and cell adhesion molecules (VCAM-1, ICAM-1, and P-selectin) (Section 5.3.7), and coagulation mediators (TF, Serpin-C1 (antithrombin), TFPI, thrombomodulin, and Serpin-E1 (PAI-1)) (Section 5.3.9).

All genes measured and release of the markers were unaltered following Pim kinase inhibition in HCAECs, this was not expected based on the observations in the transcriptomic data presented in Chapter 4 (Figure 4.17). Exceptions were VWF release, which was increased following Pim inhibition with PIM447 (Figure 5.9Bii), and TFPI release, which was decreased by AZD1208, PIM447, and TP3654 treatment of HCAECs (Figure 5.17A). These observations were associated with a high variability between the baseline expression of different HCAEC batches. Although VWF-A release was increased following treatment of HCAECs with PIM447, suggesting that the inhibition of Pim kinases may promote the release of pro-thrombotic VWF, it was not altered by AZD1208 or TP3654 (Section 5.3.6.2), indicating a variable inhibitor response. This is in contrast to transcriptomics results from Chapter 3 indicating an increase in VWF following $PIM1^{-/-}$, with TP3654 being a selective PIM1 inhibitor, a similar response would be expected.

As there were not many differences in gene expression following Pim kinase inhibition in HCAECs, protein expression of the mediators should be measured, as that would give a better indication of phenotypic functional biological activity.

5.4.1 Endothelial contribution to platelet activation

NO mainly secreted by ECs is the most powerful endogenous molecule with vasodilator and anti-thrombotic properties (Costa et al., 2019). The association between Pim kinases and eNOS activity are limited to a single study in the literature that demonstrates that PIM1 regulates eNOS activity in HUVECs, phosphorylating eNOS at Ser-633 (Chen et al., 2016). This prompted the investigation of the effects of Pim kinase inhibition on HCAEC eNOS gene expression and nitrate release, as a measure of eNOS activity and NO synthesis/production in HCAECs, which demonstrated no effects. This could be due to the conditions used in this set of experiments, differing EC type, and treatment timings.

ADAMTS13 is a metalloprotease that cleaves VWF and prevents spontaneous platelet aggregation and pathological thrombosis (Sartori et al., 2025). Beyond haemostasis, ADAMTS13 has important roles in vascular biology, where various studies suggest that it modulates endothelial activation, leukocyte recruitment, and inflammation, and may influence angiogenesis through VEGF signalling and endothelial gene regulation (Woods et al., 2023, Randi, 2017, Gragnano et al., 2017). The VWF/ADAMTS13 axis is implicated in the pathogenesis of atherosclerosis, promoting plaque formation and inflammation (Gragnano et al., 2017). An increase in VWF release may indicate vascular dysfunction following the inhibition of Pim kinases with PIM447; however this is highly unlikely given the small increase (~25%) with only PIM447 (Figure 5.9Bii). This is also at odds with the transcriptomics data from Chapter 4 (Figure 4.17) which demonstrated decreased gene expression of VWF following HCAEC PIM447 treatment. Further markers need to be investigated to confirm these observations.

Although the expression of *PTGS1* (COX-1) and *PTGS2* (COX-2) mRNA was evaluated in this study, additional transcripts downstream of the COX enzymes that could have been investigated are TxA2 synthase and PGI2 synthase, which would alter the ratio of TXA2, a platelet activator and vasoconstrictor, and PGI2, a platelet inhibitor and vasodilator.

5.4.2 Endothelial contribution to leukocyte recruitment and adhesion

Inflammatory cytokines play major roles in endothelial function, where IL1β contributes to increased adhesion molecule expression, increased permeability, and inflammation, while IL-6 contributes to inflammation and EC proliferation (Carbone and Failla, 2021). TNFα induces gene expression of various inflammatory cytokines and chemokines, either dependently or independently of the activation of transcription factors including NF-κB. This TNFα-mediated signalling initiates and accelerates atherothrombosis, vascular remodelling and inflammation, endothelial apoptosis, oxidative stress and impaired NO bioavailability, which contribute to vascular dysfunction (Zhang et al., 2009a). It has been demonstrated in various studies that IL-6 can activate the JAK2/STAT3 signalling pathway, upstream of Pim kinases, resulting in an upregulation of the expression of PIM1 (Jin et al., 2014, Gao et al., 2019, Block et al., 2012). Endothelial release of inflammatory cytokines IL1β, IL-6, IL-8 and TNFα was measured following Pim kinase inhibition in HCAECs, and not altered (Figure 5.11), suggesting that Pim kinase inhibition does not induce an inflammatory phenotype or modulate endothelial inflammatory cytokine secretion in HCAECs.

A study using apolipoprotein E (apoE)-deficient mice found that reductions in the expression of P-selectin, ICAM-1, or E-selectin provide direct protection from atherosclerotic lesion formation (Collins et al., 2000). Although soluble VCAM-1, ICAM-1, and P-selectin levels were unaltered following Pim kinase inhibition in HCAECs (Figure 5.12), it is possible that surface expression may be altered as transcriptomic analysis in Chapter 4 indicated reduced gene expression of *VCAM1* and *SELP* (p-selectin) following PIM477 treatment. The measurement of soluble molecules assumes that shedding is a surrogate marker for surface expression, however concentrations in the media do not mirror the surface expression of HCAECs in culture therefore future work should be to validate this using flow cytometry, fluorescence microscopy or western blotting of proteins levels.

5.4.3 Endothelial contribution to coagulation and fibrinolysis

As seen previously with plasma from PIM1$^{-/-}$ mice in Chapter 3 (Section 3.3.5), thrombin induced clotting time was not altered following the addition of conditioned media treated with Pim kinase inhibitors to PPP (Section 5.3.8.2), however clotting time was delayed via the intrinsic pathway measured by an aPTT assay (Section 5.3.8.1). This indicates that all three Pim kinase isoforms may play a role in coagulation, affecting endothelial derived factors involved in the intrinsic pathway of coagulation (FVIII). No effects on fibrinolysis (Figure 5.15) indicate that Pim kinases contribute to coagulation, with fibrinolysis processes unaltered. Interestingly, these effects were seen both *in vitro* and *ex vivo*.

Following vascular injury, TF is exposed to the blood and binds FVIIa. The TF-FVIIa complex initiates the coagulation cascade by activating FX which contributes to the cleavage of fibrinogen to fibrin by thrombin (Wood et al., 2014). Endothelium-derived TFPI is an inhibitor of TF-FVIIa–mediated activation of FX (Hubbard and Jennings, 1987). TFPI release was decreased following Pim kinase inhibition with all 3 inhibitors (Figure 5.17A), in addition to a decrease in gene expression following HCAEC treatment with PIM447 demonstrated in Chapter 4 (Figure 4.9c). Reduced soluble TFPI in the supernatant could reflect reduced total TFPI production, increased retention on the cell surface or reduced shedding/release. Further assays are required to elucidate the exact cause of reduced TFPI. To determine whether total TFPI is reduced, total cellular TFPI should be determined using western blotting, and surface TFPI using flow cytometry. Moreover, as TFPI was decreased, but there was no effect observed on TF release, a TF surface activity assay in HCAECs could also be performed to confirm that the mRNA findings translate into TF protein expression.

Haemostasis and fibrinolysis are the consequence of a complex series of events. Serine proteases involved in these processes are regulated by feedback loops, local cofactors, and serine protease inhibitors (Serpins). The balance between proteolytic and inhibitory reactions in haemostasis and fibrinolysis

(coagulation, protein C and fibrinolytic pathways), can be disrupted, resulting in either pathological thrombosis or abnormal bleeding (Rau et al., 2007). Serpin-C1 (antithrombin) and Serpin-E1 (PAI-1) are involved in the regulation of coagulation. While AT predominantly inhibits thrombin, it can also inhibit coagulation factors, FIXa, FXa and FXIa. In addition to its anti-coagulant activity, AT has been shown to have anti-inflammatory and anti-angiogenic functions (Rau et al., 2007). PAI-1 regulates both tPA and uPA and is considered the main inhibitor of plasminogen activation (Cesarman-Maus and Hajjar, 2005). Both Serpin-C1 (Figure 5.17B) and Serpin-E1 (Figure 5.18) release was unaffected by Pim kinase inhibition in HCAECs, demonstrating no notable effects on coagulation or regulators of fibrinolysis.

Thrombomodulin-mediated binding of thrombin leading to protein C activation has anti-coagulant, anti-fibrinolytic, and anti-inflammatory properties for the vessel wall (Conway, 2012). It has previously been shown that thrombin downregulates thrombomodulin expression and activity in ECs (Seguin et al., 2008). Although thrombin-induced clotting time was unaffected by Pim kinase inhibition, thrombomodulin levels are a key indication of endothelial anti-coagulant function and therefore thrombomodulin release was measured in HCAECs following Pim kinase inhibition, showing no notable effects (Figure 5.17C).

5.4.4 Limitations

1. HCAECs used in experiments were from healthy Caucasian male donors with no comorbidities between the ages of 50-65 years old. It has been shown in various studies that responses can vary based on donor differences or disease states, where donor comorbidities result in differential endothelial inflammatory responses. A study has shown that HCAECs exposed to plasma derived small extracellular vesicles (EVs) from donors with diabetes show altered inflammatory and endothelial dysfunction markers indicated by reduced phosphorylated eNOS expression and NO levels, increased reactive oxygen species (ROS)

production, and elevated ICAM-1–mediated inflammatory pathways in HCAECs (Abd-Elmoniem et al., 2024) indicating that donor metabolic disease status can modulate inflammatory responsiveness. Additionally, another study comparing diabetic HCAECs vs non-diabetic HCAECs has shown baseline differences in proteomic and immune signalling pathways and altered cellular responses to therapeutic mediators where key metabolic, growth, and stress/starvation cellular responses were significantly altered in DM-HCAECs in comparison to that of HCAECs at baseline (Xu et al., 2023). Differing effects of inflammatory mediators of HCAECs may be observed based on differences in the comorbidities of the donors and donor metabolic status. This should be investigated in future.

2. RT-qPCR experiments demonstrated that TNFα and IL-6 failed to induce dysfunction in expected mediators of thrombosis, haemostasis, and endothelial activation, including VWF, PTGS1, and VCAM-1. This is in contrast to the literature where it has previously been shown that the adhesion genes (E-selectin, VCAM-1, and ICAM-1), inflammatory cytokines (IL-6, IL-1β, and TNFα), chemokines (MCP-1 and IL-8), and TF were upregulated following the induction of inflammation in HUVECs with inflammatory cytokines TNFα, IL-6, and IL1β (Gao et al., 2018). Additionally, it has been shown in the literature that TNFα suppresses transcription of the thrombomodulin gene in HUVECs (Conway and Rosenberg, 1988). eNOS, PTGS2, and thrombomodulin gene expression was also not altered as expected following 24-hour treatment of HCAECs with TNFα or IL-6. Attempts were made to overcome the limitation by trying different batches and manufacturers of TNFα and IL-6. Failure to evoke reproducible endothelial dysfunction severely limited the investigation of whether Pim kinase inhibition protects against endothelial dysfunction.
3. Despite the resistance of HCAECs to TNFα and IL-6, HCAECs are still the most disease-relevant cell model to investigate the role of Pim kinase

inhibition in the arterial endothelium. It has been shown in the literature that TNFα stimulation of HCAECs significantly upregulated gene expression of CRP, tPA, PAI-1, TNFR1, and TNFR2, however IL-6 consistently had no significant impact on all genes assessed (Humphreys et al., 2025). No significant differences were reported in gene expression of fibrinogen α chain, thrombomodulin, uPA, VWF, vitronectin, or IL-6 receptors (Humphreys et al., 2025). This demonstrates that TNFα did affect key HCAEC thromboinflammatory mediators, but not many, whilst IL-6 did not alter gene expression of any mediator, confirming our observations. This could be due to a lack of SS induced in HCAECs in both studies. It has been shown that the transcription profile changes significantly with flow, as shown by a study comparing SS effects across vascular EC types where SS caused significant changes in global gene expression patterns, with haemostatic and thrombotic genes differentially regulated (Cox and Ng, 2025).

Various studies have been conducted to demonstrate the differential transcriptomic and individual gene profiles of ECs in static culture compared to ECs introduced to flow. A study demonstrated using transcriptomic profiling that among 16,313 well-expressed genes identified in the HCAEC transcriptome, 8,177 were detected to be differentially expressed in OS vs. LS conditions and 9,369 in static vs. LS conditions. Notably, only 1,618 were differentially expressed in OS vs. static conditions (Qiao et al., 2016) demonstrating that HCAECs cultured under static and oscillatory SS conditions are similar (Qiao et al., 2016). They also confirmed using qPCR data, a comparable fold change of gene expression level in ECs under static and OS conditions compared with those under LS conditions (Qiao et al., 2016).

Another study conducted by Afshar *et al* demonstrated that over 43% of the HUVEC transcriptome was significantly changed by the *in vitro* environment, and subjecting cultured HUVECs to long-term SS significantly rescued the expression of approximately 17% of genes

(Afshar et al., 2023). It was therefore considered whether this lack of EC activation by TNFα and IL-6 may be due to the HCAECs being cultured under static conditions, as it has been shown in the literature that the transcriptome is significantly changed by lack of exposure to shear stress (SS). The cause of the resistance of HCAECs to TNFα and IL-6 has yet to be elucidated but along with the lack of SS applied, it could be related to batch or passage variability, as the cells used are primary cells from 50 to 70-year-old males.

4. Additionally, levels of the endothelial regulators of haemostasis and thrombosis may differ between sexes, as demonstrated by various studies where sex differences have been shown in levels of haemostatic regulators and endothelial dysfunction markers (Ossei-Gerning et al., 1998, Ellery et al., 2018), and it has been demonstrated that sex hormones affect endothelial signalling and consequently modulate endothelial function (Chen et al., 1999). Whilst the intention had been to include cells sourced from both sexes, this was not possible due to stock availability. Culturing HCAECs from both males and females under flow would be appropriate to re-assess the mRNA and protein levels of the endothelial regulators of haemostasis and thrombosis evaluated in this chapter.

5.4.5 Chapter conclusions

The main findings in this chapter indicate that gene expression of all 3 Pim kinase isoforms is increased following HCAEC treatment with inflammatory mediators TNFα and IL-6, suggesting a role for Pim kinases in endothelial dysfunction. The most significant functional finding was a prolongation of clotting time via the intrinsic pathway, demonstrated by an increased aPTT clotting time, consistent with observations in Chapter 3 where PIM1 deletion produced a similar delayed clotting phenotype in INTEM. These results suggest that whilst Pim kinase activity is not required for thrombin induced clot formation, Pim kinase inhibitors offer endothelial anti-coagulant potential by modulating intrinsic coagulation pathway activity. No changes in fibrinolysis were observed when comparing PPP exposed to EC conditioned media from Pim kinase inhibitor treated versus control treated cells. Investigation of the endothelial expression of platelet, coagulation and fibrinolytic regulators of thrombosis did not identify any alterations in these regulators following treatment with Pim kinase inhibitors, highlighting the need for further investigation into the specific biological roles of Pim kinases within endothelial regulation of thrombosis. Further investigations are required to identify the mechanisms by which Pim kinases regulate endothelial contributions to thrombosis.

5.5 Chapter 5 summary abstract

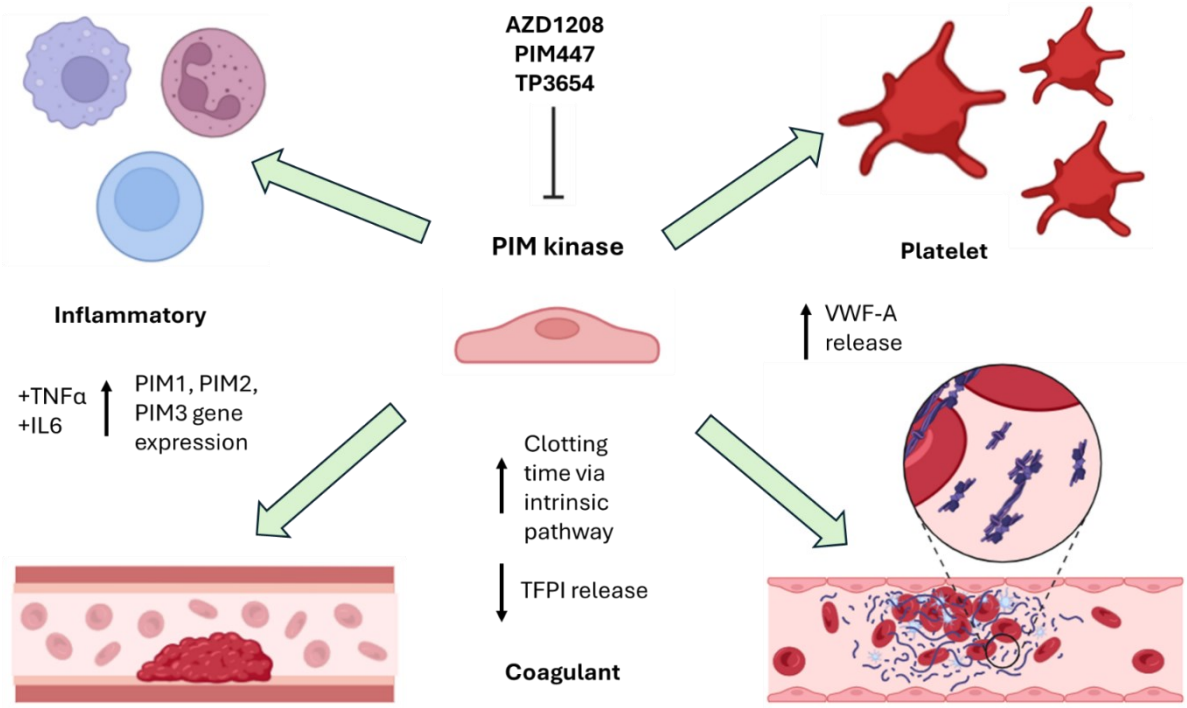

Figure 5.19: Summary abstract of the anti-thrombotic, anti-coagulant, and anti-inflammatory effects of Pim kinase inhibition on HCAECs experiment results in this chapter. The anti-thrombotic, anti-coagulant, and anti-inflammatory properties of HCAECs experiment results summarised in this Chapter. Created in BioRender and PowerPoint.

6. General discussion

6.1 Introduction

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality globally, influenced by an interplay of various genetic, environmental, and atherothrombotic factors (Alrahimi et al., 2024). Atherosclerosis, a process underpinned by the complex interaction of various cardiovascular cell types, is at the core of many CVDs (Alrahimi et al., 2024).

Due to the wide understanding of Pim kinases and their contribution to cell survival, proliferation, and migration (Santio et al., 2020, Torres-Ayuso et al., 2024), Pim kinases have been extensively investigated in the context of cancer in various studies (Popivanova et al., 2007, Keeton et al., 2014). More recently, a role for Pim kinases in endothelial functional processes including angiogenesis has been established (Chen et al., 2016). While there have been several publications on Pim kinases and their role in HUVECs (Yang et al., 2011c, Siemerink et al., 2012), there has been limited investigation on the contribution of Pim kinases to arterial vasculature and atherothrombotic processes described to date.

The most widely used ECs in research are HUVECs because of their convenience and ease of culturing along with their use as cost-effective alternatives to HCAECs, as HCAECs are roughly 3x more expensive than HUVECs to purchase (Promocell, 2025). ECs have been shown to be heterogeneous in their behaviour, depending on the vascular bed they originate from (Larionov et al., 2024). The most relevant blood vessels in human arterial thrombosis are the coronary arteries. Although HUVECs are cheap and more easily obtainable, HCAECs are a more representative model of the arterial contribution that is associated with atherosclerosis and expression of Pim kinases in HCAECs was established. To further characterise the contribution of PIM1 to thrombosis, this project used a genetically modified mouse model, using ECs isolated from WT and PIM1$^{-/-}$ mouse lungs (PECs).

6.1.1 Project aim

This thesis aimed to investigate the role of Pim kinase in the arterial endothelium and its contribution to atherothrombosis. The experiments carried out in this project to date incorporate a multi-model approach, using *ex vivo* pulmonary endothelial cells (PECs) from a PIM1$^{-/-}$ mouse model, and *in vitro* HCAECs treated with pharmacological Pim kinase inhibitors, AZD1208, PIM447, and TP3654.

6.2 Key findings

In this project, several novel findings were described following the investigation of the role of Pim kinases in HCAECs. In the following sections, the main findings of this project from results chapters 3, 4, and 5 will be summarised along with a discussion of the repurposing potential of currently existing Pim kinase inhibitors. The key findings from this project support the hypothesis of this work where Pim kinase inhibition promotes the anti-thrombotic capacity of the endothelium and reduces endothelial activation and dysfunction.

6.2.1 Pim kinase gene expression is affected by pharmacological inhibition and PIM1 deletion

PEC gene expression of PIM2 and PIM3 was decreased in *ex vivo* experiments following PIM1 genetic deletion, demonstrating PIM1 expression positively regulates expression of the other isoforms. Interestingly however, *in vitro*, HCAEC gene expression of PIM1, PIM2 and PIM3 was observed to increase following pharmacological inhibition, indicating cellular compensation mechanisms to recover kinase activity following partial blockage kinase activity, which has implications for prolonged treatment and could lead to drug resistance. The differences in observations could be explained by differences between mouse and human Pim kinase regulatory mechanisms. Another explanation could be that pharmacological inhibition will likely be partial, so HCAECs are able to increase gene expression, whereas in PIM1$^{-/-}$ mice there can be no compensation for lack of PIM1 following genetic deletion, and if PIM1

controls PIM2 and PIM3, the cell won't be able to compensate. HCAECs are a more representative cell type for the study of atherothrombosis, however *in vitro* static conditions trigger stress responses which may activate intracellular signalling pathways that significantly modify gene expression, therefore are not completely representative of *in vivo* conditions of healthy HCAECs exposed to flow and laminar shear stress (Qiao et al., 2016, Afshar et al., 2023), therefore this should be considered.

6.2.2 PIM1 kinase is a regulator of coagulation and thrombosis

It has previously been shown that Pim kinases regulate platelet biology, reducing thrombus formation, but not impairing haemostasis (Unsworth et al., 2021, Nock et al., 2025, Nock et al., 2024). In these studies, clotting time was prolonged via the intrinsic pathway, demonstrated by ROTEM in Chapter 3 (Section 3.3.4) using whole blood from PIM1$^{-/-}$ mice and aPTT assay using human blood treated with conditioned media collected from pan-Pim kinase inhibitors AZD1208, PIM447, and TP3654 treated HCAECs in Chapter 5 (Section 5.3.8.1), but the extrinsic pathway and clot breakdown by fibrinolysis was not affected. Interestingly, although there was no measured effect on fibrinolysis, in the assays performed. Gene expression of PLAT (tPA), PLAU (uPA), and PLAUR (uPA receptor) were upregulated following both PIM1 genetic deletion and PIM447 treatment of HCAECs, demonstrating a possible protective effect of the Pim kinase inhibition on fibrinolytic processes.

It has also recently been shown in the literature that PIM1 promotes upregulation of TF and coagulation processes in sepsis (Wang et al., 2026). This indicates that Pim kinases, specifically PIM1, plays a role in coagulation processes in health and disease states, and therefore presents as a potential target for the prevention and treatment of thrombotic events. However, Pim kinase mediated regulation of other pro-thrombotic factors is not straight forward as in this study, transcriptomic analysis of PECs, identified increased VWF mRNA expression following PIM1 deletion, but decreased in HCAECs following PIM447 inhibition treatment. This indicates potential isoform specific roles with the roles of PIM2

and PIM3 requiring further investigation but does suggests that pan Pim kinase inhibition may actually be required to limit thrombotic marker expression.

6.2.3 Pim kinase is a regulator of the coronary arterial endothelium

In Chapter 4, a simplistic approach to investigate the role of the Pim kinases in HCAEC biology was undertaken using HCAECs cultured under static conditions to investigate the effects of AZD1208, TP3654, and PIM447 treatment on HCAEC functions.

HCAEC viability was investigated due to the previously established effects of Pim kinase phosphorylation on cancer cell proliferation, cell cycle progression, and evasion of apoptosis. Based on previous reports stating that Pim kinases promote cell cycle progression and evasion of apoptosis signals (Santio and Koskinen, 2017), there was concern that Pim kinase inhibitors would reduce proliferation rate and initiate active cell death (Lee et al., 2019), limiting their potential for repurposing. In contrast to findings in cancer cell lines, Pim kinase inhibitors were not observed to affect HCAEC survival at physiologically achievable *in vivo* concentrations (up to 10μM) (El Chaer et al., 2022, Foulks et al., 2014). These concentrations were subsequently used in functional experiments.

Whilst no impact on endothelial proliferation, adhesion, morphology or migration was observed following Pim kinase inhibition or deletion, decreased angiogenesis was observed in both PIM1$^{-/-}$ mice PECs and pan-Pim kinase inhibitor treated HCAECs, indicating potential for targeting Pim kinases in neovascularisation in atherosclerosis and cardiovascular events, along with cancer metastasis. Additionally, expression of IL-33 has shown to promote angiogenesis (Yu et al., 2024), and was observed to be significantly decreased following PIM447 treatment of HCAECs, further indicating anti-angiogenic potential of Pim kinase inhibition.

Transcriptomic analysis from Chapter 4 (Section 4.3.8) identified an upregulation of flow regulatory genes KLF2 and KLF4 following PIM447

treatment, indicating targeting potential of Pim kinases for maintaining endothelial function in atherosclerosis and other diseases.

6.2.4 Pim kinase regulates the inflammatory properties of the endothelium

Pim kinases are important intermediates in the cytokine signalling pathways of inflammatory diseases, and PIM1 particularly has been shown to play an important role in inflammatory responses (Baek et al., 2020). Gene expression levels of PIM1, PIM2, and PIM3 in damage induced HCAECs was therefore determined and compared in HCAECs following treatment with TNFα and IL-6, inflammatory modulators of endothelial dysfunction. TNFα significantly increased PIM1 and PIM3 gene expression, and IL-6 significantly increased PIM1, PIM2, and PIM3 gene expression, highlighting a potential role for Pim kinase in endothelial dysfunction. In addition, IL-6 gene expression was observed to be downregulated following both PIM1 deletion and PIM447 treatment in ECs, suggesting that targeting Pim kinases may be an appropriate strategy for regulating the expression of inflammatory cytokines, and preventing dysfunction of the endothelium.

P-selectin, similar to PECAM-1 was increased in ECs following genetic deletion of PIM1; however, it was decreased following pan-Pim inhibition, indicating that inhibition of all 3 Pim kinase isoforms is required for a protective effect on inflammatory properties and the regulation of inflammatory cytokines and adhesion molecules of the endothelium.

6.3 Repurposing potential of Pim kinase inhibitors

Based on key findings extrapolated from this thesis, the repurposing potential has been highlighted for Pim kinase inhibitors. The roles for Pim kinase in cancer, mechanisms of thrombosis, present Pim kinase inhibition as an appropriate and attractive targeting strategy for both the prevention and treatment of atherosclerotic processes and cancer-associated thrombosis (CAT).

In Chapter 3 (Section 3.3.2), it was demonstrated that PIM1 deletion does not affect mouse body weight or size. This is instrumental as it indicates that functional metabolic processes are not affected, and therefore a lack of PIM1 is safe and tolerable. Additionally, it was demonstrated in Chapter 4 (Section 4.3.3) that Pim inhibitors AZD1208, PIM447 and TP3654 are not cytotoxic to HCAECs after 24 hours of treatment. Again, this importantly highlights that the functional blockade of all three Pim isoforms is tolerable by HCAECs, and their survival is not compromised. This safety profile is crucial as Pim kinase inhibitors have been largely developed to target uncontrollable cell proliferation and promote apoptosis in cancer cells (Rathi et al., 2021), and this would not be appropriate in the context of ECs. Although this has potential, further studies are necessary to determine the cardiotoxicity profiles of Pim kinase inhibitors in disease-relevant cohorts.

6.3.1 Pim kinase inhibition to prevent Atherosclerosis and Atherothrombosis

Atherosclerosis is a chronic inflammatory process, initiated by endothelial activation, followed by a cascade of events including the accumulation of lipids, oxidation, monocyte recruitment and differentiation, and foam cell formation (Ross, 1999), which triggers vessel narrowing and activation of inflammatory pathways, resulting in atherosclerotic plaque formation and calcification (Jebari-Benslaiman et al., 2022). Atherothrombosis, the process of thrombus formation on disrupted atherosclerotic plaques, is a major player in CVD related deaths, being the leading cause of cardiovascular morbidity and mortality globally (Bhatt et al., 2006, Asada et al., 2020).

Current therapies that exist including statins, oral anti-coagulants (Vitamin K antagonists (VKAs)/direct oral anti-coagulants (DOACs)), anti-platelet therapies, and PCSK9 inhibitors reduce risk, however residual inflammatory and thrombotic risk remains, with limited effectiveness (Singh et al., 2024). Furthermore, the effects of anti-platelet therapy are clinically variable in reducing the incidence of death, MI, stroke, and/or the need for further

intervention (Antithrombotic Trialists et al., 2009). There is therefore a requirement for novel upstream signalling-targeted therapies that modulate endothelial dysfunction, smooth muscle proliferation, macrophage phenotype, and thrombotic signalling simultaneously.

It has been evidenced from this project and the literature that Pim kinase activity is linked to various pathological processes contributing to atherosclerosis and atherothrombosis.

6.3.1.1 Endothelial dysfunction/plaque initiation

Increasing evidence suggests that EndMT contributes to the occurrence and progression of atherosclerotic lesions and plaque instability. Pim kinases, which were shown to be increased in this study following TNFα and IL-6 stimulation of HCAECs (Section 5.3.1), have demonstrated to increase endothelial activation. A study using an endothelial cell-specific PIM1 knockdown demonstrated PIM1 deletion negatively regulated atherosclerosis progression and the EndMT process (Xue et al., 2025). The same study reported increased expression of PIM1 in ox-LDL stimulated HUVECs and in human and mouse atherosclerotic lesions, indicating that PIM1 plays a role in oxidative stress pathways, which drive endothelial injury, lipid oxidation, and plaque initiation (Xue et al., 2025). These findings support the potential for PIM1 as a suitable target for the attenuation of atherosclerotic processes.

6.3.1.2 Monocyte recruitment

Findings from this study demonstrated a decrease in VCAM-1, PECAM-1, P-selectin, IL-6, and VWF gene expression following pharmacological Pim inhibition, indicating that Pim kinases play a role in increased adhesion molecule expression and thromboinflammatory signalling, promoting monocyte recruitment. This is supported by a study which has demonstrated a role for PIM1 in monocyte activation, showing that PIM1 knockdown in macrophage-like THP-1 cells suppressed LPS-induced upregulation of pro-inflammatory cytokines (Baek et al., 2024). Additionally, another study has identified PIM1 to act as a key

upstream regulator of CD36, promoting ox-LDL binding, uptake, and foam cell formation, driving atherosclerosis (Beg et al., 2025).

Analysis of blood cell profiles in our study identified that the proportion of the mixed population of WBC (particularly monocytes) was decreased following FBC of PIM1$^{-/-}$ mice, which could have implications for atherosclerotic plaque progression, through reduced monocyte production. This proposes beneficial effects of lack of PIM1 in alleviating monocyte recruitment and foam cell formation, and contribution to the pathophysiology of atherosclerosis.

6.3.1.3 VSMC proliferation and plaque development

It has been demonstrated by Willert *et al* that PIM1 contributes to VSMC proliferation and migration, contributing to plaque growth. The study investigating PIM1 expression and regulation in murine VSMC in response to factors typically present within the atherosclerotic plaque showed that $PDGF_{bb}$, a major mitogen within atherosclerotic plaques, caused a 3-fold increase in PIM1 mRNA expression in VSMCs, followed by robust protein induction, demonstrating that $PDGF_{bb}$-induced proliferation of VSMC is partly attributed to transcriptionally upregulated PIM1 which is essential for downstream atherogenic signalling (Willert et al., 2010). Targeting PIM1 provides a potential strategy for limiting VSMC proliferation in atherosclerosis progression.

6.3.1.4 Neovascularisation and plaque instability

EC angiogenesis-driven neovascularisation of plaque material contributes to atherosclerotic plaque growth and instability (O'Brien et al., 1994). Neovascularisation of plaque regions is associated with a 1.5-fold increased risk of cardiovascular events compared to plaque regions with lower levels of vessel density (Hellings et al., 2010). A decrease in angiogenesis parameters following pharmacological Pim inhibition in our study demonstrates potential for the targeting of neovascularisation and potential for limiting plaque development in the arterial endothelium.

6.3.1.5 Thrombosis

This project identified a role for Pim kinases in coagulation demonstrated by a delay in the intrinsic pathway following Pim inhibition and the regulation of fibrinolysis demonstrated by an increase in tPA and uPA following HCAEC treatment with PIM477, building on previously reported studies elucidating Pim kinase involvement in platelets (Unsworth et al., 2021). These findings are supplemented by the literature where it has been shown that PIM1 upregulates TF, driving pro-thrombotic endothelial activation (Wang et al., 2026).

Findings from this project demonstrate Pim kinases as therapeutic targets that when inhibited would address processes that drive atherosclerosis, including inflammation, endothelial dysfunction, plaque formation and instability and thrombosis. Pim kinase inhibitors could therefore form a new class of dual-acting cardiovascular therapeutics, providing anti-inflammatory and anti-thrombotic benefits simultaneously.

6.3.2 Cancer-associated thrombosis

Cancer-associated thrombosis (CAT) is a major cause of morbidity and mortality in patients with cancer (Tsantes et al., 2024). It is well established that Pim kinases are involved in cancer pathology and cancer progression, supporting cellular proliferation, survival and cancer metastasis. Findings from this project and others have demonstrated that Pim kinases contribute to angiogenic processes. Dysregulated angiogenesis presents a risk of the development of pathological conditions including cancer and thrombotic processes, where a hallmark feature of cancer is its capacity to induce the development of new blood vessels, contributing to tumour growth and disease progression (Han et al., 2025). Furthermore, the dysfunctional endothelium plays a prominent role in both tumorigenesis and CAT, through the expression and release of various inflammatory cytokines (TNFα, IL-6) and thrombotic markers (TF, VWF) which promote platelet activation and activation of coagulation and fibrin formation. Impaired fibrinolysis is also associated with CAP. Cancers with high thrombotic risk including pancreatic, prostate, lung, and haematologic malignancies have

demonstrated to have high Pim kinase expression (Torres-Ayuso et al., 2024, Toth and Warfel, 2021). Targeting Pim kinases therefore presents a novel strategy for limiting both cancer progression and CAT.

There is a requirement for novel prophylactic strategies that go beyond anti-coagulants and act on tumour-driven thrombogenic pathways. The work from this project and previous observations positions Pim kinase inhibition as a dual-action strategy targeting both anti-cancer and anti-thrombotic without relying solely on anti-coagulants. This provides possible avenues for the exploration of the potential use of Pim kinase inhibitors in combination with other cancer therapy in high-risk patients, their use in combination with current anticoagulation strategies, or the need for future mechanistic studies and early-phase clinical evaluation focusing specifically on CAT outcomes which have not yet been done in clinical trials of Pim kinase inhibitors.

6.4 Limitations

There were limitations associated with some of the methods used in this project as highlighted in respective chapters, due to the novelty of some protocols not yet well established, along with the time and resources available for the development of this project.

1. **HCAEC batch variation**

Although different batches of HCAECs were used for experiments in this project, only HCAECs obtained from male donors were used, due to the lack of commercially available HCAECs from female donors. Moreover, the age of the donors was between 50-65 years old. Differences in cardiovascular morbidity and mortality exist between males and females of different ages where in both men and women although the risk of CHD increases with age, a more prominent increase is observed in women after the age of 50 (Mudrovcic et al., 2017, Jousilahti et al., 1999). In future, using a more inclusive research approach to test the effects of Pim kinase inhibition in arterial ECs from women and other age populations would provide an age and sex-specific insight. A study investigating

intrinsic transcriptomic sex differences in human endothelial cells from birth to adulthood analysed HUVEC gene expression data from boy–girl twins at birth and HAECs in non-twin adults to detect sex differences at different stages of life, and measured 2,528 DEGs (25% of the interrogated transcriptome) between the sexes at birth, and 1,798 DEGs (14% of the interrogated transcriptome) between the sexes in adults, demonstrating that 14–25% of the EC transcriptome is sex-biased (Hartman et al., 2020). By combining data from both stages of life, sex differences were identified that are present at birth and maintained throughout life, and those that are acquired over life (Hartman et al., 2020), highlighting that EC studies should take both sex and age into account. Both data from this study and genome-wide association studies (GWAS) revealed that genes with an intrinsic sex difference in ECs are enriched for CAD GWAS hits, emphasising the need for treating sex as a biological variable in cardiovascular research (Nelson et al., 2017).

2. Static culture

ECs used in this project were cultured under static conditions for a simplistic approach in line with most *in vitro* studies focusing on the molecular mechanisms of endothelial function. Some findings, however, are not recapitulated in subsequent translational studies, mostly likely due to the missing biomechanical environment. However, it has been demonstrated that static conditions are more representative of a dysfunctional endothelium, unlikely to elucidate normal endothelial physiology this simple model holds some use for the investigation of disease conditions (Qiao et al., 2016). It would be useful to culture cells under flow/apply physiological shear stress to mimic the *in vivo* environment and the forces that ECs are subjected to. Additionally, with direct relevance for this project and Pim kinase biology, it has been demonstrated that SS directly induces PIM1 expression in HUVECs, regulating the phosphorylation of eNOS Ser633 and Ser1177 (Da-peng et al., 2023), indicating that Pim kinases are mechanosensitive and the regulation of proteins downstream of Pim is impacted by flow. This provides further rationale to investigate the role of Pim kinases in ECs exposed to SS.

3. Lack of heterotypic cell interactions

As for all cells, endothelial biology is dependent on its native environmental which includes cell-cell interactions, soluble factors, and physical forces (Choi and Seo, 2019, Larionov et al., 2024). This context is removed when cells are placed *in vitro* (Afshar et al., 2023). ECs were cultured alone, without SMC and ECM basement membrane proteins which are present *in vivo* as a simplistic approach. It has been shown that co-culture of ECs with SMCs further rescues the *in vivo* transcriptional profile of ECs (Afshar et al., 2023). ECs could be cultured on collagen/laminin/fibronectin to recapitulate the ECM to accurately model *in vivo* conditions as demonstrated in other studies (Hall et al., 2025). Additionally, co-culture with other cell types present in the vasculature would more accurately model an *in vivo* environment. 3D co-culture multicellular systems including 'Vessel-on-a-chip' models exist which enable the more precise modelling of vascular physiology, inflammation, permeability, remodelling, and disease, through mimicking the *in vivo* multicellular environment incorporating flow dynamics and an ECM basement membrane (Bulut et al., 2022, Vila Cuenca et al., 2021).

4. Lack of HCAEC stimulation with TNFα and IL-6

The lack of response from HCAECs to conventional inflammatory mediators in thrombosis and inflammation in Chapter 5 is one of the main limitations of this study and requires troubleshooting. TNFα (5ng/mL) and IL-6 (100ng/mL) failed to induce or reduce the expression of the genes evaluated, which are widely considered markers of endothelial dysfunction. However, the same concentration and batch of TNFα (5ng/mL) and IL-6 (100ng/mL) successfully induced increased Pim kinase isoform gene expression (Section 5.3.1). This is at odds with a study that has shown that TNFα significantly increased the HCAEC surface expression of ICAM-1 and VCAM-1 and significantly enhanced the mRNA expression of IL-6 after both 6hr and 24hr (Qiu et al., 2016). This study however used 10ng/mL TNFα, indicating that a higher dose may be required for stimulation. A dose response of TNFα treatment in HCAECs may be required to assess responsiveness of measured genes of interest.

The expression of all genes was evaluated at the same time point using the same concentration of TNFα (5ng/mL) and IL-6 (100ng/mL), while the time required for up or downregulation is variable depending on the specific gene. This has been demonstrated by a study profiling global changes in the EC transcriptome following TNFα stimulation of HUVECs over an extended time course (0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, or 72hr) identified that TNFα induces EC transcriptome modifications over time (Struck et al., 2024). Genes were grouped into two regulation profile categories for both upregulated and downregulated genes. Upregulated gene categories included group 1, early induction (1-4hr after stimulation) where several genes with well-established roles in the initial stages of inflammation including those encoding for components of the NF-κB signalling pathway, leukocyte adhesion receptors, such as VCAM1 and SELE, and various chemokines such as CXCL8, CX3CL1, CCL2, and CCL20 responded at the earliest timepoint, and group 2, delayed induction (12-24hr after stimulation), whereas downregulated genes included group 1, inhibition maintained over time, and group 2, inhibition resolved over time, where there was a trend back toward baseline level after reaching maximum downregulation (Struck et al., 2024). Therefore, performing a time course with healthy and treated cells to identify the mRNA peak or bottom would be necessary to obtain significant changes in all the genes of the panel. Moreover, culturing HCAECs under arterial flow might help to maximise transcriptional changes, as observed in investigations with TNFα (Satta et al., 2023).

5. HCAEC variability and reactivity

The isolation process of HCAECs is complex and depending on the number of successive divisions required to reach confluency cells can be less reactive, which causes high variability in the results. An appropriate marker to measure HCAEC reactivity would be ICAM-1/VCAM-1 as these are sensitive markers of endothelial activation and strongly upregulated on activated endothelium in response to various cytokines and can be measured to quantify endothelial activation (Videm and Albrigtsen, 2008).

6. **Lack of biological repeats (n numbers)**

Unfortunately, due to lab time lost during ongoing construction works at the University and moving to a new institute, time constraints and issues with the use of some equipment meant that some experiments including mouse PEC experiments in Chapter 3, and thrombus formation experiments in Chapter 5 have fewer biological repeats than others.

6.5 Future directions

6.5.1 Investigation of the role of Pim kinase in the regulation of EC dysfunction

6.5.1.1 Measurement of biomarkers of vascular endothelial function: Endothelial microparticles (EMP), which play a role in coagulation, inflammation, endothelial function, and angiogenesis, and are emerging as an indicator of endothelial damage can be measured via flow cytometry as a surrogate marker of endothelial function (Dignat-George and Boulanger, 2011). As Pim kinases have been demonstrated to be upregulated in HCAECs treated with inflammatory mediators in this study and shown to promote endothelial activation and pro-thrombotic signalling, EMP release would be an appropriate quantitative and sensitive measure of the role of Pim kinase in the endothelium.

Measurement of protein expression: The measurement of protein expression using western blotting should be carried out to further investigate the effects of Pim kinase inhibition on endothelial function as gene expression does not always correlate with protein. This has been demonstrated by a study where VSMC stimulation with PDGFbb lead to a transient increase in PIM1 mRNA at 1hr however, the protein expression profile did not match this time course with protein levels peaking much later at 24hr (Willert et al., 2010). The measurements of protein expression suggested would therefore be a more accurate representation of endothelial function and help further the understanding of the contribution of Pim kinases to vascular endothelial function and thrombosis as demonstrated by a study where PIM1 was shown to upregulate TF in HUVECs (Wang et al., 2026).

*6.5.1.2 Endothelial barrier integrity**:*** Transcriptomic data from Chapter 4 demonstrated that gene expression of F11R (JAM-A), a tight junction protein, was decreased following PIM447 treatment of HCAECs. Additionally, it has previously been shown in the literature that endothelial PIM3 protects the vascular barrier during lung metastasis (Santio et al., 2024). Future work should investigate the

effects of Pim kinase on barrier function and other endothelial junction proteins by studying EC gap junctions e.g. adherins (Ve-Cadherin) and tight (claudins/occluding/JAMs) junctions using fluorescence microscopy to interrogate these findings further.

6.5.1.3 Sex differences: PIM2 is located on the X-chromosome therefore it would be interesting to compare sex differences in the effects observed in the endothelium using HCAECs from females. Additionally knocking down PIM2 in both male and female mice would allow for a comparison of the effects of sex differences.

6.5.1.4 EndMT: It has recently been shown that PIM1 expression increased in ECs with the progression of atherosclerosis and EndMT processes using qPCR, western blotting, and immunohistochemistry techniques (Xue et al., 2025). The role of PIM1 in EndMT processes should further be investigated using functional assays and mechanobiology, along with the two isoforms, PIM2 and PIM3, by measuring characteristic endothelial markers including CD31, VE-Cadherin, Tie2, and VWF along with mesenchymal markers including fibroblast-specific protein-1 (FSP1), alpha 2 smooth muscle actin (α-SMA), type I/III collagen, and vimentin, and incorporating flow, as disturbed flow is a dominant inducer of EndMT in atherosclerosis (Choi et al., 2020, Kidder et al., 2023).

6.5.2 Further investigation of the role of Pim kinase in the regulation of EC thrombotic profile

6.5.2.1 Coagulation Factor assays: Following an observation of prolonged clotting time via the intrinsic pathway using both blood from PIM1$^{-/-}$ mice (Chapter 3) and human blood treated with Pim kinase inhibitors (Chapter 5), specific factor assays should be carried out including Factor VIII and IX assays using releasate from ECs to deduce specific factor defects.

6.5.2.2 VWF release: As VWF expression was decreased following PIM447 treatment of HCAECs, but release was increased, it would be interesting to investigate VWF further for example measuring VWF-platelet strings following

Pim kinase inhibition to further elucidate the role of Pim kinases in coagulation and thrombosis processes, as demonstrated by Wang *et al* using fluorescent microscopy to visualise VWF strings and platelet binding (Wang et al., 2012).

6.5.2.3 PIM2 and PIM3 knockout mouse models: These are commercially available and have been used in various studies (Wang et al., 2023, Spirli et al., 2019), and would be useful to compare to acquired PIM1-specific knockout data to deduce isoform-specific effects.

6.5.3 Investigation of the role of Pim kinase in the regulation of other cellular contributors to atherothrombosis

6.5.3.1 Leukocyte activation: Based on the changes in WBC count, and effects observed in immune cells in other studies, the role of Pim kinase in the leukocyte-mediated interactions with the endothelium involved in atherothrombosis should further be elucidated. Endothelial activation markers such as ICAM-1/VCAM-1/E-selectin/PECAM-1 could be measured using flow cytometry/microscopy/western blot/qPCR/ELISA as an indicator of leukocyte-endothelial interactions including leukocyte adhesion, rolling and TEM. Additionally surface activation markers of leukocyte degranulation, activation, adhesion, and migration including CD63, CD66b, and CD11b could be investigated. Furthermore, the effects of Pim kinase inhibition on platelet-neutrophil interaction and NET formation could also be explored, establishing the overall effect of Pim kinase inhibitors on the interaction between different cell types involved in atherothrombosis and inflammation.

6.6 Conclusions

Pim kinases play a coordinated and complex role in the regulation of endothelial biology, coagulation, inflammation, and cardiovascular pathology. Genetic deletion of PIM1 and pharmacological inhibition of all three Pim isoforms reveal inhibitory effects on intrinsic coagulation without impacting fibrinolysis, suggesting specific endothelial-driven anti-coagulant properties.

PIM1 deletion and Pim kinases inhibitors were also observed to reduce angiogenesis and alter endothelial gene expression of modulators that control EC platelet interactions, inflammatory signalling, and EC barrier function.

The identification of anti-angiogenic effects and increases in anti-inflammatory protective factors following Pim kinase inhibition and deletion, upregulation of Pim kinase isoforms in response to inflammatory mediators and increased expression of Pim isoforms in patients with cardiovascular pathologies position PIM1 and the other Pim kinase isoforms as promising yet complex targets in atherothrombosis and cardiovascular disease. Further emphasising the therapeutic potential of Pim kinase inhibition, particularly in vascular inflammation relevant to atherosclerosis and thrombosis.

6.7 Chapter 6 summary abstract

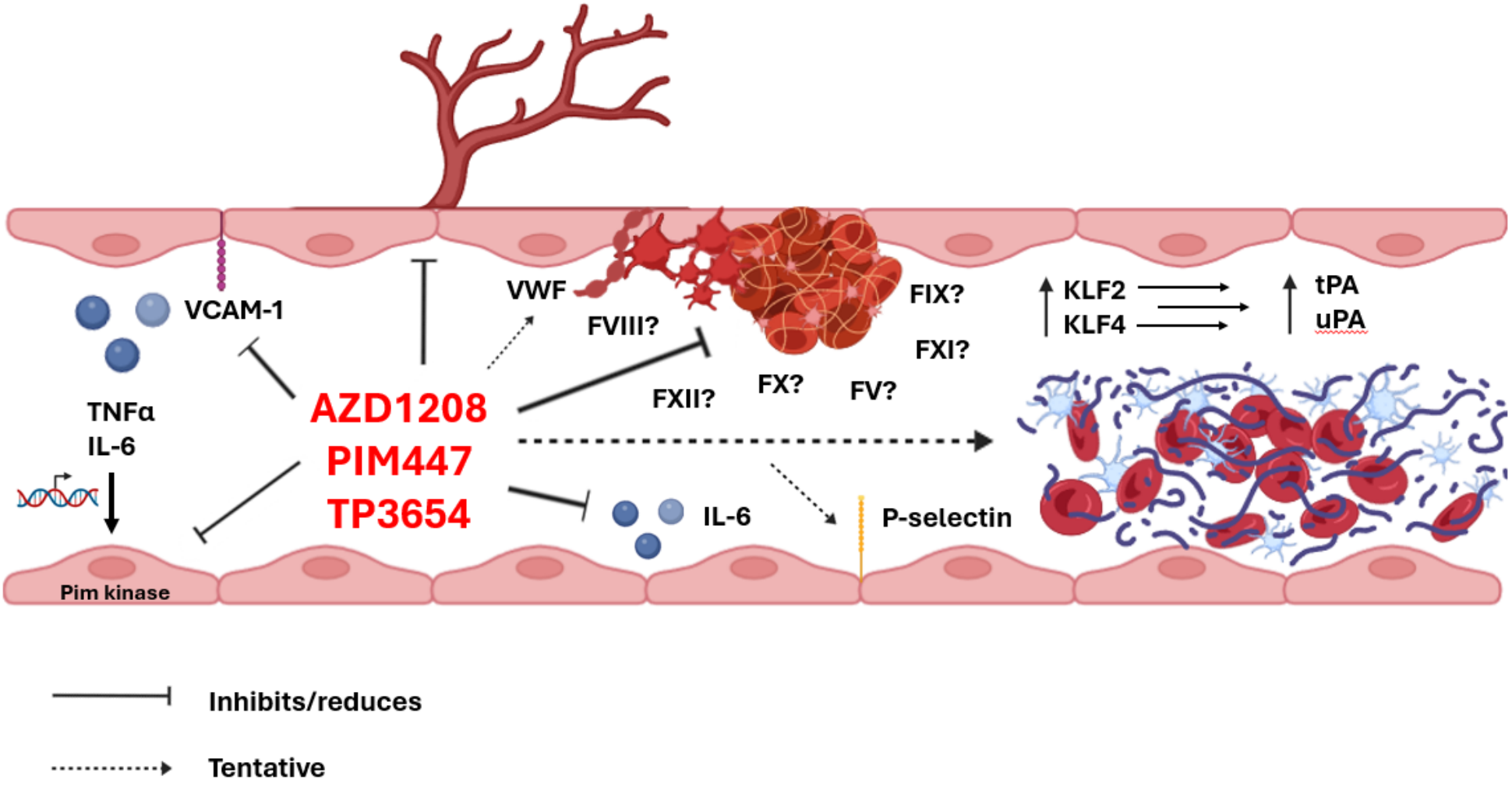

Figure 6.1: Chapter conclusions summary abstract. TNFα and IL-6 increased mRNA levels of PIM1, PIM2, and PIM3 in HCAECs demonstrating that Pim kinase isoforms are upregulated following endothelial dysfunction. Transcriptomic data revealed PIM447 (1μM) induced downregulation of endothelial adhesion molecule VCAM-1, and inflammatory cytokine IL-6, whilst flow regulatory genes KLF2 and KLF4 were upregulated. Both VWF and p-selectin gene expression was increased in PIM1$^{-/-}$ mice PECs, however a decrease was observed in HCAECs, potentially indicating that all three Pim kinase isoforms require targeting to induce anti-inflammatory and anti-thrombotic effects. Intrinsic coagulation was delayed following Pim kinase inhibition, whilst fibrinolysis was unaffected, although tPA and uPA were demonstrated to be upregulated. The mechanism behind this effect is still unknown and requires further investigation of coagulation factors. Angiogenesis was decreased following treatment of HCAECs with all three Pim kinase inhibitors, presenting targeting potential for neovascularisation in atherosclerosis and cancer-associated thrombosis. Overall, Pim kinase inhibition has an anti-thrombotic effect on the arterial endothelium, which was demonstrated in *in vitro* arterial thrombus formation experiments on both HCAECs and collagen, presenting Pim kinases as a novel appropriate target for the prevention and treatment of atherothrombotic processes. Created in BioRender and PowerPoint.

Appendices

Appendix A: Participant consent form.

Manchester Metropolitan University

CONSENT FORM

Investigating Pim kinase as a novel anti-thrombotic endothelium targeting strategy.

Participant Identification Number:

	Please tick your chosen answer	YES	NO
1.	I confirm that I have read the participant information sheet for the above study.	☐	☐
2	I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐	☐
3	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my legal rights being affected.	☐	☐
4	I agree to participate in the project to the extent of the activities described to me in the above participant information sheet.	☐	☐
5	I consent to blood sample being taken for the study as detailed in the accompanying participant information sheet	☐	☐

Name of participant	Date	Signature
Name of person taking consent	Date	Signature

Appendix B: Ethical approval.

16/11/2022
Project Title: Investigating Pim kinase as a novel anti-thrombotic endothelium targeting strategy

EthOS Reference Number: 48062

Ethical Opinion

Dear Eima Karim,

The above application was reviewed by the Science and Engineering Research Ethics and Governance Committee and, on the 16/11/2022, was given a favourable ethical opinion. The approval is in place until 02/10/2025 .

Conditions of favourable ethical opinion

Application Documents

Document Type	File Name	Date	Version
Consent Form	Consent-form	10/10/2022	1
Information Sheet	Participant-Information-Sheet 2022	10/10/2022	1
Project Protocol	New protocol	10/10/2022	1

The Science and Engineering Research Ethics and Governance Committee favourable ethical opinion is granted with the following conditions

Adherence to Manchester Metropolitan University's Policies and procedures

This ethical approval is conditional on adherence to Manchester Metropolitan University's Policies, Procedures, guidance and Standard Operating procedures. These can be found on the Manchester Metropolitan University Research Ethics and Governance webpages.

Amendments

If you wish to make a change to this approved application, you will be required to submit an amendment. Please visit the Manchester Metropolitan University Research Ethics and Governance webpages or contact your Faculty research officer for advice around how to do this.

We wish you every success with your project.

Science and Engineering Research Ethics and Governance Committee

Science and Engineering Research Ethics and Governance Committee

For help with this application, please first contact your Faculty Research Officer. Their details can be found here

Appendix C: Mice PEC RNA-Seq samples correlation heatmap.

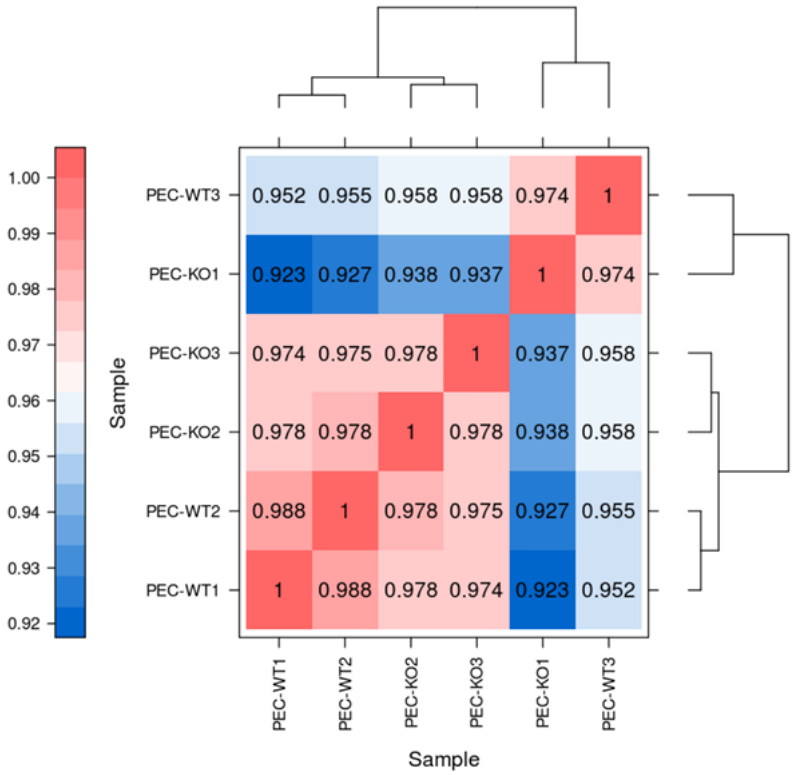

Figure 1: Correlation heatmap between RNA-Seq mice PEC samples demonstrates variability in two samples, requiring exclusion from analysis. Three sets of mice PEC samples were prepared for RNA-Seq, 3 WT and 3 KO (n=3). RNA-Seq analysis reported by BMKGene suggested that WT1/WT2 and KO2/KO3 cluster tightly, demonstrating high correlation and similarity in biological repeats between samples within the same conditions. KO1 and WT3 formed a distinct subgroup and demonstrated low correlations with their biological repeat samples, suggesting sample-specific variation. These samples were therefore excluded from transcriptomic analysis.

Appendix D: aPTT (Pim kinase inhibitor-treated PPP data).

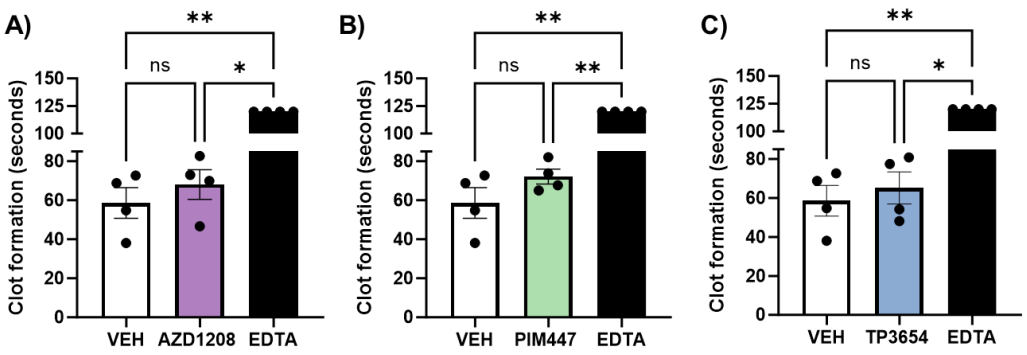

Figure 2: Pim kinase inhibitor treatment does not affect clot formation time via the intrinsic pathway in the absence of the endothelium. PPP was treated with (A) AZD1208 (1µM), (B) PIM447 (1µM), and (C) TP3654 (0.1µM) treatment for 10 minutes before the addition of aPTT reagent and calcium chloride and clot formation time in seconds measured on a Helena C-4 Coagulation Analyser. EDTA was used as a positive anti-coagulant control. Technical repeats were done in duplicate. Results are expressed as mean + SEM for n=4. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism.

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