Investigating endothelial cell Pim kinase as a novel anti-thrombotic target

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Abbreviation	Definition
ACS	Acute coronary syndromes
ANOVA	Analysis of Variance
ARE	Antioxidant response element
CAD	Coronary artery disease
CAM	Cell adhesion molecules
CD39	Cluster of Differentiation 39
CD73	Cluster of Differentiation 73
CRP	C-reactive protein
CSE	Cigarette smoke extract
EC	Endothelial cells
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase

HCAEC	Human coronary artery endothelial cells
HUVEC	Human umbilical vein endothelial cells
iNOS	inducible nitric oxide synthase
ICAM-1	Intercellular cell adhesion molecule-1
IL-6	Interleukin 6
MI	Myocardial infarction
NO	Nitric oxide
PAH	Pulmonary arterial hypertension
PGI2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
Pim	Proviral Integration Site for MuLV (Murine Leukaemia virus)
RNAi	RNA interference
S.E.M	Standard Error of the Mean
SMC	Smooth muscle cells
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNFα	Tissue necrosis factor alpha
t-Pa	Tissue plasminogen activator
u-Pa	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand Factor
WPB	Weibel-Palade bodies

Abstract

Cardiovascular disease is the most common cause of mortality worldwide, presenting a significant burden on healthcare costs globally. Atherothrombosis, the development of an occlusive clot in an artery, is triggered by atherosclerotic plaque rupture/erosion, and involves the interaction of multiple cell types in the blood and vasculature, with endothelial cells and platelets playing significant roles. Blood clots formed during thrombotic events are rich in platelets, making them a suitable target for anti-thrombotic therapies. However, the most widely prescribed anti-platelet drugs for arterial thrombosis prevention, aspirin and clopidogrel, only provide ~20% protection against cardiovascular disease related events. Simultaneously targeting platelets and the endothelium could provide an effective novel anti-thrombotic therapeutic approach. Pim kinase, a family of serine/threonine kinases, have shown to modulate platelet function, and whilst their expression is confirmed in endothelial cells, their role in the endothelium remains unknown. This project aimed to investigate the role of Pim kinase in regulating the inflammatory pathways involved in endothelial cell control of thrombus formation. The role for Pim kinase in endothelial cells in response to cigarette smoke extract and Tumour Necrosis Factor alpha, initiators of endothelial cell damage, was determined using human umbilical vein endothelial cells as a model platform of endothelial cell function. Human umbilical vein endothelial cells were treated for 24 hours with cigarette smoke extract and/or Tumour Necrosis Factor alpha +/- pan Pim kinase inhibitor AZD1208, and techniques including enzyme-linked immunosorbent assay, fluorescence microscopy, qPCR, and Western Blotting used to monitor gene and protein expression of Pim kinases and mediators of thrombo-inflammation. mRNA expression of all three Pim kinase isoforms, and protein expression of Pim-1 was confirmed. Human umbilical vein endothelial cells treated with cigarette smoke extract and Tumour Necrosis Factor alpha combined demonstrated a decrease in endothelial nitric oxide synthase levels, a protective mediator of cardiovascular homeostasis. Human umbilical vein endothelial cells treated with AZD1208 demonstrated a decrease in the expression of von Willebrand factor, a pro-coagulant mediator, and release of inflammatory markers, Interleukin-6, and

Interleukin-8 were observed. Collectively, these findings identify a potential role for Pim kinase in atherothrombosis and indicate that Pim kinase inhibitors could be repurposed for use alongside other anti-thrombotic agents for the prevention of cardiovascular-related events.

Introduction

Cardiovascular Disease

Cardiovascular disease (CVD) describes conditions affecting the heart or circulatory system, including high blood pressure, stroke, and vascular dementia (BHF, 2022c). CVD continues to be the number one cause of death globally, taking approximately 17.9 million lives a year, an estimated 32% of all deaths worldwide (WHO, 2022). In the UK, the CVD burden of all heart and circulatory diseases accounted for 87,790 deaths in males and 80,529 in females in 2020 (BHF, 2022a), with annual healthcare costs in England attributed to CVD estimated at £7.4 billion (PHE, 2019).

Cardiovascular events such as myocardial infarction (MI) and stroke are triggered by atherosclerosis, causing narrowing of the arteries, blood vessel occlusion, and consequent thrombosis, following atherosclerotic plaque rupture (Ashorobi et al., 2022), restricting blood and oxygen supply to the heart and brain (BHF, 2022d). Thrombotic events are initiated by the formation of clots within blood vessels through platelet activation and aggregation, limiting the flow of blood (Ashorobi et al., 2022). The ability of blood to flow freely in vessels relies on the interaction between components of the vasculature; blood cells, coagulation factors, inflammatory cytokines, and the endothelium within the lumen of blood vessels (Ashorobi et al., 2022). It is important that the causes and mechanisms of cardiovascular pathology are understood to enable the development of treatment strategies to improve survival and reduce premature deaths and the health burden associated with CVD.

The Vasculature

Blood vessels are responsible for the movement of blood, supplying tissues with nutrients and oxygen, and removing waste products. The network of blood vessels constituting the vasculature surround almost all body tissues, and

therefore cells comprising the vasculature are able to interact with many other cell types (Logsdon et al., 2014).

Arteries are a major part of the circulation, playing a crucial role in maintaining homeostasis throughout the body. This is done by controlling blood pressure through constriction and dilation, allowing the distribution of blood flow at high pressure to the site of tissue exchange at the capillaries (Mercadante and Raja, 2022). Three distinctive layers form the artery (Figure 1), including the tunica intima, the inner layer which contains a single layer of endothelial cells (EC) and an internal elastic lamina, creating a tube for oxygen-rich blood to move through to its site of perfusion (Mercadante and Raja, 2022). The second layer, the tunica media, is made up of smooth muscle that can constrict or dilate to control vascular tone and an external elastic lamina (Mercadante and Raja, 2022). The outer layer, the tunica adventitia, consists of collagen fibres and connective tissue and is crucial for connecting the arteries to other body tissues (Mercadante and Raja, 2022).

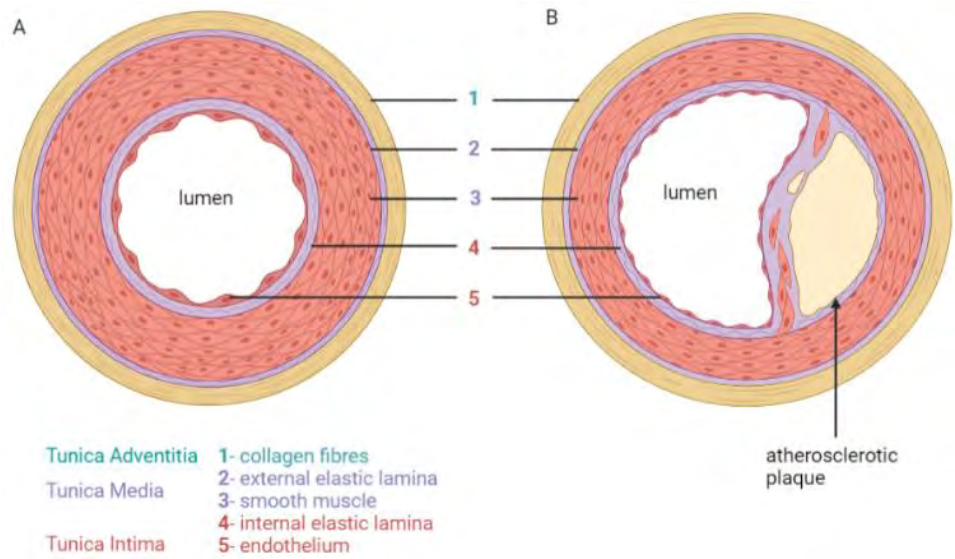

Figure 1: Artery three-layer structure in health and disease. A. *Structure of a normal large artery consisting of three layers. The intima is the extremely thin inner layer, consisting of a monolayer of ECs and a sheet of elastic fibres, the internal elastic lamina. The media, the middle layer, consists of smooth muscle cells and an external elastic lamina, consisting of extracellular connective tissue matrix and collagen. The adventitia, the outer layer, consists of connective tissue with collagen and elastic fibres.* ***B.*** *Structure of an atherosclerotic artery with ECs overlying a plaque. Adapted from (Lusis, 2000; Mercadante and Raja, 2022).*

The inner lining of blood vessels, a monolayer of ECs, forms the vascular endothelium and is directly in contact with blood components and cells (Kruger-Genge et al., 2019). It functions as a barrier between blood and tissues (Lusis, 2000), playing a crucial role in maintaining oxygen and nutrient

supply to the heart (Luxan and Dimmeler, 2022). The endothelium generates cell surface markers and paracrine messengers that regulate other cells in the blood and vasculature, playing roles in thrombosis, inflammation, and control of vascular tone (Lusis, 2000). Although only a single monolayer, the healthy endothelium is able to respond to physical and chemical signals by the production of factors that regulate cell adhesion, thromboresistance, smooth muscle cell (SMC) proliferation, and vessel wall inflammation (Deanfield et al., 2007). Additionally, ECs regulate angiogenesis, a complex process of vasodilation, degradation of the basement membrane, EC migration, chemotaxis, increasing vascular permeability, EC proliferation, and the eventual formation of new blood vessels (Kruger-Genge et al., 2019; Lu et al., 2017), through changes in shape elicited by the cytoskeleton (Bayless and Johnson, 2011).

ECs are essential mediators of normal haemostasis. The roughly 10^{14} ECs of the vasculature provide protection against atherosclerosis and thrombosis, maintaining a balance between coagulation and fibrinolysis, whilst playing a role in the regulation of immune response and inflammation (Forstermann and Munzel, 2006). This is attributed to their key role in controlling platelet adhesion and aggregation, and leukocyte activation, adhesion, and transmigration (Kruger-Genge et al., 2019).

During vascular injury, EC activation increases blood vessel wall permeability to leukocytes and low-density lipids (LDL), promoting the progression of atherosclerotic plaque formation (Medina-Leyte et al., 2021). Endothelial dysfunction is a key contributor in atherosclerosis and is highly associated with changes creating a reduced output in the production of nitric oxide (NO) (Satta et al., 2019), a chemical mediator with anti-thrombotic properties (Lusis, 2000).

Atherosclerosis

Atherosclerosis initiates via damage or dysfunction of ECs lining the vessel wall. Atherosclerosis is a chronic, progressive, inflammatory disease characterised by the accumulation of lipids and fibrous components in the large arteries, narrowing the arterial lumen through advanced plaque formation (Weber and Noels, 2011). Atherosclerosis develops at regions of disturbed flow within arteries, which further activates the endothelium, priming

ECs for inflammation (Hahn and Schwartz, 2009; Warboys et al., 2011; White et al., 2011).

Atherosclerosis initiates the recruitment of lymphocytes and monocytes to the artery wall, triggered by the accumulation of minimally oxidised LDL (ox-LDL), which stimulates the overlying ECs to produce pro-inflammatory molecules, including adhesion molecules and growth factors (Lusis, 2000). Uptake of ox-LDL by recruited monocyte-derived macrophages as well as SMCs contributes to foam cell formation, further driving cytokine expression, inflammatory cell recruitment, endothelial dysfunction, and plaque expansion (Y. Yang et al., 2017). Although plaques can grow sufficiently large to block blood flow, the most significant clinical complication is occlusion due to rupture or erosion of the plaque, resulting in the formation of a thrombus, causing tissue ischaemia and consequently MI or stroke (Lusis, 2000).

The Endothelium and thrombosis

The cardiovascular system is maintained by a range of haemostatic processes that sustain its integrity. Two key components in arterial haemostasis are platelets and ECs (Knowles and Warner, 2019). Platelets respond to arterial wall damage by sensing the exposed subendothelial matrix proteins, adhering, activating, and attracting more platelets to rapidly build a platelet plug. Damage to the endothelium enhances this process, which in turn activates the clotting cascade via the release of tissue factor (TF) to add insoluble fibrin strands to strengthen and bind the growing thrombus. On the other hand, this cascade of factors is limited by the generation of anti-clotting processes that limit clot growth and excessive unwarranted thrombosis (Knowles and Warner, 2019).

ECs keep the vasculature in a vasodilating phenotype in the healthy state (Goncharov et al., 2020), expressing and releasing anti-platelet and anti-thrombotic factors that prevent platelet aggregation, coagulation, fibrin formation and thrombosis (Yau et al., 2015), playing a vital role in the maintenance of cardiovascular homeostasis (Kruger-Genge et al., 2019). The vascular endothelium functions as a barrier, separating blood from highly reactive components of the sub-endothelium, including collagen and TF (Kanthi et al., 2014). A healthy endothelium provides a continuous source of

the major anti-platelet agents, NO and prostacyclin (PGI2), which act in concert to maintain platelets in their inactive state, synergistically increasing platelet cyclic GMP (cGMP) and cyclic AMP (cAMP) content, respectively, preventing their aggregation and limiting the formation of thrombi (Kruger-Genge et al., 2019; Bye et al., 2016).

ECs further express and release CD39 and CD73, which rapidly break down platelet agonists ATP and ADP to adenosine, inhibiting platelet activation (Bye et al., 2016). The endothelium expresses a variety of inhibitors of the coagulation pathway such as tissue factor pathway inhibitor (TFPI), and thrombomodulin, as well as tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), which promote fibrinolysis (Yau et al., 2015), as displayed in Figure 2. All of these mechanisms function to maintain blood flow, preventing unwarranted platelet activation and thrombosis.

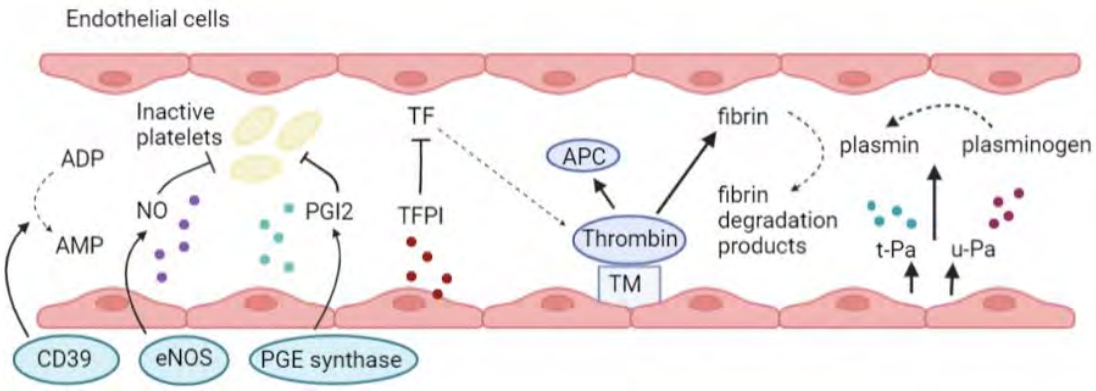

Figure 2: Inhibitory properties of healthy ECs preventing thrombus formation. *ECs express nitric oxide (NO) and prostacyclin (PGI2) which inhibit platelet adhesion and aggregation. Tissue factor pathway inhibitor (TFPI) is expressed by ECs, which limits the action of tissue factor (TF). Thrombomodulin (TM) binds thrombin, activating protein C which inhibits thrombosis and inflammation and promotes fibrinolysis. Fibrinolysis is triggered by tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Adapted from (Neubauer and Zieger, 2021).*

Alternatively, when normal function is compromised, endothelial dysfunction promotes platelet activity and thrombosis through the release of pro-coagulant mediators and inhibition of anti-platelet and anti-coagulant mediators (Neubauer and Zieger, 2021), as shown in Figure 3. Atherosclerotic plaque rupture or erosion causes loss of the endothelium overlying a plaque, resulting in platelet adhesion to components of the sub-endothelium, activation, and aggregation, initiation of the coagulation cascade, and fibrin formation (Yau et al., 2015). At sites of vascular injury, Von Willebrand factor (VWF) binds to sub-endothelial collagen, which is exposed alongside other extracellular

matrix (ECM) components, and captures platelets from the circulation via interaction with platelet glycoprotein GPIb, initiating thrombus formation (Denorme et al., 2019). The secretion of mediators following platelet activation can lead to EC activation, initiating a pro-thrombotic state (Kruger-Genge et al., 2019). The chronic, pro-inflammatory pathology of atherosclerosis leads to EC dysfunction, which disrupts the haemostatic balance and contributes to the increased risk of thrombosis-related events (Bonetti et al., 2003), with occlusive clot formation reducing blood flow to tissues and organs, restricting nutrient and oxygen supply (Kruger-Genge et al., 2019).

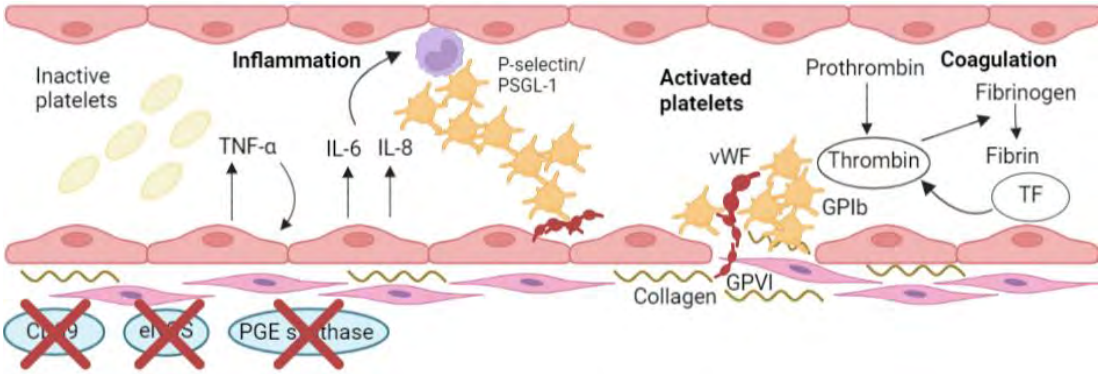

Figure 3: Pro-coagulant properties of ECs. *At sites of vascular injury, Von Willebrand factor (VWF) binds to exposed subendothelial collagen. VWF captures platelets from the circulation via interaction with GPIb on the platelet. Tissue factor initiates the formation of thrombin from prothrombin which then acts to convert fibrinogen to fibrin, initiating the process of coagulation with platelets. Inflammatory factors such as IL-6, IL-8, and TNFα are released from the activated endothelium, promoting leukocyte-platelet interaction and thrombo-inflammation. Adapted from (Neubauer and Zieger, 2021).*

ECs significantly influence the initiation and progression of atherothrombosis, with the ECs overlying advanced atherosclerotic plaques frequently expressing markers of activation and dysfunction (Quillard et al., 2017), such as acute-phase proteins (C-reactive protein (CRP)), inflammatory cytokines (Tumour Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6)), cell adhesion molecules (CAM) (Intercellular adhesion molecule 1 (ICAM-1), Vascular cell adhesion molecule 1 (VCAM-1), E-selectin, P-selectin), and cell markers (extracellular vesicles) (Medina-Leyte et al., 2021).

Mediators of thrombosis and inflammation

Platelets, together with ECs and circulating coagulation proteins, are crucial mediators of haemostasis and thrombosis (Johnston-Cox et al., 2012). There are various proteins involved in modulating thrombosis and inflammation through interaction with the endothelium, either promoting or inhibiting cardiovascular events. Under normal physiological conditions, ECs secrete

various signals and mediators which are important for the regulation of blood coagulation and platelet function in order to prevent unwarranted platelet adhesion and aggregation (Kruger-Genge et al., 2019).

Endothelial nitric oxide synthase **(eNOS)** plays a critical role in regulating and maintaining a healthy cardiovascular system, exerting its protective action (Tran et al., 2022). eNOS activity is regulated by shear stress, particularly through Ca^{2+} signalling, but also through phosphorylation cascades to modulate vessel tone of large arteries, dependent on blood supply needs (Weber and Noels, 2011). eNOS produces NO which acts directly on ECs and underlying SMCs (Weber and Noels, 2011), suppressing cellular adhesion to the endothelium, and attenuating vascular SMC proliferation and migration (Vallance and Chan, 2001). Alongside supressing inflammation regulator NFκB, NO also regulates platelet function and contributes to the anti-thrombotic state of the endothelium, inhibiting platelet adhesion to the vessel wall by promoting the production of cGMP (Freedman and Loscalzo, 2003).

CD39 is a critical mediator in the regulation of vascular inflammation and thrombosis, hydrolysing ADP (an inducer of platelet aggregation) to AMP (Kanthi et al., 2014). Microparticles from mice lacking CD39 have shown to induce a pro-thrombotic and pro-inflammatory response from ECs *in vitro* with enhanced expression of ICAM-1 and VCAM-1, and release of TNFα (Banz et al., 2008). In addition to CD39, ECs express CD73, converting AMP to adenosine which limits thrombosis by blocking platelet activation and acts as an anti-inflammatory mediator (Johnston-Cox et al., 2012).

Thrombomodulin is activated through laminar shear stress and has been demonstrated to regulate coagulation, initiating thrombin-induced activation of protein C to activated protein C (APC) and catalysing the inhibition of thrombin by anti-thrombin to prevent fibrin formation and activation of platelets (Yau et al., 2015). Through the expression of thrombomodulin, ECs can regulate protein C activation, responsible for regulating thrombosis, and decreasing inflammatory responses (Yau et al., 2015). Thrombomodulin also holds direct anti-inflammatory activity where it reduces cytokine formation in the endothelium whilst decreasing leukocyte-EC adhesion (Esmon, 2003).

Alternatively, ECs release markers of inflammation and activate markers of thrombosis, contributing to the onset of atherothrombosis and CVD (Goncharov et al., 2020). Many factors are involved in inflammatory processes, forming links between haemostasis, thrombosis, and inflammation (Denorme et al., 2019).

VWF is produced by ECs and synthesised by megakaryocytes and is known to play a major role in haemostasis (De Meyer et al., 2009). As well as its well-established role in haemostasis, VWF is recognised as an effective mediator of inflammatory responses, recruiting leukocytes to inflamed tissues after binding platelets (Denorme et al., 2019). When synthesised, VWF is either constitutively secreted into the blood or stored in endothelial Weibel-Palade bodies (WPBs) and platelet α-granules (Mourik and Eikenboom, 2017). Alongside VWF, WBPs store other molecules involved in inflammation and angiogenesis (including angiopoietin-2, Factor H, IL-6, IL-8, and P-selectin) (Denorme et al., 2019).

Selectins are inflammatory markers that act to mediate the adhesion of leukocytes to ECs and platelets. Selectins play important roles in leukocyte trafficking to sites of inflammation (McEver, 2015). The rolling of leukocytes along the endothelium is mediated by selectins, whereas the adhesion and trans-endothelial migration of leukocytes require interaction between the integrin family and CAMs such as ICAM-1 and VCAM-1 (N. Wang, 2014). Studies of mice deficient in P- and E-selectins provide evidence that selectins play a critical role in both early and late stages of atherosclerotic plaque development, highlighting their importance in assisting the rolling of blood cells (Dong et al., 1998). When vascular function is impaired following atherosclerosis, cardiovascular complications can be initiated such as atherothrombosis (Hahn and Schwartz, 2009).

Atherothrombosis

Atherothrombosis, a major cause of acute coronary syndromes and cardiovascular death, is characterised by atherosclerotic plaque disruption with superimposed thrombus formation (Viles-Gonzalez et al., 2004). Atherothrombosis, a multicellular process affecting various cell types and factors within the blood and vasculature (Yau et al., 2015), is initiated by EC

dysfunction (Asada et al., 2020). Atherosclerotic plaque disruption via rupture or erosion activates ECs, triggering the process of inflammation (Viles-Gonzalez et al., 2004; Yamashita and Asada, 2012). This initiates the production and release of cytokines (IL-6, IL-8), and expression of adhesion molecules such as ICAM-1, VCAM-1, selectins, VWF, and TF (Viles-Gonzalez et al., 2004; Yamashita and Asada, 2012). In turn, these factors recruit leukocytes, induce platelet adhesion, aggregation, and activation of the coagulation cascade, resulting in arterial thrombus formation and subsequent atherothrombosis (Yamashita and Asada, 2012) (Figure 4). Coronary atherothrombosis due to atherosclerotic plaque rupture or erosion is commonly associated with acute coronary syndromes (ACS) (Da Silva et al., 2015).

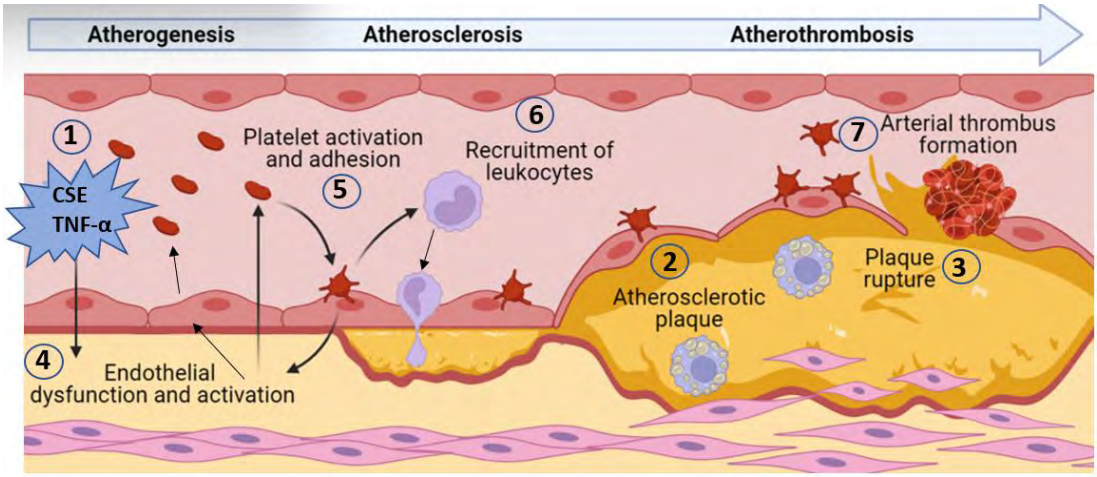

Figure 4: The pathogenesis of atherothrombosis. *1. Increased ROS in plasma as a result of pathological factors 2. atherosclerotic plaque formation 3. plaque rupture leads to 4. endothelial cell dysfunction and activation- activated endothelial cells have altered function becoming more adhesive by expressing cytokines and factors including tissue factor and VWF 5. platelet activation, adhesion, and aggregation 6. recruitment of leukocytes and inflammatory factors 7. vessel occlusion due to arterial thrombus formation.* Adapted from (Gawaz, 2004; Kattoor et al., 2017).

Current anti-thrombotic therapies

Rupture or erosion of an atherosclerotic plaque precipitates thrombus formation, the major cause of MI (Braganza and Bennett, 2001). Platelets play a key role in the pathogenesis of vascular inflammation and thrombosis, forming occlusive blood clots (Yau et al., 2015). Understanding the role of platelets in thrombosis and CVD has enabled the development of therapeutic agents with the potential to reduce morbidity and mortality, with an aim to separate reduced thrombotic events from increased bleeding events (Michelson, 2010). Anti-platelet therapy is therefore currently the main

treatment strategy for the secondary prevention of MI, with 45 million prescriptions for anti-platelet medication dispensed in the UK yearly (Lettino et al., 2017).

Aspirin and clopidogrel are the most commonly prescribed therapies for the prevention of CVD related events (BHF, 2022b). Aspirin mediates its effects through the irreversible inhibition of platelet COX-1 which prevents the production of TXA_2 (Warner et al., 2011). For 'at risk' patients, low dose aspirin reduces thrombotic events by around 30% (Patrono et al., 2005). Clopidogrel is an irreversible $P2Y_{12}$ receptor antagonist acting to reduce arteriosclerotic events in patients with recent MI or stroke, and peripheral arterial disease (PAD) (Kelly et al., 2012). For many patients at risk of coronary thrombosis, pharmaceutical protection is supplied by dual anti-platelet therapy (DAPT) (Knowles and Warner, 2019). DAPT with aspirin and clopidogrel has shown to reduce cardiovascular events in patients with ACS (Jiang et al., 2015), representing the cornerstone of therapy for the treatment of atherothrombosis, however 10% of ACS patients still experience recurrent thrombotic events and DAPT increases the risk of bleeding (Knowles and Warner, 2019).

Both aspirin and clopidogrel display good anti-thrombotic activity, however they are commonly associated with issues of resistance and an increase in bleeding events (Cattaneo, 2004). Despite clear experimental evidence of the efficacy and safety of aspirin, the use of aspirin continues to be less than optimal with effects such as intolerance, and increased bleeding (Mekaj et al., 2015), specifically gastrointestinal bleeding, which is reported in approximately 3% of elderly patients treated with aspirin (Thorat and Cuzick, 2015). Some patient groups such as individuals with Type 2 diabetes mellitus and obesity demonstrate high on-treatment (aspirin) platelet reactivity, which has been shown to associate with an increased risk of ACS (Cattaneo, 2009). Although clopidogrel is safe and effective in many patients, there is substantial variability in treatment response between individuals (Jiang et al., 2015), with a third of patients not achieving satisfactory platelet inhibition, and some patients continuing to have cardiovascular events (Gurbel et al., 2003). This lack of efficacy results in high on-treatment platelet reactivity and the development of

atherothrombotic complications (Angiolillo et al., 2007). Clopidogrel resistance is shown to affect 5-44% of patients receiving treatment (Gurbel et al., 2003). Alternatively, some patients experience drug-induced bleeding due to excessive platelet inhibition (Yusuf et al., 2001). A novel approach achieving an optimal balance between thrombosis and bleeding is therefore necessary.

Pleiotropic effects of anti-platelet agents enhance EC anti-platelet action, which could be beneficial if balanced with reduced bleeding risk (Warner et al., 2016). EC dysfunction prevents this effect by reducing EC dependent anti-platelet activity, especially in high-risk CVD individuals (Cattaneo, 2013). Considering this, simultaneously targeting platelets and the endothelium poses an effective strategy for an anti-thrombotic therapeutic approach. Single therapies that target the multi-faceted nature of atherothrombotic pathology could effectively offer a multi-factorial approach to the treatment and prevention of MI and stroke. A therapy that inhibits platelet action whilst promoting the anti-thrombotic properties of ECs could therefore be more effective than current anti-platelet agents for the prevention of CVD-related events.

Pim kinase

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: Pim-1, Pim-2, and Pim-3 (Nawijn et al., 2011), as shown in Figure 5, and have been identified in numerous cell types in the body, contributing to many functions (Morishita et al., 2008).

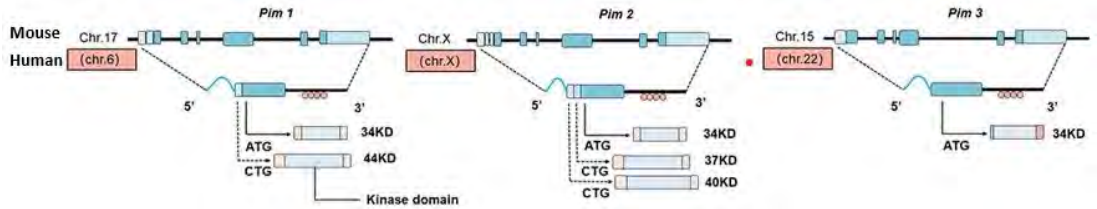

Figure 5: Pim kinase family isoforms. *Pim genes are located in different chromosomal locations in the mouse and human genome. The Pim isoforms have different molecular masses but hold their serine threonine activity.* Adapted from (Nawijn et al., 2011).

Pim-1 mRNA expression levels are found to be highest in the thymus and testes, Pim-2 in the thymus and brain, and Pim-3 is most abundant in the kidney (Mikkers et al., 2004). Although Pim-3 can be detected in several normal tissues including the brain and heart, it is particularly expressed in high

levels in tumour tissues of endoderm-derived organs including the pancreas, liver, colon, and stomach (Y. Y. Li and Mukaida, 2014). Despite Pim family member genes being located on different chromosomes (Figure 5), they encode proteins with a high degree of sequence homology (Figure 6).

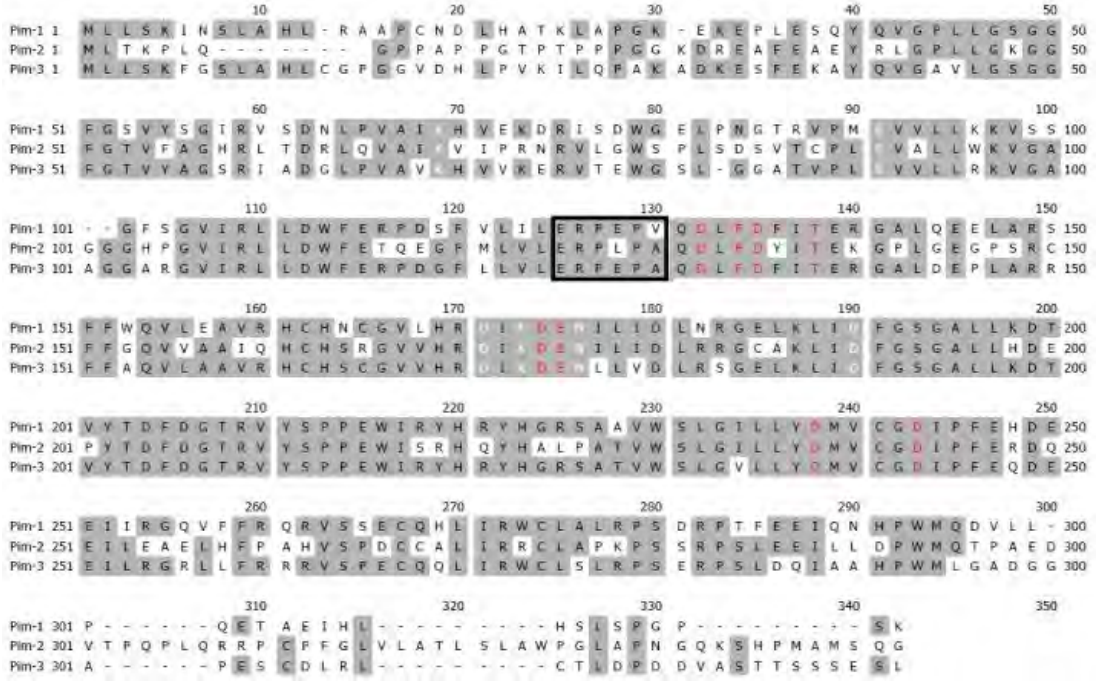

Figure 6: Amino acid alignment of human Pim kinase family proteins. *The amino acid sequences of Pim kinase family members are shown. The box indicates the hinge region. Residues marked with white, and red are important for ATP and substrate selectivity, respectively. Adapted from (Y. Y. Li and Mukaida, 2014).*

Whilst classed as serine threonine kinases, the Pim family members exclusively lack 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 Pim kinases contain a novel two stranded β-sheet predating the αc helix which differentiates them from other kinases (Kumar et al., 2005). Another distinctive feature of Pim kinases is their unique hinge region (Kumar et al., 2005; Qian et al., 2005), presenting as an obvious target. The hinge region has two inserted proline residues widening the ATP binding site, this does not allow for the typical hydrogen bonds

between ATP and kinases, and therefore results in high Km values for ATP (Bullock et al., 2009) (Figure 7).

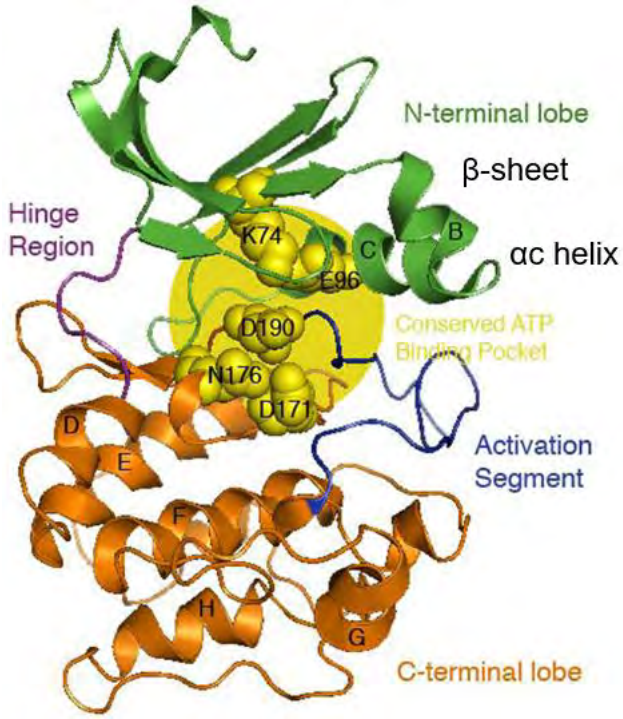

Figure 7: Consensus global model structure of Pim kinase. *The model structure of human Pim kinase shows the basic two-lobe kinase fold, with the N- and C-terminal lobes (green and orange respectively) joined by a unique hinge region (magenta). Substrate recognition is through interaction with the activation segment (blue). ATP binds at a site between the two lobes (yellow). Two stranded β-sheet is shown predating the αc helix. Adapted from (Izarzugaza et al., 2011).*

Pim kinases are primarily activated by the janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway which results in the phosphorylation of various proteins at serine or threonine sites (Jimenez-Garcia et al., 2016), with regulatory functions in cell processes (Nawijn et al., 2011), playing a role in transcription, translation, apoptosis, and the cell cycle (Cervantes-Gomez et al., 2019). In turn, Pim kinases regulate cell survival, proliferation, differentiation, and migration (Mikkers et al., 2004), processes which are fundamental to their pro-survival and cancer supporting activity. Although Pim kinases are expressed in multiple cell types and therefore implicated in the pathogenesis of various diseases, they are best established for their contribution to haematological and solid tumour malignancies (Arrouchi et al., 2019). Pim kinases demonstrate a growth advantage through a variety of mechanisms, promoting growth factor independent proliferation through the phosphorylation of cell cycle factors (Bullock et al., 2009).

Pim-1

Pim-1 is the most characterised Pim kinase family member and has two isoforms (34 and 44kDa) due to alternative translation initiation points at an upstream CUG codon (Liang and Li, 2014). Pim-1 has a two-lobed kinase fold structure with a deep cleft between the N- and C-terminal lobes connected via the hinge region, which has specific residues (121-126) identified as ATP-binding sites (Liang and Li, 2014). The ATP-binding pocket in Pim-1 remains open, indicating that Pim-1 constitutively resides in an active conformation (Qian et al., 2005). It has been established in some cell types that the 44kDa variant of Pim-1, Pim-1L is localised to the cell membrane, while the 32kDa variant, Pim-1S is predominantly localised to the cytoplasm and nucleus (Xie et al., 2006). Although this is the case, both isoforms have comparable kinase activity.

The Pim-1 gene is expressed in a wide variety of cell types and tumours, enhancing the growth and survival of cells (Qian et al., 2005). Pim-1 is involved in differentiation and the protection of cells from apoptosis (Bhattacharya et al., 2002) as Pim-1 inactivates pro-apoptotic protein BAD by phosphorylating it on Ser112 (Aho et al., 2004), inducing the release of anti-apoptotic protein Bcl-X(L) (UniProt, 2022a). Pim-1 also promotes cell cycle progression through phosphorylation and activation of Cdc25 (Bachmann et al., 2004; Mochizuki et al., 1999) and phosphorylation and inhibition of Cdc25-associated kinase 1 (C-TAK1) (Bachmann et al., 2004), an inhibitor of Cdc25C. It is able to promote tumorigenesis through phosphorylation of the c-Myc proto-oncogene (van Lohuizen et al., 1989; Y. Zhang et al., 2008), and by sustaining the activity of mTORC (Rebello et al., 2018). Phosphorylation of Myc leads to an increase in Myc protein stability and therefore an increase in its transcriptional activity (UniProt, 2022a). Cancer metastasis can additionally be promoted by Pim-1 through the phosphorylation of CXCR4, promoting cell migration (Santio et al., 2015).

Pim-2

Pim-2 is located in the cytoplasm (UniProt, 2022c), has 3 isoforms, 34, 37, and 40kDa, and is highly induced by growth factors and cytokines through STAT5 activation (Bullock et al., 2009). The amino acid sequence of Pim-2 is only

55% similar to Pim-1, with the most considerable differences being within the last 23 residues (Bullock et al., 2009). Compared with Pim-1, the last 23 residues of Pim-2 are disordered, and share little sequence identity, with the Pim-2 end terminus containing 6 proline residues that are not predicted to form a helical structure (Bullock et al., 2009). This lack of helical structure may increase flexibility to the N-terminus, causing structural changes to Pim-2 (Bullock et al., 2009).

Pim-2 is highly expressed in leukaemia and lymphomas and has been shown to positively regulate the survival and proliferation of tumour cells (Bullock et al., 2009). Pim-2 is a proto-oncogene with serine threonine kinase activity, exerting its oncogenic activity through cell cycle progression and cell survival signalling (UniProt, 2022c). Pim-2, like Pim-1, can also phosphorylate c-Myc (Y. Zhang et al., 2008) and maintain mTORC signalling by maintaining inhibitor phosphorylation on eukaryotic initiation factor 4E binding protein (4E-BP1) and BAD protein, allowing for cell growth and survival (Fox et al., 2003). Pim-2 also promotes cell survival in response to a variety of proliferative signals via positive regulation of the I-kappa-B kinase/NF-kappa-B cascade, and promotes growth factor independent proliferation through the phosphorylation of cell cycle factors such as CDKN1A and CDKN1B (UniProt, 2022c).

Pim-3

Pim-3 is a highly conserved serine threonine kinase (Liu et al., 2018) located in the cytoplasm and has only one isoform (34kDa) (UniProt, 2022b). The open reading frame of Pim-3 mRNA encodes a protein consisting of 326 amino acids (Fujii et al., 2005). The Pim-3 protein shares a high percentage of sequence homology with other members of the Pim family, Pim-3 is most alike to Pim-1 in relation to structure and amino acid sequence, being 71% identical at the amino acid level, and Pim-3 and Pim-2 are 44% identical (Y. Y. Li and Mukaida, 2014). The crystal structure of the Pim-3 protein has not yet been established, however given the high sequence similarity (Figure 6), it is highly likely that Pim-3 can adopt a similar three-dimensional conformation to Pim-1 and Pim-2 (Y. Y. Li and Mukaida, 2014).

The Pim-3 proto-oncogene demonstrates various biological activities such as cell survival and proliferation, protein translation, and anti-apoptotic effects,

contributing to cancer progression (Liu et al., 2018). Similar to Pim-1 and -2, Pim-3 can contribute to tumorigenesis through the phosphorylation of BAD at Ser112, as Pim-3 knockdown has been shown to reduce phosphorylation at this site (Y. Y. Li et al., 2006; Popivanova et al., 2007). Myc activity can also be enhanced by Pim-3, along with control of protein synthesis (Beharry et al., 2011). Additionally, Pim-3 can also negatively regulate insulin secretion by inhibiting the activation of MAPK1/3 (ERK1/2) through SOCS6 (UniProt, 2022b).

Alongside their oncogenic potential, Pim kinases are involved in normal cell functions including the regulation of early B lymphopoiesis (Domen et al., 1993), the self-renewal of mouse embryonic stem cells (Aksoy et al., 2007), and myocardial regeneration (Fischer et al., 2009). While most established for their pathological role in cancer progression, there is increasing evidence for wider pathological roles of Pim kinases within the context of CVD, thrombosis, and inflammation. The Pim kinase isoforms have widespread expression in cardiovascular tissues including the heart, coronary artery, aorta, and blood, and have been demonstrated to be upregulated in a number of co-morbidities and risk factors for CVD such as smoking (de Vries et al., 2014). Pim-1 mRNA expression is increased 11-fold in monocytes of patients with coronary artery disease (CAD) (Chittenden et al., 2006), has shown to be increased in the plasma of pulmonary arterial hypertension (PAH) patients (Renard et al., 2013), and has demonstrated an increase in the aorta of diabetic rats (K. Wang et al., 2017). Pim-2 has shown to be upregulated in atherosclerotic arteries in CAD (Archacki et al., 2003).

Pim kinase, 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). It has recently been shown that Pim-1 is expressed in human and mouse platelets (Unsworth et al., 2021), and plays an important role in haematopoietic stem cell (HSC) proliferation and survival (An et al., 2013a).

Investigation of the effect of Pim kinase on platelet indices demonstrated that Pim-1 and Pim-2 single gene deficient mice do not display any differences in

platelet number (Laird et al., 1993; An et al., 2013b), whereas Pim triple KO mice displayed a reduced platelet count (Mikkers et al., 2004; An et al., 2013b), suggesting some but not total compensation of Pim-3 for platelet production, demonstrating functional redundancy between the three Pim isoforms. Bone marrow HSCs in Pim triple KO mice also show a reduced ability to differentiate into megakaryocytes *in vitro* (Mikkers et al., 2004) and have reduced gene expression of platelet transcription factor GATA1 (An et al., 2013b). This is supported by observations that Pim-1 mRNA is upregulated in response to thrombopoietin, an essential mediator of thrombopoiesis (An et al., 2013b). Reported thrombocytopenia in clinical trials with pan Pim kinase inhibitors (Cortes et al., 2018; Iida et al., 2021) provide further evidence that Pim kinases mediate platelet production. These findings indicate that Pim-1 could be exploited therapeutically as a novel target for thrombocytosis.

As well as the regulation of platelet number, Pim kinases play a role in mediating platelet function. It has been shown that treatment of human platelets with a pan Pim kinase inhibitor (AZD1208) caused a reduction in surface expression levels of the thromboxane A2 receptor (TPaR), leading to reduced signalling events downstream of TP-coupled G proteins (Gq and Ga13), and therefore reduced platelet activation and thrombus formation (Unsworth et al., 2021).

Platelets rely on G protein-coupled receptors (GPCR) to mediate their activation in response to blood vessel damage and Pim-1 has been shown to play a role in the regulation of GPCR function (Grundler et al., 2009). Pim kinase inhibition attenuated CXCR4 mediated platelet responses, inhibiting platelet aggregation to SDF-1a (Unsworth et al., 2021). Additionally, observations from studies using Pim-1 deficient mice show reduced thrombus formation *in vitro* and reduced thrombosis *in vivo* following acute treatment of mice with a pan Pim kinase inhibitor (Unsworth et al., 2021). Despite Pim-1 deficient mice demonstrating reduced thrombotic capacity, normal haemostatic functions were maintained with no adverse effects on bleeding (Unsworth et al., 2021), suggesting that Pim-1 inhibition may be a suitable target for anti-platelet therapy. The roles for Pim-2 and Pim-3 in platelet function have yet to be explored and described.

Pim kinase and the Endothelium

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. The contribution of Pim kinases in the regulation of ECs and atherothrombotic pathology has not been investigated or characterised to a large extent. More CVD-relevant endothelial models have identified that Pim-1 is present in human coronary artery endothelial cells (HCAECs), rat endothelium (Z. Zhang et al., 2020), and mouse aortic ECs (Walpen et al., 2012).

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 human umbilical vein ECs (HUVECs) within the context of cancer. HUVECs are more often used *in vitro* as a model of EC function due to their lower cost, ease of growth, and simple and quick isolation process (Lau et al., 2021). All three isoforms of Pim kinase are expressed in HUVECs and have been shown to play regulatory roles in their adhesion, inflammation, and migration (Min et al., 2012; Walpen, 2012; H. Yang et al., 2011; X. Zhang et al., 2017). The role of Pim kinase in the EC functions that are most relevant to ACS and CVD is currently unknown.

A variety of roles have been identified for both Pim-1 and Pim-3 kinases in ECs in relation to angiogenesis (Min et al., 2012; Walpen et al., 2012; H. Yang et al., 2011). Although the endogenous expression of Pim-1 in HUVECs appears low, it has been shown to increase in response to vascular endothelial growth factor (VEGF) treatment (M. Chen et al., 2016; Zippo et al., 2004) and pharmacological inhibition of phosphoinositide 3-kinase (PI3K) (Min et al., 2012). Quiescent ECs rapidly switch to an activated state gaining the ability to sprout, migrate, and proliferate in response to VEGF signalling during angiogenesis (Muhleder et al., 2021). Pim-1 has been shown to mediate VEGF signalling and endothelial differentiation downstream of the Flk1 receptor in mice endothelial precursor cells (Zippo et al., 2004). Pim-1 knockdown prevents the differentiation of the endothelial precursors, demonstrating that Pim-1 is required for EC differentiation (Mikkers et al., 2004). In addition to EC

Flk1 mediated differentiation, studies using HUVECs have also identified a role for Pim in the regulation of VEGF-dependent eNOS signalling downstream of Flk1 (M. Chen et al., 2016). siRNA-mediated knockdown of Pim-1 in HUVECs reduced VEGF-induced eNOS phosphorylation at Ser-663 (M. Chen et al., 2016), suggesting roles for Pim kinase in EC differentiation, eNOS activation, and angiogenesis.

Loss of EC adhesion is also necessary for the initiation of angiogenesis. During atherosclerotic plaque rupture or erosion, ECs detach from the vascular wall, contributing to the pathogenesis of atherothrombosis. It has been demonstrated that Pim-1 regulates the adhesive phenotype of mouse aortic ECs, with the loss of Pim-1 associating with increased EC adhesion (Walpen et al., 2012; Walpen, 2012), indicating the potential for Pim kinase inhibitors to reduce atherosclerotic plaque erosion-linked atherothrombotic events that occur when ECs detach from the vascular wall. Additionally, RNA interference (RNAi) of Pim-3 has shown to reduce HUVEC spreading and migration but does not alter endothelial adhesion to cell matrices *in vitro* (P. Zhang et al., 2009), highlighting Pim-3 gene silencing as an effective targeting strategy.

The mechanisms behind the contribution of Pim-1 and Pim-3 to EC attachment and spreading may be related to their function in relation to the cytoskeleton. Pim-1 has been shown to regulate cytoskeletal organisation in cancer cells (Bhattacharya et al., 2002; Santio et al., 2015), and siRNA mediated Pim-1 knockdown in HUVECs has been shown to attenuate EC sprouting (Zippo et al., 2004). Pim-3 has been observed to be localised at the leading edge of lamellipodia in specialised actin focal complexes within ECs (P. Zhang et al., 2009), supporting its role in the regulation of the actin cytoskeleton. Pim-3 silencing was shown to reduce the ability of ECs to form stress fibres (P. Zhang et al., 2009), suggesting that Pim-3 plays a role in regulating the endothelial cytoskeleton.

The formation of actin stress fibres, filopodia, and membrane ruffles in ECs can be induced by TNFα, an inflammatory cytokine which is known to mediate

EC dysfunction (H. Yang et al., 2011), increase vascular permeability (Friedl et al., 2002), and influence EC migration (Gao et al., 2002). Pim-3 has been shown to regulate TNFα signalling in ECs, with TNFα treatment associated with increased Pim-3 mRNA expression in HUVECs (H. Yang et al., 2011). siRNA mediated knockdown of Pim-3 significantly impairs TNFα-induced formation of EC membrane protrusions *in vitro*, and Pim-3 silencing also inhibited EC sprouting *in vivo* (H. Yang et al., 2011). These results support a positive role for Pim-3 in TNFα mediated cytoskeleton rearrangements and TNFα induced angiogenesis. Loss of Pim-1 has also shown to increase actin polymerisation (Walpen et al., 2012). TNFα is a key inflammatory mediator and activator of EC damage. These findings demonstrate the potential of targeting Pim-1 and Pim-3 to protect against pathological activation of the endothelium, preventing detachment of ECs, and unwarranted angiogenesis.

Pim kinase inhibitors

Designing novel Pim kinase inhibitors is now a very active area of research (Arrouchi et al., 2019), with the development of several novel inhibitors of Pim kinases for the treatment of haematological and solid tumour malignancies (Arrouchi et al., 2019). Phase I clinical trials using Pim kinase inhibitors for the treatment of several malignancies have demonstrated drug efficacy and shown AZD1208, a pan Pim kinase inhibitor to be safe and tolerable at therapeutic doses, without bleeding complications (Cortes et al., 2018; Raab et al., 2019), indicating the potential to repurpose Pim kinase inhibitors for the treatment of relevant diseases.

Pim kinase inhibitor AZD1208, in addition to its anti-cancer properties, has demonstrated the ability to inhibit platelet function and platelet-mediated thrombosis but is not associated with impaired haemostasis (Unsworth et al., 2021), indicating that drugs targeting Pim kinase activity could offer an anti-thrombotic strategy that is not associated with increased bleeding, a common side effect of current anti-platelet therapies. Although Pim kinase inhibitor anti-platelet properties are promising, there is a need to identify a class of drugs that do not interfere adversely with haemostasis, simultaneously targeting the endothelium to boost its anti-thrombotic properties in order to avoid the problems of variability and lack of efficacy that current anti-platelet drugs

present. A further deepened understanding of Pim kinase and its role within the vascular endothelium is essential for novel approaches in the treatment of CVD.

Hypothesis

Pim kinase will represent a novel anti-thrombotic targeting strategy as Pim kinase inhibitors will restore the anti-thrombotic capacity of the endothelium.

Aim

The aim of this research is to investigate the role of the Pim kinase isoforms (Pim-1, Pim-2, and Pim-3) in EC biology and their contribution to atherothrombosis using HUVECs as a model of EC function to determine the anti-thrombotic potential of Pim kinase inhibitors.

Objectives

1. **Elucidate the contribution of the Pim kinase isoforms (Pim-1, Pim-2, and Pim-3) to the regulation of EC function.**
    - Determine Pim kinase isoform expression using *in vitro* models of EC activation.
    - Determine the roles of Pim kinase in EC function in health and disease.
2. **Characterise the contribution of the Pim kinase isoforms to the anti-platelet and anti-coagulant properties of ECs.**
    - Explore the effect of Pim kinase inhibitor treatment on EC production and release of mediators of thrombosis.

Materials and methods

Ethical statement

Procedures and experiments were conducted following appropriate approval by The Research Ethics and Governance Committee (REGC) at Manchester Metropolitan University (EthoS approval- 37094).

Materials

Endothelial cells

For our set of experiments, three separate batches of HUVECs were purchased from Promocell (Heidelberg, Germany) and maintained in EC growth medium MV2 supplemented with 5% foetal calf serum (FCS) (Promocell) and 100U/mL penicillin/streptomycin.

Pim kinase inhibitor preparation

AZD1208 was purchased from Cambridge BioScience (UK) and dissolved in dimethyl sulfoxide (DMSO) (Thermo Scientific, US), to make 10mM and 100mM stock solutions, which were stored at -20°C. AZD1208 was used at a range of concentrations including 0.1, 0.3, 1, 3, 10, 30, and 100μM. 0.1% DMSO was used as vehicle control in all experiments conducted.

Mediators of endothelial cell activation/dysfunction

Cigarette smoke extract (CSE), an initiator of endothelial erosion, was prepared by infusing EC medium with cigarette smoke. A retort stand, stripette, tubing, and pump were set up, as shown in Figure 8. The pump was set to draw in air and 10mL media was aspirated into the 25mL stripette. The pump was then stopped, and tubing was attached from stripette to the cigarette holder with the cigarette attached firmly onto the end. CSE was produced using ~385ml of mainstream smoke (~7–8 average puffs) from a Marlboro Gold cigarette (7mg tar, 0.6mg nicotine). The pump was started to draw in air at 70ml/min and the cigarette was lit before fume hood was closed three-quarters. Cigarette ash was knocked off the end of the lit cigarette into a waste beaker full of water as the cigarette burned. The tubing attached beneath the stripette was removed and air flow was reversed in the pump in order to collect the CSE-infused media into a fresh tube. The pump was then stopped, and cigarette safely disposed of. In a Class II cabinet, collected media was taken into a 10mL syringe and passed through a 0.2μM filter to remove any potential contaminants. CSE was used at a 1:10 dilution for cell treatment and applied to cells within 15 minutes of collection.

Figure 8: Setup for the collection of cigarette smoke extract. *Retort stand, tubing, stripette, and pump were set up with a Marlboro Gold cigarette to collect CSE-infused media for cell treatment.*

Tumour necrosis factor (TNFα) was purchased from Peprotech (UK) and stored at -20°C. TNFα was dissolved in 1% DMSO at a 1:100 dilution and used at 5ng/mL.

Methods

Endothelial cell culture

HUVECs were cultured at 80% confluency up to passage 7 in MV2 media + 5% FCS and 100U/mL penicillin/streptomycin at 37°C with 5% carbon dioxide (CO_2). HUVECs were treated with AZD1208 (0, 0.1, 0.3, 1, 3, 10, 30, and 100μM), and/or mediators of EC activation/dysfunction (TNFα (5ng/mL), CSE (1:10 v/v), and both combined), then cultured for 24 hours prior to further experiments being carried out. The cells were routinely checked for infection.

Gene expression- qPCR

RNA lysate preparation

To enable investigation of the expression of the Pim kinase isoforms and endothelial mediators of thrombosis, ECs were cultured under conditions described above. RNA lysates were prepared from 24-well plates where 200μL buffer RL (P/N 90055) obtained from the Total RNA Purification Kit (Norgen Biotek Corp) 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.

Total RNA extraction- RNA purification

RNA was extracted using the Total RNA Purification Kit following the manufacturer's instructions. In short, genomic DNA was removed from 600µL lysate using a gDNA removal column and centrifuged at 14,000xg (~14,000 RPM) for 1 minute (flowthrough was retained for RNA purification and stored on ice). An RNA purification column was assembled, and 600µL of lysate applied onto the column with ethanol (60µl of 96-100% ethanol added to every 100µL of lysate), then centrifuged for 1 minute at 3,500xg (~6000 RPM). The column was washed three times with 400µL of wash solution A and centrifuged for 1 minute, flowthrough was discarded. RNA was eluted using 50µL of elution solution A and centrifugation of the column for 2 minutes at 200xg (~2000 RPM) followed by 1 minute at 14,000xg (~14000 RPM). The purified RNA sample was stored at -20°C for a few days. For long term storage, samples were stored at -80°C. The concentration and purity of RNA were measured using the Nanodrop OneC Spectrophotometer (Thermo Scientific, US), values obtained shown in Table 1. An acceptable limit of purity was set at ~2.0, a purity of ~1.8 suggested DNA contamination (ThermoScientific, 2022).

Table 1: *RNA sample purity and concentration*

Repeat number (N)	**Sample**	**Purity**	**Concentration (ng/µL)**
1	Healthy	1.93	68.7
	CSE	1.96	72.8
	TNFα	1.94	71.9
	Both (CSE and TNFα)	1.96	75.7
	Healthy (+AZD1208)	1.92	68.7
	CSE (+AZD1208)	1.90	63.7
	TNFα (+AZD1208)	1.92	64.4
	Both (+AZD1208)	1.93	59.1

2	Healthy	1.95	55.8
	CSE	1.93	55.0
	TNFα	1.96	59.4
	Both	1.98	51.1
	Healthy (+AZD1208)	1.95	48.4
	CSE (+AZD1208)	2.04	46.4
	TNFα (+AZD1208)	2.05	46.0
	Both (+AZD1208)	2.08	41.2
3	Healthy	1.92	50.8
	CSE	1.91	44.7
	TNFα	1.90	47.9
	Both	1.96	43.0
	Healthy (+AZD1208)	2.08	47.8
	CSE (+AZD1208)	1.96	47.3
	TNFα (+AZD1208)	1.97	45.6
	Both (+AZD1208)	1.90	34.2

Reverse transcription- RNA to cDNA

Purified RNA from samples was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (200) (QIAGEN, Germany) according to the manufacturer's instructions, and as 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 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, then immediately put back on ice. A mastermix was then made up: per tube- 1µL RT, 1µL Primer mix, 4µL RT buffer. The samples were then placed in the

TurboCycler 2 Thermal Cycler (Blue-Ray Biotech), conditions are listed in Table 2.

Table 2: *Thermal Cycler setting for reverse transcription reaction*

Stage	Temperature	Time	Cycles
1	42.0	30:00	1
2	95.0	3:00	1
3	4.0	Hold	1

At the end of the run, 20µL RNAse free water was added to each sample (to yield a final concentration of 5ng/µL – ready for qPCR). 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 three independent sets of samples.

Quantitative Real-Time PCR

Primers were designed and purchased from Sigma-Aldrich (US) (primer sequences listed in Table 3). Gradient PCR was carried out prior to qPCR to optimise primer annealing temperatures (Appendix 1 and 2). qPCR reactions were performed in 96-well plates using the QuantiNova SYBR Green PCR kit. Each 10µL reaction contained 5µL of SYBR Green, 3µL DNAse free water, 1µL primer mastermix (392µL RNAse free water, 4µL forward primer, 4µL reverse primer), and 1µL of cDNA sample. PCR conditions are listed in Table 4 and summarised in Figure 9. All qPCR analyses were performed in triplicate (technical repeats) using the CFX Connect Real-Time PCR Detection System (Bio-Rad, US).

Table 3: *Primer sequences for qPCR*

Primer name	Forward sequence	Reverse sequence
GAPDH (housekeeping gene)	CGGATTTGGTCGTATTGGGCG	GTCTTCACCACCATGGAGAAGGC

RPLPO (housekeeping gene)	GCAGCAGATCCGCATGTCCC	TCCCCCGGATATGAGGCAGCA
eNOS	TCGGCCGGAACAGCACAAGA	AAAGGCGCAGAAGTGGGGGT
VWF	TGCCATGGAACGTGGTCCCG	ACCACCGCCTTTGAGGCTCC
CD39	ACACATCCATGTGCCCATCACA	GGTGCCTTCCTCTGGATGCACT
Pim-1	TTCTGGCAGGTGCTGGAGGC	GCGGATCCACTCTGGAGGGC
Pim-2	CTTCGCAGGACACCGCCTCA	AAAGGCCGCTCGAGGACCAG
Pim-3	TTCGGGTGCGCTGCTCAAGG	GAGAAGCACGCCCAGCGACC

Table 4: *qPCR set up*

Temperature	Cycles
95.0	1
95.0 62.0	40
62.0	1
95.0	1

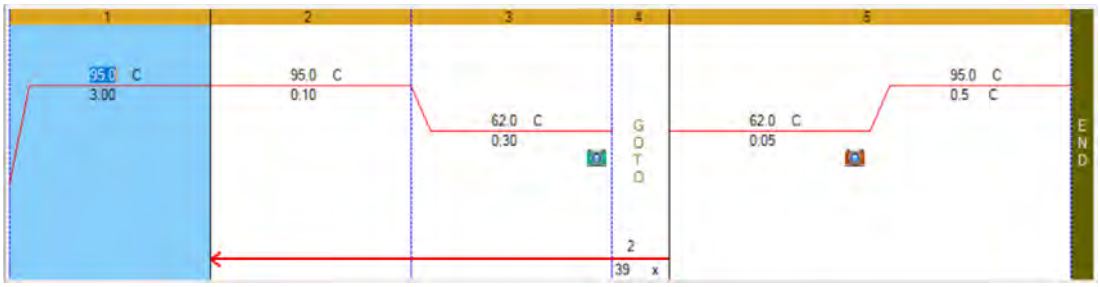

Figure 9: Conditions for qPCR reaction. *Samples were placed on the Real-time PCR machine and qPCR reaction was carried out. All analyses were performed in triplicate.*

Protein expression- Western blotting

Protein lysate preparation

To enable investigation of the protein expression of Pim kinase isoforms, HUVECs were cultured under conditions described previously. Protein lysates were prepared by extracting total protein from ECs using RIPA protein extraction reagent (Sigma-Aldrich, US), supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, US), 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). Plate was put on ice and supernatant aspirated out of each well of plate and into 2mL eppendorf tubes. PBS was added to each well of plate to wash, aspirated, then 200µL RIPA/protease/phosphatase inhibitor mastermix 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.

Sample preparation

A Pierce BCA protein quantification assay (Thermo Scientific, US) was performed to determine the protein concentration of each HUVEC cell lysate. The appropriate quantity (30µL) of protein lysates were then prepared by the addition of Laemmli sample buffer (1M Tris-HCl, 8% Sodium Dodecyl Sulfate (SDS), 40% glycerol, β-mercaptoethanol, 0.5M Ethylenediaminetetraacetic acid (EDTA), and bromophenol blue (pH 6.8)). To reduce and denature the samples, each cell lysate in sample buffer was heated to 90°C in a Accublock Digital Dry bath incubator (Labnet International) for 5 minutes before use.

SDS-PAGE Gel Electrophoresis

Protein transfer

Equal amounts of prepared lysates (30µL) at 10ug were loaded into the wells of a 10% Mini-PROTEAN pre-cast polyacrylamide gel, along with 4µL of a molecular weight marker (GeneRuler 1 kb DNA Ladder) (Thermo Scientific, US). The gel was run with Tris-glycine running buffer (192mM glycine, 25mM tris, 1% SDS (pH 8.3)) at 60V for 30 minutes then increased to 120V for 60 minutes to ensure sufficient separation of the proteins.

Blocking

Polyvinylidene difluoride (PVDF) membrane was activated by soaking in methanol for 1 minute then rinsed with 1% transfer buffer (tris glycine 10x transfer buffer- 192mM glycine, 25mM tris, 20% methanol) before the transfer stack (gel and membrane embedded in filter paper) was prepared. Samples were then transferred from gel to membrane in a transfer tank by blotting at 10V for 35 minutes. Following transfer, the PVDF membrane was blocked with 5% BSA for 1 hour at room temperature.

Antibody incubation

Primary antibodies raised against Pim-1 (C93F2), Pim-2 (D1D2), and Pim-3 (D17C9) (Cell Signaling Technology, US) were prepared at 1:1000 (5µL in 5mL of BSA), and the membrane was incubated with primary antibody for 1 hour at room temperature. The membrane was then washed 3 times with Tris buffered saline tween-20 (TBST) (10mM Tris, 150mM NaCl, 0.2% tween-20) for 10 minutes each. HRP-conjugated secondary anti-rabbit IgG antibody was then prepared at 1:10000 (1µL of antibody in 10mL BSA), and membranes incubated with secondary antibody for 1 hour at room temperature on a rocker. The membranes were washed again 3 times with TBST for 10 minutes each.

Actin (mouse) antibody (ab6276, Abcam, UK) was prepared at 1:2500 (4µL of actin and 10mL BSA) and used as a loading control to normalise the levels of protein detected.

Protein Immunodetection and visualisation

Membranes were imaged using the Li-COR Odyssey Fc to detect antibody binding using an enhanced chemiluminescence system. Band density was quantified using ImageJ software.

Endothelial Cell function assays

Cell growth inhibition assay (MTS)

HUVEC proliferation and cytotoxicity was measured using an MTS colorimetric assay (Abcam, UK). ECs cultured on 96-well plates were incubated for 24 hours with increasing concentrations of AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100µM), vehicle control (0.1% DMSO), or 10% Sodium Dodecyl Sulfate (SDS). SDS was included as a positive control for cell lysis. SDS has biochemical and

physical effects on cells membranes, and is considered a cell wall perturbing agent, triggering the cell wall integrity (CWI) signalling pathway, a kinase cascade to maintain cell integrity (Cao et al., 2020). SDS effects are concentration dependent and range from loss of barrier function and increased permeability to complete cell lysis (Cao et al., 2020), therefore a concentration of 10% was chosen to demonstrate cell lysis.

After 24-hour treatment, 100µL of MTS tetrazolium reagent (abcam-ab197010) was added to each well and the plate was incubated for 4 hours at 37°C before absorbance was read at 490nm with a Promega GloMax Discover microplate reader. Absorbance correlates with cell proliferation, with increased absorbance indicating increased proliferation. Two replicate wells (technical duplicates) were included for each analysis, and three independent experiments were performed.

Fluorescence microscopy- Cytoskeleton imaging

HUVEC count and cytoskeleton dynamics were determined using phalloidin staining and fluorescence microscopy. Jasplakinolide is a commonly used actin filament polymerising and stabilising drug, which competes with phalloidin for filamentous actin (F-actin) binding (Holzinger, 2009), and was included as a positive control for cytoskeleton disruption.

Endothelial cells cultured on 24-well plates were incubated for 24 hours with AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100µM), Jasplakinolide (100µM) (Merck, Germany), or vehicle control (0.1% DMSO). After treatment, cells were washed 3 times with PBS before being fixed with 10% formalin (Merck, Germany) and kept at 37°C for 15 minutes. Fixed cells were permeabilised in 0.1% Triton for 5 minutes before washing 3 times with PBS. Cells were then stained with DAPI (1:1500) (Sigma-Aldrich, US), which stains the nuclei (Tarnowski et al., 1991), and FITC-conjugated-phalloidin (1:750) (Thermo Scientific, US), which stains F-actin filaments of the cytoskeleton (Abcam, 2022), before being imaged using a 10x lens on a Celena S Digital Imaging System (Logos Biosystems). Three images were taken per well and representative images were analysed to determine nuclei number and fluorescence intensity (FI) using ImageJ software.

Magnetic bead based Luminex Multiplex Assay

Supernatant preparation

Pre-treated cell culture media was pipetted into eppendorf tubes and centrifuged at 1,500 RPM for 10 minutes. Supernatant was immediately aliquoted, and samples were stored at -80°C. It was ensured that freeze/thaw cycles were minimised in order to preserve sample quality.

Multiplex Assay

The release of mediators of thrombosis and inflammation from ECs was measured using a bespoke Magnetic Luminex multi-analyte kit (R&D systems) to detect the following analytes: IL-6, and IL-8, and TNFα.

Frozen EC sample supernatants were defrosted, brought to room temperature, and centrifuged at 16,000xg for 4 mins, immediately prior to use in the assay. The assay was performed as per the manufacturer's protocol. In short, 25µL samples and standards were diluted (1:2) in Calibrator Diluent RD6-52 provided and mixed with 50µL magnetic microparticle cocktail (1:1 v/v). The Luminex plate was incubated for 2 hours in the dark at room temperature on a horizontal orbital plate shaker (0.12'' orbit, set at 800 +/- 50 RPM). Wash buffer was then added to remove unbound substances (antibodies immobilised by magnetic microparticles and magnetic platform). 50µL of biotinylated antibody cocktail (specific to analytes) was added to each well and incubated in the dark for 1 hour on an orbital shaker (800 +/- 50 RPM) at room temperature. After incubation the plate was washed to remove unbound biotinylated antibodies. 50µL of Streptavidin-phycoerythrin (PE) conjugate was added to each well and incubated in the dark for 30 mins on an orbital shaker set (800 +/- 50 RPM) at room temperature. After this incubation, the plate was washed to remove unbound streptavidin-PE. Microparticles were resuspended in 100µL wash buffer (2 minutes of shaking). The plate was read within 90 minutes using the Promega GloMax Discover plate reader.

Statistics

Data was analysed and quantified using ImageJ software and GraphPad Prism Version 8.0.1. Results are expressed as mean ± standard error. A p-value of less than 0.05 was considered to be statistically significant. When comparing two sets of data, an unpaired, two-tailed Student's t-test was used.

If more than two means were present, significance was determined by a one-way or two-way ANOVA followed by Bonferroni correction (multiple comparisons). Where data was normalised, statistical analyses were performed prior to normalisation. Assays were performed with 3 experimental repeats (n=3).

Results

HCAECs are considered the most relevant cell type for the study of atherosclerosis because of their relevance to arterial thrombosis and CVD. However, HCAECs are expensive to buy and are supplied at passage 2, limiting the number of passages possible before they undergo replicative senescence. For this reason, we chose to use HUVECs in our research, which are comparatively cheap and more easily obtainable. Results of HUVEC-based studies are assumed to be transferrable to other EC types, such as HCAECs (Lau et al., 2021).

Expression of Pim-1 in HUVECs

Pim kinase isoforms, Pim-1, -2, and -3 have shown to be expressed in HUVECs (Min et al., 2012). To confirm and verify Pim isoform expression in HUVECs, we first performed western blot analysis of HUVEC lysates. Pim-1 western blot analysis identified protein bands of 34 and 44kDa molecular weight indicating the expression of both Pim-1 variants, Pim-1L and Pim-1S in HUVECs, validating previously published data. Platelet lysates were included as a positive control (Figure 10).

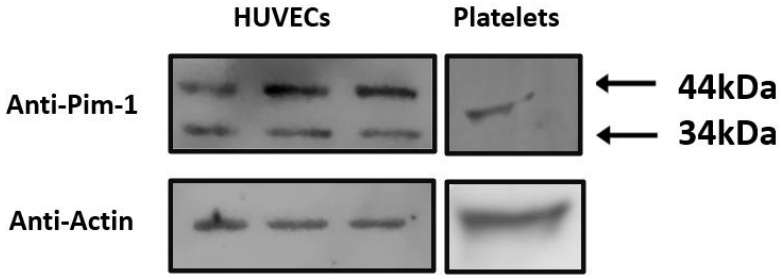

Figure 10: Protein expression of Pim-1 in HUVECs. *Healthy HUVEC lysates were prepared with the addition of Laemmli sample buffer separated on SDS PAGE gels and transferred to PVDF membranes before immunoblotting with Pim-1 antibody for 1 hour. Platelet lysates were included as positive controls. Actin was included as a loading control. Representative blots are shown.*

Unexpectedly, under the experimental conditions used and reagents available, Pim-2 and Pim-3 protein expression in HUVEC lysates appeared to be below levels of detection. While Pim-2 and Pim-3 isoforms were not

identified in our assays, expression of either paralog in ECs cannot be ruled out and requires further investigation.

Physiological concentrations of AZD1208 do not alter EC viability

Pim kinases have previously been shown to play important roles in cell survival promoting cell evasion of apoptosis signals (L. S. Chen et al., 2009), and it has been shown that the inhibition of Pim kinase enables the activation of pro-apoptotic BAD and the initiation of apoptosis (Macdonald et al., 2006). Therefore, AZD1208,a potent, selective, ATP-competitive pan Pim kinase inhibitor that affects all three Pim isoforms at a low nanomolar range; IC_{50} values of 0.4, 5, and 1.9nM for Pim-1, 2, and 3, respectively (Cortes et al., 2018; Cervantes-Gomez et al., 2019; Lee et al., 2019), was used to determine the effects of Pim kinase inhibitor treatment on HUVEC proliferation and survival. HUVECs were treated with either 0.1% DMSO (vehicle control), 10% SDS (positive control), or increasing concentrations of AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100μM) for 24 hours and cell proliferation determined via the MTS assay. As expected, treatment of cells with 10% SDS caused a significant reduction in cell proliferation (P=0.0039) (Figure 11). In contrast, following treatment with increasing physiological concentrations of AZD1208 (up to 10μM), and even at an increased 30μM concentration, no effect on EC proliferation was observed (Figure 11), indicating that Pim kinase inhibition does not initiate EC apoptosis at therapeutically relevant concentrations (Cortes et al., 2018). The upward trend in absorbance could suggest that the ECs are proliferating faster at increasing concentrations of AZD1208 up to 30μM. Treatment with a supraphysiological concentration (100μM) suppressed the proliferation of HUVECs with obvious growth inhibition (P=0.0047), suggesting the induction of EC death.

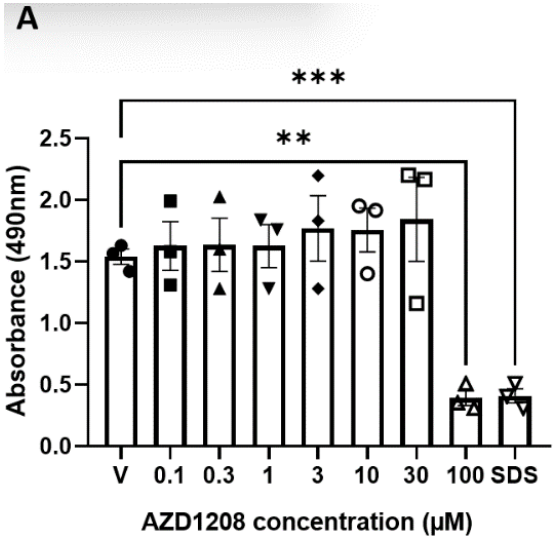

Figure 11: AZD1208-treated HUVEC absorbance determined via MTS assay. *HUVECs were pre-treated with a range of concentrations of the pan Pim kinase inhibitor AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100μM for 24 hours. MTS reagent (abcam- 197010) was added 4 hours before absorbance was read at 490nm on a GloMax explorer microplate reader. Absorbance at 490nm was determined. Results are expressed as mean + S.E.M for n=3. ** indicates P≤0.01, ***≤0.001. Data was analysed using One-Way ANOVA and graph plotted using GraphPad Prism.*

AZD1208 alters EC cell count and actin cytoskeleton dynamics

To further investigate the effects of Pim kinase on cell survival, HUVECs were treated with 0.1% DMSO, Jasplakinolide (100μM), or increasing concentrations of AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100μM) for 24 hours. A DAPI stain was used to visualise cell nuclei through the use of immunofluorescence. No significant difference in nuclei number (cell count) was observed following treatment with increasing physiological concentrations of AZD1208 when compared with vehicle control, however 100μM AZD1208 decreased cell number significantly, suggesting EC death or EC detachment, supporting the MTS data (Figure 12B).

Pim kinases have shown to contribute to EC attachment and spreading (P. Zhang et al., 2009), suggesting a role in the regulation of the cytoskeleton. FI of FITC-conjugated phalloidin staining (which binds to F-actin) was then used to determine whether Pim kinases play a role in EC cytoskeleton arrangement and dynamics. HUVECs were treated with increasing concentrations of AZD1208 for 24 hours and FI of phalloidin binding explored. Interestingly, a significant decrease in FI of HUVECs treated with 0.1μM AZD1208 was observed when compared to vehicle control (P=0.0023), but not at other physiological concentrations (up to 10μM) (Figure 12C). Following treatment with 30μM and 100μM AZD1208, FI was also decreased, although

not significantly with 100μM AZD1208-treated cells when compared with vehicle control (P=0.0460 and 0.2366). Together these observations suggest that Pim kinase inhibitors alter HUVEC survival (supraphysiological concentrations) and regulation of the cytoskeleton. Representative fluorescence microscopy images (vehicle, 1, 10, 100μM, and Jasplakinolide) are shown in Figure 12A.

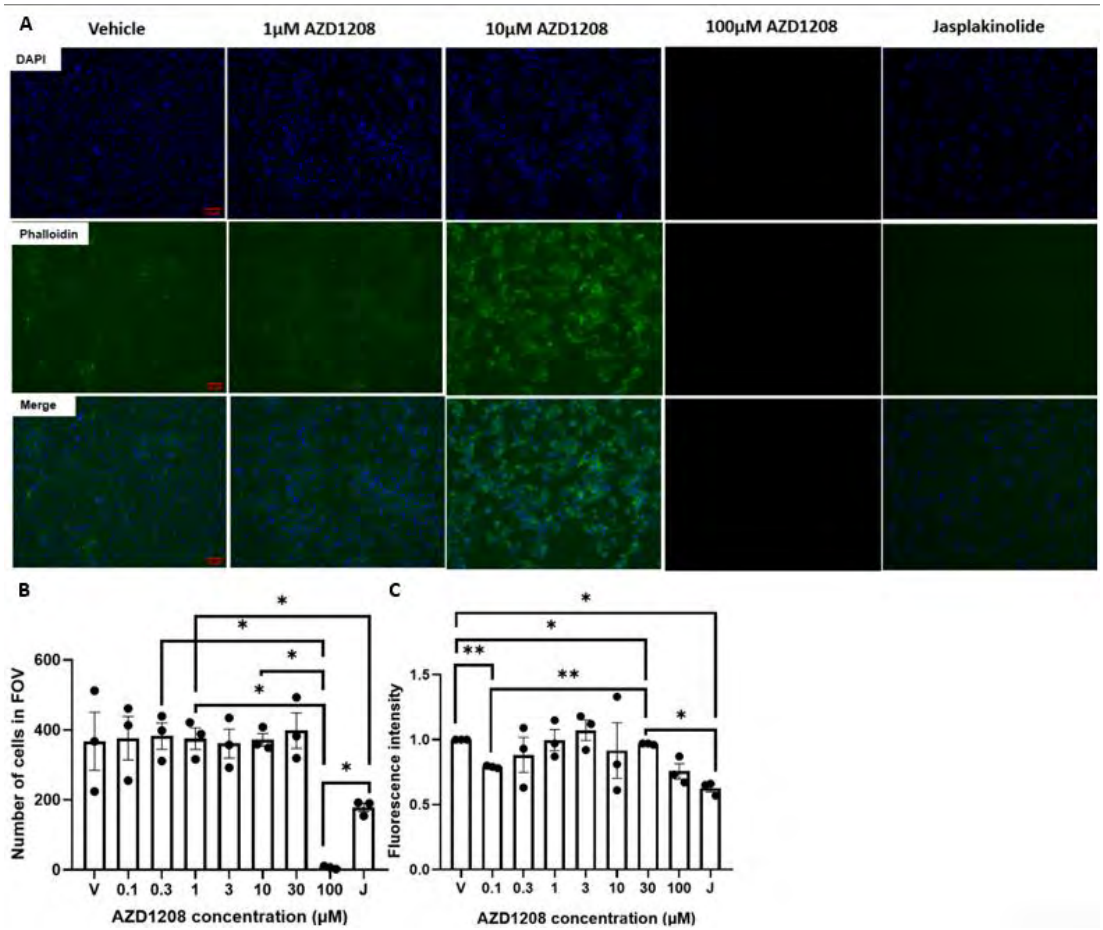

Figure 12: Fluorescence microscopy determining the effect of AZD1208 on EC number and the F-actin cytoskeleton. *HUVECs were pre-treated for 24 hours with or without increasing concentrations of AZD1208 (0.1, 0.3, 1, 3, 10, 30, and 100μM), Jasplakinolide (100μM), or vehicle control (0.1% DMSO). HUVECs were then fixed, stained, and imaged.* **A.** *Representative images were taken at 24 hours. HUVECs were labelled with DAPI and phalloidin for visualisation.* **B.** *Number of cells were counted manually using a cell counter on ImageJ software.* **C.** *Cytoskeleton organisation was assessed by labelling F-actin filaments with phalloidin and analysing FI on ImageJ software. Images of HUVECs were captured using the 10x lens on a Celena S logos imaging system. Scale bar represents 50μm across all images. Results shown are expressed as mean + S.E.M for n=3. * indicates P≤0.05, **≤0.01. Where normalised data is shown, statistics were performed prior to normalisation. Data was analysed using a One-Way ANOVA and graphs plotted using GraphPad Prism.*

Pim kinase gene expression in HUVECs in response to mediators of EC damage

Previous studies have shown that CSE triggers EC dysfunction *in vitro* by initiating oxidative stress and an inflammatory response (Bernard et al., 2019). TNFα is a pro-inflammatory cytokine that initiates vascular injury, leading to EC dysfunction (Madge and Pober, 2001). Pim kinase is upregulated in various cell types downstream of mediators of inflammation. This provides a rationale to investigate its effects in HUVECs infused with either CSE to mimic physiological concentrations of smokers as a model of EC erosion, TNFα to demonstrate EC dysfunction, or both (CSE+TNFα), to establish a model of atherothrombosis. qPCR was used to determine the effect on Pim kinase gene transcription of exposing HUVECs to mediators of cell damage (CSE/TNFα/both) for 24 hours. There were no significant changes noted in Pim gene expression in HUVECs exposed to either cell damage indices (CSE or TNFα) (Figure 13).

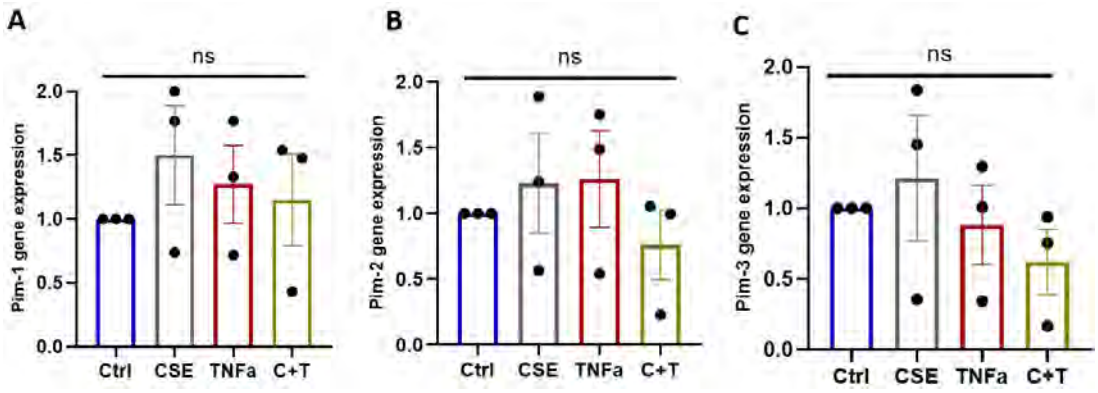

Figure 13: qPCR determining Pim-1, Pim-2, and Pim-3 gene expression levels. A. *Pim-1* ***B.*** *Pim-2* ***C.*** *Pim-3 gene expression was analysed in HUVECs damaged with CSE/TNFα/both for 24 hours. qPCR experiments were carried out in triplicate and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean + S.E.M for n=3. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism.*

Pim kinase protein expression in HUVECs in response to mediators of EC damage

Gene expression levels do not always correlate with protein expression levels. Therefore, protein expression of the Pim kinases in response to mediators of EC damage was determined. Pim-1 was shown to be expressed in damage-induced HUVECs (Figure 14A), and two bands were observed, representing both the 34 and 44kDa isoforms. There was no significant difference observed between Pim-1 protein bands in damage-induced HUVECs between the two

isoforms (Figure 14B) following treatment with either CSE, TNFα or both combined.

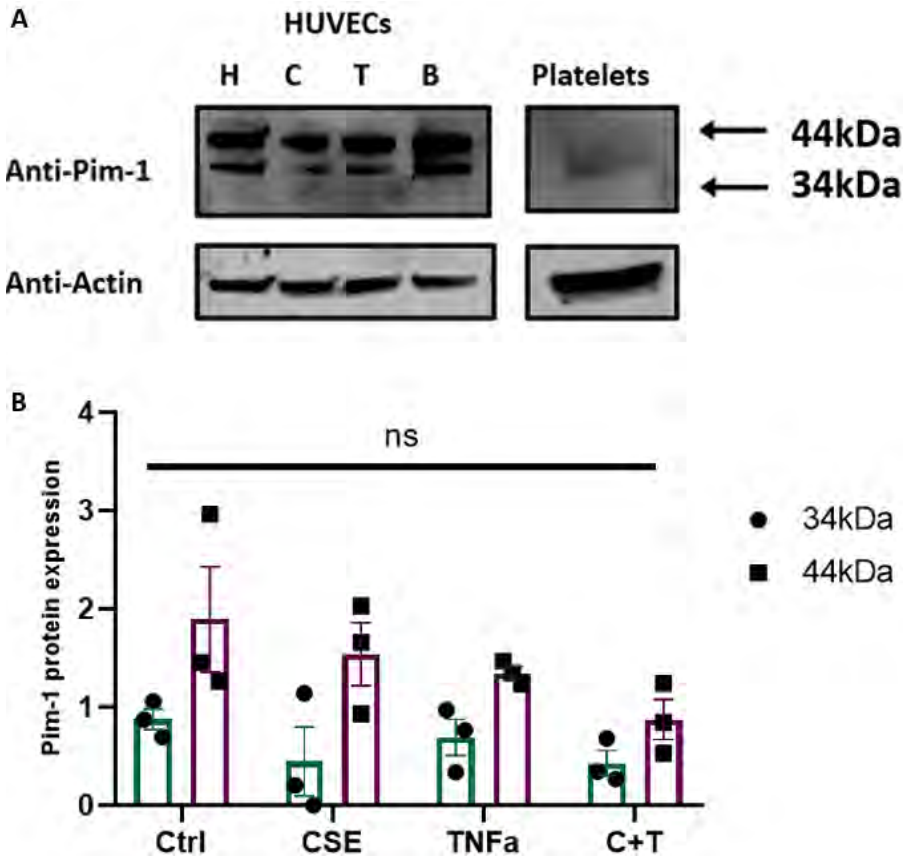

Figure 14: Pim-1 protein expression is confirmed in damage-induced HUVECs and remains unchanged between isoforms. *HUVEC lysates induced with CSE, TNFα, and both (CSE, TNFα) were prepared with the addition of Laemmli sample buffer, separated on SDS PAGE gels and transferred to PVDF membranes before immunoblotting with Pim-1 antibody. Platelet lysates were included as positive controls. Actin was included as a loading control.* ***A.*** *Representative blots are shown. B. Pim-1 isoform protein expression (34kDa = Green, 42kDa = purple) was analysed in HUVECs damaged with CSE/TNFα/both for 24 hours. Experiments were carried out in triplicate. Results are expressed as mean + S.E.M for n=3. Data was analysed using Two-Way ANOVA and graphs plotted using GraphPad Prism.*

Similar to our earlier observations, Pim-2 and Pim-3 expression was below levels of detection under the experimental conditions used. This could indicate that neither isoform is upregulated in response to mediators of EC damage. However, while Pim-2 and Pim-3 isoforms were not identified in our assays, expression of either paralog in activated or damaged ECs cannot be ruled out entirely and requires further investigation.

Changes in gene expression of mediators of thrombotic propensity in response to HUVEC activation

There are various mediators of thrombosis and inflammation that are involved in atherothrombotic pathology. To characterise the effect of CSE and TNFα on the thrombotic propensity of ECs, HUVECs were treated with CSE/TNFα/both

for 24 hours, and qPCRs carried out to determine gene expression of several mediators of thrombotic propensity including eNOS and CD39, negative regulators of thrombosis, and VWF, a pro-thrombotic mediator.

NO is an important protective molecule in the vasculature, and eNOS is responsible for most vascular NO produced (Forstermann and Munzel, 2006), playing a crucial role in regulating and maintaining a healthy endothelium. NO, generated by EC, plays critical roles in the regulation and maintenance of vascular tone, migration, proliferation, and maturation of cells, leukocyte adhesion, and platelet aggregation (Tran et al., 2022). Treatment of HUVECs with both CSE and TNFα combined caused a significant decrease in the levels of eNOS expression (P=0.0476) (Figure 15A). CSE alone also decreased eNOS expression levels, although not significant.

CD39 is a critical mediator in the regulation of vascular inflammation and thrombosis, inhibiting platelet activation. Following treatment of HUVECs with CSE alone, no significant difference was observed in CD39 expression. Whilst a slight decrease in CD39 expression is observed following treatment with TNFα (P=0.9581), and both CSE+TNFα combined (P=0.4646), this is not statistically significant (Figure 15B).

VWF released by ECs promotes platelet adhesion to components of the sub-endothelium and the process of thrombosis during vascular injury and is therefore an indicator of endothelial activation and damage (Kawecki et al., 2017). A slight decrease in VWF expression was observed in HUVECs treated with CSE (P=0.2732) and CSE+TNFα (P=0.1276) compared to vehicle control however this was not found to be statistically significant (Figure 15C).

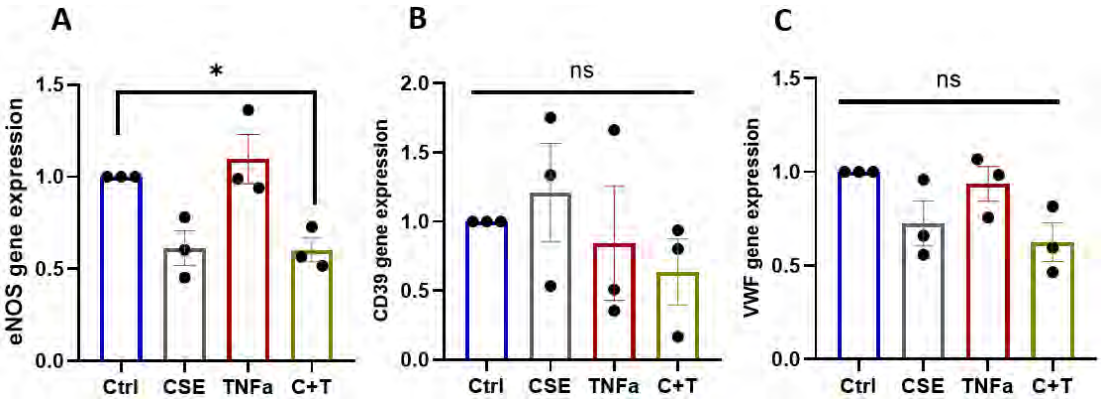

Figure 15: Gene expression levels of thrombotic mediators. A. *eNOS* ***B.*** *CD39 and* ***C.*** *VWF expression was analysed in HUVECs damaged with CSE/TNFα/both for 24 hours. qPCR experiments*

*were carried out in triplicate and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean + S.E.M for n=3. Where normalised data is shown, statistics were performed prior to normalisation. * indicates P≤0.05 in comparison to vehicle control. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism.*

Pim kinase alters gene expression of mediators of EC thrombotic propensity

We next set out to determine whether Pim kinase plays a role in the regulation of the thrombotic profile of ECs. HUVECs were treated with 1μM AZD1208 to determine the effect of Pim kinase inhibitor treatment on gene expression of mediators of thrombotic propensity in the endothelium. 1μM AZD1208 was chosen as a physiologically relevant concentration based on clinical trial data (Cortes et al., 2018) that demonstrates AZD1208 can reach plasma concentrations of up to 10μM following high dosing, 1μM represents lower dosing strategies and plasma concentrations at 24 hours post dosing. Interestingly, in untreated (healthy) HUVECs there was an increase in eNOS gene expression following treatment with AZD1208 compared with vehicle control however this is not significant (P=0.9056) (Figure 16A). In contrast, AZD1208 demonstrated a significant decrease in eNOS gene expression in damaged HUVECs treated with TNFα compared to vehicle treated control (P=0.0065) (Figure 16A). An AZD1208-mediated decrease in eNOS expression was also observed in CSE treated HUVECs however this was not significant (P=0.5451). There were no alterations in CD39 gene expression following treatment with AZD1208 between damage-treated and untreated HUVECs (Figure 16B).

Of particular interest was the observation that the treatment of HUVECs with AZD1208 decreased VWF expression in healthy control HUVECs (P=0.414) as well as HUVECs treated with CSE (P=0.0033), and TNFα (P=0.0001) (Figure 16C), highlighting a role for Pim kinase in VWF expression and potential release, and supporting the use for AZD1208 as a potential anti-thrombotic. Surprisingly, AZD1208 is inhibitory when CSE and TNFα are used alone but not in conjunction. The reason for this is unclear and requires further investigation.

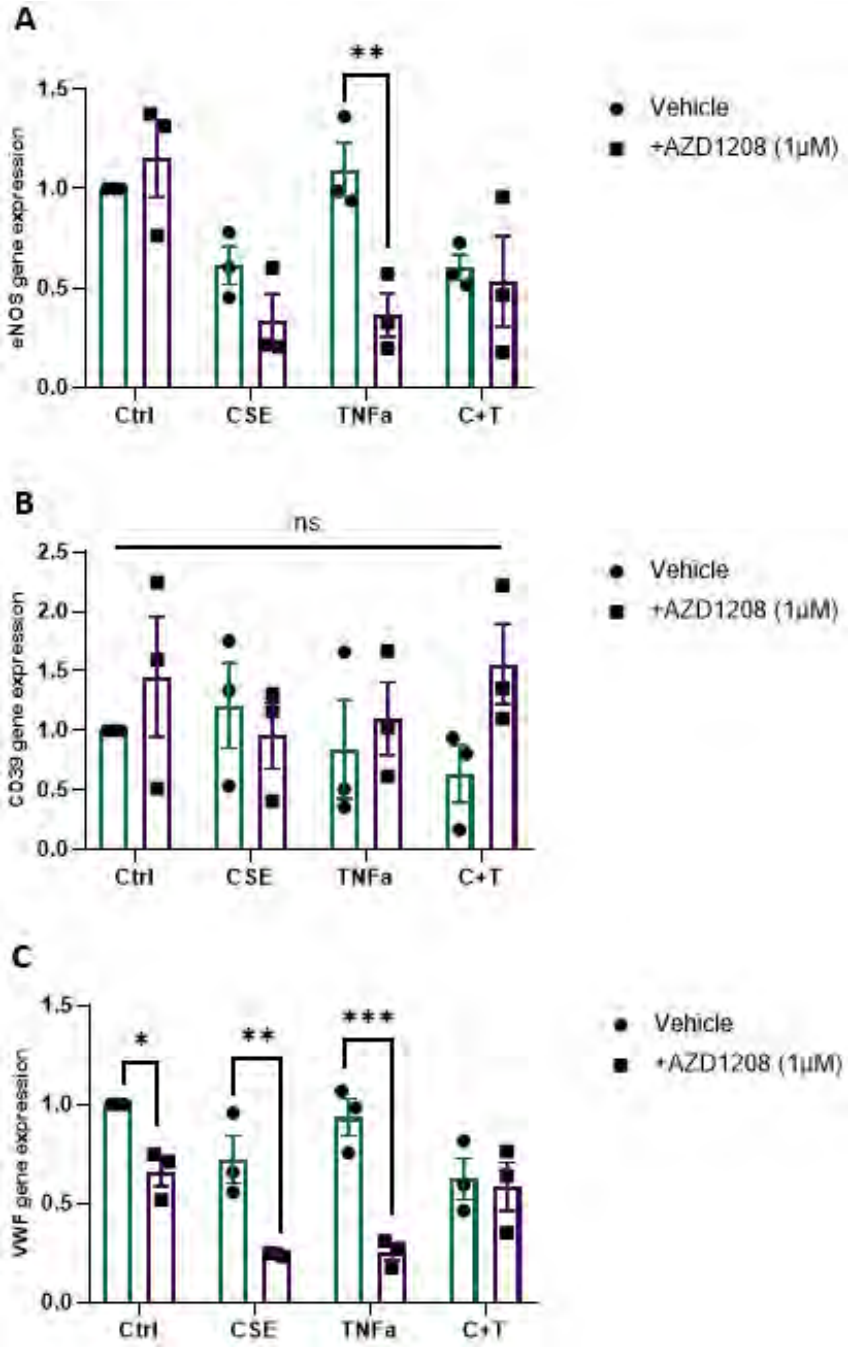

Figure 16: Gene expression of thrombotic mediators following treatment with AZD1208. *HUVECs damaged with CSE/TNFα/both were pre-treated with AZD1208 (1μM).* ***A.*** *eNOS* ***B.*** *CD39* ***C.*** *VWF gene expression with and without Pim inhibitor AZD1208. qPCR experiments were carried out in triplicate and results were plotted as fold change in comparison with untreated cells. Results are expressed as mean + S.E.M for n=3. * indicates P≤0.05, **≤0.01, ***≤0.001. Data was analysed using Two-Way ANOVA and graphs plotted using GraphPad Prism.*

Release of inflammatory markers in HUVECs induced by CSE/TNFα

Cigarette smoke has been shown to increase the production of pro-inflammatory cytokines such as TNFα, IL-6, and IL-8 (Rom et al., 2013), which promote EC damage and recruit leukocytes enhancing thrombosis. Levels of these released inflammatory markers were therefore determined from HUVECs treated with inducers of EC damage through an ELISA assay of cultured cell media. Following treatment with modulators of EC damage, IL-6 and IL-8 levels were shown to be increased in HUVECs induced with CSE damage compared to untreated control, although these increases were not found to be statistically significant (P=0.1965 and 0.4147, respectively) (Figure 17A and B). As expected, levels of TNFα were increased following stimulation with TNFα (P=0.0233) and with CSE+TNFα damage (P=0.0256) when compared to untreated control, to approximately 10ng/mL. As cells were treated with 5ng/mL exogenous TNFα, an increase in TNFα release is indicated following treatment with mediators of EC damage (Figure 17C).

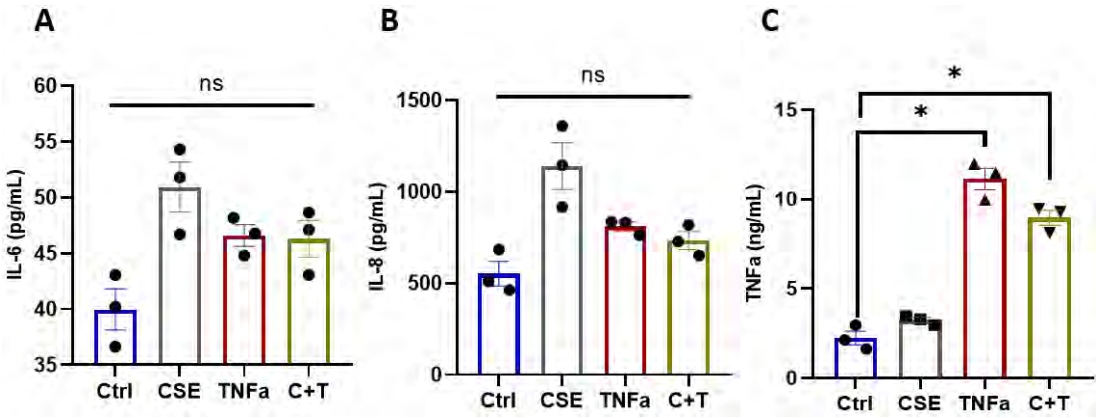

Figure 17: Release of inflammatory markers in response to endothelial cell activation. A. *IL-6* ***B.*** *IL-8 and* ***C.*** *TNFα levels were analysed in HUVECs damaged with CSE/TNFα/both for 24 hours using an ELISA assay. Results are expressed as mean + S.E.M for n=3. * indicates P≤0.05 in comparison to vehicle control. Data was analysed using One-Way ANOVA and graphs plotted using GraphPad Prism.*

AZD1208 reduces EC release of inflammatory markers following damage

To determine the effect of Pim kinase inhibition on EC release of inflammatory modulators, healthy HUVECs and HUVECs induced with CSE and TNFα were treated with 1μM AZD1208 for 24 hours and the release of IL-6, IL-8 and TNFα was determined. AZD1208 was shown to significantly decrease levels of released IL-6 in healthy control HUVECs (P=0.0029) (Figure 18A). A decrease was also observed in the release of IL-6 (P=<0.0001), IL-8 (P=0.0007), and endogenous TNFα levels (P=<0.0001) in TNFα damage induced HUVECs following treatment with AZD1208 (Figure 18A, B, and C). This work identifies

a role for Pim kinase in inflammation, highlighting Pim as a potential novel target in atherothrombosis.

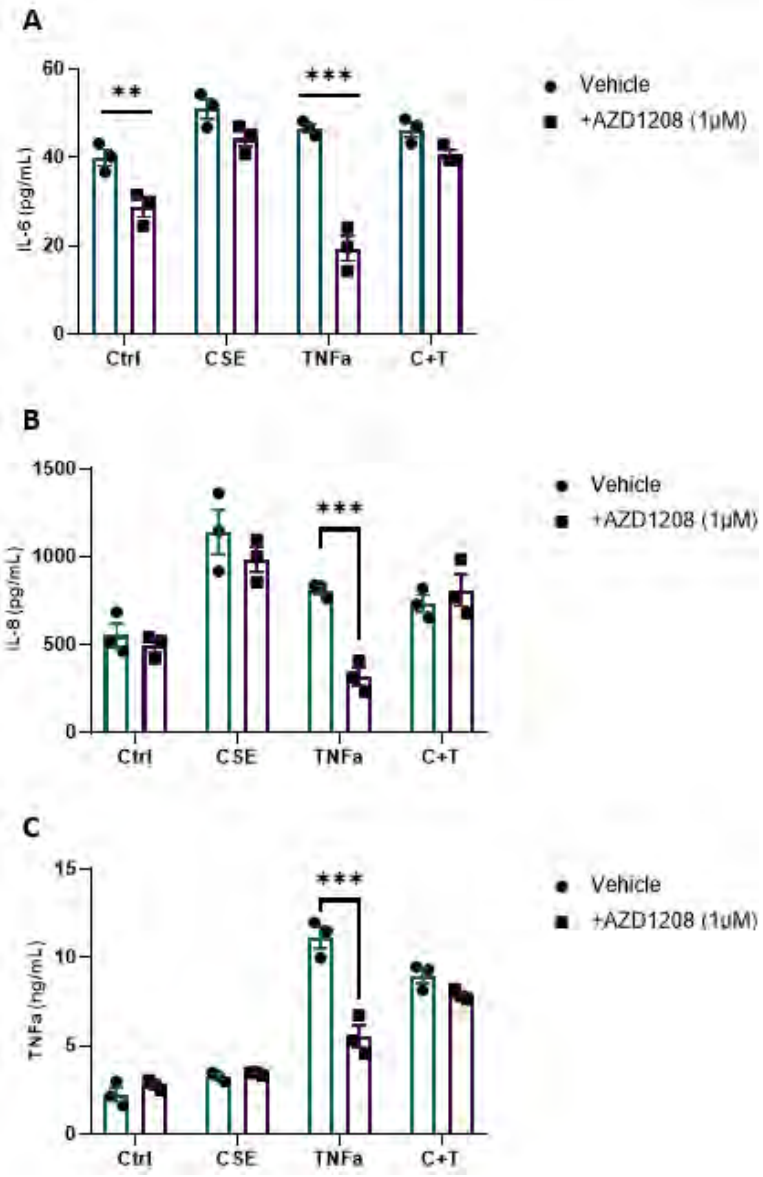

Figure 18: Inflammatory marker levels in response to endothelial cell activation +/- AZD1208. *HUVECs damaged with CSE/TNFα/both were pre-treated with AZD1208 (1μM).* ***A.*** *IL-6* ***B.*** *IL-8* ***C.*** *TNFα, with and without Pim inhibitor AZD1208. Levels of inflammatory markers were determined through the use of a Multiplex ELISA assay. Results are expressed as mean + S.E.M for n=3. ** indicates P≤0.01, ***≤0.001. Data was analysed using Two-Way ANOVA and graphs plotted using GraphPad Prism.*

Discussion

In this project the role for Pim kinase in EC control of atherothrombosis was investigated through the use of a pan Pim kinase inhibitor, AZD1208. The effects of Pim kinase inhibition on gene expression of thrombotic mediators and levels of released inflammatory markers in the presence and absence of TNFα and CSE, modulators of endothelial dysfunction, was determined by

treating HUVECs with AZD1208 for 24 hours. Pim kinase inhibition was shown to downregulate the expression of VWF, a pro-thrombotic mediator, and decrease inflammatory modulators, IL-6, and IL-8 in TNFα and CSE-activated HUVECs following treatment with AZD1208. These findings suggest a role for Pim kinase in thrombo-inflammation and support the use of AZD1208 as a potential novel anti-thrombotic.

The need for more efficacious anti-thrombotic therapies

Cardiovascular disease is the leading cause of death worldwide. MI and stroke occur due to the formation of a thrombus following atherosclerotic plaque rupture and erosion, initiating platelet activation and interaction with various cell types in a pro-inflammatory environment. Anti-platelet therapies are therefore commonly administered for the prevention and treatment of thrombosis, targeting different pathways of platelet function to prevent excessive thrombus formation.

Aspirin is the 'gold standard' anti-platelet therapy for the prevention of arterial thrombosis, and one of the most commonly used anti-platelet treatments acting through irreversibly targeting platelet TPαR signalling by inhibiting COX-1, which controls synthesis of TxA2, an important secondary mediator of platelet activation (Awtry and Loscalzo, 2000). However, aspirin has dose-limiting off-target effects on COX-2 which can increase the risk of thrombosis when repressed (Warner et al., 2011). Clopidogrel works by irreversibly inhibiting the ADP receptor $P2Y_{12}$, blocking ADP binding and signalling, and the primary agonist response and therefore preventing unwarranted platelet aggregation (Warner et al., 2011). Despite widespread use and their successes, both of these therapies provide limited protection against CVD related events (~20%) (Antithrombotic Trialists et al., 2009) and are associated with variable outcomes and adverse side effects with meta-analyses suggesting that the harms of aspirin outweigh the cardiovascular benefits in low-risk patients (Warner et al., 2011; Antithrombotic Trialists et al., 2009; Desborough and Keeling, 2017).

For this reason, this study explored an alternative approach to limiting thrombosis by identifying novel multicellular targets. Developing new strategies that target different cell types could be promising in creating

successful efficacious anti-thrombotic therapies that are required to overcome the disadvantages of current anti-thrombotic therapies. As platelets interact with various components within the endothelium, targeting EC appears an appropriate strategy.

Pim kinase and the Healthy Endothelium

As established proto-oncogenes, Pim kinases have been well characterised in regard to their roles in cancer progression and cell survival. All three Pim kinases, Pim-1, Pim-2, and Pim-3 have been investigated in EC function within the context of cancer, in angiogenesis, cell sprouting, and tumorigenesis (H. Yang et al., 2011; X. Zhang et al., 2018), however their role in the normal function of ECs and thrombosis has not yet been explored. The expression of Pim kinase in ECs has been confirmed (Min et al., 2012), although their physiological role is unclear. This project therefore aimed to investigate the relationship between Pim kinase and ECs in order to determine its wider effects on atherothrombosis and CVD, exploring Pim kinase as a potential target for a novel anti-thrombotic therapeutic approach.

HUVECs were our chosen model ECs as they are frequently used in studies *in vitro* as a model of general EC function due to their ease of growth and low maintenance costs. HUVECs have also been used in various studies to investigate arterial thrombosis (Lau et al., 2021; Njoroge et al., 2021).

We first set out confirm Pim-1, -2, and -3 protein expression in healthy HUVECs. Pim-1 protein was shown to be expressed in HUVECs however Pim-2 and 3 protein expression was undetectable. This was unexpected based on previously published literature which confirms the expression of all three Pim isoforms in HUVECs (Min et al., 2012). This could be due to differences in experimental conditions influencing HUVEC phenotype and Pim kinase detection, as media conditions (media, and supplements) and antibodies (no longer available) used in the methodology by Min *et al* were different to our study. In addition, Pim isoform expression has most often been observed in cancer models where Pim kinases are shown to be highly or over-expressed and their protein levels increased. This is not representative of the 'normal' situation and therefore lower levels of Pim protein expression are commonly observed in other cell types. It is possible that Pim kinase expression below

levels of detection in our study could be due to various experimental factors. Other studies analysing Pim protein expression using Western blotting have used different antibodies and reagents to the ones used in our study. In one study, their membrane was blocked with 5% milk in TBST where 5% BSA was used in our study, and incubated overnight at 4°C where ours was only incubated for 1 hour at room temperature (Song and Kraft, 2012). Future work would involve an optimisation and comparison of western blotting protocols to confirm Pim kinase expression. Manufacturers' instructions were followed for the antibodies used in our study, but we anticipate that higher concentrations of protein (10µg used in our study) and antibody (5µL at 1:1000 used in our study) could work better and would therefore be used in future.

The effect of AZD1208 treatment on HUVEC viability was investigated due to the well-established effects of Pim kinase phosphorylation on cancer cell proliferation, cell cycle progression, and evasion of apoptosis. Several studies have shown that AZD1208 treatment induces apoptosis in cancer cell lines. One study showed that the *in vitro* incubation of primary CLL cells (n=7) with 1, 3, and 10µmol SGI-1776 for 24 hours resulted in an average increase in apoptosis of 10%, 22%, and 38% (L. S. Chen et al., 2009). Another study investigating Pim kinase in prostate cancer cell growth revealed a clear dose-dependent decrease in phosphorylated BAD protein with increasing doses of SGI-1776 along with cell cycle arrest in the G1 phase (Mumenthaler et al., 2009). Based on previous reports stating that Pim kinases promote cell cycle progression and evasion of apoptosis signals, there was concern that AZD1208 would reduce proliferation rate and initiate active cell death (Lee et al., 2019). However, reassuringly, in our study, there was no effect on HUVEC proliferation and no apoptotic cell death after 24-hour treatment with physiologically relevant concentrations of AZD1208 (Figure 11).

Other studies have used AZD1208 to treat cancer cells where there is extreme cell growth and proliferation, and apoptosis is favourable in that context. This has been shown in a study exploring AZD1208-induced cell cytotoxicity where cells were incubated with DMSO, 3µM, or 10µM AZD1208 for 24 hours and evaluated for cell death by flow cytometry. Treatment of CLL lymphocytes with AZD1208 resulted in moderate cell death, whereas practically no cytotoxicity

was observed in healthy lymphocytes (Cervantes-Gomez et al., 2019). Our findings support these previous findings of no effect on cell proliferation and no observed cell death in healthy HUVECs treated with physiologically achievable concentrations of AZD1208. A study by Walpen *et al* has shown that deletion of Pim-1 imposes a hyper adhesive phenotype on ECs. This could also suggest that Pim kinase inhibition impacts on EC attachment to the culture plate, enhancing adhesiveness (Walpen et al., 2012). Our results are promising for the potential repurposing of Pim kinase inhibitors as anti-thrombotic therapies; however, further characterisation should be performed.

In addition to its well-established role in the regulation of cell cycle progression and prevention of apoptosis, Pim kinase has also been shown to play roles in cell migration via regulation of the cytoskeleton. Pim-1 has been identified to promote prostate cancer cell migration through the phosphorylation of GSK3B and FOXP3, inhibiting their tumour suppressive effects and resulting in increased migration (Santio et al., 2020). Physical forces that occur during migration are generated by the dynamic F-actin cytoskeleton, and adhesion is regulated by complex assemblies of structural and signalling proteins that couple the F-actin cytoskeleton to the ECM (Gardel et al., 2010). Cytoskeletal rearrangement that occurs due to F-actin turnover and polymerisation is essential for the regulation of cell adhesion and migration. A study has previously shown that Pim kinases are able to induce changes in cytoskeletal actin dynamics to support cell adhesion and movement (Santio et al., 2020). F-actin polymerisation was therefore quantified in our study in HUVECs treated with AZD1208 by fluorescence microscopy of phalloidin staining. High (supraphysiological) concentrations of AZD1208 (30 and 100μM) decreased FI of phalloidin and therefore actin polymerisation, indicating a potential disruption of cytoskeletal rearrangements in HUVECs following 24-hour incubation with high concentrations of Pim kinase inhibitors. We postulate that targeting Pim kinase in EC's could be a vital pathway to inhibit the development of pathological angiogenesis and neovascularisation through regulating the cytoskeleton, which controls EC adhesion and migration.

Initiators of Endothelial Dysfunction

Previous studies have shown that TNFα, a pro-inflammatory cytokine, initiates vascular injury leading to EC dysfunction. Vascular injury is initiated directly from pro-apoptotic actions exerted by TNFα on ECs, leading to endothelial dysfunction (Madge and Pober, 2001). TNFα imposes a significant pro-inflammatory effect on ECs, inducing the expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) and chemokines primarily through the activation of NFκB (Rajan et al., 2008). TNFα also increases the production of reactive oxygen species (ROS), maintaining endothelial dysfunction (Teasdale et al., 2017). Smokers have been found to have significantly increased serum levels of pro-inflammatory cytokines such as TNFα (Barbieri et al., 2011).

Smoking has widely been recognised as a leading risk factor for clinical CVD (W. Wang et al., 2021). Cigarette smoke exposure interferes with all stages of atherosclerotic plaque formation and development, and pathological thrombus formation, increasing the formation of plaques in blood vessels and causing ECs to become swollen and inflamed (CDC, 2022). CSE triggers EC dysfunction *in vitro* by initiating oxidative stress and an inflammatory response (Bernard et al., 2019). ROS in CSE contributes to oxidative stress (Teasdale et al., 2017), upregulation of inflammatory cytokines, and endothelial dysfunction by reducing the bioavailability of NO and further by increasing the expression of adhesion molecules including ICAM-1 and VCAM-1 (Messner and Bernhard, 2014). In addition to direct physical damage to EC, smoking induces pro-thrombotic processes with simultaneous activation of the release of inflammatory cytokines such as IL-6 and TNFα (Messner and Bernhard, 2014), through the increased adherence of platelets and macrophages. Additionally, exposure to cigarette smoke results in platelet activation and stimulation of the coagulation cascade (Csordas and Bernhard, 2013).

HUVECs were therefore treated with TNFα and CSE to induce EC dysfunction. Treatments with TNFα and CSE, alone and in combination, were used to mimic the effects of atherothrombosis. CSE to model EC erosion, TNFα to initiate EC dysfunction, and both (CSE+TNFα), to model atherothrombosis.

mRNA and protein expression levels of Pim-1, Pim-2, and Pim-3 in damage induced HUVECs compared with healthy untreated HUVECs was therefore

determined using qPCR analyses and western blotting. Differential gene expression of all three Pim family members was observed, however this was not found to be significant. It has previously been shown that Pim-3 is regulated by TNFα, demonstrated by an increase in Pim-3 mRNA expression with increasing TNFα in ECs (H. Yang et al., 2011), therefore a difference was expected when compared to healthy cells. High levels of variation observed could be due to genetic drift between cell culture passages, resulting in altered phenotypes of the cells, and subsequent gene expression profiles. Future work would involve additional repeats of this experiment at similar cell culture passages and Pim expression monitored throughout HUVEC culture.

Protein expression was analysed, and Pim-1 protein expression was confirmed in HUVECs treated with CSE, TNFα, and both combined. Protein expression levels do not appear to change on treatment with EC activators compared to healthy HUVECs. Interestingly, although levels of Pim-1 protein were detected, Pim-2 and Pim-3 protein expression was undetectable in healthy untreated HUVECs along with HUVECs following treatment with EC activators. This could indicate that neither isoform is upregulated in response to mediators of EC damage. However, this is at odds with multiple studies which have suggested that Pim kinase family member expression is regulated by inflammation. Pim-1 expression has shown to be upregulated in SCA-1+ cardiac resident stem cells by inflammatory cytokines IL-6 and IL-27 (Tanaka et al., 2015; Iwakura et al., 2011), Pim-2 protein expression has shown to be upregulated in ox-LDL treated THP-1-derived macrophages (Liao et al., 2021), and Pim-3 expression has been shown to be regulated by TNFα in HUVECs (H. Yang et al., 2011).

Our unexpected finding could indicate that the experimental conditions used were not optimal to investigate the role of Pim kinase in EC dysfunction. As such, whilst Pim-2 and Pim-3 isoforms were not identified in our assays, expression of either paralog in activated or damaged ECs cannot be ruled out entirely and require further investigation.

Pim kinase and the thrombotic properties of the endothelium

Endothelial dysfunction is one of the earliest abnormalities observed during the development of atherosclerosis, and eNOS is a key player in regulating

endothelial dysfunction (Gimbrone and Garcia-Cardena, 2016). Both CSE and TNFα affect eNOS activity, resulting in altered eNOS expression. CSE reduces eNOS mRNA and protein expression, protein quality, and impairs eNOS activity, which consequently decreases NO synthesis (W. Wang et al., 2021). TNFα may also selectively turn off the expression and synthesis of eNOS (Madge and Pober, 2001). Analysis of eNOS gene expression in HUVECs following treatment with CSE+TNFα confirms these previous findings as a decrease in eNOS gene expression was observed following 24-hour treatment with CSE+TNFα. Interestingly, an increase in eNOS gene expression in healthy HUVECs following treatment with AZD1208 compared with vehicle control was observed, supporting the idea that Pim kinase plays a role in eNOS gene expression, and that Pim kinase inhibitors could offer an additional protective effect in healthy ECs. TNFα has also been shown to decrease eNOS activity and lead to leukocyte recruitment and adhesion to EC *in vivo* and *in vitro* (Aguilar et al., 2021), enhancing inflammation. We would therefore propose that treatment with Pim kinase inhibitors may be beneficial in this context.

Surprisingly however, treatment with AZD1208 demonstrated a decrease in HUVEC eNOS gene expression in TNFα-treated (significant) and CSE-treated (not significant) cells compared to vehicle treated controls. This implies that under conditions of EC activation, eNOS gene expression is reduced by Pim kinase inhibition. These observations are supported by previously published work that have shown that transfection of HUVECs with Pim-1 specific siRNA inhibited eNOS phosphorylation at Ser-663 and eNOS activity downstream of VEGF in *in vitro* and *ex vivo* conditions (M. Chen et al., 2016), identifying a role for Pim kinase in the regulation of EC eNOS expression and activity, requiring further characterisation. In the context of platelets, a decrease in eNOS would be concerning regarding the development of atherothrombosis and reduction in NO generation, however in EC, eNOS can become dysfunctional in atherosclerosis, promoting super oxide production detrimental to EC function (Kawashima and Yokoyama, 2004), therefore in regard to endothelial phenotype and damage, a decrease caused by Pim kinase inhibition may be desirable.

Further investigation could include measuring levels of EC synthesised NO and Ser663 phosphorylation of eNOS. Additionally, other NO synthesising enzymes such as inducible nitric oxide synthase (iNOS) and how this contributes to endothelial NO production in response to EC dysfunction could be explored to investigate and validate the role of Pim kinase in the inflammatory processes that contribute to atherothrombosis.

In addition to eNOS and the synthesis of NO, other endothelial derived 'inhibitory' regulators of thrombosis include CD39 and CD73. CD39 and CD73 are ectonucleosides that are viewed as 'immunological switches' shifting ATP-driven pro-inflammatory immune cell activity toward an anti-inflammatory state and preventing platelet activation by degrading endothelium derived ATP, ADP, and AMP to adenosine (Antonioli et al., 2013). When investigating CD39, under the conditions used, we observed no significant differences in CD39 gene expression in HUVECs treated with mediators of endothelial damage or following Pim kinase inhibition. These observations indicate that Pim kinase does not regulate CD39 gene expression and CD39-mediated anti-inflammatory and anti-thrombotic effects.

Following vascular injury or atherosclerotic plaque rupture, the ECM is exposed, and VWF, released from the Weibel Palade bodies of EC, binds to exposed sub-endothelial collagen and initiates platelet tethering to the site of damage. This tethering occurs via interaction with platelet glycoprotein 1b (GP1b) receptors, triggering platelet activation and a series of events to form a plug at the site of damage (Lancellotti et al., 2019). The 'thrombo-inflammatory' nature of VWF-platelet interaction is recognised in different cardiovascular pathologies, making it a potential therapeutic target for thrombo-inflammation (Denorme et al., 2019). Serum taken from cigarette smokers has been shown to contain higher levels of VWF and is cytotoxic to EC *in vitro* (Blann and McCollum, 1993), indicating increased EC release of VWF in response to CSE. Gene expression of VWF was therefore investigated in this study in response to HUVEC treatment with mediators of EC dysfunction and following treatment with AZD1208. Interestingly, a slight decrease in VWF gene expression was observed in HUVECs treated with CSE, and CSE+TNFα

for 24 hours compared to vehicle control but this was not found to be significant. Whilst gene expression wasn't affected, EC release of VWF wasn't investigated and would be interesting to investigate further as the release of VWF is more pathological. When investigating the effect of Pim kinase inhibition in this study, treatment with AZD1208 was found to cause a decrease in VWF gene expression in healthy HUVECs as well as HUVECs treated with CSE, and TNFα, highlighting a role for Pim kinase in the regulation of VWF expression, and supporting the use of AZD1208 as a potential anti-thrombotic by targeting and reducing VWF levels. It has been demonstrated by Unsworth *et al* that AZD1208 inhibited platelet α-granule secretion (Unsworth et al., 2021). Investigating the effects of Pim inhibition on EC granule release would give a clearer indication of the effects of Pim kinase in thrombosis between the interaction of different cell types, to aid the development of novel multicellular anti-thrombotic therapeutic targets.

In addition to promoting the pro-thrombotic properties of ECs, both smoking and CSE exposure has shown to increase vascular mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Orosz et al., 2007). To investigate the endothelial contribution to inflammation, release of inflammatory mediators IL-6, IL-8, and TNFα from HUVECs treated with CSE and TNFα for 24 hours was determined. Consistent with previous studies (Chi et al., 2001; Yeh et al., 2001), IL-6 and IL-8 levels were shown to be increased in HUVECs induced with mediators of EC damage compared to untreated controls, although not statistically significant. Lack of statistical significance is likely due to insufficient repeats (n=3), and we cannot rule out other confounding factors such as different CSE preparation, concentration (not comparable between studies, due to preparation differences), and times of incubation. Similarly, levels of secreted TNFα were also increased following stimulation with TNFα, and with CSE+TNFα damage compared to untreated controls. Caution should be taken however, as this could be explained by its exogenous addition. To investigate the role of Pim kinase in inflammation, the effects of treatment with AZD1208 on HUVEC secretion of IL-6, IL-8 and TNFα was determined. AZD1208 treatment reduced the levels of secreted IL-6 in healthy control HUVECs and TNFα treated HUVECs. A decrease was also

observed in IL-8, and endogenous TNFα release in TNFα damage induced HUVECs, suggesting that AZD1208 may play a role in the down-regulation of TNFα stimulated pro-inflammatory genes. This warrants further research to explore the complex relationship between Pim kinase, TNFα, IL-6, and IL-8 in inflammation and its contribution to atherothrombosis. As Pim kinase is downstream of IL-6 (STAT3) mediated signalling (Iwakura et al., 2011), Pim kinase inhibition reducing IL-6 may therefore reduce IL-6 mediated signalling on ECs, demonstrating a promising novel therapeutic strategy in targeting inflammation.

Pim kinase as a novel endothelial thrombo-inflammatory target?

The findings in our study suggest that Pim kinase inhibition is a promising potential endothelium directed strategy for targeting pro-thrombotic and pro-inflammatory components of atherothrombosis. Furthermore, our findings provide significant insights into the mechanism underlying the effects of Pim inhibition, decreasing VWF expression in healthy, CSE, and TNFα treated cells (Figure 16C) and levels of IL-6 in healthy and TNFα treated cells (Figure 18A). Results from the study illustrate the importance of exploring endothelial dysfunction *in vitro*. These data hint at the potential for using local downregulation of Pim kinase activity in ECs as a new avenue for treating atherothrombosis-related events. Although our results show promising potential for Pim kinase inhibitors with inhibitory actions on CSE and TNFα inflammatory and prothrombotic responses when used alone, these inhibitory properties are not observed when CSE and TNFα are used in conjunction. This is likely due to their contradicting roles when combined and may relate to the conflicting effects both mediators can have on downstream signalling pathways, such as NRF2 signalling where TNFα has demonstrated a protective role in antioxidant response element (ARE) signalling (Namakkal-Soorappan, 2017), whilst CSE has shown to potentiate cardiovascular dysfunction through inducing NRF2 activation by enhanced oxidative stress, leading to abnormal vasculature formation (Xue et al., 2022). The reasons for this require further investigation to determine the future utility of Pim kinase inhibitors, especially in patients presenting with multiple risk factors.

Additionally, this may be due to varying responses in HUVECs when exposed to CSE, where HCAECs could be used as a more arterial relevant system.

Regulation of Pim kinase gene expression and kinase activity in ECs may represent a novel approach to modulate endothelium mediated pro-inflammatory and pro-thrombotic events and focus on novel and more effective therapeutic approaches to treat CVD. In support of this, it has been shown that the loss of all three Pim kinase isoforms do not affect embryo viability (Mikkers et al., 2004; An et al., 2013a), with mouse knockouts lacking all three Pim genes remaining fertile and viable (Bullock et al., 2009), indicating that Pim kinase inhibition is likely to be tolerable for use as a drug therapy.

Limitations of the study

Use of HUVECs

Although HCAECs might be considered the gold standard for a study of arterial thrombosis, these cells are expensive and HUVECs, which are cheaper, have been acknowledged as a useful model for research on the endothelium. HUVECs are a representative model for the study of vascular endothelium properties and the main biological pathways involved in endothelium function and pathophysiological mechanisms involved in CVD (Baudin et al., 2007). Although this is the case, while EC behaviour is modulated by culture conditions, some differences in endothelial phenotype may be retained *in vitro*, making it difficult to predict comparative responses of EC from different origins, even when conditions are matched. Additionally, because HUVECs are fetal and not maternal tissue, gender differences must be considered. Sexual dimorphism shown by HUVECs indicate that sex differences exist (Addis et al., 2014), suggesting that HUVECs of both sexes should be used as a model to reduce variability in results.

Our study has generated some preliminary data where HCAECs have been shown to behave differently to HUVECs in response mediators of cell damage (Appendix 3). Although not significant, a decrease in VWF gene expression was observed in HUVECs treated with CSE (P=0.2732), TNFα (P=0.8390), and CSE+TNFα (P=0.1276) compared to vehicle control (Appendix 3A), however VWF gene expression was shown to be increased in damage-

induced HCAECs with CSE (P=0.3513), TNFα (P=0.2299), and both (CSE+TNFα) (P=0.3422) (Appendix 3B). HCAECs should therefore be investigated in arterial studies as they have shown to respond and behave differently to HUVECs. As Pim kinase has shown to play a role in HUVECs, this study would be enhanced by investigation into the effects of Pim kinase inhibitors under arterial shear in HCAECs as a more representative model of arterial atherothrombosis.

Interestingly, increased expression and release of IL-6 is indicative of venous disease to a greater extent than arterial, with IL-6 contributing to DVT (Y. Zhang et al., 2020). As HUVECs are venous ECs, further work could be done to follow up the role of Pim kinase in regulating VTE and other venous disease, including investigation of TF release, and regulation of coagulation.

Limitations of Methodology

CSE was generated by passing filtered cigarette smoke through tissue culture media. This captured the water-soluble components of cigarette smoke, which was filtered to remove particulate matter and immediately applied to HUVECs to prevent the quenching of dissolved free radicals. The water-soluble components of cigarette smoke have more relevance for the study of the systemic effects of cigarette smoke, although it is not possible to measure if CSE generated in this way captures the same components of cigarette smoke that are capable of passing through the lungs into the bloodstream (Teasdale et al., 2017). An *in vivo* model may therefore be more appropriate, exposing mice directly to cigarette smoke using a specific rodent ventilator (Budani et al., 2021) due to the *in vitro* limitations of our model. Further research using animal models is necessary to confirm our *in vitro* observations.

In this study a treatment time of 24 hours was selected based on other studies using the same timepoint (Cervantes-Gomez et al., 2019). However, throughout the literature various different timepoints for EC treatments are used. One study by Chen et al investigating the effects of SMI-4a on HUVECs applied inhibitor treatment at different timepoints between 2 and 24 hours (L. S. Chen et al., 2009). In our study, HUVECs were treated for 24 hours (to impose a chronic treatment), further studies should be performed at shorter time points to investigate potency of inhibitor treatment. Alternatively, even

longer exposure would be interesting to investigate the effects of severe chronic exposure. These differences in treatment times could explain why some of our results are inconsistent with previous studies.

Static vs flow

All of our experiments were done under static conditions however flow rates also impact biology and influence EC behaviour in health and disease (Y. S. Li et al., 2005; Chistiakov et al., 2017). A study has shown that shear stress and flow changes initiate mechanisms that play a central role in haemostasis and thrombosis through the induction and release of EC mediators of thrombosis (Yau et al., 2015). A more physiologically relevant study would therefore include experiments performed under shear (arterial: 15 dynes/cm^2, venous: 5 dynes/cm^2, pathological: 75 dynes/cm^2), subjecting ECs to flow conditions to gain a truer representation of the role of Pim kinase in EC contribution to venous or arterial thrombosis and give better insight into the mechanisms of EC biology.

Statistical Power

Our study used 3 repeats in each experiment (n=3). This could be why a large amount of variation was observed and a lack of statistical significance based on the literature. EC studies carried out typically use a larger number of repeats (around n=5) to account for this variability (Khawaja et al., 2020), allowing smaller changes to be detected. In future, a larger number of repeats (at least n=6) would be considered to increase precision and better measure variation in the experiment.

Pim kinase inhibitors

Whilst it may be beneficial to target Pim kinases in atherothrombosis using Pim kinase inhibitors within the context of the endothelium, various factors should be taken into account. Although pan Pim kinase inhibitors such as AZD1208 explored in our study are promising, it should be considered that the lack of helical structure in Pim-2 may increase flexibility to the N-terminus, causing structural changes, often why pan Pim kinase inhibitors appear to be less potent on Pim-2 compared to Pim-1 and Pim-3 (Bullock et al., 2009). This has made the development of pan Pim inhibitors difficult because Pim-2 has a low K_m for ATP and therefore requires an extremely potent inhibitor to

effectively block its kinase activity at the ATP levels in cells (Garcia et al., 2014). There are various other Pim kinase inhibitors in clinical trials (SMI4-4a, LGH447) (X. Zhang et al., 2018) that should be investigated in the context of atherothrombosis and CVD. Alternatively, selective individual Pim inhibitors should be explored to investigate individual isoforms of Pim and their potential compensatory effects and mechanisms. Additionally, knockdown experiments would be useful in this context to explore the effects of each individual Pim isoform when deleted.

Directions for further research

Further insight is required to characterise the role that Pim kinase plays in the regulation of endothelial control of thrombosis.

Further experiments to explore live cell functions such as cell viability, cell morphology, and cell motility following treatment with a drug dose response would help to further elucidate the role of Pim kinase in thrombosis.

EC activation leads to increased expression of multiple inflammatory cytokines in addition to IL-6 and IL-8 that we investigated, and several adhesion molecules that initiate leukocyte homing, adhesion, and migration into the subendothelial space, processes fundamental to atherosclerotic plaque initiation and progression. Activation of cultured EC with TNFα is known to increase ICAM-1 and VCAM-1 expression (Wong and Dorovini-Zis, 1995), and levels of E-selectin, ICAM-1, VCAM-1, and P-selectin are positively associated with cardiovascular risk factors such as smoking, and have been associated with functional measures of atherosclerosis, as well as with adverse cardiovascular prognosis (Hwang et al., 1997). The expression of adhesion molecules, ICAM-1, VCAM-1, P-selectin, and E-selectin could therefore be measured using a Multiplex ELISA assay to further explore the effects of Pim kinase in inflammation associated with atherothrombosis.

Additionally, to further investigate the contribution of Pim kinase to endothelium driven thrombosis, coagulation mediators, thrombomodulin, TF, and TFPI could be measured using qPCR, and validated using western blotting and flow cytometry to further investigate the roles of Pim kinase in

other pathways associated with EC and thrombosis. To compare Pim inhibitor anti-thrombotic effects, gene expression of COX-1, COX-2, and thromboxane synthase could be explored to investigate the role of Pim kinase in the pathway that aspirin targets to compare its anti-platelet properties and their effectiveness including side effects.

Prolonged and repeated exposure to cardiovascular risk factors can deplete the protective effects of processes within EC. Consequently, the endothelium becomes dysfunctional, and EC can lose integrity, progress to senescence, and detach into the circulation (Woywodt et al., 2002). Circulating markers of EC damage include endothelial extracellular vesicles derived from activated or apoptotic cells, and whole EC (Deanfield et al., 2007). Flow cytometry could be used in HUVEC studies to measure and quantify endothelial microparticles in response to Pim inhibitor treatment to determine the effects on vascular injury and investigate the effects of AZD1208 on cell viability and death to confirm results generated from our cell proliferation experiments. Additionally, endothelial barrier integrity could be measured using V-cadherin which would give a measure of how intact the endothelial junctions are following treatment with Pim kinase inhibitors.

A study by Riley *et al* has previously explored the contribution of ECs to platelet mediated thrombosis using an *in vitro* thrombosis model where HUVECs exposed to TNFα were cultured on ibidi chamber slides, and whole blood perfused through the HUVEC lined channel to analyse thrombus generation (Riley et al., 2018). This *in vitro* thrombosis assay could be carried out to investigate the effects of Pim kinase on the endothelium along with other blood cells and components involved in the process of thrombosis on collagen, incorporating EC into the assay. As all of the assays in our study are carried out under static conditions, it would be useful to explore the effect of Pim kinase inhibition on ECs under different shear conditions such as arterial (15 dynes/cm^2) and pathological shear rates (75 dynes/cm^2) which are more relevant to atherothrombosis.

Conclusion

In conclusion, this study has identified Pim kinase as a novel drug target in HUVECs that is capable of downregulating markers of thrombosis and inflammation following endothelial activation by TNFα, and aqueous CSE through Pim kinase inhibition. Although Pim kinase inhibitor treatment has identified roles for Pim kinase in the regulation of various markers of inflammation and thrombotic propensity, it is still unclear as to what specific cellular roles Pim kinase itself plays in atherothrombosis. The cellular mechanisms underlying this requires further investigation. Nevertheless, Pim kinase inhibition offers an exciting novel endothelium-targeting strategy for the prevention of thrombosis.

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Appendix

Appendix 1: Pim-1, Pim-2, Pim-3 gradient PCR

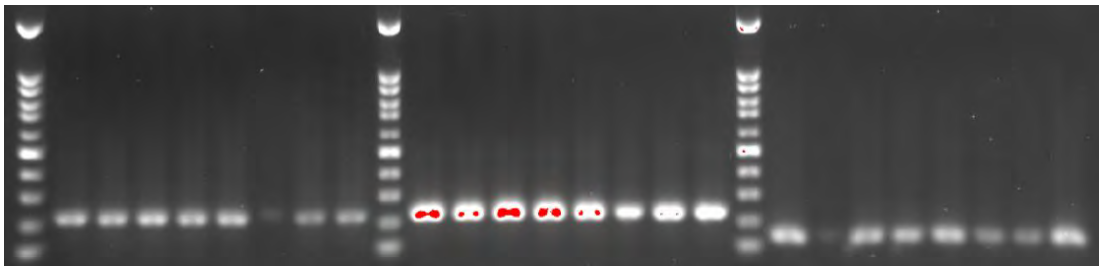

Figure 1: Pim-1, Pim-2, and Pim-3 gradient PCR, respectively. Optimum annealing temperature for Pim primers in qPCR was determined through gradient PCR.

Appendix 2: VWF gradient PCR

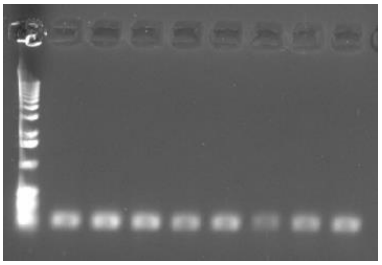

Figure 2: VWF gradient PCR. Optimum annealing temperature for VWF primers in qPCR was determined through gradient PCR.

Appendix 3: HUVEC vs HCAEC VWF gene expression

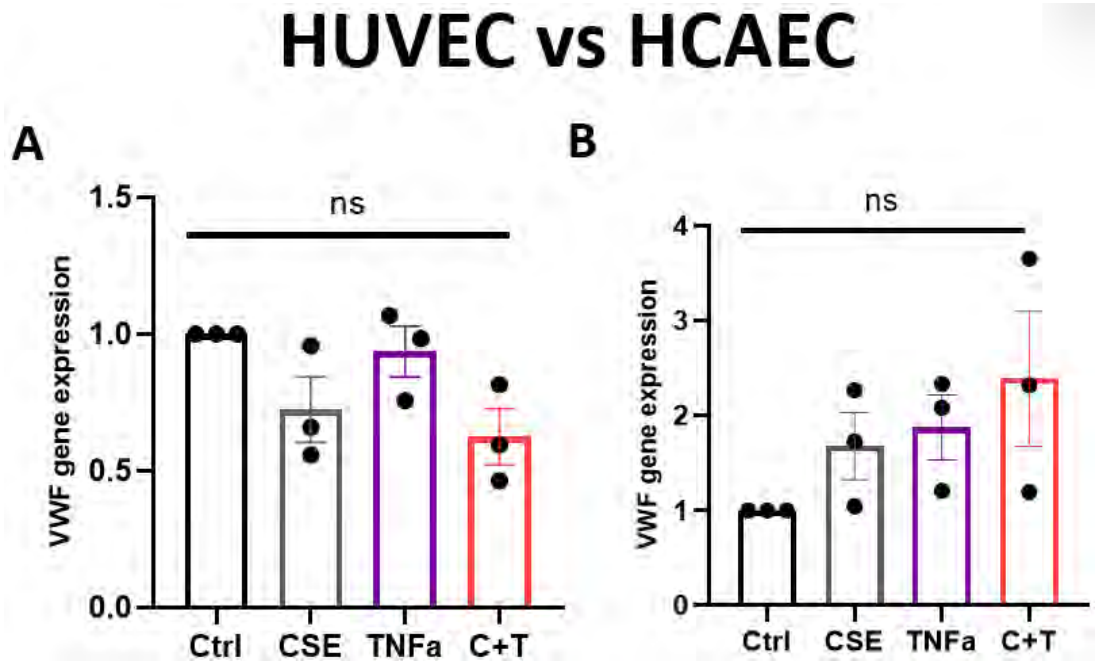

Figure 3: Difference between damage-induced HUVEC and HCAEC VWF gene expression A. VWF gene expression is decreased in HUVECs treated with CSE, TNFα, and both combined when compared to vehicle control. **B.** VWF gene expression is increased in HCAECs induced with damage, suggesting that CSE and TNFα play a role in arterial thrombosis through the increase of thrombotic modulators.

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