

Targeting pathological vascular smooth muscle cell remodelling to develop new therapeutics for abdominal aortic aneurysm

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Abstract

Abdominal aortic aneurysm (AAA) is a silent but progressive disease with no pharmacological treatment. Available treatment options – open surgical and endovascular repair carry significant risks. We hypothesize that vascular smooth muscle cell (VSMC) remodelling driven by platelet derived growth factor (PDGF) may contribute to AAA pathogenesis which could be targeted for pharmacological therapy. We used transgenic models to understand this pathological VSMC remodelling in aortic disease.

We demonstrated SMC (spatial) and tamoxifen-inducibility (temporal) specificity of the Cre-recombination in our transgenic lines and set-up the porcine pancreatic elastase (PPE)-AAA model in our animal facility. Lineage tracing studies in PPE-AAA showed an increase in lineage traced SMC number and reduced expression of classical SMC markers indicating VSMC dedifferentiation with subsequent re-differentiation and healing.

SM22CrePDGFR β^{D849V} mice developed generalised aortopathy from 4 weeks. Transcriptomic analysis of aortic tissue showed 2721 differentially expressed genes mapped to important signalling pathways and cellular processes including extracellular matrix homeostasis and inflammation. Using quantitative RT-PCR, we confirmed the upregulation of key AAA-related genes *Mmp2*, *Mmp12*, *Timp1*, *Clec3b*, and *Spp1* and inflammation-related genes *Bmp2* and *Ccr5* in SM22CrePDGFR β^{D849V} aorta. While the (inducible) SM-MHCCreER^{T2}PDGFR β^{D849V} mice did not develop any major aortic phenotype, following PPE application, AAA development was similar at 14 days but then became significantly larger and progressive compared to SM-MHCCreER^{T2}PDGFR β^{WT} littermates.

Store-operated calcium entry (SOCE) was enhanced in PDGFR β^{D849V} VSMC compared to littermate controls and inhibited by the small molecule Orai1 inhibitor, JPIII. *In vivo* administration of JPIII failed to alter the progression of PPE-AAA in SM-MHCCreER^{T2}PDGFR β^{D849V} mice. However, *Orai1* genetic deletion in SM22CrePDGFR β^{D849V} mice partially rescued the aortic dilatation.

In conclusion, these data suggest VSMC remodelling driven by PDGFR β is involved in AAA development and progression and Orai1/SOCE may be a potential downstream target for therapeutics.

Abbreviations

Abdominal aortic aneurysm	AAA
Alpha smooth muscle actin	α -SMA
Angiotensin converting enzyme	ACE
Anterior-posterior diameter	AP diameter
Apolipoprotein E null	ApoE ^{-/-}
Base pairs	bp
Bone morphogenetic proteins	BMP
Calcium ions	Ca ²⁺
Cardiovascular disease	CVD
Collagen Type XIX Alpha 1 Chain	Col19a1
Complementary DNA	cDNA
Cyclization recombinase	Cre
Deoxy-nucleotide triphosphate	dNTP
Disability-adjusted life year	DALY
Dulbecco's modified eagle medium	DMEM
Endoplasmic reticulum	ER
Endothelin 1	ET-1
Endovascular aortic repair	EVAR
Extra cellular matrix	ECM
Green fluorescent protein	GFP
Immunoglobulin G	IgG
Insulin-like growth factor binding protein	IGFBP
Interleukin	IL
Krüppel-like factor 4	KLF4
Laser scanning microscope	LSM
Low density lipoproteins	LDL
Low-density lipoprotein receptor	LDLR
Low-density lipoprotein receptor-related protein 1	LRP1
Magnetic resonance angiography	MRA
Matrix metalloproteinases	MMP
Mesenchymal stem cell	MSC
Messenger RNA	mRNA
Micro-computed tomography	μ CT
Mitogen-activated protein kinases	MAPK
Nuclear factor of activated t-cell	NFAT
Oestrogen receptor T2	ER ^{T2}
Open aortic repair	OAR

Paraformaldehyde	PFA
PDGF receptor beta	PDGFR β
Peripheral arterial disease	PAD
Phosphate-buffered saline	PBS
Phosphatidylinositol-3-kinase	PI3K
Platelet-derived growth factor	PDGF
Polymerase chain reaction	PCR
Probability value	P-value
Protein kinase C	PKC
qRT-PCR	Quantitative reverse transcriptase PCR
Reactive oxygen species	ROS
Region of interest	ROI
RNA-sequencing	RNAseq
Sarco/endoplasmic reticulum Ca ²⁺ -ATPase	SERCA
Secreted Phosphoprotein 1	SPP1
Single nucleotide polymorphisms	SNP
Smooth muscle 22 alpha	SM22 α
Smooth muscle cell	SMC
Smooth muscle myosin heavy chain	SM-MHC
Standard error of the mean	SEM
Stem cell antigen 1	Sta-1
Store-operated calcium entry	SOCE
Stromal interaction molecule 1	STIM1
Tissue inhibitor of MMP	TIMP
Transforming growth factor beta	TGF β
Transgenic	Tg
Tumor necrosis factor-alpha	TNF α
Ultrasound Scan	USS
Vascular smooth muscle cell	VSMC
Wildtype	WT
World health organization	WHO

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Chapter 1 : Introduction

1.1 Cardiovascular system

The cardiovascular system comprises of the heart, blood and the blood vessels making up the vascular network. The heart is a complex organ that pumps oxygenated blood to the body and deoxygenated blood to the lungs through an intricate system of muscle layers, chambers, valves and nodes. Blood is the vital fluid consisting of plasma, red blood cells, white blood cells and platelets. It carries oxygen and the nutrients required for the tissues and removes carbon dioxide and the waste from the tissues. The vascular/circulatory system is made up of blood vessels that have a central lumen for the flow of blood through which oxygen, nutrients, and heat are transported throughout the body.

1.1.1 Blood vessels

The blood vessels contain diverse cellular components and interact with a large variety of nonvascular parenchymal cell populations that differ among various organs. The vascular network comprising of the blood vessels, plays an important role in physiological processes such as organ development, tissue morphogenesis, inflammation, and wound healing. The blood vessels also contribute to pathological events in a variety of diseases. Occlusion, inflammation, or weakness in the blood vessels cause vascular diseases which can be fatal.

Five main types of blood vessels are arteries, arterioles, capillaries, veins and venules. The aorta is the main and largest artery in the body that carries oxygenated blood from the left ventricle of the heart through systemic circulation to all over the body. The blood flows through the arterial system through subsequent branches into smallest arteries and capillaries and is delivered to cells. The deoxygenated blood returns to the right ventricle via veins and venae cavae and is pumped to

the lungs by the pulmonary circulation. The walls of the arteries are thick and strong and have specialised elastic fibres that propagate the pulse wave from the heart and help pump blood throughout the body under high “arterial” pressure. The capillaries are single cell layered tiny blood vessels that merge into smaller veins called venules and then into larger veins that has thinner wall with large lumen which maintains low blood pressure that returns the deoxygenated blood to the heart.

The walls of arteries and veins are made up of three layers of tissue- internal *tunica intima*, middle *tunica media* and outer *tunica adventitia*. The tunica intima is the thinnest layer comprising of connective tissue and a single layer of flat endothelial cells which continues throughout the entire vascular system, including the lining of the heart chambers. The endothelium forms the interface between the blood in the lumen and the vessel wall and performs keys functions. Endothelial dysfunction, a classical hallmark of cardiovascular diseases (CVDs), can cause hypertension and atherosclerotic plaque development (discussed later). The *tunica media* is the thickest layer of the artery wall consisting of spindle shaped vascular smooth muscle cells (VSMC) arranged as a circular framework between the layers of wavy elastic fibres supported by collagen. Together, with the release of local chemical mediators, VSMC within the layers of the elastic fibres facilitate vasoconstriction and vasodilation which regulates the blood pressure and vascular tone. The *tunica adventitia* is the outermost layer of the vessel wall consisting primarily of collagenous fibres supported by the elastin fibres maintaining the structural integrity of the wall. The vessel wall is continuously exposed to biomechanical and biochemical stress which resulting to a multitude of biological processes like pathological vascular remodeling, inflammation and oxidative stress leading to CVDs.

1.1.2 Embryology of blood vessels

Development of vascular networks *de novo*, called vasculogenesis, is driven by the recruitment of epiblast cells through the primitive streak. There those epithelial cells transdifferentiate into mesenchymal cells (called epithelial to mesenchymal transition (EMT)) to form mesoderm. The process starts within the yolk sac and then continues in embryo (Udan et al., 2013). Precursor

vessels, which ultimately differentiate into arteries, veins and lymphatic vessels, are formed in the embryo. They are surrounded by primitive VSMC derived from mesoderm. Platelet-derived growth factor-B (PDGF-B) and transforming growth factor- β (TGF- β), are the two most important chemokines that are responsible for recruiting and differentiating VSMC pre-cursor cells (G. Wang et al., 2015). The precursors of the endothelial cells are primarily driven from the splanchnic mesoderm, whereas VSMC precursor cells are driven from multiple mesodermal sources including secondary heart field, neural crest, somites and pleural and splanchnic mesoderm (G. Wang et al., 2015; Yao et al., 2014).

Upon remodelling, the VSMC in the thoracic aorta are replaced by VSMC derived from neural crest. However, the VSMC in mature abdominal aortic segment closely resemble mesoderm derived primitive VSMC. Neural crest VSMC are believed to be more suitable to adapt and withstand the higher blood pressure and ejection volume in the thoracic aorta by laying down more elastic lamellae during development and growth. Moreover, the VSMC in the two different segments of the aorta respond to various cytokine and growth factor stimuli differently. For example, while the amino acid homocysteine induces proliferation of neural crest derived thoracic aortic VSMC and TGF- β induces DNA synthesis and collagen production in them, VSMC of mesodermal origin in abdominal aorta were unaffected by either of them (Pinard et al., 2019; Ruddy et al., 2013). Also, compared to the thoracic aortic VSMC, those in descending aorta are more responsive to the cytokines interleukin-1 β (IL-1 β) which results in protease expression and matrix degradation (Wasteson et al., 2008).

Though both thoracic and abdominal aorta have intimal, medial, and adventitial layers they differ structurally too. The media of the thoracic aorta has ~60 lamellar units comprising avascular and vascular regions. The medial layer of abdominal aorta instead consists of ~30 units and is completely avascular and thus is completely dependent on trans-intimal diffusion of nutrients for the survival of VSMC. Moreover, distinct fibromuscular layers called lamellar units, which help the aorta to distribute stress and provide elasticity, are less substantial in abdominal aorta. It is likely that the presence of avascular media containing fewer lamellar units make the abdominal aorta more prone to aneurysmal degeneration compared to thoracic counterpart (Kuivaniemi et al., 2015; Tromp et al., 2010). Such differences in embryological origin and architecture leads to

differences in the pathophysiology of diseases such as aneurysms that occur in different parts of the aorta (Qian et al., 2022).

1.1.3 Endothelial cells

The squamous cells lining the inner surface of the blood vessels are called endothelial cells. Apart from acting as a barrier layer between the blood and other cells/tissues, they play multiple active and essential roles, including in angiogenesis (the formation of new vessels), vascular tone maintenance, and clotting regulation and aiding active/passive movement of substances to/from blood (Godo and Shimokawa, 2017). The barrier function of the endothelial layer depends on many factors such as shear stress, inflammation, pathogens, and disease states (Komarova and Malik, 2010). Under physiological conditions, endothelial cells contribute to the inhibition of vascular inflammation; in various pathological states, they may stimulate an inflammatory response by interacting with leukocytes and producing adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and proinflammatory cytokines like tumour necrosis factor-beta (TNF- β) and macrophage colony-stimulating factor (M-CSF) (Pober and Sessa, 2007). Angiogenesis, or the sprouting of new vessels from pre-existing vessels, is achieved essentially by endothelial cell migration and proliferation, which is regulated by chemotactic, haptotactic, and mechanotactic stimuli (Chia et al., 2020). Endothelial cells regulate vascular tone by releasing vasodilators, such as nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF), and prostacyclin, in addition to vasoconstrictors, such as endothelin-1 (ET-1) and angiotensin II (AngII) (Sandoo et al., 2010). Also, endothelial cells regulate thrombosis through their interaction with platelets by producing pro-thrombotic molecules, such as thromboxane, plasminogen activator inhibitor-1 (PAI-1), von Willebrand's factor (vWF), and tissue factors (Neubauer and Zieger, 2022). Endothelial cells exhibit extensive heterogeneity across different tissues (Aird, 2012).

1.1.4 Vascular smooth muscle cells

VSMC are the main cellular constituent of blood vessels and the major component of the medial layer. VSMC are mononucleated, spindle shaped, non-striated cells that are tightly packed/aligned in an organised structure with surrounding VSMC and present in the medial layer of the walls of arteries and veins (Owens et al., 2004).

The most crucial function of mature VSMC is maintaining the tone of blood vessels and regulating blood flow (through vasoconstriction and vasodilation), thus ensuring optimal blood pressure for adequate tissue perfusion throughout the body (Yap et al., 2021). VSMC have physical and biochemical characteristics optimised to perform this role. Structurally, they are elongated in shape, aligned in an organised structure with surrounding VSMC. Biochemically, they express specific calcium handling proteins, ion channels, cell surface receptors and contractile proteins to enable vasoconstriction and dilatation. Healthy, mature VSMC are quiescent and divide with a half-life ranging from 270 – 400 days (Neese et al., 2002). They also provide the structure to the vessel wall by controlled proliferation and secretion along with extracellular matrix (ECM) synthesis. Apart from a multitude of physiological functions they play a vital role in vascular repair after injury. VSMC are not terminally differentiated cells and exhibit an ability to de-differentiate to perform additional tasks. In healthy blood vessels, the VSMC exhibit a quiescent contractile phenotype with extremely slow proliferation, show little synthetic activity and express a distinct set of contractile components, namely α -smooth muscle actin (α -SMA), smooth muscle myosin heavy chain (SM-MHC), smooth muscle protein 22a (SM22 α) and calponin. This contractile phenotype is essential for maintaining the tone of the blood vessels and also for contraction/dilatation of blood vessels according to the physiological cues and needs (Bennett et al., 2016; Chistiakov et al., 2015; Gomez and Owens, 2012; Owens et al., 2004). However, in pathological states of injury to blood vessels, they switch to a synthetic state characterised by differences in morphology - they adapt a flat, rhomboid shape. Also, they lose expression of key SMC contractile proteins and instead produce inflammatory cytokines and new matrix products resulting in activation of proliferation, migration, and secretion. This process, called VSMC phenotype switching is a key feature of the VSMC plasticity (Rzucidlo et al., 2007; Yap et al., 2021). While this de-differentiation was evolutionarily preserved as it allowed repair of blood vessels, as humans evolved/developed it has turned into an adverse phenomenon following a trauma/injury. In modern society, this “injury” is due to western lifestyle, oxidative stress,

smoking, hypertension etc that leads to chronic activation of this mechanism leading to pathology (Owens et al., 2004). Typically, this process is studied in the direction of contractile to synthetic and is vital in allowing a rapid response to vascular injury and fluctuating environments, permitting remodelling and re-vascularisation (Alexander and Owens, 2012). However, this description of two distinct VSMC phenotypes is an over-simplified interpretation of the mature VSMC as in reality each phenotype occupies extreme ends of a spectrum. Thus, the VSMC spectrum ranges from the ‘contractile’ phenotype at one extreme to the ‘synthetic’ phenotype at the other.

1.1.5 VSMC phenotype switching

Switching of VSMC phenotype from the contractile and quiescent state to a proliferative and synthetic state is a hallmark of various vascular diseases including atherosclerosis, neointimal hyperplasia, hypertension, and vascular aneurysms (Rzucidlo et al., 2007) (Figure 1.1a). While few investigators differentiate phenotype switching (reversible changes in phenotype) from remodeling (irreversible morphological changes), in this thesis both terms are used interchangeably (Bkaily et al., 2021).

Development of lineage-tracing studies in mouse models and the high-throughput transcriptome sequencing studies paved a way to identify the different phenotypes of VSMC involved in the development and progression of vascular diseases (Liu and Gomez, 2019). For over several decades, activation and de-differentiation of VSMC has been believed to be the adoption of a single synthetic, proliferative phenotype (Liu and Gomez, 2019). However, Yap and colleagues classified six possible phenotypes of VSMC and the impact of phenotype switching on different vascular diseases by reviewing the data of single-cell RNA sequencing (scRNA-seq) studies suggesting a much more complex regulation of the VSMC characteristic phenotypes. The six phenotypes are the contractile, mesenchymal-like, fibroblast-like, macrophage-like, osteogenic-like, and adipocyte-like VSMC (Yap et al., 2021).

Contractile phenotype:

As mentioned earlier, the tunica media of the aortic wall is made of multiple layers of the VSMC, elastin and collagen fibres which are contractile type. The contractile phenotype, expression of specialised contractile proteins and the surrounding ECM with collagen and elastin fibres are optimised for maintaining arterial tone and the contraction and dilation of blood vessels in response to the physiological cues and needs to control the supply of blood to organs and tissues (Bennett et al., 2016; Chistiakov et al., 2015; Gomez and Owens, 2012; Yap et al., 2021). Interestingly, expression of the contractile proteins is regulated by the transcription factors myocardin and serum response factor which regulates the differentiation of VSMC to the contractile phenotype (Long et al., 2008; Miano et al., 2007). Additionally, phosphatase and tensin homolog (PTEN), myocyte enhancer factor 2C (MEF2C), microRNA-143/145 and external factors like TGF- β and heparin were all known to induce the contractile phenotype of VSMC thus involved in the proper regulation of vascular structure and function (Beamish et al., 2010; Horita et al., 2016). Krüppel-like factor 4 (KLF4), a transcription factor that is essential for the maintenance of pluripotency in embryonic stem cells, has been shown to play a critical role in regulating phenotypic transitions of VSMC not only in cultured VSMC treated with PDGF, but also *in vivo* during development and cancer metastasis, and after carotid injury (Deaton et al., 2009; Jiang et al., 2008; Murgai et al., 2017; Shankman et al., 2015).

Mesenchymal-Like VSMC:

Several transcriptomics studies confirmed the presence of mesenchymal-like VSMC (Conklin et al., 2021; Dobnikar et al., 2018). They were found to exhibit the signature characteristics of the mesenchymal stem cells/ stromal cells such as ability to proliferate and self-renew. They also lack the expression of the contractile proteins. Interestingly, such VSMC phenotype was found in the tissues of atherosclerotic plaques or aortic aneurysms suggesting the possible role of mesenchymal-like VSMC in vascular diseases including aortic aneurysms (Chen et al., 2020; Shankman et al., 2015; Yap et al., 2021). As mentioned before, KLF4 induces VSMC proliferation and de- differentiation leading to phenotype switching to the mesenchymal-like phenotype (Garvey et al., 2010; Yoshida et al., 2008). Supporting this, activation of KLF4 in VSMC induced the expression of SCA1/LY6A, CD34, and CD44 which are the well-known mesenchymal markers (Chattopadhyay et al., 2021).

Fibroblast-Like VSMC:

Transcriptomics studies have reported a subset of SMCs that shifted towards but clustered separately from the authentic fibroblasts (Conklin et al., 2021; van Kuijk et al., 2019). Such clusters were identified as fibroblast-like VSMC (Wirka et al., 2019). Fibroblast cells are responsible for the generation of ECM framework. In case of an injury, these cells switch to myofibroblasts and repair the wound by producing collagen-rich scar tissue (Micheletti and Alexanian, 2022). Therefore, this type of VSMC are often found in vascular anomalies such as atherosclerosis and aneurysms. For instance, single cell-transcriptomic profiling of the thoracic aortic aneurysm of a Marfan syndrome mouse model confirmed the presence of fibroblast-like VSMC (Pedroza et al., 2020; Yap et al., 2021) (Pedroza et al. 2020; Yap et al. 2021). Further, these cells were found to express both smooth muscle and fibroblast gene markers such as ACTA2, SCA1/LY6A and lumican (LUM), biglycan (BGN), and decorin (DCN). Contrastingly, the expression of contractile gene markers like TAGLN and CNN1 were significantly reduced. Several factors such as KLF4, TCF21, cholesterol or oxidized phospholipid exposure and ER unfolded protein response (UPR) were shown to be involved in the regulation of this fibroblast-like VSMC phenotype switching (Chattopadhyay et al., 2021; Iyer et al., 2018).

Other VSMC phenotypes

Using genetic lineage tracing models, it has been shown that in various proliferative vascular disorders like atherosclerosis, neointimal hyperplasia and pulmonary hypertension, VSMC phenotypic switching leads to the formation of less-differentiated VSMC such as macrophage-like, osteogenic-like and adipocyte-like phenotypes. Contractile VSMC were proved to undergo phenotype switching to a macrophage-like VSMC via the mesenchymal-like VSMC and contribute to the development of atherosclerosis. Further, this type of VSMC is characterized by the lack of contractile VSMC factor MYOCD and the phenomenal expression of macrophage markers (CD11b, CD45, CD68 and CD116) and galectin-3 gene which is known to induce the differentiation of monocytes and VSMC into macrophages (Bennett et al., 2016; Depuydt et al., 2020; Vengrenyuk et al., 2015). Dedifferentiation of VSMC into chondrocyte-like and osteoblast-like VSMC were identified in scRNA-seq studies (Chen et al., 2020; Dobnikar et al., 2018; Worssam and Jørgensen, 2021). Both these types of dedifferentiated VSMC are classified as

osteogenic-like VSMC and are characterized by the loss of SM22 α and ACTA2 and increased expression of osteogenic and chondrogenic transcription factors such as MSX2, RUNX2, Sp7/Osterix and SOX9 (Durham et al., 2018; Voelkl et al., 2019). Interestingly, deposition of calcium phosphate was found to induce such phenotype switching thus contributing to various vascular abnormalities such as atherosclerosis. Adipocyte-Like VSMC was observed in scRNA-seq study of aortic aneurysms (Chen et al., 2020). Such VSMC are categorized as beige adipocytes or inducible brown adipocytes that regulate thermogenesis and express the crucial beige adipocyte marker UCP1 along with PPARGC1A, TMEM26, PRDM16 and TRPV1 markers (Shamsi et al., 2021; Yap et al., 2021). However, not much is explored about this phenotype.

1.1.6 Platelet derived growth factor signalling

PDGF is a potent mitogen to a variety of cells including VSMC, fibroblasts and monocytes. It is one among the numerous growth factors that have been found to stimulate cell growth and division. The PDGF signalling pathway consists of two receptors and four ligands. All the ligands function as homodimers whereas PDGF-A and B form an additional heterodimer thus generating a family consists of five different isoforms: PDGF-AA, -AB, -BB, -CC and -DD. Their tyrosine kinase receptors (PDGFR- α and - β) occur in three isoforms: PDGFR- $\alpha\alpha$, - $\beta\beta$ and - $\alpha\beta$. PDGF-AA binds only to $\alpha\alpha$ receptor, PDGFR-BB binds to all three receptor isoforms, PDGFR-AB and -CC bind to $\alpha\alpha$ and $\alpha\beta$ receptors and PDGFR-DD binds to $\alpha\beta$ and $\beta\beta$ receptors (Fredriksson et al., 2004; Heldin and Lennartsson, 2013). Further, the dimerization results in the activation and autophosphorylation of the receptors which in turn triggers conformational changes in the intracellular parts of the receptors thus facilitating the binding and activation of SH2-domain-containing signal transduction molecules such as Src, Ras, SHP-2, STAT5, PI3K, Grb2 and PLC- γ etc. Therefore, PDGF is the key regulator of the complex signalling network that promotes cell growth, survival, and migration (Heldin and Lennartsson, 2013). While activation of the PDGFR- α receptor stimulates cell growth and differentiation, PDGFR- β activation stimulates migration and proliferation. Inhibition of both the receptors results in reduced proliferation and migration rate of VSMC leading to reduced neointimal formation. Interestingly, an elevated expression of PDGF was reported in elastase-induced aortic aneurysm model which might indicate its

contribution in AAA development/progression (Jiao et al., 2010). Adding to this, loss of PDGF receptor signalling in AAA showed a reduction in collagen deposition and an increase in MMP-2 activity (French et al., 2008). As described before, various genetic studies, showed significant association between low-density lipoprotein receptor-related protein gene LRP1 and AAA (Au et al., 2018; Bown et al., 2011). Interestingly, LRP1 inactivation or selective deletion was found to limit the PDGFR signalling which in turn resulted in reduced VSMC proliferation, aneurysm formation and susceptibility to cholesterol-induced atherosclerosis (Boucher et al., 2003). However, LRP1 and PDGFR- β act as co-receptors and direct binding of PDGF-BB was shown to result in transient tyrosine phosphorylation of the LRP1 cytoplasmic domain (Au et al., 2018). Thus, further studies are required to fully understand the role of LRP1-PDGF signalling interplay in AAA and elucidate the therapeutic implications (Wild et al., 2012).

Blood vessel development is guided by receptor ligand interactions that allow for crosstalk between the endothelial cells and the surrounding mesenchyme. The role of PDGF in recruiting mural cells during blood vessel formation has been demonstrated through gene targeting of the ligand (PDGF-BB) and the PDGFR β (Hellström et al., 1999; Soriano, 1994). The dependence of pericyte/VSMC recruitment on PDGF signalling was shown to be variable between different tissues, with vascular mural cells expressing PDGFR β in most tissues except liver, as determined by *in situ* hybridization. PDGF signalling also plays important roles in arteries and in the response to arterial injury by promoting migration of VSMC from the media into the developing neointima (Fan et al., 2022; Owens et al., 2004). Elevated levels of PDGFR β were observed in activated and dedifferentiated SMC in vascular injury (Andrae et al., 2008).

It is well established in VSMC and multiple other cell types that PDGF-BB application causes phospholipase C gamma (PLC γ) activation and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃, through IP₃R activation induces ionic calcium (Ca²⁺) release from the intracellular calcium store, the endoplasmic reticulum (ER) into the cytosol. When the ER Ca²⁺ sensor stromal interaction molecule 1 (STIM1) detects the depletion of the intracellular Ca²⁺ stores, entry of Ca²⁺ from extracellular space into the cytosol is induced. This Ca²⁺ entry is called store operated calcium entry (SOCE). A well-established candidate protein which forms the pore upon induced by and

interacting with STIM1 to produce SOCE is called Orai1 (Collins et al., 2013; Feske et al., 2006; Gruszczynska-Biegala et al., 2021). SOCE and Orai1 have indispensable role in VSMC remodelling in various vascular diseases including atherosclerosis, neointimal hyperplasia and calcification (Martín-Bórnez et al., 2022; Shower et al., 2021).

VSMC remodelling to a proliferative phenotype has been shown to be associated with increase in expression and activity of Orai1 (Shower et al., 2021). The contractile VSMC expresses lower level of Orai1 and thus have lower SOCE. However proliferating VSMC in culture have abundant SOCE and Orai1 expression (Martín-Bórnez et al., 2022; Shower et al., 2021). Inhibition of Orai1/SOCE by pharmacological agents or genetic silencing in VSMC from various sources including human aorta and saphenous veins and rabbit, rat and mice aorta have shown the pro-migratory and proliferative effects of Orai1 and demonstrated that SOCE/Orai1 can be successfully targeted to inhibit the VSMC phenotype switching process. Orai1 signalling promotes the activation of the transcription factor, NFAT, which induces the transcription of pro-proliferative genes such as IL-6, cyclin A and cyclin D in VSMC. Upon Orai1 silencing, NFAT activation and nuclear translocation was reduced (Shower 2021; Spinelli and Trebak, 2016). Orai1 activation can be induced by PDGF in human saphenous vein and pulmonary arterial VSMC. The potentiation of Orai1 activity, via the Orai1 enhancer IA65, was reported to augment PDGF-induced VSMC migration, supporting the role of Orai1 signalling in PDGF-induced VSMC remodelling. In addition to PDGF-induced remodelling, Orai1 has been shown to play essential roles also in AngII and Urotensin-II -stimulated VSMC proliferation in human coronary and rat aortic VSMC. In addition, synthetic VSMC showed higher sphingosine-1-phosphate (S1P)-induced Orai1 mediated SOCE than the contractile phenotype (Martín-Bórnez et al., 2022; MA Bailey PhD Thesis 2016 and H Shower PhD Thesis 2023). These reports clearly demonstrate the indispensable roles of Orai1 activation and signalling in VSMC remodelling (Figure 1.1b).

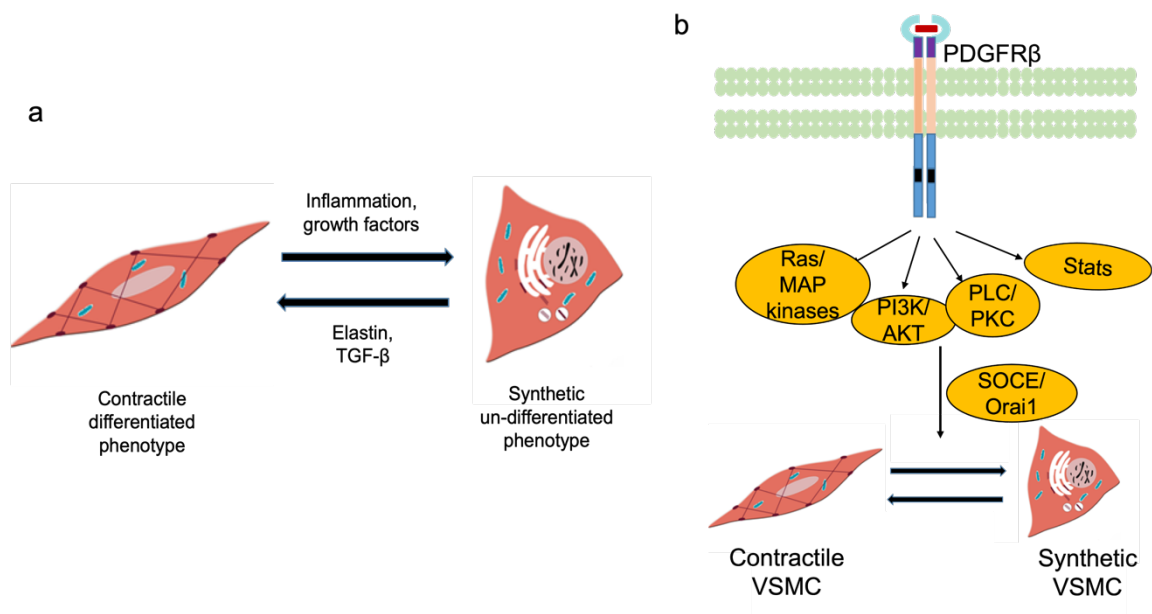


Figure 1.1 VSMC phenotypic switching and PDGF signalling

Diagrammatic representation of reversible phenotypic switching of contractile VSMC and synthetic VSMC induced by various factors (**a**) and PDGF signalling in VSMC phenotype switching (**b**). TGF- β : transforming growth factor beta; PI3K: phosphoinositide 3-kinase; PLC: phospholipase C; PKC: protein kinase C; SOCE: store operated calcium entry; Stats: signal transducer and activator of transcription

1.2 Cardiovascular diseases

CVDs – incorporating atherosclerosis of the coronary, cerebral and peripheral arteries - are the leading cause of mortality globally accounting for 31 per cent of all fatalities. Around 17.8 million people died from CVDs in 2017 with 80% of the deaths from low- and middle-income countries – now overcoming infectious diseases (Roth et al., 2020). The World Health Organization (WHO) estimates that by adopting a healthier lifestyle, 80 per cent of CVDs-related deaths may be avoided (WHO, 2016). Around 30 per cent of deaths in England are linked with CVDs and in the UK, one death from CVDs happens every three minutes (BHF CVD statistics fact sheet, Jan 2020). The 'disability-adjusted life years' (DALYs) that measures the years of healthy life lost from death and illness, due to CVDs has been significantly increasing worldwide since 1990s (Roth et al., 2020). (. CVDs is correlated with blood vessel damage in organs including the heart, brain, kidneys, eyes, and limbs. The broad group of CVDs includes coronary/ischæmic heart disease (CHD / IHD, myocardial infarction, angina), cerebrovascular disease (stroke, transient ischaemic attack), peripheral artery disease (PAD) and aortic disease. IHD and stroke account for 80 percent of CVDs. Risk of CVDs starts from foetal period, and it is more prevalent in men compared to women. Smoking, alcohol, stress, obesity, high blood pressure, high cholesterol and diabetes are the major risk factors linked CVDs (i.e., a typical Western lifestyle). Managing the condition is accomplished by a healthy “Mediterranean” lifestyle, reducing clinical risk factors such as elevated blood pressure, high cholesterol rates and by taking cardioprotective medications (Vaduganathan et al., 2022). Two of the major CVDs – atherosclerotic vascular disorders and aneurysm are discussed in this chapter.

1.2.1 Atherosclerotic cardiovascular disorders

Atherosclerosis is the primary cause of occlusive vascular diseases by generating deposits of cholesterol, fat, and other substances called ‘atheroma’ in the inner walls of arteries that limits oxygen rich blood to the heart. It is a progressive disease that, over time, can lead to advanced lesions with distinct structural complexity (Ross, 1999). Atherosclerosis often begins early in childhood and remains asymptomatic until significant arterial lumen narrowing occurs impeding

the perfusion to the distal tissue which often takes several decades. A typical atherosclerotic lesion is characterized by thickening of the intimal layer following sustained local injury to vulnerable regions of blood vessels that drive an inflammatory response. The development and progression of atherosclerotic plaques has been proposed to occur in five main steps: 1) Endothelial injury, dysfunction, and sub-endothelial accumulation of low-density lipoproteins; 2) Infiltration of leukocytes and formation of foam cells; 3) VSMC proliferation and de-differentiation; 4) Connective tissue accumulation; 5) Plaque stabilisation or erosion/rupture leading to symptomatic complications (Libby et al., 2019). However, plaque development is not necessarily orderly, neither does it always follow the linear classification system of plaques (type I to VI) defined by The American Heart Association Committee (Stary, 2000). Regions of high sheer stress, such as branches, and vessel arches are particularly vulnerable to focal endothelial injury.

Coronary heart disease is the commonest CVD which arises when the blood supply to the heart is reduced by the development of atherosclerotic plaques in the coronary arteries leading to impaired blood supply to parts of heart which can cause angina. The plaque rupture and induce thrombosis and thus lead to complete occlusion of the vessel causing myocardial infarction. Stroke arises from reduced blood flow to the brain and causes damage to the brain cells. It is the second-largest killer disease in the world and is the fourth killer disease in the UK (King et al., 2020). People over the age of 55 are most prone to develop stroke. Ischemic stroke secondary to atherosclerosis involves complex pathophysiological mechanisms which include energy depletion, oxidative and nitrative stress, calcium overload and ion imbalance, inflammation leading to apoptosis (Sacco et al., 2013). Stroke is the leading cause of disability relative to all other disease conditions which involve weakening and numbness of arms and legs, paralysis, slurred speech, vision problems, which can even contribute to vascular dementia (King et al., 2020). Early recovery of blood supply by supportive treatment and interventions such as thrombolysis/thrombectomy, may reduce post-stroke brain damage (Benjamin et al., 2019).

Peripheral artery disease (PAD) is the world's third leading cause of CVD-related morbidity caused by widespread atherosclerosis build-up in arteries which contributes to the occlusion of the extremity-supplying artery (Marbach, 2020). In the UK almost one in five people over the age of 60 have PAD. Nearly 50 per cent of individuals with PAD have moderate to no symptoms. Most

often, PAD affects arteries in the legs but can affect arms, neck, and arteries in the kidneys. The most common risk factor for PAD is diabetes, and smoking (Kyle et al., 2020). Diagnosis of PAD is by doppler vascular examination assessing the ankle-brachial index which is the ankle-to-arm systolic blood pressure. Stopping smoking, daily exercise, and medication help improve the arteries that have been partially occluded.

1.2.2 VSMC phenotypic switching in atherosclerotic CVD

VSMC phenotype switching plays a major pathogenic role in various vascular diseases. Following phenotype switching and excessive proliferation, VSMC undergo replicative senescence and become apoptotic (Bennett et al., 2016; Libby et al., 2011). VSMC senescence, apoptosis, and VSMC-derived macrophage like cells may further promote inflammation in atherosclerosis other related disorders (Bennett et al., 2016). Elucidation of molecular mechanisms of regulating VSMC differentiation marker genes contributes for better understanding of disease process and to the development of novel therapeutic targets that can inhibit disease formation and progression. An overview of the VSMC remodeling that occurs in few of those common disorders is given below.

I have introduced the importance of atherosclerosis in a range of CVDs above. Traditionally atherosclerosis has been thought as a disease caused by endothelial dysfunction leading to accumulation lipid-laden macrophages (foam cells) and VSMC migration from media to intima ending in fat deposition, hardening and calcification (Hansson, 2005). Recent work by various investigators challenged that view and emphasised that VSMC significantly contribute to the pathogenesis of atherosclerosis (Bennett et al., 2016; Grootaert and Bennett, 2021; Shankman et al., 2015). However, there is still an ambiguity about the exact origin of different VSMC-like cells in atherosclerotic plaques, participation and essentiality of those cells in atherogenesis is clear. VSMC apoptosis, senescence, and VSMC-derived macrophage-like cells may promote inflammation and thus induce progression of disease and plaque instability. Transcriptional repression of VSMC contractile proteins -such as α -SMA and SM-MHC were induced by increased levels of growth and inflammatory factors including PDGF-BB, oxidized phospholipids, or IL1 β that are present in atheroma lead to VSMC phenotype switching (Bennett et al., 2016).

Though apoptosis of VSMC at the fibrous cap of atheroma is conspired as a major cause of plaque rupture, some investigators continue to advocate proliferative VSMC may play protective roles in advanced lesions by preventing fibrous caps from rupturing and promoting plaque repair (Bennett et al., 2016; Harman and Jørgensen, 2019). For harnessing this knowledge for clinical application, further studies are needed to clearly demarcate specific role of VSMC in each stage of atherogenesis. Balloon angioplasty / stenting, venous bypass grafts, and fistula creation are used to treat a wide range of occlusive vascular (and other) diseases. However, the endothelial injury and arterialisation of vein bypass grafts can lead to treatment failure through re-stenosis and thrombotic occlusion. Typically, this process is driven by neo-intimal hyperplasia. This also involved VSMC dedifferentiation. Similar to atherosclerosis, local inflammatory signals act as chemoattractant for VMSCs resulting in their migration, de-differentiation and proliferation, ECM secretion and intimal thickening (Chakraborty et al., 2021; Chappell et al., 2016; Déglise et al., 2023).

Vascular remodeling in hypertension involves changes to VSMC in the vessel wall of both large and small arteries, as well as other cellular components of the vascular wall. Unlike atherosclerosis, hypertension is a disease of contractile VSMC that are regulators of vascular tone. This type of remodeling of contractile VSMC is mainly due to an increase of vasoconstrictive factors such as AngII and ET-1 (Bkaily et al., 2021). In pulmonary hypertension, the key pathologic feature is muscularization of normally non-muscular distal pulmonary arterioles leading to elevated pulmonary arterial resistance (Frid et al., 2006). The molecular mechanisms include downregulation of MYH11 and upregulation of PDGF signalling leading to de-differentiation and clonal expansion of VSMC (Chakraborty et al., 2021). Though reducing the accumulation of intracellular Ca^{2+} using pharmacological agents are shown to partially reverse such remodeling in contractile VSMC and reduce blood pressure, further studies are essential to understand remodeling of contractile VSMC in hypertension.

1.2.3 Aneurysm

The term ‘Aneurysm’ comes from the Greek word ἀνεύρυσμα (aneurysma), meaning dilatation. An aneurysm (A) is the pathological weakening of an arterial wall that causes enlargement or bulge

in the artery. Aneurysms tend to grow in size eventually resulting in biomechanical failure of the vessel wall and rupture or in other cases, thrombosis due to altered flow through the dilated segment. Aneurysms can occur in any artery of the body. Most common are – cerebral aneurysm that occurs in the brain, aortic aneurysm (AA) sub-classified as thoracic aortic aneurysm (TAA) in the ascending or descending thoracic aorta or aortic arch and abdominal aortic aneurysm (AAA) that develops in the abdominal region of the aorta. In addition, aneurysms can also occur in the iliac arteries, the popliteal artery (behind the knee), and mesenteric arteries (the arteries that supplies blood to the intestine). Among all types of aneurysms, AAA which typically occurs in the infra-renal region of the aorta is the most common. The rupture of an AAA is a catastrophic event that causes internal bleeding with a high mortality rate. An arterial dissection can be defined as a tear in the inner lining of an artery leading to entry of blood between the intima and media. When a tear occurs in a major artery that supplies brain or heart, it can result in stroke or myocardial infarction. Aortic dissection is a rare but life-threatening condition which require immediate repair surgery.

There are different ways of subcategorising AA. For example, it can be based on etiopathogenesis (atherosclerotic, degenerative, genetic, inflammatory etc), morphology (fusiform, saccular, false; Figure 1.2; (LaRoy et al., 1989)), or anatomical site within the arterial tree (TAA vs AAA). Further, TAA is subdivided into ascending TAA and descending TAA (Saeyeldin et al., 2019). AAA are more common than the TAA. Based on the proximity to renal arteries, AAA are again classified into three types namely, infrarenal, juxtarenal and pararenal/paravisceral. Aneurysm that arises one or two centimetres away from the renal vessels or if the transverse aortic diameter is more than 3.0 cm, it is called an infrarenal AAA. Juxtarenal AAA are the aneurysms that extent to the renal orifices but do not involve them. Finally, aneurysms involving the visceral vessels and lacking the normal aorta between their upper extent and renal arteries are either pararenal (involving renal artery) or paravisceral (involving both renal and visceral artery). Proximal aneurysms (e.g., root, arch etc) are more often due to genetic causes (such as Marfan's) or infection (syphilis) that distal aneurysms (e.g., AAA) which are most commonly “degenerative”.

Finally, pseudoaneurysm also described as false aneurysm is an aneurysm characterized by small irreversible ruptures of the artery that are contained by the surrounding tissues. Such ruptures can

be caused by an aortic root surgery or a trauma or a primary infection of a healthy artery wall or through a secondary infection of an already existing aneurysm. Unlike the true aneurysm, pseudoaneurysm lacks all the three components of the arterial wall. i.e., in case of true aneurysm, wall of the artery maintains the normal trilaminar structure (intima, media and adventitia) whereas a false aneurysm lacks all the three components. Pseudoaneurysms are associated with a very high risk of rupture and mortality (Hussain and Roche-Nagle, 2013).

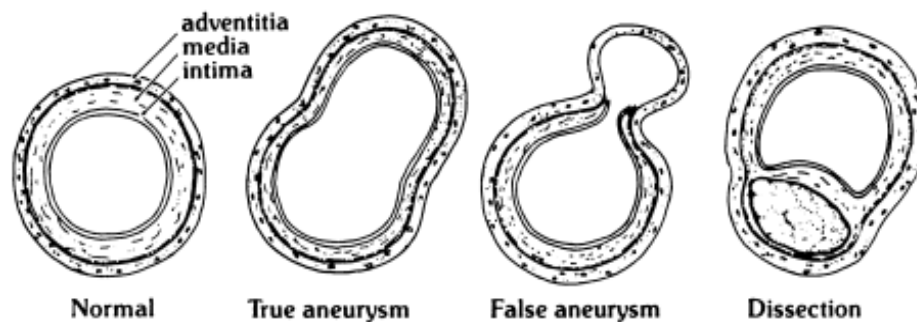


Figure 1.2 Schematic of aneurysms and dissection

Diagrammatic representation of true, false aneurysms and dissection (reproduced from LaRoy et al., 1989)

1.3 Abdominal aortic aneurysm (AAA)

A permanent focal dilation of the abdominal aorta to more than 1.5 times of its normal diameter or ≥ 3.0 cm is termed an AAA (Figure 1.3). AAA is a relatively common aortic degenerative disease which becomes more common with age. It can become a life-threatening medical condition as the aneurysm weakens the aortic wall which eventually fails to withstand the luminal blood pressure ultimately leading to wall rupture resulting in severe haemorrhage. The risk of rupture increases with increase in the AAA diameter. In the UK alone, more than 4000 deaths are attributed to AAA every year (Kuivaniemi et al., 2015; Meecham et al., 2021; Shaw et al., 2023).

AAA are usually asymptomatic and detected by routine screening or during various imaging studies (e.g., abdominal ultrasound, and computed tomographic angiography) obtained for other unrelated pathologies (Ullery et al., 2018). Enlarging aneurysms can cause symptoms like severe and persistent pain in the back, abdomen, flank, or groin area which may also indicate erosion of the aneurysm into surrounding structures, infection of the aortic wall, or rupture. Further, compression of the nearby viscera by the enlarging aneurysm would result in the gastrointestinal (GI) or renal complications (Shaw et al., 2023). Thromboembolization, atheroembolization, aortic rupture, or development of an arteriovenous or aortoenteric fistula are rare complications of AAA (Shaw, 2022; Ullery et al., 2018).

As per the recent reports from National Abdominal Aortic Aneurysm Screening Programme (NAAASP) the rupture risk at 3 cm is 0.03% per year and at 5-5.5 cm 0.5% per year in the UK. Though ruptures are relatively rare, mortality rate of AAA is more than 90% once the aneurysm ruptures, signifying the seriousness of this disease (Jeanmonod, 2022). Therefore, it is crucial to develop novel therapies for the prevention and stabilization of AAA.

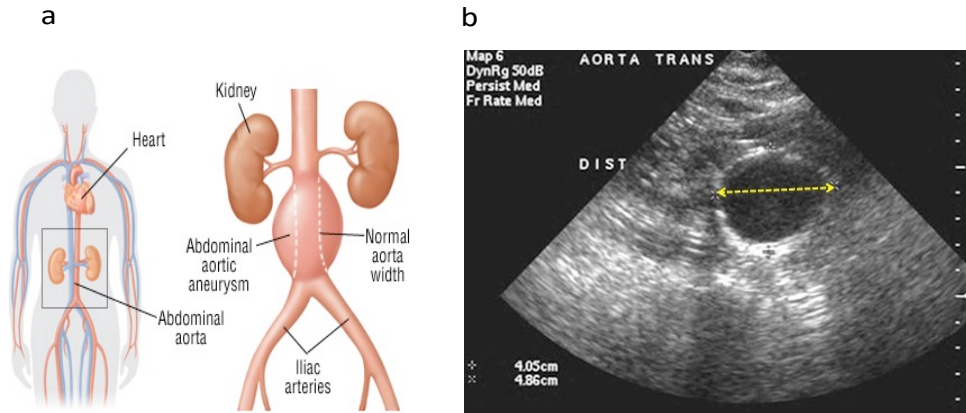


Figure 1.3 Abdominal aortic aneurysm.

(a) Localised dilation or bulge in the abdominal aorta. (b) AAA is detected by USS from which the measurement of aortic diameter & location of the aneurysm are obtained (Source: Medscape)

1.3.1 Pathophysiology of AAA

Historically insights were gained into the pathology of AAA based on samples taken from the aneurysm at the time of open surgery which is usually done when the aneurysm is large (5.5 cm) and the aneurysm has been present for many years. Thus, these samples represent end-stage disease which may well be different to the earlier events that define development and progression of the disease. These end-stage samples demonstrated characteristic features which include loss of elastin, elevated expression of MMPs, loss of medial VSMC (“medial degeneration”) and inflammation (Boddy et al., 2008; Kuivaniemi et al., 2015) (Figure 1.4). Development of aneurysm is regarded as a result of collective effects of various degenerative, mechanical, hemodynamic and inherited factors (Eleftheriades et al., 2017; Nordon et al., 2011; Singh et al., 2018). Historically, AAA was considered as an “atherosclerotic” vascular disorder as were found to share common risk factors and histopathological features. However, recent epidemiological, clinical, and histological evidence suggests that these two pathologies are more distinct than previously believed (Toghill et al., 2017).

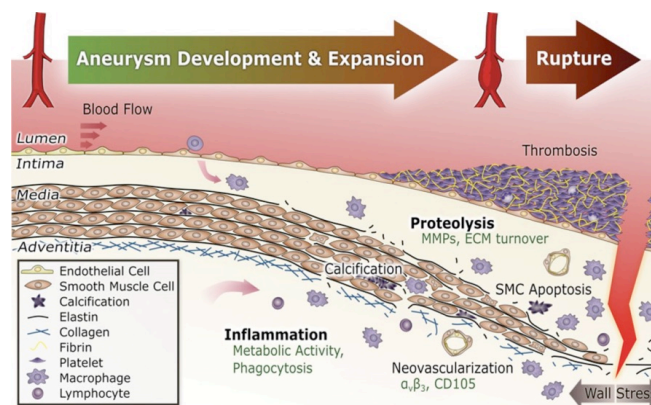


Figure 1.4 Schematic representation pathogenesis of AAA.

The key characteristics shown in end-stage AAA are inflammation, proteolysis, smooth muscle cell (SMC) apoptosis, neovascularization. MMPs: matrix metalloproteinases; ECM: extra-cellualr matrix; CD105: cluster of differentiation 105; $\alpha_v\beta_3$: integrin alpha v beta 3. Source: (Toczek et al., 2016)

Degeneration of arterial wall components

Aneurysms are characterized as the progressive degeneration of the arterial wall components such as collagen, elastin and smooth muscle cells which leads to the wall dilatation and risk of aortic rupture. Hence, these aneurysms are termed as “degenerative” (Calero and Illig, 2016; Golledge et al., 2006). A proper dynamic equilibrium between the formation and destruction of ECM is the prime factor for the maintenance of an intact aortic wall. However, in case of aneurysm this equilibrium is highly affected as a result of various factors (Singh et al., 2018). As mentioned before, tunica media of aorta has highly organized concentric layers of medial lamellar units (MLUs) and their number is directly proportional to the diameter of the wall (Petit et al., 2019). However, the abdominal aorta contains only around 28 layers of MLU despite the larger diameter and tangential tension. Therefore, there is more pressure on each layer. Further, no vasa vasorum in the media to supply nutrients to these layers. This increased lamellar tension and limitation of vassal supply makes the abdominal aorta more susceptible to aneurysm (Petit et al., 2019; Zatina et al., 1984).

Elastin is responsible for the elastic properties of arteries and collagen gives the tensile strength. Therefore, imbalance in the levels of these proteins causes the aortic wall dilation. For instance, it has been reported that, in aneurysms, elastin level is decreased, and collagen level is increased thus increasing the collagen to elastin ratio thus decreasing the elasticity of the wall. Further, the matrix-remodelling proteins like MMPs are reported to be involved in the degradation of aortic walls through the destruction of the elastin fibres and smooth muscle cells. In addition to the activation of MMPs, downregulation of tissue inhibitors of MMPs (TIMPs) are also observed in AAA disturbing the MMP to TIMP activity ratio resulting in an increased proteolytic activity which in turn affected the integrity of the aortic wall by degrading the elastin fibres (Calero and Illig, 2016; Singh et al., 2018; van Vlijmen-van Keulen et al., 2002). Interestingly, destruction of the elastin fibres results in the dilation whereas destruction of collagen is often associated with rupture (Sakalihasan et al., 2018). In addition, increased levels of collagenases (MMP-1 and MMP-13) are observed in human AAA and accompanied with interstitial collagen dissolution. Similar to, the collagenases, levels of elastase enzymes (MMP-2, MMP-9 and MMP-12) are also elevated in AAA. MMP-9 knockout mice are unable to develop experimental aneurysm signifying its importance in the AAA pathogenesis (Ailawadi et al., 2003). In addition to the MMPs, serine proteases are also found to be involved in the either direct or indirect degradation of ECM

(Sakalihasan et al., 2018). For instance, plasmin, a serine protease, induces VSMC detachment and apoptosis through degradation of the intermediate adhesive proteins and activates the pro-MMPs. Likewise, another serine protease named leukocyte elastase damages the fibrillar ECM contributing to wall destruction (Sakalihasan et al., 2018).

Infiltration of immune cells

It is well established that inflammation plays an essential role in the development of AAA with an excessive infiltration of wide range of immune cells such as macrophages and lymphocytes (Ailawadi et al., 2003; Satoh et al., 2009; Wanhainen et al., 2005). Release of elastin degradation products in the aorta can facilitate this infiltration via chemotaxis. The infiltrating immune cells can also induce the secretion of various cytokines which in turn activate the proteases responsible for wall destruction (Ailawadi et al., 2003). Upregulation of IL-1 β , IL-6, TNF- α , and monocyte chemoattractant protein (MCP)-1 have been shown in the *in vivo* and clinical samples of aneurysm (Juvonen et al., 1997; Middleton et al., 2007). Additionally, analysis of tissue samples from patients and animal models have also revealed the accumulation of macrophages and mast cells which are the prime source of matrix-degrading enzymes and pro-inflammatory cytokines which in turn are responsible for the VSMC cell death and aortic wall dilation (Q. Wang et al., 2015). Further, due to the excessive infiltration of lymphocytes and monocytes in the adventitia and the deposition of immunoglobulin G in the aortic wall, AAA is assumed to be an autoimmune disease (Ailawadi et al., 2003).

Receptor-interacting protein kinase 3 (RIP3), a protein responsible for the initiation of necroptosis, is known to be activated by pathogen-associated molecular patterns (PAMPs), inflammatory cytokines and oxidative stress, factors that are also associated with the development of AAA. Interestingly, an increased level of RIP3 was observed in aneurysm tissues suggesting RIP3 to play an important role in the induction of VSMC destruction in AAA. According to Wang and colleagues RIP3 triggers necroptosis of SMCs and secretion of inflammatory cytokines through the activation of NF- κ B (Q. Wang et al., 2015). All these reports suggests that anti-inflammatory drugs may be useful for the treatment for AAA. There have been a small number of clinical trials using anti-inflammatory drugs (all negative) and some studies using large clinical data sets looking

at patients on anti-inflammatory drugs for other reasons including full immunosuppression (Miyake and Morishita, 2009). None of these link to reduced AAA growth. Thus, the data clearly suggest that inhibiting inflammation alone does not benefit AAA patients.

Oxidative stress

Oxidative stress generated by the reactive oxygen and nitrogen species (ROS and RNS) is one of the key factors responsible for the development of AAA (Ailawadi et al., 2003; McCormick et al., 2007). Superoxide (O_2^-) levels are found to be 2.5-times higher in the AAA tissues than the adjacent, non-aneurysmal aortic tissue and 10-times higher than that of the normal aorta suggesting a strong influence in AAA (Ailawadi et al., 2003). Additionally, it is well-known to trigger inflammatory response in the tissues. Further indeed, ROS are involved in the activation of various MMPs and are responsible for apoptosis of VSMC (Ailawadi et al., 2003; McCormick et al., 2007; Satoh et al., 2009). Furthermore, there have been evidence showing the involvement of ROS and RNS in the development of AAA in both humans and animal models. Also, the NADPH oxidase is found to be the major source of oxidative stress in the pathogenesis of AAA (McCormick et al., 2007). Adding to this, a significant change is observed in the expression pattern of genes regulating oxidative stress in experimental AAA models thus showing the importance of the management of oxidative stress in AAA (Ailawadi et al., 2003). Moreover, modulation of oxidative stress could also suppress the formation of AAA in rodents (McCormick et al., 2007).

Hemodynamic stress

Hemodynamic conditions and indices vary markedly along the length of the aorta. For example, Reynolds number is higher at the aortic root and low and oscillatory shear conditions are found in the aortic bifurcation. Moreover, wall shear stress (WSS) markedly differs between thoracic and abdominal aorta. In suprarenal aortic parts, blood flow is always antegrade and thus results in continuous antegrade laminar WSS. However, in the infrarenal aorta, reverse flow is present in late systole and diastole and WSS values are lower. When distal arterial resistance reduced leading to increased flow (e.g., lower extremity exercise) WSS turns antegrade and laminar in the infrarenal parts too, mimicking proximal aortic segments. Such distinct regional differences in

hemodynamic parameters may partially account for regional differences in aneurysm risk between different parts of the aorta. Also, change in the haemodynamic stress dissemination is found to be a driving factor for the infiltration of immune cells and atherosclerosis which increases the wall damage and stress (Golledge, 2019). Several *in vivo* studies showed the segmental stiffening of the aorta to be an early mechanical event that increases the wall stress and induces AAA development in experimental models (Golledge, 2019; Raaz et al., 2015). Development of an aneurysm weakens the wall structure and increase the stress load which further accelerates the dilation and rupture risk. Supporting this, small molecules that reduce the wall stress namely, β -blockers are shown to exhibit a protective effect against aneurysm and rupture in animal models (Ailawadi et al., 2003). Mechanical stress beyond the strength of the wall tissue leads to the wall rupture. Therefore, monitoring the wall stress is an important factor in the management of this disease. For instance, ruptured or symptomatic AAA are found to have a higher peak wall stress compared to the diameter-matched asymptomatic AAA. Interestingly, aneurysm volume, rather than its diameter, is considered to be the best indicator of peak wall stress (Fillinger et al., 2002; Golledge, 2019; Raghavan et al., 2000). Thus, better understanding of aortic hemodynamic risk profiles can present an opportunity to optimise the activity patterns of AAA patients to minimise the risk of AAA development and growth (Dua and Dalman, 2010).

1.3.2 Risk Factors, Comorbidities and Aetiology

Though exact etiopathogenesis has not yet been clearly elucidated, several modifiable and non-modifiable risk factors and comorbidities have been identified. Apart from age and male sex, the most notable risk factors are smoking, race, family predisposition, hypertension, dyslipidaemia, obesity, and atherosclerosis which are all found to predispose an individual to vascular aneurysm. Additionally, it can also be directly caused by trauma, acute and chronic bacterial infections like brucellosis, salmonellosis and tuberculosis, inflammatory diseases like Behçet and Takayasu disease and connective tissue disorders like Marfan syndrome and Ehlers-Danlos syndrome type IV etc. and such aneurysms are called non-specific (Anagnostakos and Lal, 2021; Erentuğ et al., 2003; Sakalihasan et al., 2018). As mentioned before, in contrast to its adverse roles in other CVDs, diabetes mellitus imparts some protection against aneurysm.

Atherosclerosis

For over a half century, atherosclerosis was thought to be the prime driver of AAA since patients with aortic aneurysm often have atherosclerosis (Golledge and Norman, 2010; Kaschina et al., 2009). Indeed, many medical textbooks describe AAA as “atherosclerotic aneurysms.” Atherosclerotic disease and AAA often co-exist and clearly share common risk factors and some aspects of pathogenesis. However, the most striking discrepancy is the conflicting role of diabetes mellitus—adverse in atherosclerosis and beneficial in AAA. Due to the presence of inflammatory infiltration, arterial remodelling and connective tissue lysis in both diseases, it is possible that they share a similar pathogenic root and all AAA start as atherosclerosis. In fact, the most successful small animal-model of AAA is the AngII infusion in atherosclerosis-prone strains of mice (i.e., ApoE^{-/-} or LDLR^{-/-}) (Golledge and Norman, 2010).

More recently, the atherosclerotic aneurysm theory has been questioned. AAA and atherosclerosis could co-exist sharing common risk factors and similar pathological features but with independent developmental mechanisms (Golledge and Norman, 2010; Toghiani et al., 2017; Trollope and Golledge, 2011; Xanthouleas et al., 2009). Many studies have shown that a history of atherosclerotic disease increases the risk of developing an AAA about fourfold, and this risk is amplified by having multiple other CVDs. Though no strong association between atherosclerosis and AAA progression and rupture has been found, AAA patients with severe atherothrombotic diseases are prone to higher severity of AAA due to the risk of other cardiovascular events such as myocardial infarction and stroke. Conversely, AAA patients are found to be at greater risk for newly diagnosed or worsening peripheral arterial diseases and showed increased rates of hospitalization from atherothrombotic events. A patient with even a small AAA is about 1.5 times more likely to suffer a cardiovascular event than a patient without it. It is estimated that risk of cardiovascular death increases by about 4% to 6% for every mm increase in the infrarenal aortic diameter above 23 mm (Norman et al., 2011).

In general, MACE such as stroke and myocardial infarction are more common in AAA patients and the aneurysm patients have 3.0% per year chance of death from MACE which is similar to patients who have already experienced a major cardiac event (Bath et al., 2015; Kwan et al., 2023).

This risk persists even post-operatively in AAA patients (Diender et al., 2022; Hammo et al., 2022). Hence the European Society for Vascular Society recommends that antiplatelet therapy and lipid-lowering therapy (LLT) should be considered in all AAA patients (Wanhainen et al., 2019). In summary, while the presence of common pathogenic mechanism between atherosclerosis and AAA is not fully clear, there is strong evidence that both diseases co-exist and possibly exacerbate each other.

Age and Sex

Age is one of the most important non-modifiable risk factors for the development of an AAA. When compared to a 40–44-year-old man, a 75–79-year-old man has 200-fold increased risk of developing AAA (incidence rate of 0.83 versus 164 per 100,000). While the comparable incidence data is sparse among women, various epidemiological meta-analyses of population screening data clearly indicate that in women also, age increases the risk of AAA albeit to a lower level than same-age men. Overall prevalence rate of AAA in women is around 0.74–1.6% and it doubles in women over 70 indicating that women develop AAA later in life probably due to immunomodulating effects of female sex hormones including estrogen (Lo and Schermerhorn, 2016; Wu et al., 2009). However, AAA grows faster, and rupture occurs at smaller diameter in women compared to men. So, despite the lower incidence of AAA in women, one third of all AAA ruptures occur in women (Boczar and Coutinho, 2018; Lo and Schermerhorn, 2016).

Smoking

Along with the age, the current smoking status is a strongest modifiable risk factor (Aune et al., 2018). The duration and dose of nicotine consumption seems to be directly proportional to the risk of developing AAA. Each year of smoking increases the RR by 4%. Smoking increases AAA growth rate by 15–24% and is associated with an increased risk of rupture, regardless of diameter (HR 2.02). Epidemiological surveys and screenings have shown that more than 75% of ruptures occur in smokers. Thus, smoking not only contributes significantly to development of AAA but also to the growth rate, and rupture risk. The Odds Ratio for AAA development between smokers and non-smokers range between 2.3 and 13.72. Hence this is a much stronger association than

between smoking and coronary heart disease or chronic obstructive pulmonary disease (COPD). The effect of smoking on AAA development seems to be stronger in women. By activating tissue plasminogen activator and production of MMPs, smoking disrupts collagen. Laboratory studies showed that benzopyrene, a chemical found in cigarette smoke, can increase VSMC apoptosis and inflammatory infiltration leading to induction of MMP-2, 9 and 12 expressions resulting in formation of AAA in AngII-induced hypertension (Ji et al., 2014). Moreover, rat models demonstrated that nicotine weakens the vascular wall by promoting the degradation of elastin and collagen in the abdominal aorta resulting in aortic dilatation (Ji et al., 2014). Also, by increasing the risk of hypertension and COPD – which both are independent risk factors for AAA, smoking can also indirectly increase the risk of AAA. Current smokers have a hazard ratio (HR) of 5.55 compared to that of 1.91 for former smokers. Cessation of smoking reduces the risk of AAA formation, growth, and rupture rates. Statistical models have predicted that the risk of AAA halves in about 10 years post-quitting and it takes more than 25 years for it to approach the risk level of non-smokers (Kessler et al., 2022).

Ethnicity and Socioeconomic status

The prevalence of AAA is higher in White Caucasian men compared to other races including African American, Hispanic, and Asian men. While adjusting for all other known risk factors did not eliminate such difference in prevalence, both individual/genetic and environmental factors are thought to contribute towards it (Sandiford et al., 2012). Notably, though their risk of AAA development is lower, non-white ethnic groups have worse outcomes which may be due to disparities in the level of education, occupation, and socioeconomic status rather than the anatomical or genetic differences between the ethnicities (Khashram et al., 2017). As expected, health awareness and attendance at screening and follow-up scans are shown to be significantly associated diseases outcomes which partially explains the worse outcomes in non-white population (Jacomelli et al., 2017).

Genetic predisposition

Family history is considered as an important non-modifiable risk factor for AAA. People having first degree relatives with AAA are at a higher risk of developing an aneurysm due to the possible genetic predisposition (odds ratio, OR 1.96–3.8) (Kuivaniemi et al., 2015; Sakalihasan et al., 2018; van Vlijmen-van Keulen et al., 2002). Around 10% of the AAA are familial with the prevalence of 13% among the family members and 25% among siblings suggesting AAA to have a degree of heritability (Kuivaniemi et al., 2015; Sakalihasan et al., 2018).

Though TAA and AAA share various pathogenic components and risk factors, they have definitive differences in prevalence among various population, modes of inheritance, and genetic associations. About 20% of TAA patients have dominant pattern of inheritance which implies that TAA may result from a mutation in the single gene. However, AAA does not typically demonstrate such inheritance indicating it is a complex polygenic disease (Pinard et al., 2019).

About 6–20% of AAA patients have a positive family history. However, some reports suggest an autosomal dominant inheritance with incomplete penetrance. Particularly, mutations in the genes encoding the structural components of the connective tissues are seemed to increase the susceptibility to AAA (van Vlijmen-van Keulen et al., 2002). Two large scale genetic epidemiological analysis of twins have concluded that genetic factors may contribute as much as 77% to development of an AAA and the remaining 23% can be attributed to individual and environmental factors (Joergensen et al., 2016; Wahlgren et al., 2010).

Over the last twenty years, many groups attempted to identify genetic and epigenetic factors including microRNAs and long noncoding RNAs that contribute to AAA pathogenesis (Kessler et al., 2022). Through meta-analysis of 6 genome-wide association studies (GWAS) and validation of the results using more than 10,000 cases and controls a consortium of investigators identified 4 new AAA risk loci: 1q32.3 (SMYD2), 13q12.11 (LINC00540), 20q13.12 (near PCIF1/MMP9/ZNF335), and 21q22.2 (ERG). While these associations were consistent with known AAA pathogenic mechanisms such as lipoprotein metabolism, inflammation, and MMPs, they also identified potentially novel mechanisms relating to genes ERG and FGF9. In addition, through network analyses they have identified *ERG*, *IL6R*, and *LDLR* as modifiers of *MMP9* (Jones et al., 2017). Various genetic studies, including Genome-Wide Association Studies

(GWAS), exome sequencing, and single nucleotide polymorphism (SNP) genotyping assays showed significant association between SNPs in low-density lipoprotein receptor-related protein gene LRP1 and AAA (Au et al., 2018; Bown et al., 2011; Chasman and Lawler, 2017). Hence, the gene variations associated with AAA are multiple and may partly be shared with other cardiovascular disease.

A group from Stanford University employed whole genome sequencing, electronic health records and machine learning algorithms to understand the genomics of AAA. Using a novel analytical framework, they identified increased mutations in 60 key genes which are involved in diverse signalling processes including ECM synthesis and regulation and metabolic and developmental pathway and implicated 40 distinct functional modules for the development of AAA (Li et al., 2018). They also experimentally validated those results using human AAA tissues and murine models.

Arterial hypertension

Systemic hypertension, especially diastolic blood pressure significantly increases the risk of AAA development, and this relationship is more pronounced in women than men (Kobeissi et al., 2019). Though the evidence for adverse impact of hypertension on AAA growth is insufficient, there are reports supporting an increased risk of rupture 1.11-fold for every 10 mmHg increase in mean blood pressure (Sweeting et al., 2012).

Dyslipidaemia and obesity

While increased blood level of total cholesterol, triglycerides, and low-density lipoproteins (LDL) are clearly identified as major risk factors for atherosclerosis and CVDs, their role in AAA development and progression is less clear. Retrospective clinical epidemiological studies and meta-analysis have given conflicting results – from no effect to adverse effect of LDL (Weng et al., 2018). However, high density lipoprotein (HDL) was clearly shown to reduce the AAA risk (OR 0.7–0.83). AAA growth or rupture rates are not significantly impacted by lipids (Kessler et al., 2022). Interestingly, as mentioned above, it remains true that GWAS of AAA found that a lipid

regulating gene LRP1 was the most significant gene associated with AAA. Effect of obesity on AAA risk and growth has also been not fully understood. As obesity is often associated with other risk factors of CVD- age, hypertension, dyslipidaemia, and atherosclerosis, which are all also major risk factors for AAA, it is often believed that obesity is also a AAA risk factor. However, experimental, and epidemiological studies have produced conflicting results. Paradoxically there exists a ‘obesity paradox’, i.e., an association between cardiovascular risk factors and an improvement in clinical outcomes in AAA (Eckstein and Maegdefessel, 2020).

Inflammatory diseases

Immune cells are known to play crucial role in the development of AAA. Therefore, people with inflammatory diseases are at a high risk of developing AAA. For instance, aneurysm is an important clinical manifestation of Behcet’s disease which is characterized by the inflammation of the human vasculature (Davatchi et al., 2017; Hamzaoui and Hamzaoui, 2005). Similarly, Takayasu arteritis is also an inflammatory disease of the larger arteries that weakens the arterial wall leading to aneurysmal destruction (Russo and Katsicas, 2018). Giant cell arteritis (GCA) is another type of primary vasculitis that is most common in Western countries. Aneurysm is one of the important long-term complications associated with GCA (Camellino et al., 2020). A fibro-inflammatory process involving the abdominal aorta and the iliac arteries is categorized as chronic periaortitis. Initially considered to be a localized inflammatory disease, periaortitis is later proved to affect other arteries as well. As a result, the arterial walls dilate and become weak causing aneurysms (Vaglio et al., 2011).

Connective tissue disorders

Though TAA is more common in patients with connective tissue disorders, rarely AAA also occurs. Marfan syndrome is an inherited autosomal dominant connective tissue disease caused primarily by mutations of ECM protein fibrillin-1 gene responsible for the elasticity and strength of the connective tissues. If affected, the aortic wall loses its integrity resulting in the development of aneurysmal dilation which becomes life-threatening due to the high possibility of rupture (Coelho and Almeida, 2020; Judge and Dietz, 2005) (Coelho and Almeida, 2020, Judge and Dietz,

2005). Loeys–Dietz syndrome (LDS) is another familial autosomal dominant connective tissue disorder diagnosed by mutations in any of the genes namely, TGFBR1, TGFBR2, SMAD3 and TGFBR2 affecting the TGF- β signalling. LDS is also shown to increase the risk of an individual to develop aortic aneurysm (MacCarrick et al., 2014). Ehlers–Danlos syndromes (EDS) are a group of hereditary connective tissue disorders with common manifestations like joint hypermobility, soft and hyperextensible skin, abnormal wound healing, and easy bruising etc. Certain EDS like Kyphoscoliotic (disorder of collagen folding and crosslinking) and Spondylo-dysplastic (disorder of glycosaminoglycan biosynthesis) diseases are found to be strongly associated with the development of aneurysm (Malfait et al., 2020).

The paradox of Diabetes Mellitus

Diabetes mellitus is an undisputed major risk factor for atherosclerosis and CVD. However, extensive evidence generated by multiple large clinical epidemiological studies show that it offers protection against development of AAA. Moreover, diabetes is the only known risk factor to slow AAA annual growth rate - by about 25% (Raffort et al., 2018). In addition, diabetes also reduces rupture risk significantly. The pathological mechanisms behind such a phenomenon have been investigated using various laboratory models and human tissues. Hyperglycaemia can increase synthesis and decrease the degradation of ECM. Also, high glucose can increase advanced glycation end products which also can reduce the degradation of ECM (Astrand et al., 2007; Portik-Dobos et al., 2002). The resultant thickening of intimal and medial layers of the aortic wall and hyperglycaemia-mediated inflammation -which make the vessel more prone for atherosclerosis, are proposed as possible explanations for the protective effect seen in diabetic patients (Ning et al., 2020; Raffort et al., 2018). Hyperglycaemia has also been shown to reduce the AAA growth. Various clinical and laboratory studies showed that a common group of antidiabetic medications-thiazolidinediones, which are peroxisome proliferator-activated receptor agonists, inhibit the growth of aortic aneurysms (Pirianov et al., 2012). Metformin, a commonly used anti-diabetic drug has been shown to reduce clinically important AAA events such as requirement of surgery, rupture, and mortality in patients with diabetes (Golledge et al., 2019, 2017; Unosson et al., 2021).

1.3.3 VSMC phenotype switching in AAA

VSMC de-differentiation and remodelling has been introduced in section 1.1.5. Traditionally VSMC loss and medial degeneration were considered as the key VSMC observations in AAA. In end-stage AAA, the majority of the VSMC are lost (called medial degeneration) and the remaining cells were larger than control cells and presented apoptosis/senescence cues such as reduced proliferation, increased DNA damage and shortened telomeres (Cafueri et al., 2012; Liao et al., 2000; Ramadan et al., 2018; Riches et al., 2018, 2013). The infiltrating immune cells were thought to be responsible for the destruction of VSMC and the ECM. Hence, the VSMC have been considered “innocent bystanders” in AAA pathology.

VSMC phenotype switching has been consistently observed – as reduction in the expression of classical SMC marker genes and increase in non-SMC gene expression, by many investigators in AAA tissues (Ailawadi et al., 2009; Goodall et al., 2002; Lu et al., 2021; Petsophonsakul et al., 2019). Emerging evidence from studies on human AA samples and animal models also suggest that such VSMC phenotype switching could play an active role in early-stage AAA pathogenesis (Ailawadi et al., 2009; Petsophonsakul et al., 2019; Si et al., 2022). The synthetic type VSMC are highly proliferative, secrete more ECM degrading enzymes (MMPs), induce oxidative stress, produce extracellular vesicles that enhance local inflammation and promote vascular calcification and thus lead to vascular dysfunction and AAA initiation (Airhart et al., 2014; Jia et al., 2017; Lu et al., 2021; Schurgers et al., 2018). Further direct evidence comes from *in vivo* studies using animal models of AAA in which genetic modifications that prevent VSMC phenotype switching were found to protect the animals from AAA. Using 2 different animal models of AAA, Salmon and colleagues showed that deletion of *KLF4* gene can protect against AAA formation by significantly reducing elastin degradation and cytokine production in addition to increasing SMC marker genes expression (Salmon et al., 2013). Similarly, a recent elegant study showed that miR-23b protects against AAA in animal model by inhibiting VSMC de-differentiation through suppressing transcription factor forkhead box O4 (FoxO4) (Si et al., 2022). In summary, simple apoptosis/destruction of VSMC alone may not be enough to initiate AAA formation and VSMC are not innocent bystanders during early stages of AAA pathogenesis and their phenotype switching can be targeted for preventing AAA formation. However, the exact role of VSMC

phenotype switching in AAA development warrant further investigations as it will provide clues for new therapeutic target identification.

1.4 Management of AAA

The treatment of AAA has come a long way from the early days of open aneurysmorrhaphy and cellophane wrapping as performed on Albert Einstein. However, with the absence of any significant pharmacological therapies, surgical repair remains the only viable option. The current clinical management strategy is to detect them when they are asymptomatic (through surveillance programs or incidentally), continue to survey them until such a time as they become large enough to pose a significant risk of rupture. At this point patients are considered for planned repair of the aneurysm to remove the risk of rupture. This is challenging as the operation itself carries a risk of death and reduced quality of life. Notably, AAA is now less common than 10 or 20 years ago and although the mortality rate from rupture is high, in most aneurysms the chance of rupture is actually very low and therefore the risk of death from AAA for most individuals is actually very small. Hence it is also essential to understand the public health and economic context of such longer-term surveillance. Apart from the developed world countries in which AAA screening program is widely implemented, AAA is still diagnosed as an incidental finding when patients are examined/investigated for other unrelated symptoms and disorders (Shaw et al., 2023).

1.4.1 Clinical diagnosis

Until recently, around 30% of AAA used to be diagnosed during routine physical examinations as a pulsatile expansible abdominal mass at or above the umbilicus (Aggarwal et al., 2011) (Aggarwal et al. 2011). Large aneurysms in thin people can be suspected by incidental physical examination. However, this is more difficult in obese individuals (Aggarwal et al., 2011; Chervu et al., 1995). Plain X-ray scan also detect aneurysms as most of the affected aortic walls present curvilinear calcifications (Aggarwal et al., 2011; LaRoy et al., 1989). Ultrasound Scan (USS) is an imaging technique that uses high-frequency sound (ultrasound) waves to generate the pictures of internal organs and tissues. It is the most commonly used imaging modality not only for the screening and diagnosis but also for the follow-up of AAA and after repair with endovascular aneurysm repair (EVAR) (Aggarwal et al., 2011; LaRoy et al., 1989; Shaw et al., 2023; U.S. Preventive Services Task Force, 2005). However, it is not suitable for the aneurysms located above renal arteries since

the presence of overlying lungs and viscera may interfere with accurate diagnosis and measurement. Along with an arterial contrast, a computed tomography (CTA – CT angiography) is more appropriate to find the exact location and size of the aneurysm and the involvement of other vessels (Gozzo et al., 2022; Moxon et al., 2010; Shaw et al., 2023). It also gives better imaging of the suprarenal aneurysms and provides in depth anatomical details of the involvement of mesenteric and iliac arteries (Aggarwal et al., 2011). It can delineate the anatomy of the aneurysm so the surgeon can determine if it is suitable for endovascular repair. Plain CT is also an excellent technique for pre- and post- repair evaluation of aneurysms (Andersson et al., 2022; Smith and Quencer, 2020). Though rarely used, magnetic resonance angiography (MRA) is an excellent procedure, well suited for imaging the aorta and more accurate in the evaluation of aneurysms. Besides accuracy, the main advantages of MRA include non-invasiveness, multiplanar display and the natural contrast between flowing blood and wall structures (Aggarwal et al., 2011; Calero and Illig, 2016; LaRoy et al., 1989; Niknejad and D’Souza, 2008).

1.4.2 Screening

Early diagnosis of AAA is essential to prevent mortality occurring due to the rupture. As most AAA are asymptomatic, relying on incidental detection through clinical examination or imaging is suboptimal. Screening of a high-risk population can better identify such asymptomatic aneurysms and allow surveillance and targeted intervention to prevent rupture.

During the year 2000 ruptured AAA caused about 6800 deaths (2.1% of all deaths) in England and Wales. As majority of those were in males over the age of 65 years, a national AAA screening for men in their 65th year was proposed. Evidence for the proposal was obtained through The Multicentre Aneurysm Screening Study (MASS) – a randomised controlled trial. This trial concluded that men who attended AAA screening had a 53% reduction in aneurysm-related deaths compared to those in the control group (Ashton et al., 2002)a. Based on the findings of the MASS trial, the NHS AAA Screening Programme was introduced in England in 2009 and was extended to include all four nations by 2014. Current definition of an AAA as in the MASS trial, is a maximum aortic diameter of ≥ 3 cm as measured by ultrasound scan (USS) using the inner-to-inner wall method of measurement. If an AAA is detected at initial screening, regular surveillance USS

is advised to monitor the growth of aneurysm. The current surveillance intervals, in the UK, reflect the intervals in the MASS trial. Thus, AAA with a diameter of 3.0 cm–4.4 cm are scanned every 12 months, and AAA with a diameter of 4.5 cm–5.4 cm are scanned every 3 months. Every year around 300,000 men above 65 yrs of age are being screened for AAA in the UK. As per the NICE guidelines, aortic diameter <3.0 cm is considered as normal, diameter between 3.0 and 5.4 cm are considered as small aneurysms and recommended for USS surveillance and diameter ≥ 5.5 cm are regarded as large aneurysms and recommended for surgical intervention (Davis et al., 2013; Stather et al., 2013).

Several other clinical studies also proved that screening for possible AAA significantly reduced the AAA-related mortality in men above 65 years of age (Meecham et al., 2021; Stather et al., 2013). Acknowledging this, several countries across the globe including the USA, Sweden and Australia introduced nationwide AAA screening programmes (Stather et al., 2013). A one-time ultrasound-based screening for AAA is recommended in the USA for men aged between 65-75 years old who have ever smoked by the U.S. Preventive Services Task Force (USPSTF). Following this, the U.S. congress also added a free Medicare benefit for one-time AAA screening in men above 65yrs having smoking history and for people with a family history of AAA (Aggarwal et al., 2011; U.S. Preventive Services Task Force, 2005). An overview of national screening programs and trials are given in the Table 1.

Country	Start date trial	End date trial	National screening implemented	Population screened in trial	Population covered by screening programme	Total screened thus far	Age group invited	Gender
Western Australia	1996	1998	No	12 203	N/A	12 203	65–79 years	Male
Denmark (2 trials)	1994–2008	1995–2010	No	4843–25 000	N/A	29 843	65–74 years	Male
England (2 trials)	1997–1988	1999–1994	Yes started 2009	27 147–5394	51 000 000	52 000	65th year (65–74 years in trial)	Male
Finland*	N/A	N/A	No	N/A	N/A	N/A	N/A	N/A
Norway	2011	2029	Oslo only	N/A	2000/year	1116	65 years	Male
Scotland	N/A	N/A	Yes, started July 2012	N/A	5 200 000	Unknown	65th year	Male
Sweden	N/A	N/A	Yes, started 2006	N/A	8 100 000	Attendance rate 85%	65 years (65–74 years in trial)	Male
Northern Ireland	N/A	N/A	Yes, started July 2012	N/A	1 800 000	Unknown	65th year	Male
USA (Society for Vascular Surgery guidelines)	2007	2008	Recommends if ever smoked >100 cigarettes	2918	311 000 000	Unknown	60–85 years Male 60–85 years Female with cardiovascular risk Over 50 if family history	Male and Female

Table 1.1 Aneurysm screening programmes and trials worldwide (adapted from Stather et al., 2013)

1.4.3 Clinical management

Currently, there are no medical therapies available for the prevention or treatment of AAA except prophylactic surgical repair to remove the risk of rupture (Lindeman and Matsumura, 2019). As the surgery itself carries significant risk, the management requires careful assessment and weighing of the surgical risk versus aneurysm rupture risk. As long as the risk of elective surgical repair is above the risk of rupture, conservative management which includes watchful waiting while following the current guidelines on AAA follow-up and cardiovascular disease control is prescribed. Many large trials established the safety of approach over early elective repair. In fact, early elective repair offers no advantage to the patients with small AAA. Hence careful surveillance along with medical care of CVDs and risk limitation by lifestyle modification is considered safe and cost-effective. Data from RESCAN consortium studies showed that the surveillance intervals be safely increased to several years in small AAA (RESCAN Collaborators et al., 2013). As per their analysis of 15,000 patients including data from Leeds, the typical time taken by a 3 cm aneurysm to reach the intervention threshold of 5.5 cm is about 6-13 years (RESCAN Collaborators et al., 2013). However, this has not yet led to a change in clinical practice guidelines.

Lifestyle modification and Pharmacological Therapy

Exercise is generally safe and well tolerated by patients with small AAA (Nakayama et al., 2018). Earlier thoughts that exercise or physical activity should be avoided due to risk of rupture is no longer considered true and in fact pre-operative moderate to high intensity regular exercise is suggested to improve cardiopulmonary fitness and quicken the post-operative recovery in AAA patients (Tew et al., 2017; Weston et al., 2017). However, the beneficial effects of regular exercise on AAA development and growth are not clear. There is evidence supporting and against the positive impact of exercise on AAA (Aune et al., 2020). There is better evidence to suggest that regular exercise may reduce the risk of AAA rupture, probably by indirectly by reducing blood pressure and improving general cardiovascular health (Aune et al., 2020; Kato et al., 2019). A deficiency of vitamin D has been associated with AAA as shown by a recent mouse study (Nsengiyumva et al., 2020). In this study, AngII infusion-induced AAA in ApoE^{-/-} mice showed

significantly promoted the development of large rupture-prone aneurysms. However, further clinical studies and trials are needed to elucidate the therapeutic potential of vitamin D in AAA patients. While similar to other CVDs, a well-balanced diet may reduce the risk of AAA, further focused studies are needed to identify and understand the beneficial effects of diet quality in AAA patients. After the confirmation of the presence of an aneurysm, medical management is imperative in order to suppress the further growth and rupture. However, no such drugs are currently available for clinical use (therapies in development are discussed in next section).

In summary, probably only 2 factors impact AAA growth – ongoing smoking makes the aneurysm grow more quickly and diabetes makes the aneurysm grow more slowly. Otherwise, so far there is no definitive evidence that specific changes to blood pressure or any medications do in fact slow AAA growth in patients despite some positive findings in studies.

Surgical management

Size of the aneurysm is the only crucial anatomical criterion used for clinical decision making. AAA rupture risk increases with AAA size, but the relationship is not perfect. Many trials including The UK Small Aneurysm Trial (UKSAT) and the American Aneurysm Detection and Management (ADAM) have established the threshold diameter for intervention. The minimum cut-off size for a surgical intervention is 5.5 cm in men and 5 cm in women (Brown et al., 2008; Filardo et al., 2015; Moll et al., 2011; Powell et al., 2007). However, co-morbidities, general health status and surgical fitness of the patients have to be taken into account to make a decision on intervention. Furthermore, the presence of symptoms (e.g. back pain), rapid growth of >1 cm in imaging necessitate urgent repair and obviously if an aneurysm is ruptured, emergency repair is offered. Thus, the clinical management of AAA involves: 1. Detection, 2. Surveillance 3. Repair upon reaching intervention threshold 4. Post Repair monitoring.

Thus far, only two methods of surgery are known for treating end-stage aortic aneurysm diseases namely, open AAA repair (OAR) and endovascular aneurysm repair (EVAR). In OAR, through laparotomy the aneurysmal sac is opened followed by the removal of thrombus and repair using a Dacron graft. Considering most AAA patients are of elderly age and high-risk category, the highly

invasive nature of OAR carries significantly high morbidity/mortality rate (Kieffer et al., 2012; Malina, 2015). Hence, a minimally invasive surgical option known as EVAR was developed in the early 1990s in which a stent-graft is delivered percutaneously through the ilio-femoral arteries. OAR involves infrarenal aorta clamp placement for the repair of infrarenal AAA. Juxtarenal AAA can be repaired through inter-renal or suprarenal clamping, with reconstruction of the infrarenal aorta (Calero and Illig, 2016; Chiesa et al., 2013). Pararenal or paravisceral aneurysms require branch artery re-implantation or a complex fenestrated EVAR (Calero and Illig, 2016; Wang et al., 2018).

Compared to OAR, EVAR carries significantly shorter hospital stay and low perioperative mortality and morbidity (Tzani et al., 2018). Many clinical trials including the Dutch OVER trials, which compared OAR with elective EVAR clearly concluded that there is increased early survival with EVAR (Lederle, 2009). Moreover, in patients who have extreme obesity, major cardiac or pulmonary comorbidities, and limited life expectancy OAR is contraindicated and hence EVAR may be the only option. Similarly, the most common contraindications for EVAR are anatomic ones such as short aortic neck length, inadequate distal seal zone, and inadequate access vessels (Soo Hoo et al., 2022). However, in longer term, this survival advantage of EVAR over OAR was lost and some trials have even shown that EVAR may have worse outcome (The Lancet, 2020).

The UK-based EVAR1 trial which evaluated 1252 patients' records from 37 centres between 1999 and 2004 concluded that after about 8 years post-surgery EVAR has worse survival outcome primarily due to secondary aneurysm sac rupture (Patel et al., 2016). The Dutch trial (DREAM) which is a randomised one, concluded that after 12 years of follow-up, there was no survival difference between OAR and EVAR patients. However, they observed that increasing number of reinterventions were needed for EVAR group (van Schaik et al., 2017). EVAR2, another randomized trial done in the UK involving patients who were not eligible for OAR and hence underwent EVAR, found that EVAR was associated with significantly lower rate of aneurysm-related mortality-but not all cause mortality- compared to no repair (EVAR2, 2010, p. 2).

In the UK, the 2018 National Institute for Health and Care Excellence (NICE) guidelines (NG156) recommended clinicians not to offer EVAR to patients suitable for OAR as EVAR was shown not

to be cost effective and associated with a risk of late rupture. Moreover, it was also suggested that patients not suitable for OAR due to co-morbidities or the other risk factors should also not be offered EVAR. However, the 2020 NG156 guidelines changed those and recommended EVAR as an option for patients with a hostile abdomen or co-morbidities that would make OAR challenging and/or unsuitable. It also suggested that patients should be given a choice on the type of surgery after careful discussion on each type with them (Hinchliffe and Earnshaw, 2020).

Recent studies have shown that starting from last decade of last century or at least from the start of this century there is steady decline in the incidence of AAA and the age at which clinically-relevant aneurysms present has increased by 5–10 years (Anjum and Powell, 2012). Similarly, the rate of rupture and the resultant mortality have also shown a declining trend (Anjum et al., 2012). Another group found that though AAA mortality, rupture, and emergency repair have declined in England and Wales, non-ruptured AAA admission has remained steady, with an increasing rate in older population compensating a decreasing rate in younger population. They argued that there may be a shift in AAA presentation to the older population and suggested that present screening strategies need reassessment to include consideration for increasing the age at which to screen men for AAA (Choke et al., 2012). Recent data from National Abdominal Aortic Aneurysm Screening Programme (NAAASP) found that rupture risk of a 5.0-5.5 cm AAA is 0.4% per year, while the mortality from surgery is 2-3% (Public Health England, 2016).

The current management guideline used in most countries, as discussed above are primarily based on the trials conducted more than a decade ago. The fact that the AAA repair is a prophylactic operation to remove the risk of rupture and it carries significant morbidity and quality of life decline and mortality is being increasingly recognised. This has led to a shift in clinical decision making and there is accumulating evidence that conservative management and monitoring was being more frequently used rather than early intervention based purely on the aneurysm size (NICE, 2020; Ulug et al., 2020). Hence the need for further research into pathophysiology of AAA along with the clinical studies looking at management options are still needed to effectively manage the disease in the ageing population.

1.5 Small animal models and proposed management strategies for AAA

Over the last three decades animal models of AAA have been developed not only to study pathophysiology of aneurysms but also for testing various therapeutic targets and modalities (Busch et al., 2021). In this section I have discussed recent literature on various small animal models of AAA and how they were utilised to identify novel drug targets and treatment strategies for AAA.

1.5.1 Small animal models of AAA

Rats, mice, and rabbits are the commonly used small animals for aneurysm models. Especially the murine models offer the advantages such as low cost, ease of handling and breeding, short life cycle and also as mice have a well-studied genome which make them suitable for genetic manipulation. A key limitation in studies of human AAA is that the samples are taken from the advanced stage of the disease and hence they can't be used for studies focusing on disease initiation and early stages pathologies. Moreover, most of those specimens are complicated by calcification, thrombosis, haemorrhage, and necrotic debris and thus severely impede observation and analysis of the histopathological changes (Gertz et al., 2013). Hence to gain insight into the early stage of disease including AAA development and progression, small animal models are used. While organoids and organs-on-a-chip technology that mimics complex human tissues and diseases are available or being developed for a variety of organs and conditions, no such alternative is available for AAA yet. Hence animal models remain the gold standard for translational research in the field. Several of these models reproduce aortic inflammation, ECM destruction and cause dilatation as observed in human disease albeit at different pace and in various proportions of severity (Lysgaard Poulsen et al., 2016). A large number of inducible murine AAA models have been established out of which, three - AngII infusion, elastase perfusion/periadventitial application or CaCl₂ painting, have been widely used. Evidently, each model has its own unique advantages and limitations. Apart from those inducible types, few spontaneous models that have spontaneous or induced genetic defects leading to AAA development have also been developed. Blotchy mice line that has

a spontaneous mutation at the *Atp7a* locus resulting in decreased copper-dependent lysyl oxidase activity and defective collagen and elastin cross-linking was the first described spontaneous aortic aneurysm (primarily ascending aorta) genetic model (Andrews et al., 1975). Other transgenic models are ApoE deficient mice (ApoE^{-/-}) along with disruption of one of the following genes - LDL receptor1 (ApoE^{-/-}:LDLR^{-/-}), tissue inhibitor of metalloproteinase-1 knockout (ApoE^{-/-}:TIMP-1^{-/-}) and endothelial nitric oxide synthase (ApoE^{-/-}:eNOS^{-/-}) (Kuhlencordt et al., 2001; Silence et al., 2002; Tangirala et al., 1995). Recently, SMC-specific ablation of TGF- β signalling in ApoE^{-/-} mice on a hypercholesterolemic diet leading to AAA that exhibit all the features of human disease was described (Chen et al., 2020). There has also been a report on a xenograft in which transplantation of the infrarenal aorta from one species to another, for example guinea pig to rat, induced aneurysms (Lysgaard Poulsen et al., 2016). Though these models may be closer to real human disease, their low penetrance of the phenotype and heterogeneity in the distribution of the lesion along with the long duration taken to develop the diseases make them less popular.

CaCl₂ model

The earliest of aneurysm models – an arterial aneurysm induced in the rabbit common carotid artery by periarterial CaCl₂ application was actually developed by mistake (Gertz et al., 1988). Peri-aortic application of CaCl₂ (0.25–0.5 M for 15 min) to the infrarenal rodent aorta induces AAA. Few investigators modified the protocol by applying phosphate after CaCl₂ application to enhance calcification and thus increase the chances of AAA formation. The follow up period was 6–10 weeks with an incidence of 50–100% (Yamanouchi et al., 2012). The mechanism through which CaCl₂ induces aneurysm formation is as follows: externally applied calcium diffuses into the vascular wall and bound to the elastic lamina in intima and in between the layers of medial VSMC. This calcium–elastic tissue complex attracts multiple types of inflammatory cells including monocytes and macrophages that disrupt the elastic lamella and result in progressive vessel wall dilatation. The same method – 0.25ml/L CaCl₂ application on proximal infrarenal aorta in rabbit for 15 minutes, was used to induce AAA formation (Freestone et al., 1997). This AAA showed disruption of the media elastic framework and inflammatory response as seen in human AAA. Similar to human diseases, this model shows leukocyte infiltration, medial degeneration and

calcification. However, progressive dilatation and thrombus formation were not observed in this model (Sénémaud et al., 2017).

Elastase models

Anidjar et al first developed an aneurysm model by intraluminal elastase perfusion in rats (Anidjar et al., 1992). Intraluminal elastase application is done by perfusing the surgically isolated infrarenal aorta with porcine pancreatic elastase (PPE) for 5-10 minutes through a catheter inserted via the femoral artery. PPE degrades medial elastin fibres by increased production of several elastolytic matrix metalloproteinases (MMPs) locally and induces macrophage accumulation as seen in human disease and the AAA development can be observed in about 2–4 weeks (Lysgaard Poulsen et al., 2016). This led to the supported the idea that elastolytic activity within the medial layer has a significant role in aneurysm formation and thus provided a useful model for further investigations that is also highly reproducible. Hence, many documented risk factors of AAA including gender, hypertension and smoking were investigated using this model (Tsui, 2010). Various issues including technical complexity such as isolating aorta from adjacent inferior vena cava and small lumbar branch vessels, size of the aneurysms induced, complications such as lower limb ischemia induced by long periods of elastase perfusion and rate of aneurysm formation have been reported (Carsten et al., 2001). Hence various investigators tried altering the concentration of elastase, lowering perfusion duration and also combining it with CaCl₂ model. Alternatively, a less-invasive elastase model was developed by Bhamidipati et al by applying elastase on the periadventitial surface of abdominal aorta which induced more than 80% dilatation in 14 days (Bhamidipati et al., 2012). In these aneurysms they observed degradation of the elastic lamina, increased presence of activated macrophages, reduced smooth muscle protein expression, and increased active MMP activity. Additionally, this model showed increased cell proliferation as seen in small human AAA, which supports the role of vascular remodelling in AAA formation. This model may be better suited to study the contribution of adventitia in aneurysm formation as elastase-induced degradation of the abluminal surface induces inflammatory homing which initiates the disease.

AngII model

The most commonly used murine model of AAA - AngII model is established by infusing AngII for a period of 28 days through an implanted osmotic pump. Though wildtype animals can be used, to accelerate the development of diseases usually apolipoprotein E deficient (ApoE^{-/-}) animals are preferred. Apart from inducing hypertension, AngII can also independently stimulate vascular wall inflammation, atherosclerosis, medial hypertrophy, accumulation of macrophages, and thrombosis that results in enlargement of the suprarenal part of the aorta (Daugherty et al., 2011; Gertz et al., 2013). As AngII infusion results in development of robust aneurysms in the suprarenal aorta within 2-3 weeks in up to 85% of animals, the number of animals required to test any treatment option is significantly lesser than other models (Gertz et al., 2013). Various factors including age, blood pressure, hyperlipidaemia and AngII infusion rate influence the development of AAA in these models (Hisashi Sawada et al., 2022). Importantly male gender had higher propensity for AAA development which was clearly demonstrated by gonadectomy studies. While ovariectomy had minimal nor no effect, orchidectomy significantly reduced AAA for formation (Zhang et al., 2012). Also, AngII models induced in any genetic background animals consistently produced suprarenal AAA while infrarenal AAA is more common in humans. Despite these issues, AngII model has multiple advantages including less-invasive methodology, higher success rate, shorter duration and observation of AAA and dissections at multiple sites throughout the aorta that helps in AAA progression studies, it is the most common model used in the field (Busch et al., 2021).

Though multiple AAA models are available, for translational research none of them fully emulate human disease. Especially for translational research, 2-4 weeks of drug treatments in mice is extrapolated to 3–6 years in humans, based on lifespan differences. However, such difference was not observed in inflammatory cell and VSMC turnover between mice and humans. So, though the current AAA models have provided us valuable insights into the AAA development and progression, for translational research further refinement of the existing models or development of new models is necessary.

1.5.2 Proposed novel drug therapies

Though a vast number of drug molecules have been investigated for AAA treatment, none of them showed consistent and significant positive impact on prevention, growth or rupture in AAA patients (Yoshimura et al., 2018).

Murine studies and many retrospective studies have shown that statins can reduce AAA progression. Subsequent larger clinical studies found statins lack any beneficial effects (Lederle et al., 2015). However, recent meta-analysis studies have shown that the statins significantly reduce mortality associated with AAA (Risum et al., 2021; Salata et al., 2019; Xiong et al., 2022). Hence, due to their overall beneficial effects in cardiovascular diseases and potentially large number of mechanisms (such as combating oxidative stress, reducing inflammation, inhibiting MMP and inducing ECM synthesis) through which they may reduce AAA progression and thus mortality from rupture, they are still advocated in many centres (Hosseini et al., 2022). Also, other cardioprotective drugs such as angiotensin-converting enzyme (ACE) inhibitors and beta-adrenergic blockers showed promising results in murine studies (Brophy et al., 1988; Park et al., 2019). However meta-analysis of multiple clinical trials concluded that neither of them have any benefits in AAA patients (Siordia, 2021; Su et al., 2022).

Early studies using murine models of AAA found that doxycycline can effectively reduce AAA progression (Boyle et al., 1998; Sho et al., 2004). As *Chlamydia pneumoniae* has been implicated in AAA pathogenesis, few studies tried the antibiotics including doxycycline, roxithromycin and azithromycin (Lindholt et al., 2001; Su et al., 2022). However, there was no significant attenuation of aneurysm growth in the Pharmaceutical Aneurysm Stabilisation Trial (PHAST) in 2013, which showed there was a significant increase in growth in patients administered doxycycline (Meijer et al., 2013). But another study showed that doxycycline may have a preventative effect rather than inhibitory effect, i.e., needs administering before AAA development (Xie et al., 2012). Nonsteroidal anti-inflammatory drugs, mast cell inhibitors, calcium channel blockers and diuretics have also been investigated clinically after obtaining promising results in experimental studies. However, none of them showed any inhibitory effect on AAA growth clinically (Lindeman and Matsumura, 2019; Su et al., 2022).

Several studies are ongoing to test whether anti-platelet drugs could be used in AAA (Elbadawi et al., 2020). Another group of drugs which raised huge interest is antidiabetic medications. As previously mentioned, diabetes mellitus protects against AAA progression. Such effect may be because of treatment with oral hypoglycaemic drugs those patients are on rather than the disease diabetes itself. Out of many antidiabetic drugs investigated, as mentioned before only metformin was consistently associated with reduced AAA growth (Golledge et al., 2019, 2017; Kessler et al., 2022; Kokje et al., 2015; Unosson et al., 2021). Further ongoing trials on metformin effect may soon provide clear conclusions. Recent studies on AAA animal models showed sodium-glucose co-transporter 2 (SGLT2) inhibitors have beneficial effects (Jin et al., 2023). But further clinical studies and trials are needed to evaluate their potential for clinical application.

Thus, none of the drugs that targeted elastin degradation, inflammation, general cardiovascular risks, and ion channels have been translated to clinic. This thesis work will explore the viability of a novel target - VSMC remodelling, for AAA treatment.

1.5.3 Advances in rupture prediction

Predicting the rupture holds a great significance in the management of AAA. Interestingly, factors like the AAA expansion rate, wall tension/stiffness, peak wall stress, intraluminal thrombus thickness, diastolic blood pressure and expression and activity of MMP-9 were investigated as clinical markers to predict the AAA rupture (Hall et al., 2000; Lederle et al., 2002; Schewe et al., 1994; Stenbaek et al., 2000; Vallabhaneni et al., 2004; Venkatasubramaniam et al., 2004). Using 18F-fluoro-deoxy-glucose (18F-FDG) positron emission tomography (PET)-CT recent studies showed that the uptake of 18F-FDG can be positively correlated with AAA rupture. However, this was not consistent across the literature (Timur et al., 2015). Despite the identification of these predictive factors, until now no clear methodologies have been developed to precisely predict the rupture as none of them has translated to clinical practice. Recently, the SOFIA trial used Fluorine-18-sodium fluoride (^{18}F -NaF) and linked tracer activity to clinical outcome. The authors suggested that ^{18}F -NaF PET-CT can be utilised for identification of disease activity in AAA and is an additive predictor of aneurysm growth and future clinical events (Forsythe et al., 2018).

1.6 Hypothesis, aims and objectives

This thesis is focused on the idea that VSMC are not innocent bystanders in AAA pathology and aortic disease, rather they are active contributors to the pathological process and might be a potential therapeutic target. In this work I will use elegant VSMC lineage tracing techniques to track VSMC fate in a murine model of AAA before driving VSMC de-differentiation and remodelling through transgenic manipulation of the PDGF signalling pathway in aortic development and AAA and studying the consequences.

1.6.1 Hypothesis and Aims

Novel Hypothesis: VSMC remodelling, and proliferation are functionally important in the development and/or progression of aortic dilation / AAA.

The primary aims of the project are, to track the behaviour of VSMC in murine AAA models, and to gain mechanistic insights into PDGFR β signalling and evaluate its' potential as therapeutic target.

1.6.2 Objectives

Objective 1: VSMC lineage tracing in AAA

- (A) To validate the SM-MHCCreER^{T2} mice and generate a VSMC specific, tamoxifen inducible mTmG lineage tracing mouse line
- (B) To use the SM-MHCCreER^{T2}mTmG line to study VSMC phenotypic modulation and remodeling in PPE induced AAA

Objective 2: PDGF signalling and downstream mechanisms in AAA

- (A) To generate and phenotype a VSMC specific, constitutive and tamoxifen inducible constitutively active PDGFR β mutant mouse lines (SM22Cre PDGFR β^{D849V} and SMMHCCreER^{T2}PDGFR β^{D849V})
- (B) To study the effects of the PDGFR β^{D849V} transgene in PPE induced AAA
- (C) Evaluate Orai1/SOCE in VSMC of PDGFR β^{D849V} transgenic line.
- (D) To evaluate the effects of pharmacological inhibition of SOCE/Orai1 on AAA in the mouse model of enhanced PDGFR β signalling
- (E) To study the effects of genetic deletion (homo and heterozygous) of Orai1 on AAA in the mouse model of enhanced PDGF β signalling

Chapter 2 : Materials and Methods

2.1 Animal husbandry

All animal experimentation and procedures were performed with the guidance outlined by the UK Home Office Animals (Scientific Procedures) Act 1986 under the Home Office project licence (PPL) P606320FB (holder: DJ Beech, deputy: MA Bailey, 2016 - 2021) and personal licence (PIL) I9785E723 (P Kandavelu, Jan 2017 onwards). Ethical approval was obtained from Animal Welfare and Ethical Review Board at the University of Leeds. Mice were maintained in individually ventilated cages (GM500, Techniplast) at 21°C and 50-70% humidity in a 12-hour light/dark cycle. Mice were fed rat and mouse no.1 maintenance diet (RM1, Special Diet Services) *ad lib* and water via Hydropac pouches. All experiments were performed on males (due to the presence of Cre allele in Y chromosome as explained in section 2.2.2) and age matched littermates were used as controls. In most experiments observer was blinded towards genotype. All animals were observed regularly for signs of ill health and housed with a maximum of 5 animals per cage. All surgical procedures were performed under sterile conditions following LASA guidelines for aseptic surgery in small animals (Benavides et al., 2020). Any animals found to be in distress or showing any signs of illness, were immediately sacrificed by a Sch1 method.

2.2 Mice

All mice used for experiments were of C57BL6/J genetic background. For experiments using wild-type mice, male C57BL6/J mice were purchased from Charles River (<https://www.criver.com>, UK) where the genetic strain purity is guaranteed via their Genetic Stability Programme. The following lines were purchased and imported from The Jackson Laboratory, USA: Sm22Cre mice (B6.Cg-Tg(Tagln-cre)1Her/J, Stock No: 017491), constitutively active PDGFR β^{D849V} mice (Stock Pdgfrbtm14 (Pdgfrb) Sor/J, Stock No: 018435), SM-MHC.CreER^{T2} mice (B6.FVB-Tg (Myh11-cre/ERT2)1Soff/J, stock No: 019079), Rosa26R.LacZ reporter mice (B6.129S4-Gt (ROSA) 26Sortm1Sor/J, Stock No: 003474). mT/mG mice (B6.129 (Cg)-Gt (ROSA) 26Sortm4 (ACTB-tdTomato,-mGFP) Luo/J, stock No: 007676 were obtained from Dr Nadira Yuldasheva & Dr Richard Cubbon (Leeds), and Orai1^{fl/fl} mice were obtained from Stefan Feske lab (New York).

2.2.1 Cre –loxP technology and the transgenic lines

The Cre/loxP system is a site-specific recombination system that enables the generation of tissue and temporal-specific gene expression (Figure 2.1). Mice that express Cre protein driven by a specific promoter (i.e., driving cell type specific expression) may be crossed with mice containing a gene of interest or a stop codon flanked by a pair of short loxP (locus of x) sequences (known as “floxed” mice). If the gene of interest is flanked by loxP sites, once activated the Cre recombinase will remove the section of DNA resulting in deletion of gene expression. If a loxP flanked stop codon has been inserted the deletion of which will unveil a downstream insertion. The tissue specific promoter associated with the Cre recombinase ensures insertion/deletion of the loxP flanked sequence only in the cells expressing that promoter e.g., Smooth muscle myosin heavy chain CreERT2 will only alter gene expression in cells expressing Smooth muscle myosin heavy chain. In the inducible Cre system as used here, the Cre is fused with the mutant estrogen receptor (ER^{T2}), which becomes activated when the mouse is administered tamoxifen. The tamoxifen is metabolized to 4-hydroxy tamoxifen that binds with the CreER fusion protein causing translocation into the nucleus and cleavage of loxP sites allowing conditional expression of reporter genes driven by the Cre. No change in gene expression occurs in mice not administered

tamoxifen as the Cre protein will stay in cytosol (and is thus inactive) until tamoxifen is given (Kim et al., 2018).

To investigate the vascular remodelling behaviour in AAA, two different SMC specific Cre promoter mouse lines were utilised:

1. Constitutive activity of Cre from embryonic development and throughout post-natal life - controlled by smooth muscle protein 22 (Sm22) alpha promoter (validation is publicly available from Jax; <https://www.jax.org/strain/017491>)

2. Inducible Cre controlled by the smooth muscle myosin heavy chain (SM-MHC) promoter in response to tamoxifen (no validation was available from Jax at the start of this project, so was undertaken as part of this thesis).

These VSMC conditional Cre lines were used to generate multiple transgenic lines to study VSMC fate (lineage tracing) in the context of experimental AAA and the role of PDGFR β activation with or without Orai1 deletion in terms of aortopathy and experimentally induced AAA. The lines used are summarised in (Table 2.1) and explained in detail below.

S.No	Transgenic Line	Description	Purpose
1.	SM-MHCCreER ^{T2} X LacZ	Tamoxifen inducible SMC specific expression of LacZ	Validation of the tamoxifen inducible SMC specific gene expression
2.	SM-MHCCreER ^{T2} X mT/mG	Tamoxifen inducible SMC specific expression of GFP mG (eGFP) in mice with global mT (red) expression	Lineage tracing of SMC in AAA
3.	Sm22Cre X PDGFR β ^{D849V}	Constitutive SMC specific expression of mutant PDGFR β (constitutively active)	Study SMC remodelling upon over-active PDGF signalling from embryogenesis
4.	SM-MHCCreER ^{T2} X PDGFR β ^{D849V}	Tamoxifen inducible SMC specific expression of mutant PDGFR β (constitutively active)	Study the effect of over-active PDGF signalling initiated in adult mice and after AAA induction
5.	SM-MHCCreER ^{T2} X PDGFR β ^{D849V} X mT/mG	Tamoxifen inducible SMC specific expression of mG (mGFP; green) and mutant (constitutively active) PDGFR β in mice with global mT (mTom; red) expression	Lineage tracing of SMC subject to over-active PDGF signalling induced in adult mice
6.	Sm22Cre X PDGFR β ^{D849V} X Orail	SMC specific deletion of Orail and expression of mutant PDGFR β (constitutive expression)	Study the effect of Orail deletion in mice with over-active PDGF signalling

Table 2.1 List of transgenic lines used in the study.

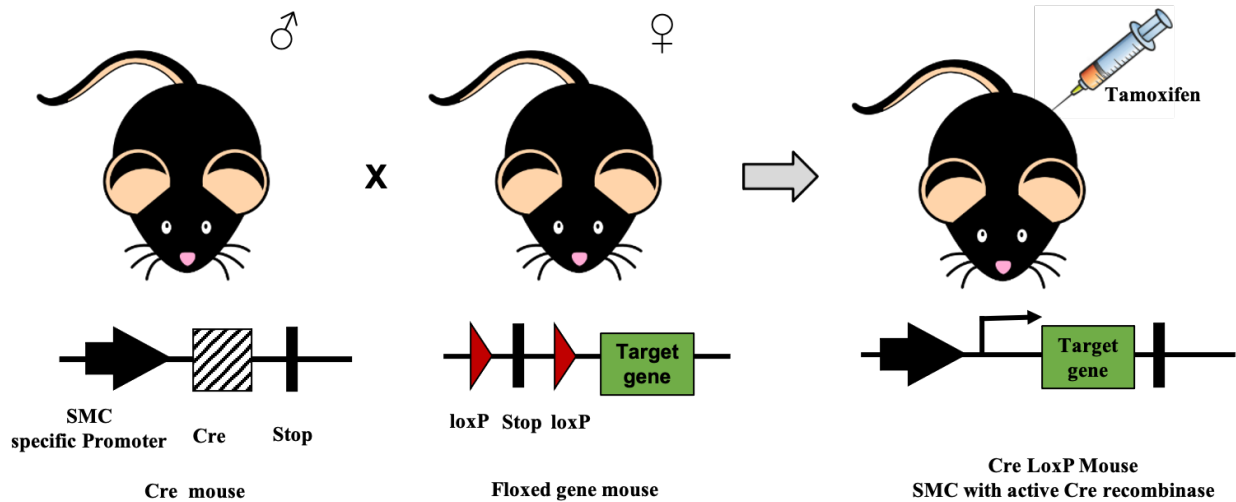


Figure 2.1 Cre-loxP system schematic.

Cre recombinase, recognizes and splices specific DNA sequences, called LoxP sites. The LoxP site consists of 34 base pairs: an 8bp spacer region and two flanking palindromic sequences 13bp-long each. The Cre with the SMC-cell specific promoter is expressed constitutively in SM22Cre lines and only after the administration of tamoxifen to the mice in inducible Cre line (SM-MHCCreER^{T2}).

2.2.2 SMC specific inducible LacZ reporter mice (SM-MHCCreER^{T2} X LacZ)

To validate the specificity of gene expression to smooth muscle cells, the male SM-MHCCreER^{T2} mice were bred with female Rosa26R.LacZ mice to generate a heterozygous colony. CreER^{T2} recombinase is under the control of mouse smooth muscle myosin, heavy polypeptide 11 (Myh11) promoter. This SM-MHCCreER^{T2} tamoxifen inducible mouse was chosen as it is the most specific for SMC compared to alternative promoters. The Cre allele is located in the Y chromosome of the male mice and therefore, smooth muscle specific gene expression can only be induced in male mice. The Rosa26R.LacZ mice have the β -galactosidase (lacZ) sequence inserted at the ubiquitously expressed Rosa26 locus. Tamoxifen was injected intraperitoneally at 8 weeks of age for 5 consecutive days and the activation of Cre resulted in removal of the stop sequence unveiling lacZ only in SMC (Figure 2.2).

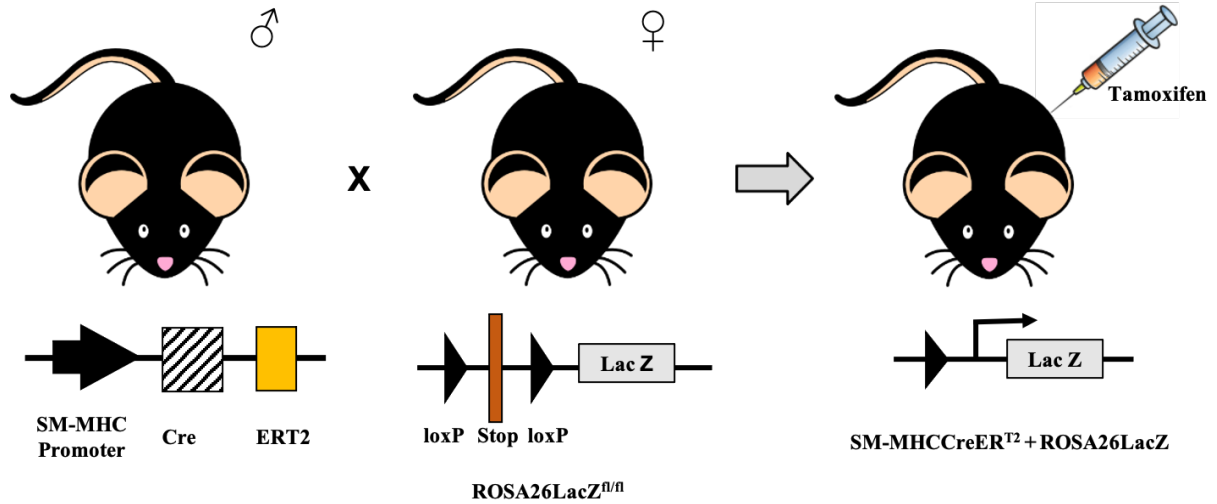


Figure 2.2 Generation of SMC specific LacZ expressing Cre mice following tamoxifen administration.

SM-MHCCreERT² mice and LacZ floxed reporter mice were bred to create transgenic offspring. The CreERT2 expression is inducible by means of tamoxifen injection.

2.2.3 SMC specific inducible mT/mG mice (SM-MHCCreER^{T2} X mT/mG)

For lineage tracing studies, double fluorescent mT/mG female mice were interbred with male SM-MHC-CreER^{T2} mice to obtain a homozygous mT colony (Figure 2.3). Tamoxifen injection was administered intraperitoneally at 8 weeks of age to specifically activate Cre recombinase. SMC specific Cre activity was validated by comparing green fluorescent protein (mG/mGFP) expression in SMC and the red fluorescent protein (mT) in non-SMC by immunofluorescence imaging (Figure 2.3).

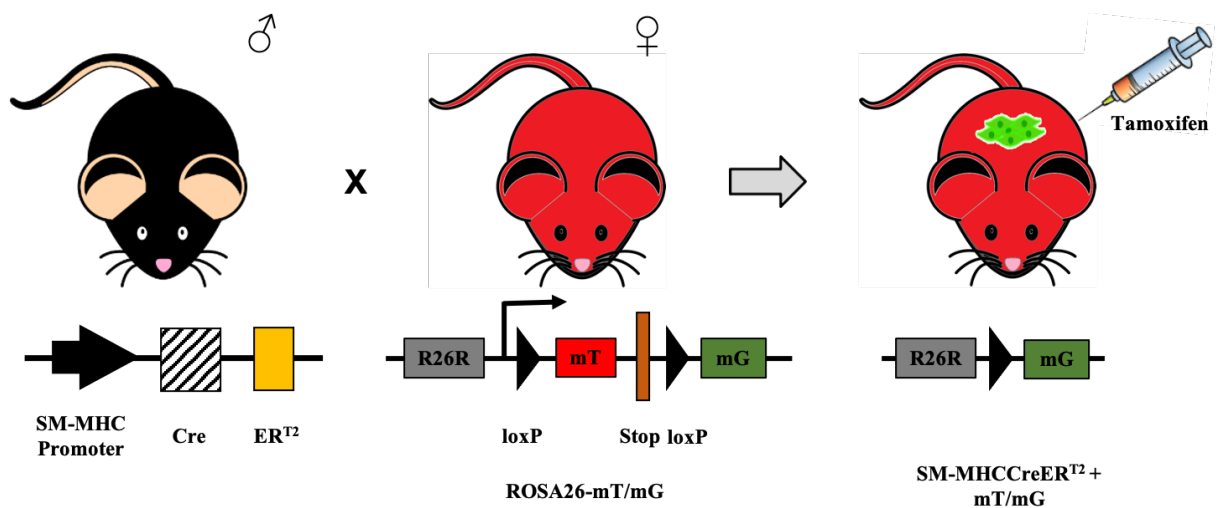


Figure 2.3 Generation of SMC specific mT/mG fluorescence expressing Cre mice following tamoxifen administration.

SM-MHCCreER^{T2} mice and Rosa26 mT/mG reporter mice were bred to create transgenic offspring which expresses membrane-targeted dTomato ("mT") prior to Cre excision and following Cre excision membrane-targeted mGFP ("mG") only in SMC.

2.2.4 SMC specific constitutive PDGFR β^{D849V} mice (Sm22Cre X PDGFR β^{D849V})

To study the effect of constitutive PDGF signalling in SMCs, the male Sm22Cre mice were bred with the female constitutive Cre PDGFR β^{D849V} transgenic mice. In PDGFR β^{D849V} conditional knock in mice, the endogenous PDGFR β sequence in the kinase domain was replaced with a floxed-STOP cassette upstream of the constitutively active PDGFR β mutant isoform. The D849V mutation introduced into the kinase domain of the activation loop of the PDGFR β sequence changes aspartic acid to valine at amino acid position 849 (D849V) providing constitutive activity of PDGFR β signalling and an enhanced response to PDGF activation. The Cre recombination resulted in knock in of PDGFR β gene in the flanked sequence in SMC. This generated a SMC specific heterozygous colony of constitutive PDGFR β mutant mice with Cre activated from embryogenesis (Figure 2.4)

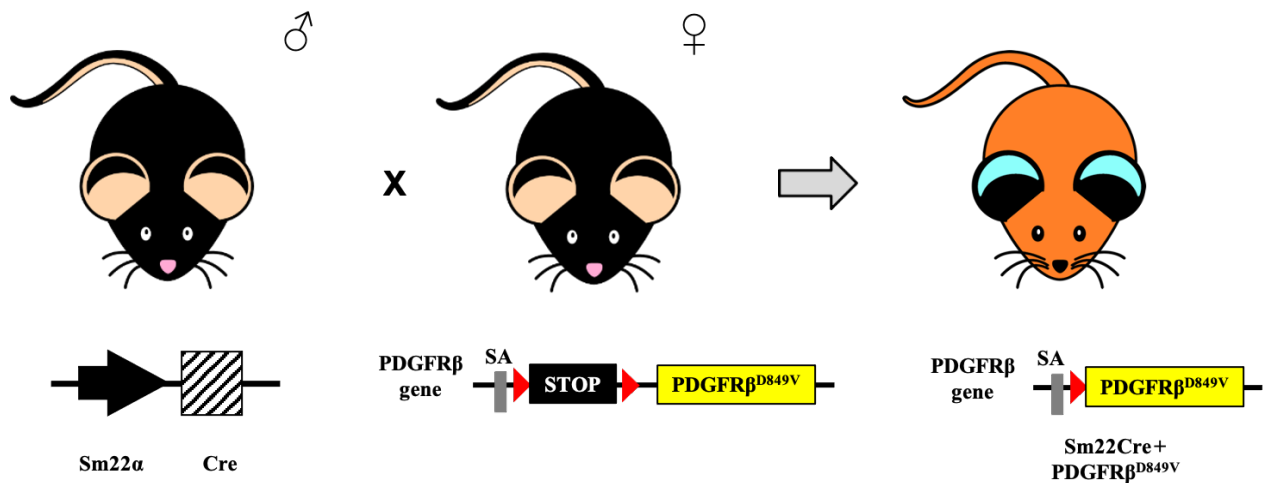


Figure 2.4 Generation of SMC specific PDGFR β^{D849V} expressing Cre mice

Sm22Cre mice and PDGFR β^{D849V} floxed mice were bred to create transgenic offspring.

2.2.5 SMC specific inducible PDGFR β^{D849V} mice (SM-MHCCreER^{T2} X PDGFR β^{D849V})

The male SMMHC X CreER^{T2} tamoxifen inducible mice were crossed with the female constitutive Cre PDGFR β^{D849V} transgenic mice to generate a conditional knock in of PDGFR β^{D849V} line. Tamoxifen induced Cre mediated recombination resulted in constitutive PDGFR β activity in SMC only in adult mice (Figure 2.5)

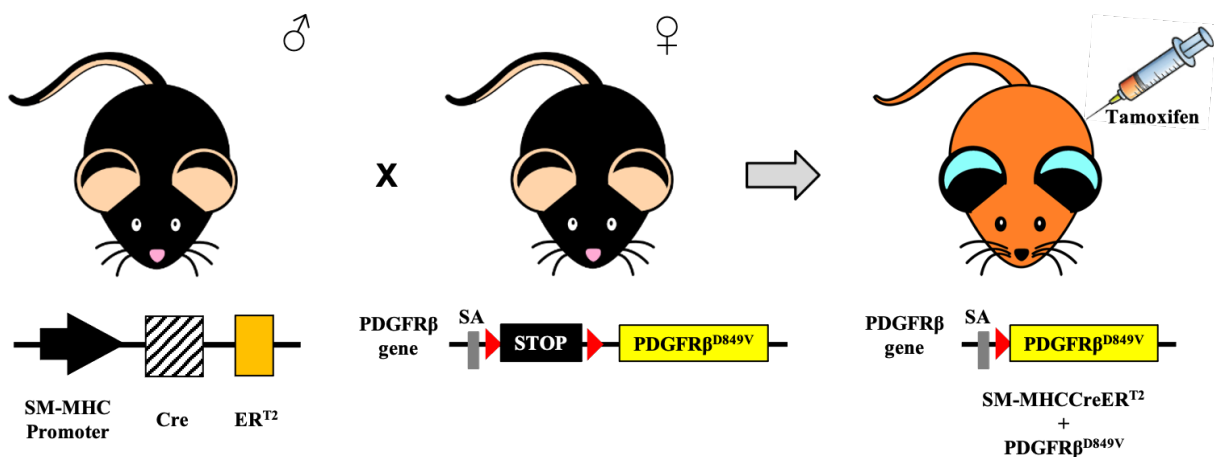


Figure 2.5 Generation of SMC specific PDGFR β^{D849V} expressing Cre mice following tamoxifen administration.

SM-MHCCreER^{T2} mice and PDGFR β^{D849V} floxed mice were bred to create transgenic offspring.

2.2.6 SMC specific inducible PDGFR β^{D849V} and mT/mG mice (SM-MHCCreER^{T2} X mT/mG X PDGFR β^{D849V})

For further lineage tracing studies in the enhanced PDGF signalling background, a triple transgenic model was created by crossing – SM-MHCCreER^{T2}PDGFR β^{D849V} with mT/mG line. The resultant line upon tamoxifen induction expressed mutant PDGFR β and mG in SMC only and mT in all other cells (Figure 2.6).

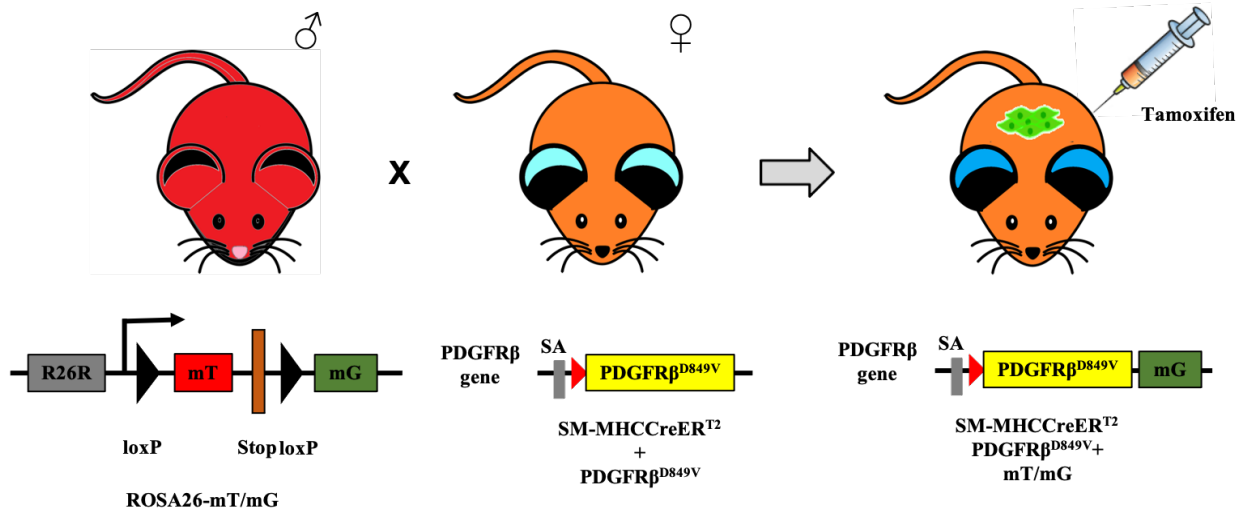


Figure 2.6 Generation of SMC specific PDGFR β^{D849V} and mT/mG fluorescence expressing Cre mice following tamoxifen administration.

SM-MHCCreER^{T2}PDGFR β^{D849V} mice and Rosa26 mT/mG reporter mice were bred to create transgenic offspring which expresses membrane-targeted dTomato (“mT”) prior to Cre excision and following Cre excision, membrane-targeted mGFP (“mG”) only in SMC and PDGFR β^{D849V} .

2.2.7 SMC specific constitutive PDGFR β^{D849V} and Orai1^{-/-} mice (SM22CrePDGFR β^{D849V} X Orai1^{fl/fl})

To understand the effect of knocking down Orai1 in the constitutive Cre PDGFR β^{D849V} mice, the male Orai1^{fl/fl} mice were crossed with the SMC specific constitutive Cre PDGFR β^{D849V} mice (Figure 2.7). In the Orai1^{fl/fl} mice, the first loxP site was inserted into intron 1 at ~1 kb upstream of exon 2 and the second loxP site, along with the Frt-PGKneo-Frt cassette, was inserted ~0.3 kb 3' of exon 3 downstream of the polyA signal sequence and 3' UTR. The heterozygous (Orai1^{+/-}) litters were used for phenotyping from 8 weeks of age as well as expanded the breeding to obtain homozygosity (Orai1^{-/-}).

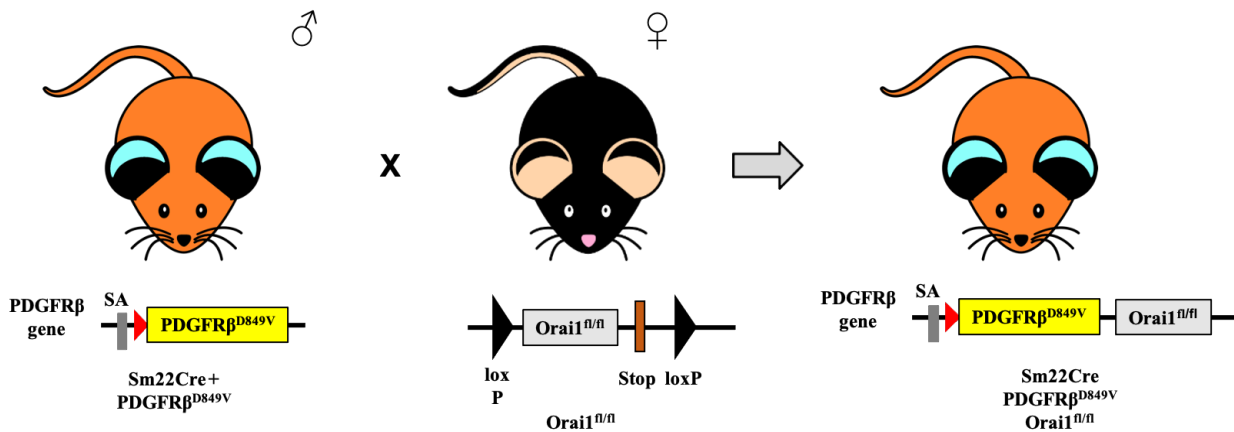


Figure 2.7 Generation of SMC specific PDGFR β^{D849V} expressing Cre mice with Orai1 knockout

SM22CrePDGFR β^{D849V} mice and Orai1^{fl/fl} floxed mice were bred to create transgenic offspring.

2.3 Animal procedures

2.3.1 Genotyping

Mouse ear notches were collected at 4 weeks of age from the inbred mouse colonies and routine genotyping was carried out by Transnetyx Inc (Cordova, Tennessee, US) by genomic DNA PCR. The probes used for genotyping are given in Table 2.1.

Gene/ Locus	Forward and Reverse (5' to 3')	Reporter
SM- MHCCre	CTGCTGACTGTGCACCAAAAC CTTCATCAGAGGTGGCATCCA	CTGCCTGCCCTCCCTG
Rosa26 WT	TTCCCTCGTGATCTGCAACTC CTTTAAGCCTGCCCAGAAGACT	CCGCCCATCTTCTAGAAAG
mGFP (mG)	CGTCGTCCTTGAAGAAGATGGT CACATGAAGCAGCACGACTT	CATGCCCCGAAGGCTAC
PDGFRb- WT	GACCATGACGTGTGACTCTTTCT GGTCCCAGGAGCCATAACAC	CAAAGGCCAGTTGTTGCT
PDGFRb- D849V	TCTCTCTCATCATCCTCATCATGCT CTCAATGACCTTCCATCGGATCTC	ATAGCGTGGCTTCTTCT
LacZ	CGATCGTAATCACCCGAGTGT CCGTGGCCTGACTCATTCC	CCAGCGACCAGATGAT
18S	GATGCTCTTAGCTGAGTGT	GATGCTCTTAGCTGAGTGT

Table 2.2 List of primers used for genotyping transgenic animal lines.

2.3.2 Tamoxifen induction

Tamoxifen induction was optimised both *in vivo* and *in vitro* (using primary VSMC). For *in vivo* Cre activation a tamoxifen dosage of 10 mg per mouse for the adult mice of 8 weeks old was chosen based on previously published doses (Shankman et al., 2015). Tamoxifen (Sigma-Aldrich, T5648-1G) was dissolved in corn oil at a concentration of 20 mg/ml by shaking overnight at 37°C. The injection dose was determined as approximately 80 mg tamoxifen/kg body weight of adult mice of 8-9 weeks old. Adult mice of 8-9 weeks old, was injected intraperitoneally once every 24 hours for a total of 5 consecutive days with a standard dose of 2mg/100µl tamoxifen/corn oil solution. Following the final injection, mice were kept in quarantine for a period of 7 days in a dedicated room of the animal facility.

For *in vitro* Cre activation, 4-hydroxy tamoxifen (4-OHT, Cambridge Bioscience, UK) was dissolved in absolute ethanol to give a stock concentration of 10 mM. High efficiency of recombination was achieved when the primary VSMC were treated with 10 µM concentration of 4-OHT for 72 hours (replenishing with a fresh dose daily). The recombination was verified by IncuCyte which acquires the phase contrast, red and green fluorescence expressed by the cells using Zoom 2016A software (Essen BioScience, USA).

2.3.3 Surgical induction of aneurysm

Experimental AAA was induced in 12 weeks old male mice by peri-adventitial application of porcine pancreatic elastase (PPE) in the infra renal region of the aorta below the right renal artery (Bhamidipati et al., 2012; Waduud et al., 2021). Mice were first anaesthetized (2.5-5% isoflurane) and laid in supine position. 100 µl of Buprenorphine analgesia (at 0.1 mg/ml) was injected subcutaneously before surgery. Transabdominal incision in the retroperitoneum region was made to expose the abdominal aorta. The contents of the abdomen were retracted outside the abdomen with PBS wet-sterile gauze. Aorta below the right renal artery was exposed carefully till the iliac bifurcation. The superficial fascia (posterior layer of the peritoneum as the aorta is a retroperitoneal organ) that covers the aorta was carefully removed without puncturing the vessel and the exposed

aorta was soaked in 10 µl of PPE (100%) for 5 minutes. The PPE residue was washed with three times with PBS and the intestines are returned to the abdomen. The incision was sutured with a 6-0 Vicryl to cover the peritoneal layer followed by the skin with a 4-0 Vicryl suture. The mouse was recovered on a heated platform maintained at 32°C and fed with soaked diet for 48 hrs postoperatively and observed at least twice daily for a week. If any suffering was noticed, an additional dose of 50 µl analgesia was injected. In case of further discomfort, the mice were sacrificed to avoid further suffering. In inducible Cre models, to ensure that the tamoxifen had been cleared from the mice circulation before induction of the aneurysm, the surgery was performed at least 7 days after the last tamoxifen injection.

2.3.4 Drug delivery

For experiments requiring infusion of the Orail inhibitor, JPIII, an Alzet mini osmotic pump (Model 1002) which delivered the drug at the dosage of 500ng/kg/min (0.72 mg/kg/day in DMSO) was used (based on Bartoli et al. 2020 which shows a functional effect on pressure induced heart failure at this dose in mice). Pumps were inserted under general anaesthesia in prone position via mid-scapular region into a subcutaneous pocket created in the left flank. The wound was then closed with a 4-0 Vicryl suture. The mice were recovered under as per 2.3.3 and observed carefully for any suffering or discomfort.

2.3.5 Ultrasound measurement

After 2 and 4 weeks of elastase induction, B-mode ultrasound imaging (Vevo 2100, VisualSonics Inc.) was performed with the MS-550D transducer of 40MHZ frequency on the operated mice to assess the abdominal aortic diameter (AAD) – this was performed as published in (Waduud et al., 2021). The hair was shaved from the abdomen, the day prior to imaging. Mice were anaesthetised and restrained on a heated imaging platform with 2% isoflurane in oxygen delivered by mask during imaging. Respiratory rate, heart rate and core temperature were monitored through the electrodes on the platform with conductive cream applied to each paw (Parker Labs) and a rectal

probe (Indus). For imaging, the transducer was applied on the abdomen on a layer of aquasonic clear ultrasound transmission gel (Parker Labs). Transverse three-dimensional imaging was undertaken across a 12 mm region of interest (ROI) proximally from the right renal artery (which is located higher than the left in the mouse) with 157 frames with respiration gating at peak systole. A single subjective transverse electrocardiogram-gated KiloHertz Visualisation (EKV) image was recorded in the “B mode” at the largest visible section of the aorta for measurement of the maximum anterior-posterior diameter. The 3D acquired images were used to calculate the three-dimensional lumen volume using semi-automated segmentation and a reconstruction tool available with the VevoLab v1.7.0 software (VisualSonics, Netherlands; Figure 2.8). Measurement of the lumen volume was made in millimetres-squared (mm^3). The EKV image allowed for vessel distensibility to be calculated using an add-on software package, VevoVasc (VisualSonics, Netherlands). The lumen boundaries were segmented semi-automatically and refined manually, which allowed the software to track the pulsatile motion. Vessel distensibility was calculated based on changes in the aortic wall motion over the cardiac cycle and measurements were quantified in megapascal pressure unit (MPa^{-1}).

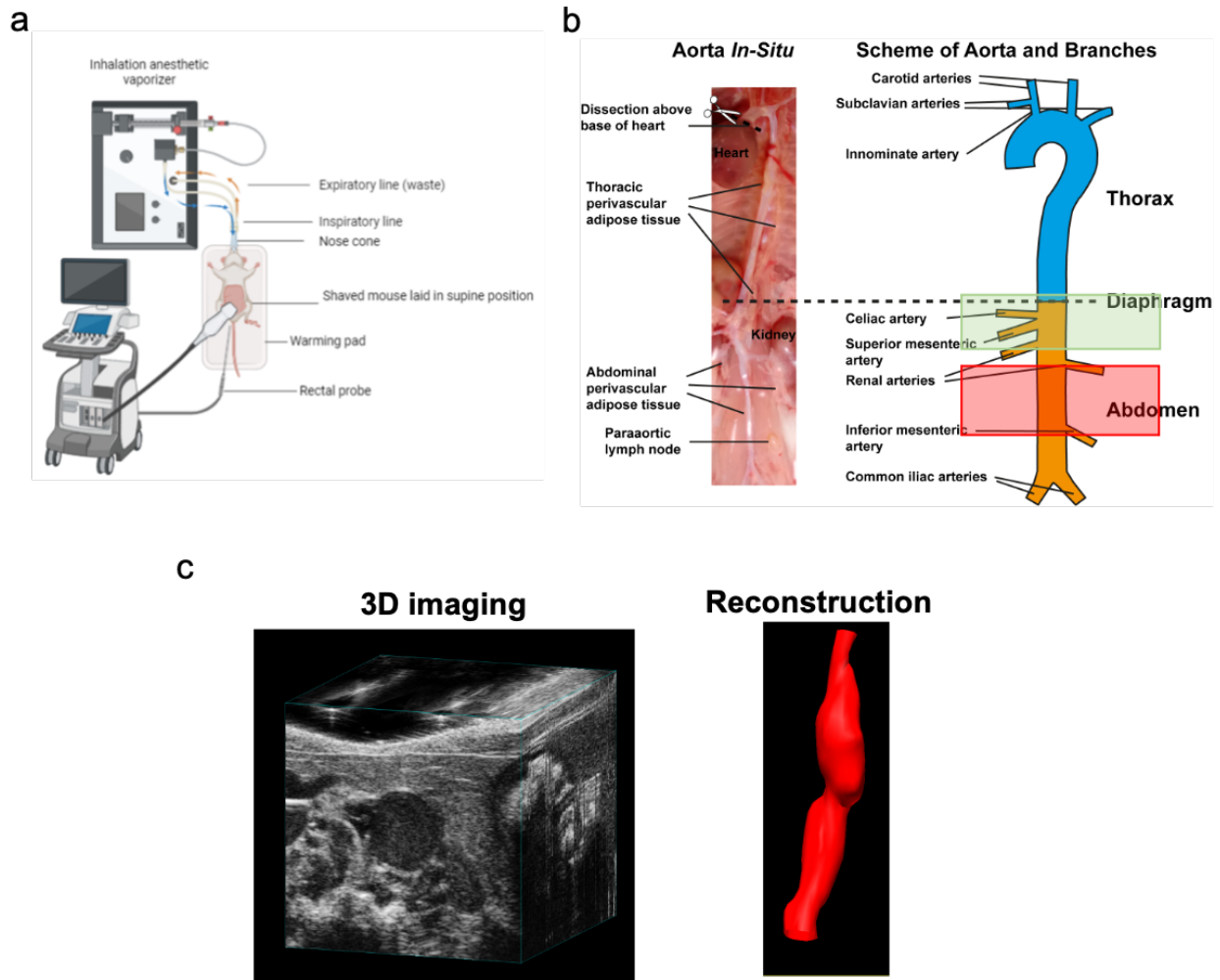


Figure 2.8 Ultrasound image acquisition of abdominal aorta of mice.

(a) Murine ultrasound imaging system including anaesthesia set-up. (b) Mouse aorta anatomy showing supra (green box) and infra (red box) renal regions of the aorta. (c) Representative acquired 3D image of the aorta which was reconstructed (Images are adapted from Bio-protocol (<https://en.bio-protocol.org/en>) and M Bailey PhD thesis 2016).

2.3.6 Micro-Computed Tomography (μ CT)

Micro-Computed Tomography (μ CT) was performed with a μ CT SkyScan 1176 (Bruker, Belgium) after induction of anaesthesia with isoflurane (5% for induction and 2-2.5% for maintenance) at 2 L/min oxygen. The contrast agent, ExiTron nano 12000 (Miltenyi Biotec, Germany) was administered via the lateral tail vein through a PE10 catheter. 100 μ L of ExiTron nano 12000 was injected using infusion pump at a rate 100 μ L/min, followed by 100 μ L bolus injection of saline. Mice were transferred to the imaging bed and scanned with μ CT using filter Al 0.5mm, 50kV, 475 μ A, pixel size 35 μ m, exposure 65 ms, rotation step 0.7°, 360° scan. The μ CT data were reconstructed with NRecon (Bruker, Belgium) software using the Feldkamp algorithm, with the following parameters: Smoothing: 4, Ring Artifact Correction: 14, Beam Hardening Correction: 40%. The analysis was performed using CTAn software (Bruker, Belgium). First, the background and skeleton were removed using thresholding, morphological and bitwise operations. The images were filtered with anisotropic diffusion (privilege wide regions), 10 iterations, gradient threshold 5. The volumes of interest were drawn to roughly cover the aorta and then the thresholding of the images were done. Using subsequent opening and closing, as well as despeckling, aortae were separated, and the volumes calculated.

2.4 Laboratory techniques

2.4.1 RNA extraction, reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from the different mouse tissues (aorta, bowel, skeletal muscle & adipose tissue) using Tri-reagent, bromochloropropane and glycogen (Sigma) according to the manufacturer's protocol. Briefly, samples were lysed in TRI-reagent with the addition of 100 μ L bromochloropropane. The aqueous phase, separated by centrifugation, was transferred into a fresh tube. After addition of 1 μ L glycogen and ice-cold isopropanol total RNA was pelleted by centrifugation and then washed in 75% ethanol. RNA precipitates were re-suspended in molecular grade water followed by Turbo DNase digestion (Ambion) at 37 °C for one hour. RNA was quantified using NanoDrop. 0.5 μ g of RNA was reverse transcribed to cDNA using the Applied Biosystems High-Capacity RNA to cDNA kit (RT+) according to the manufacturer's protocol. 0.5 μ g of RNA was also used for a genomic DNA control, in a reaction without reverse transcriptase (RT-). Real time PCR was performed using a LightCycler Carrousel system (Roche, Molecular Systems, UK) and SYBR Green (Molecular Probes, Invitrogen). The following protocol was utilised: pre-incubation at 95 °C for 5 minutes; amplification for 35 cycles of 95 °C for 30 seconds, 60°C for 30 seconds and 72 °C for 30 seconds; melt curve at 95% for 5 seconds, 65°C for 1 minute, 97°C extension; and cooling at 40 °C for 20 seconds. The reaction threshold cycle (Ct) and melt curve were determined using the Lightcycler analysis software v3.5 (Roche). The relative abundance of genes of interest amplified by RT-PCR were calculated relative to a housekeeping gene (GAPDH) in the same sample using the $2^{-\Delta\Delta C_t}$ method, where ΔC_t refers to the difference in Ct between the gene of interest and the housekeeping gene. LacZ and 18S primers were validated in-house (Table 2.2) and the rest of the validated gene-specific primers were purchased from BioRad (UK) and their assay IDs are given in Table 2.3.

Gene	Assay ID
IGFBP2	qMmuCID0006519
IL6	qMmuCID0005613
MMP2	qMmuCID0021124
MMP12	qMmuCID0018300
LRP1	qMmuCID0013464
TIMP1	qMmuCID0025322
Col19a1	qMmuCID0013448
CCR5	qMmuCID0020341
BMP2	qMmuCID0014251
Clec3b	qMmuCID0009478
Spp1	qMmuCED0061675
Gapdh	qMmuCED0027497

Table 2.3 List of Assay IDs of the primers used in RT-qPCR

2.4.2 RNA-Sequencing (RNA-Seq) transcriptomic analysis

For RNA-Seq analysis, aortae were harvested from mice and cleaned of adventitial fat under light microscope. Abdominal aorta was cleaned and used for RNA isolation and DNase treatment as described in section 2.4.1. RNA-Seq was performed on 4 aortae from each genotype (SM22CrePDGFR β ^{D849V} and WT animals).

RNA-Seq library preparation and sequencing

RNA-Seq library preparation and sequencing were performed by the Next Generation Sequencing (NGS) Facility at St. James's University Hospitals, Leeds using TruSeq Stranded mRNA kit (Illumina), according to the manufacturer's protocol. First, the mRNA molecules were purified using poly-T oligo magnetic beads and then cleaved into small fragments. cDNA synthesis was done in two steps - first strand cDNA was produced from the RNA fragments by reverse transcription and the second strand cDNA synthesis was then performed using DNA Polymerase I and RNase H enzymes. The resultant double stranded cDNA comprised of deoxythymidine (dT) residues in the first strand and deoxyuridine (dU) residues in the complementary strand as different dNTP mix were used in each step (Parkhomchuk et al. 2009). Following adapter ligation, the

dUTP-marked strand is excluded during the amplification process using a polymerase that does not incorporate dUTP, to generate a strand-specific cDNA library. cDNA libraries were sequenced using the NextSeq 500 Sequencing System (Illumina) to generate 75 bp single end reads.

RNA-Seq data analysis

Quality trimming and adaptor sequence removal was performed using CutAdapt, prior to quality assessment with fastQC (<https://github.com/s-andrews/FastQC>). Sequences were aligned to the mouse genome (mm39) using STAR aligner (<https://github.com/alexdobin/STAR>) then reads were mapped to transcripts using rSubread (Liao et al., 2019). Read count data were processed with DeSeq2 to identify differentially expressed genes (WT Vs SM22CrePDGFR β^{D849V}), after removing rRNA sequences and all transcripts which had less than 2 reads in 3 or more samples. Global transcriptomic patterns were illustrated using principal component analysis (PCA) plots and heatmaps, generated using unsupervised hierarchical clustering. The heatmaps illustrate clusters of differentially expressed transcripts, divided into several sectors according to the clades generated in dendrograms. Volcano plots were used to illustrate patterns of differential transcript expression. The list of significantly differentially expressed transcripts (at Benjamini-Hochberg false discovery rate adjusted $p < 0.05$) was then used to identify enriched gene ontology (GO) terms (<http://geneontology.org>) (i.e., in relation to terms encompassed by all detected transcripts) in the study samples. A similar analysis was performed for Reactome and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Croft et al., 2011; Kanehisa and Goto, 2000), which group transcripts/genes by biological processes and generate pathway maps for each cluster of genes. GO terms and Reactome & KEGG pathways were also considered significant if below a Benjamini-Hochberg adjusted p-value of 0.05.

2.4.3 LacZ staining

Harvested tissues were washed 2 x 5 minutes in cold phosphate buffered saline (PBS) followed by pre-fixation for 10 minutes in pre-fix solution (2% formaldehyde, 0.8% glutaraldehyde, 0.02% Igepal NP40, 1 mM MgCl₂ and 0.01% sodium deoxycholate in PBS). After 2 x 5 minute wash in PBS, tissues were incubated for 48 hours in staining solution (0.1 M K₄Fe(CN)₆·3H₂O, 0.1 M K₃Fe(CN)₆, 0.04% 5-bromo-3-indolyl-B-D-galactopyranoside (Melford Laboratories, UK), 1 mM MgCl₂ in PBS) protected from light. Following this, tissues were washed for 5 minutes in PBS before post-fixation using 4% formaldehyde in PBS.

2.4.4 Frozen section, immunohistochemistry and analysis

Animals were anaesthetised with isoflurane and terminally exsanguinated via the inferior vena cava. The aorta was flushed with 10 ml of PBS and perfuse-fixed with 5 ml of 4% PFA via left ventricle.

For frozen section embedding, the aortae were dissected out of the animal and fixed in 10 ml of 4% PFA for 2 hours at 4°C and then embedded transversely in optimal cutting temperature compound (OCT) (Cellpath, UK). Samples were stored at -80°C until use. The 10 µm thick sections were cut using Leica CM1900 cryostat onto poly-L-lysine coated slides (VWR, UK). These sections were then stained with ProLong Gold Antifade Mountant with DAPI (Thermo Scientific, UK) and imaged with a LSM 880 laser scanning confocal microscope.

For immunostaining, sections were brought to room temperature by rinsing in PBS and permeabilised with 0.3% triton-x100 in PBS for 30 minutes. After incubation with blocking buffer (10% horse serum, 0.05% triton-x100 in PBS) for 1 hour at room temperature, sections were then stained with primary antibodies (single or double in series with a wash in between) diluted in PBS with 2% horse serum and 0.05% triton-x100 for 2 hours at room temperature. After 3 washes with PBS, sections were incubated in goat anti-rabbit Alexa-514- conjugated (Invitrogen, UK) and chicken anti-rat Alexa-647-conjugated (Invitrogen, UK) secondary antibodies diluted 1:1000 in

PBS with 2% horse serum and 0.05% triton-x100 for 1 hour at room temperature. Sections were then washed and mounted in ProLong Gold Antifade Mountant with DAPI (Thermo Scientific, UK). For Orail staining, sections were incubated in anti-Orail antibody (Alomone Labs; 1:50 dilution) for 1 hour at room temperature followed by biotinylated secondary antibody for 1 hour. The sections were visualised with (3,3'-diaminobenzidine) DAB substrate using Vector labs (UK) kit following manufacturer's protocol. Primary antibodies used in the study are listed in Table 2.4.

Primary Antibody	Dilution used	Source
rabbit polyclonal anti α -SMA	1:100	Abcam, UK
rat monoclonal anti Mac2	1:1000	Cedarlane Labs, USA
rabbit polyclonal anti SM-MHC	1:200	Abcam, UK
rabbit anti-chicken anti PDGFR β	1:500	Cell Signalling Technology, UK
rabbit polyclonal anti Orail	1:20	Sigma, UK

Table 2.4 List of primary antibodies used for immunofluorescence staining.

Fluorescent imaging was performed on an LSM 880 laser scanning confocal microscopy (Carl Zeiss) using x10, x20 air objective for tiled scanning and x40, x63 oil objectives for high resolution imaging for key areas of tissues and cell imaging with the pinhole set at 1 Airy Unit providing 1.1 μ m slices. Z-stacks were obtained using 20x and 40x objectives, of around 6-7 images, at 20 μ m intervals. Images were processed using Fiji ImageJ v1.49 (<http://imagej.net/Fiji/Downloads>). Maximal intensity projection of the 20x images was used for quantification to measure the lumen and vessel size. The 40x z-stack images were used for cellular quantification and an average of at least 4 sections was used for each mouse. Representative images presented in the figures are shown as maximal intensity projection images. Both aortic sections of mTmG mice co-stained for SMC markers and macrophage marker Mac2, were counterstained with DAPI. To determine the percentage of VSMC that are stained positive for SMC marker (e.g., α -SMA), the total number of red (non-VSMC) and green (VSMC) cells in the ROI were counted first before counting the number of green (mG+, SMA+) and red (mT+, SMA+) cells that were positively stained for α -

SMA (yellow), and the number of green (mG+, SMA-) and red (mT+, SMA-) cells that were negative for α -SMA. We then calculated the ratio of the number of (mG+, SMA+) green cells to the number of (mG+, SMA-) green cells and then normalised it to the total number of green cells in the ROI to estimate the proportion of VSMC stained positively for α -SMA at different time points. Similar methodology was followed for quantifying macrophage marker positive red and green cell proportions.

2.4.5 Paraffin sections histology and analysis

For paraffin embedding, the aortae were dissected out and fixed in 5 ml of 4% PFA for 48 hours at 4°C after which they were stored in 70% ethanol at 4°C until processing and embedding. Fixed tissues were processed on a Leica ASP 200 (Leica Biosystems; https://www.histology.leeds.ac.uk/what-is-histology/histological_sections.php) using the manufacturer's overnight programme of progressive dehydration in increasing concentration of ethanol and then replacing ethanol by xylene and finally embedding in cellwax (Cellpath, UK) using a Leica EG1150H embedding station. Sections of 5 μ m thickness were cut onto slides (Solmedia, UK) with a Leica RM2235 microtome and dried overnight at 37°C. For hematoxylin and eosin (H&E) staining, the slides were deparaffinized and then hydrated by passing through decreasing concentrations of alcohol. Sections were then stained in hematoxylin for 3 minutes and counterstained with eosin for 2 minutes.

After H&E staining, imaging was performed on Leica light microscope magnification of x20. Quantification to measure the lumen size and number of cells in ROI were manually performed using ImageJ (NIH, USA) with the assessor blinded to the experimental details.

2.4.6 VSMC isolation and immunostaining

Primary VSMC were isolated from the thoracic aorta of 3-4 weeks old mice as previously described (Ray et al., 2001). Mice were killed by asphyxiation and thorax and abdomen were cut open. Dissected out aortae were cut into approximately 1-2 mm pieces and placed in the 100 mL

of enzyme solution (7.5 mg collagenase II (Millipore, UK) dissolved in 5.5 mL DMEM media) in the incubator at 37 °C, 5% CO₂, for five hours. After centrifugation the cells were plated in a 24 well plate in culture medium (Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM/F-12), Gibco, UK) supplemented with 20 % foetal calf serum and antibiotics. The cells were left undisturbed for 5 days, medium was changed on day 5 and then left until they reached confluency.

For staining, the primary VSMC with passages 4 to 6 were fixed in 4% PFA for 5 minutes at room temp, then rinsed in PBS and blocked with donkey serum for 30 minutes to avoid any non-specific binding. Cells were then incubated with primary antibody diluted in blocking buffer for 1 hour at room temp, rinsed in PBS followed by secondary antibody incubation for 30 minutes, mounted and imaged in LSM microscopy. The VSMC were stained with mouse α -smooth muscle - Cy3 monoclonal antibody (Sigma, 1:200 dilution), counterstained with anti-CD31 polyclonal antibody (Abcam, 1:25 dilution) and goat vimentin polyclonal antibody (Santa Cruz, 1:50 dilution). The corresponding secondaries used were anti-rabbit Alexa Fluor 488 conjugated (Jackson ImmunoResearch, 1:300 dilution) and anti-goat Fluorescein (FITC, Jackson ImmunoResearch, 1:100 dilution).

2.4.7 Western blotting

Expression of Orai1 protein was analysed using Western blotting of cell and tissue lysates. In brief, VSMC cultured in a 6-well plate to more than 80% confluency or the minced tissue samples were lysed in cell extraction buffer (Invitrogen, UK) with protease and phosphatase cocktail inhibitors (Sigma). The protein lysate then was transferred to 1.5 mL microcentrifuge tubes and stored at -80°C until further usage. For protein quantification, the samples were thawed on ice for 30 minutes and centrifuged at 14000 g for 15 minutes at 4 °C and the supernatant was transferred into fresh microcentrifuge tubes which were kept on ice until use. Protein quantification was conducted using a bicinchoninic acid (BCA) assay kit (Pierce Biotech, UK) in a 96-well plate. Bovine Serum Albumin Standard (BSA) (2 mg/mL) was used as protein standard. An iMark Microplate Absorbance Reader with Microplate Manager 6 Software was used to determine absorbance at a

wavelength of 562 nm. A line of best fit was obtained by plotting data from the BSA standards before quantifying the correlation coefficient (r^2), with >0.99 being the threshold for acceptance. The absorbance of each sample was then fitted to this line to estimate the protein concentration. The samples were resolved via SDS/PAGE on a 4.5% acrylamide gel. Sample loading volume for gel electrophoresis was determined according to the calculated concentration from BCA assay. Electrophoresis was conducted at 180 V for 1 hour, constantly checking to avoid buffer leaks and interruption to power supply. Once sample running was complete, the gel was removed from the tank and Criterion Blotter system was used to transfer proteins onto a polyvinylidene fluoride (PVDF) membrane. Blots were incubated in blocking solution (50mM Tris, 150mM NaCl, 5% milk, 0.25% Tween20) at room temperature for 1 hour. Blots were then incubated with rabbit anti-chicken PDGFR β antibody (1:1000; Cell Signalling Technology, UK), washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Thermo Fisher, UK) for 1 hour at room temperature. Blots were washed and bound antibody was detected by enhanced chemiluminescent HRP Substrate (Merck Millipore). Membranes were imaged using Syngene's G: BOX Chemi XT4 and GeneSys image acquisition software.

2.4.8 Intracellular Ca^{2+} (Ca^{2+}_i) measurement using FlexStation 3

Store-operated calcium entry (SOCE) was measured by quantifying the change in intracellular Ca^{2+} (Ca^{2+}_i) concentration. Thapsigargin (TG; Sigma, UK) was used to deplete the stores and evoked Ca^{2+} entry from the extracellular space. Ratiometric calcium indicator dye Fura-2 (Invitrogen, UK) and the Flex Station 3 fluorescence plate reader (Molecular Devices, USA) was used for the SOCE measurement in a 96 well plate (Figure 2.9).

Fura-2 is used to quantify intracellular free Ca^{2+} concentration in a range of cell types (Oakes, Martin et al. 1988). Fura-2 AM (acetoxymethyl ester) form is an esterified form of Fura-2 which makes it highly membrane permeable. Once inside the cytoplasm, it is cleaved by non-specific esterases, resulting in the formation of highly ionized Fura-2, which is capable of binding Ca^{2+} with high affinity. Fura-2 is excited at 340 nm and 380 nm and emission recorded at 510 nm. Fura-2 bound to Ca^{2+} is excited at 340 nm, whilst unbound Fura-2 is excited at 380 nm. An increase or

decrease in intracellular Ca^{2+} results in a change in the ratio of emission at 510 nm when excited with 340 and 380 nm ($\Delta\text{F}_{340/380}$). Increase in intracellular Ca^{2+} results in proportional increase in this ratio.

Briefly, VSMC were grown to confluency in clear-bottom, poly-L-lysine treated 96-well-plates (Corning) in the culture medium at 37°C in a 5% CO_2 incubator. Cells were treated with 2 μM Fura-2AM in 1.5 mM Ca^{2+} containing standard buffer solution (135 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2 , 8 mM glucose, 10 mM HEPES, 1.5 mM CaCl_2 , pH 7.4; SBS) with 0.01% pluronic acid for 1 hour at 37 °C. Pluronic acid is included to improve the solubility of AM dyes. This was followed by two washes with 1.5 mM Ca^{2+} SBS and a 30-minute incubation at room temperature for de-esterification of Fura- 2 AM before a final volume of 100 μL Ca^{2+} free SBS (135 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2 , 8 mM glucose, 10 mM HEPES, pH 7.4) was added to each well. During these steps, all solutions and cells in 96-well plates were protected from light. Fluorescence was quantified using a medium throughput semi-automated plate reader (Flex Station III) with 340 nm/ 380 nm excitation and 520 nm emission filters. After a minute, 1 μM TG or vehicle (DMSO) in Ca^{2+} free SBS was added to each well (through the automated solution transfer function in the Flex Station III) and fluorescence measurements were continued for 10 minutes. After that, 1.5mM Ca^{2+} containing SBS (Ca^{2+} addback protocol) was added, and fluorescence emission was measured. For Orail inhibitor studies, 10 μM JPIII or vehicle (DMSO) were added during the 30 minutes wash period. All the experiments were performed at room temperature ($21 \pm 2^\circ\text{C}$). Each measurement was performed in triplicate and presented as mean $\pm$ standard error of mean (sem). Ca^{2+} signals are presented as ratio of emitted fluorescence ($\Delta\text{F}_{340/380}$) and the results are presented as time course plot.

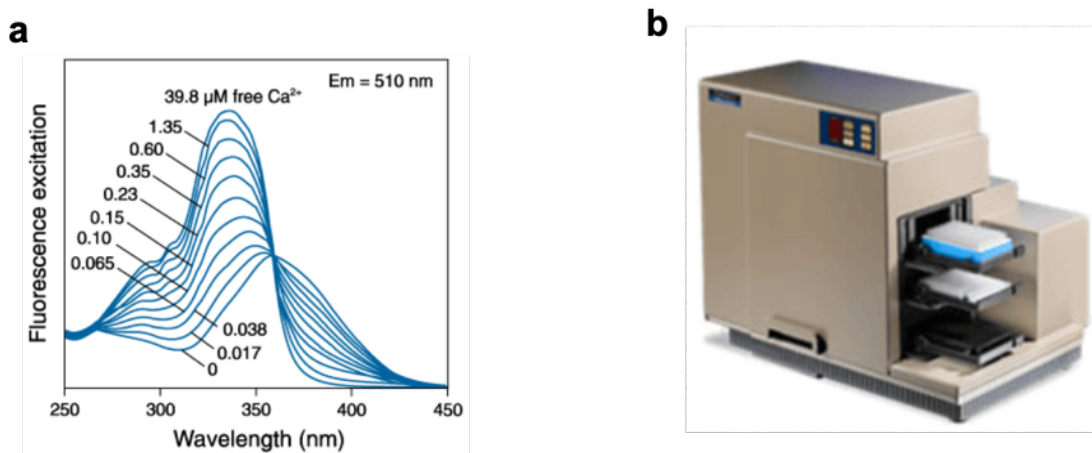


Figure 2.9 Intracellular Ca^{2+} measurement using FlexStation 3.

(a) The fluorescence excitation spectra of Fura-2 at different free Ca^{2+} concentrations. As intracellular Ca^{2+} increases, Fura-2 fluorescence emission intensity at 510 nm increases at excitation 340 nm while decreasing at 380 nm. The image indicates two excitation wavelengths. Image adapted from Life Technologies. (b) Image of Flex Station 3. Image taken from: www.moleculardevices.com.

2.5 Data collection and Statistical analysis

Data were recorded in Microsoft Excel sheet (Microsoft, USA) and images were captured as JPEG images. All data was stored on the secure University of Leeds server. Statistical analyses were performed using Origin v8.6 (OriginLab, USA). Unless specified, all data are expressed as mean values $\pm$ standard error of mean (sem). N is the total number of repeats/mice. For comparisons between two groups, statistical significance was determined using an unpaired two-tailed Student's *t*-test. Paired *t*-test were undertaken when the samples were related to one another. Multiple groups were compared using one-way or two-way analysis of variance (ANOVA) with Bonferroni correction. A value of $p < 0.05$ was considered statistically significant. *, ** and *** were used to indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Outliers with more than ± 3 standard deviations from the mean were excluded from the analysis.

Chapter 3 : Results I - VSMC lineage tracing studies in AAA mouse models

VSMC found in different locations of the vascular network are embryonically derived from different primordial layers in the developing embryo, such as mesoderm (descending thoracic aorta, abdominal aorta) and neural crest (aortic root, ascending aorta, and arch). A defining characteristic of VSMC is their flexibility to change in response to arterial injury or disease from a contractile differentiated state to a synthetic de-differentiated state. During this phenotypic transition, the expression of traditional SMC identification markers such as ACTA2, TAGLN, and SM-MHC are down-regulated (Dobnikar et al., 2018; Qian et al., 2022). As a result, they can resemble other cell types, such as macrophages (Allahverdian et al., 2018; Bentzon and Majesky, 2018). Therefore, they cannot reliably be detected by conventional methods – using antibodies raised against these SMC contractile protein markers using immunohistochemical techniques. Therefore, de-differentiated VSMC can evade detection and go unnoticed.

In this chapter, I have sought to track VSMC activity in the aorta of mice with surgically induced AAA to study this VSMC de-differentiation process. To achieve this, I have developed and validated SMC -specific lineage tracing mice.

3.1 Validation of tamoxifen inducible SMC specific CreER^{T2} mice

I sought to generate a lineage tracing mouse with temporal control so that only the mature SMC in the adult mouse would be labelled and not any cells that transiently express VSMC identification markers during embryonic development. The first step was to obtain and confirm that the SM-MHC.CreER^{T2} line from the Jackson Laboratories (USA) did indeed induce Cre recombinase activity specifically in SMC rich tissue. To this end the male SM-MHC.CreER^{T2} line was bred with Rosa26R.*LacZ* reporter females to generate a line with tamoxifen inducible LacZ expression in cells expressing the SM-MHC.CreER^{T2} (i.e., SMC lineage cells only). Under basal conditions LacZ expression is suppressed by a floxed up-stream stop sequence. When tamoxifen is

administered, Cre is expressed in SM-MHC expressing cells, which in turn leads to removal of the loxP flanked stop codon resulting in removal of repression of LacZ in vascular, bladder, and intestinal SMCs. We followed a previously published dosing regimen of 2 mg per day of tamoxifen for 5 consecutive days delivered by intraperitoneal injection (Shankman et al., 2015).

Tamoxifen treated SM-MHC.CreER^{T2}LacZ⁺ animals were compared with SM-MHC.CreER^{T2}LacZ⁻ animals. Tissues were harvested seven days after the last dose of tamoxifen. In the first set of experiments, the organs were fixed and whole mount stained for blue X-gal (Figure 3.1). Positive blue X-Gal staining was observed in the aorta and the bowel in addition to the vascular bundles of all tissues investigated. No staining was observed in skeletal or cardiac muscle. There was a slight stain in the white adipose tissue. No staining was observed in the tissues of tamoxifen injected SM-MHC.CreER^{T2}+LacZ⁻ animals (Figure 3.1). Next, Blue Gal staining of aortic cryosections was performed to confirm the location of the cells giving rise to blue stain to be between the elastic laminae, i.e., the medial layer of staining (Figure 3.2).

To further evaluate the Cre mediated recombination leading to LacZ expression, LacZ mRNA levels were measured by quantitative RT-PCR in independent experiments. Consistent with the results of the whole mount staining, LacZ mRNA expression was highest in the aorta followed by bowel compared to the LacZ⁻ littermate controls (Figure 3.3).

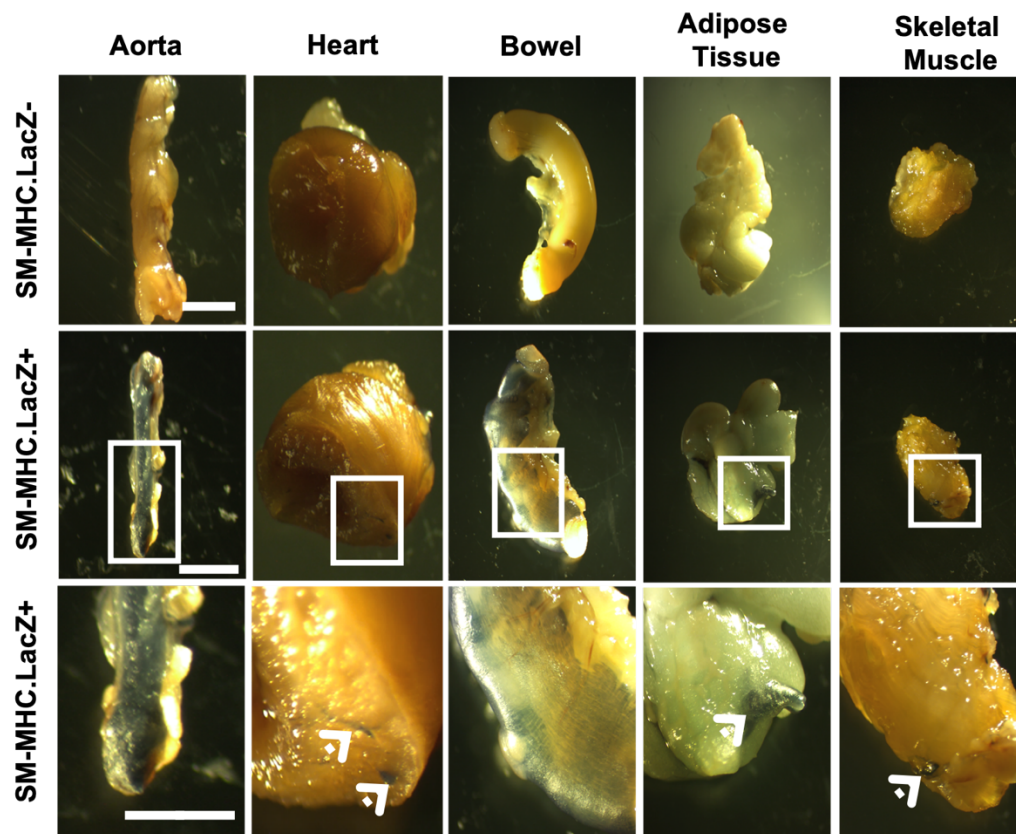


Figure 3.1 Blue X-gal staining of whole organs from tamoxifen treated SM-MHCCreER^{T2}LacZ- and SM-MHCCreER^{T2}LacZ+ animals.

Blue X-gal staining of tissues from SM-MHCCreER^{T2}LacZ- (SM-MHC.LacZ-; top row) and SM-MHCCreER^{T2}LacZ+ (SM-MHC.LacZ+; middle row) mice injected with tamoxifen. Bottom row shows the magnified images of white boxes in middle row. Regions with strong LacZ staining are indicated by arrowheads. Scale bar: 500 μ m. (work done by Dr Marc Bailey)

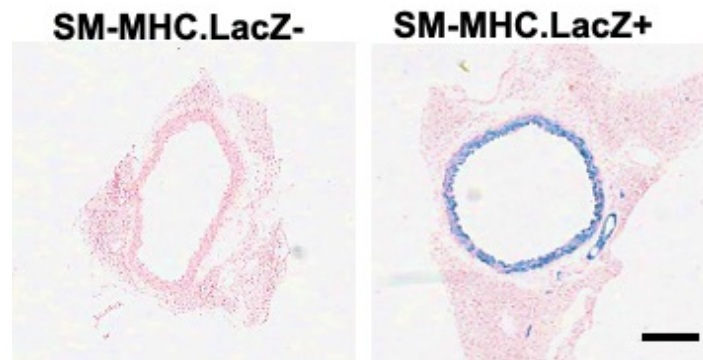


Figure 3.2 Blue X-gal staining of aortae from tamoxifen treated SM-MHCCreER^{T2}LacZ⁻ and SM-MHCCreER^{T2}LacZ⁺ animals.

Blue X-gal staining of aortae from SM-MHCCreER^{T2}LacZ⁻ (SM-MHC+/LacZ⁻; left panel) and SM-MHCCreER^{T2}LacZ⁺ (SM-MHC+/LacZ⁺; right panel) mice injected with tamoxifen. Scale bar: 300 μ m. (work done by Dr Marc Bailey)

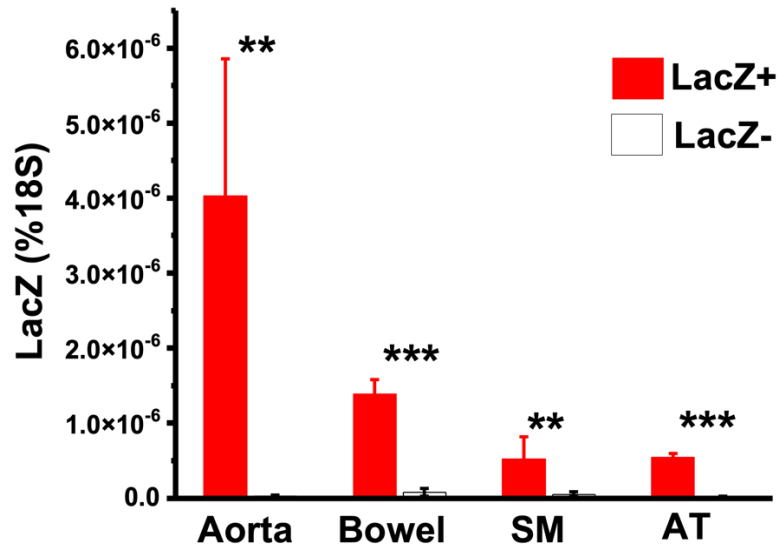


Figure 3.3 Quantitative RT-PCR analysis of LacZ mRNA expression in whole organs of SM-MHCCreER^{T2}LacZ+ and SM-MHCCreER^{T2}LacZ- mice.

Mean mRNA expression of LacZ in whole organs of SM-MHCCreER^{T2}LacZ+ (LacZ+) and SM-MHCCreER^{T2}LacZ- (LacZ-) mice (SM: skeletal muscle; AT: adipose tissue). Individual mRNA levels were normalized to that of housekeeper (18S). n=3 for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively; NS: not significant.

3.2 Validation of SMC specific mTmG lineage tracing mice

Following validation of the inducible SMC specific Cre male mice (SM-MHCCreER^{T2}; described in section 3.1) we crossed these with a double-fluorescent Cre reporter line ROSAmT/mG female mice from Jax Laboratories as described in methods section 2.2.3. This reporter uses the same basic design as the LacZ reporter with a protecting floxed stop codon before the mGFP insert, but constitutive expression of membrane localised red fluorescence (mT/mTom) in all cells. Upon Cre mediated recombination, loxP flanked stop codon was removed which switches on membrane localised GFP (mG/mGFP) expression only in cells expressing SM-MHC. Using this strategy VSMC can be detected by their green fluorescence even when they have lost expression of their standard expression markers during de-differentiation.

The SM-MHCCreER^{T2}mTmG^{+/+} dual colour reporter line was viable and fertile without any visibly apparent phenotype. Based on the results from the LacZ reporter animals four key tissue types were examined for validation of the SMC-specificity in new transgenic line: aorta, bowel, skeletal muscle, and cardiac muscle using the same tamoxifen dosing regimen as described for the LacZ mice. Male SM-MHCCreER^{T2}mTmG^{-/-} littermates treated with tamoxifen were used as controls. SM-MHCCreER^{T2}mTmG^{+/+} littermates injected with corn oil are also used as additional control. The first cohort of mTmG line were sacrificed and examined 1 week after the last tamoxifen injection. The tissues were harvested, fixed, cryosectioned and stained with nuclear dye, DAPI.

Figure 3.4 shows the fluorescence images of the tissue sections. The SM-MHCCreER^{T2}mTmG^{-/-} negative controls stained positive for DAPI, but as expected no red or green fluorescence was seen other than mild autofluorescence of the elastin (Figure 3.4 first column). Corn oil injected SM-MHCCreER^{T2}mTmG^{+/+} tissues expressed strong mTom exhibiting red fluorescence throughout irrespective of the cell type observed (Figure 3.4 middle column). In the tissues of tamoxifen injected Cre positive, SM-MHCCreER^{T2}mTmG^{+/+} mice, the red fluorescence in the SMC was entirely replaced by green fluorescence (mGFP) in the aortic and bowel layers indicating successful Cre mediated recombination (Figure 3.4c & 3.4f).

Despite some autofluorescence from the elastic laminae, the VSMC were identifiable within the aorta. No green fluorescence was seen in the skeletal and cardiac myocytes which all appeared red (Figure 3.4i & 3.4l). Unexpectedly, a small percentage of green cells indicating mGFP expression were observed in the layers of aortae and bowel sections of the corn-oil injected SM-MHCCreER^{T2}mTmG^{+/+} control animals. This suggests slightly leaky expression of the Cre recombinase in the Cre-loxP system (Figure 3.4b & 3.4e).

Furthermore, whole mount staining of abdominal aorta was prepared and examined under confocal microscopy. This analysis confirmed the presence of green fluorescence only in the VSMC and red fluorescence in the intima and adventitial layers of the aorta (Figure 3.5). These validation experiments confirmed the suitability of the transgenic line for SMC lineage tracing studies and hence they were used for further studies in this the project.

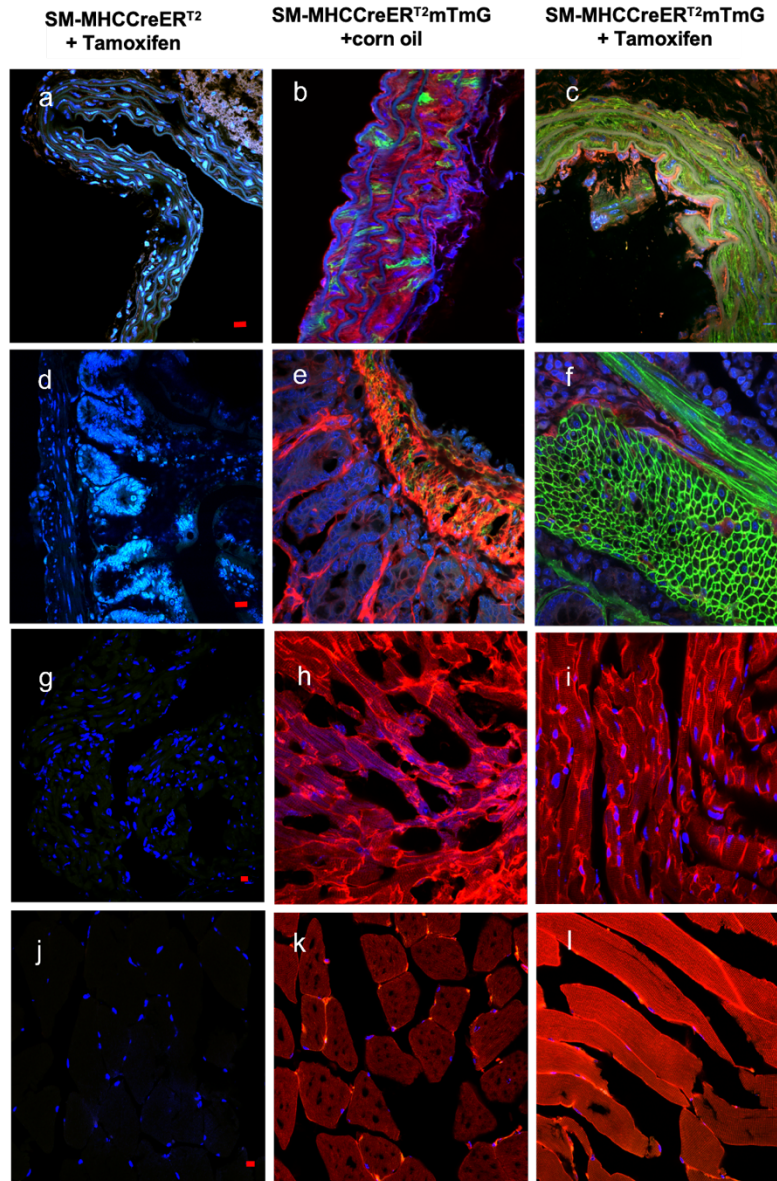


Figure 3.4 Tissue-specific and inducible Cre-mediated expression of mGFP.

(a-l) Confocal images of tissue (aorta **(a-c)**, bowel **(d-f)**, skeletal muscle **(g-i)** and cardiac muscle **(j-l)**) sections of tamoxifen injected SM-MHCCreER^{T2}mTmG^{-/-} **(a,d,g,j)**, corn oil injected SM-MHCCreER^{T2}mTmG^{+/+} **(b,e,h,k)** and tamoxifen injected SM-MHCCreER^{T2}mTmG^{+/+} mice **(c,f,i,l)**. Nuclei are stained with DAPI. Scale bar: 100 μ m.

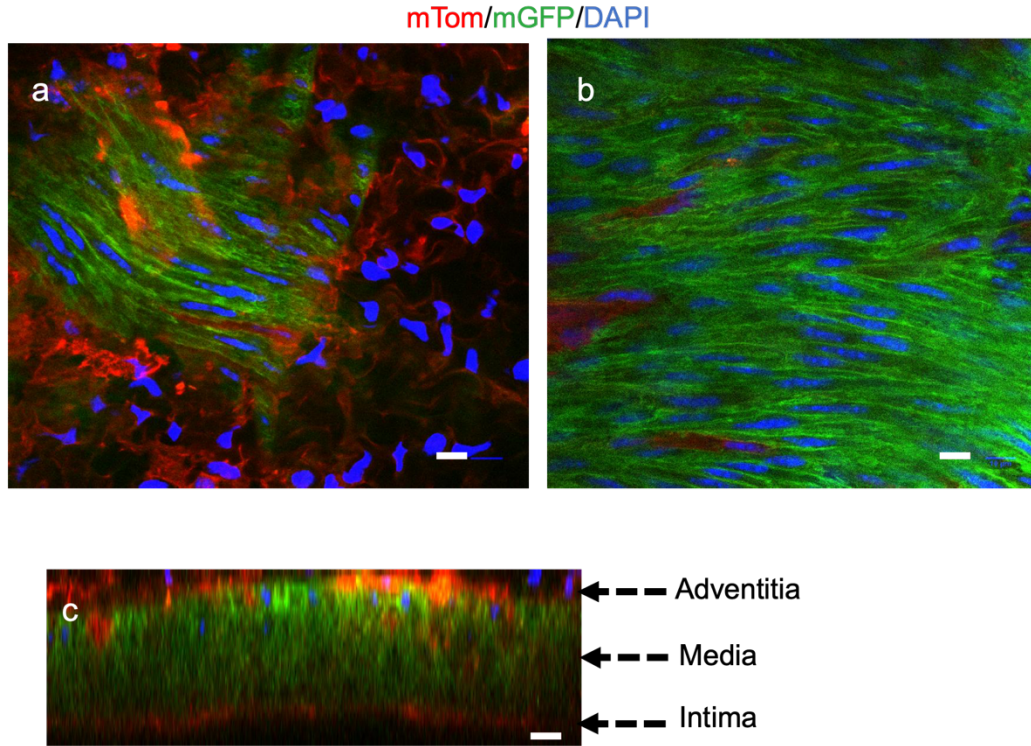


Figure 3.5 mGFP labelling of SM-MHCCreER^{T2}mTmG^{+/+} aorta after Cre- mediated recombination.

(a-c) Whole mount SM-MHCCreER^{T2}mTmG^{+/+} aorta section showing green labelling in medial vascular smooth muscle cell layer **(a & b)** and red (mTom) in adventitia and intima layers **(c)**. Nuclei are stained with DAPI. Scale bar: 50 μm.

3.3 Generation and analysis of elastase induced AAA in SM-MHCCreER^{T2}mTmG^{+/+} mice

Next, I went on to induce AAA in the SM-MHCCreER^{T2}mTmG^{+/+} line, by using the periaortic application of porcine pancreatic elastase (PPE method) and perform lineage tracing studies on them using fluorescence microscopic (confocal) analysis of aortic sections.

3.3.1 Establishment of elastase induced AAA in SM-MHCCreER^{T2}mTmG^{+/+} mice

Following tamoxifen injection of 8-week-old mice, PPE application was performed in 12 weeks old SM-MHCCreER^{T2}mTmG^{+/+} mice to induce AAA. Two weeks after PPE application, the aortae were harvested for cryosectioning and analysed under confocal microscopy after counterstaining with DAPI. Macroscopically, the aortic lumen of sham-operated control mice appeared normal (non-dilated) throughout the entire length of the aorta, while that of PPE-treated ones appeared dilated (Figure 3.6a). Only the abdominal side of the aorta was exposed to PPE and the spinal side was not exposed. Hence, only the abdominal side wall of the aorta showed significant pathological changes. Unlike the control aorta which showed thick, wavy, and layered elastin layers with organized spindle shaped VSMC arranged between, the PPE treated ones had a disorganized medial layer showing loss of elastin along with disorganised VSMC. (Figure 3.6b-e).

Next, the aortic size was assessed in the histological samples to demonstrate successful induction of AAA. Compared to sham operated control aortae, PPE aortae showed significant increase in diameter from Day 1 post PPE treatment (Figure 3.7). The dilatation progressed over time, although day 28 measurements unexpectedly showed reduction in the diameter (Figure 3.7). These data confirm successful AAA induction by PPE application in the SM-MHCCreER^{T2}mTmG^{+/+} transgenic line.

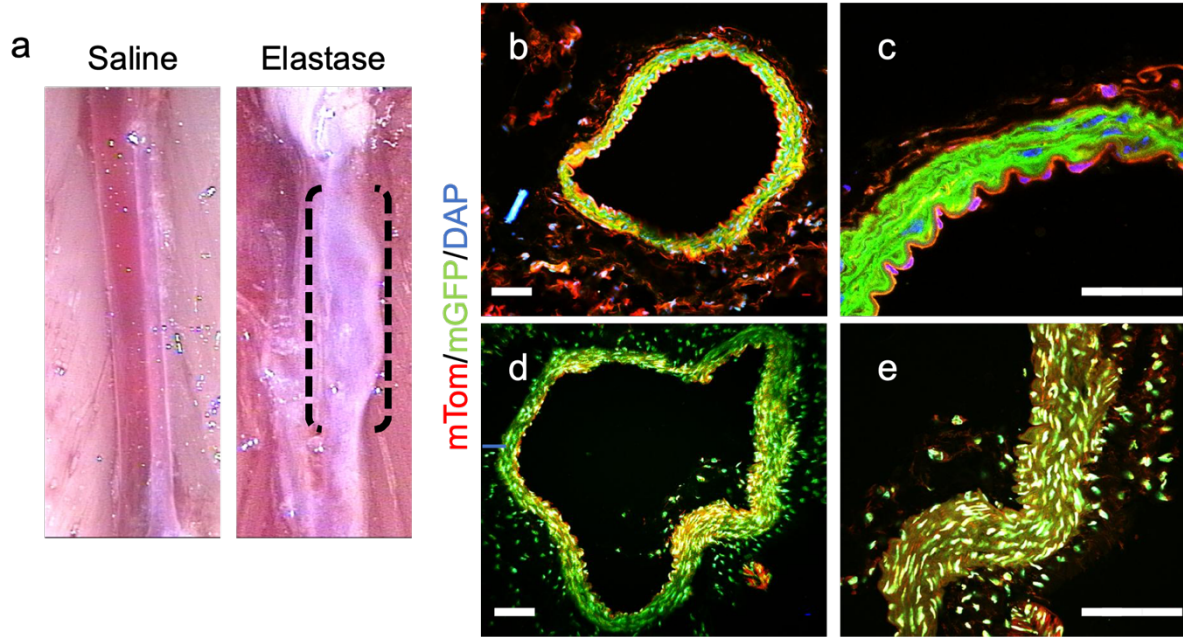


Figure 3.6 Elastase induced AAA in SM-MHCCreER^{T2}mTmG^{+/+} mice.

(a) Representative images of sham-operated (Saline) and PPE applied (Elastase) SM-MHCCreER^{T2}mTmG^{+/+} aortae. **(b&c)** Confocal images of transverse sections of saline-applied abdominal aorta of SM-MHCCreER^{T2}mTmG^{+/+} (**c**: higher magnification view). **(d&e)** Confocal images of transverse sections of PPE-applied abdominal aorta of SM-MHCCreER^{T2}mTmG^{+/+} (**e**: higher magnification view). Cells are counterstained with DAPI. Scale bar: 100 μ m.

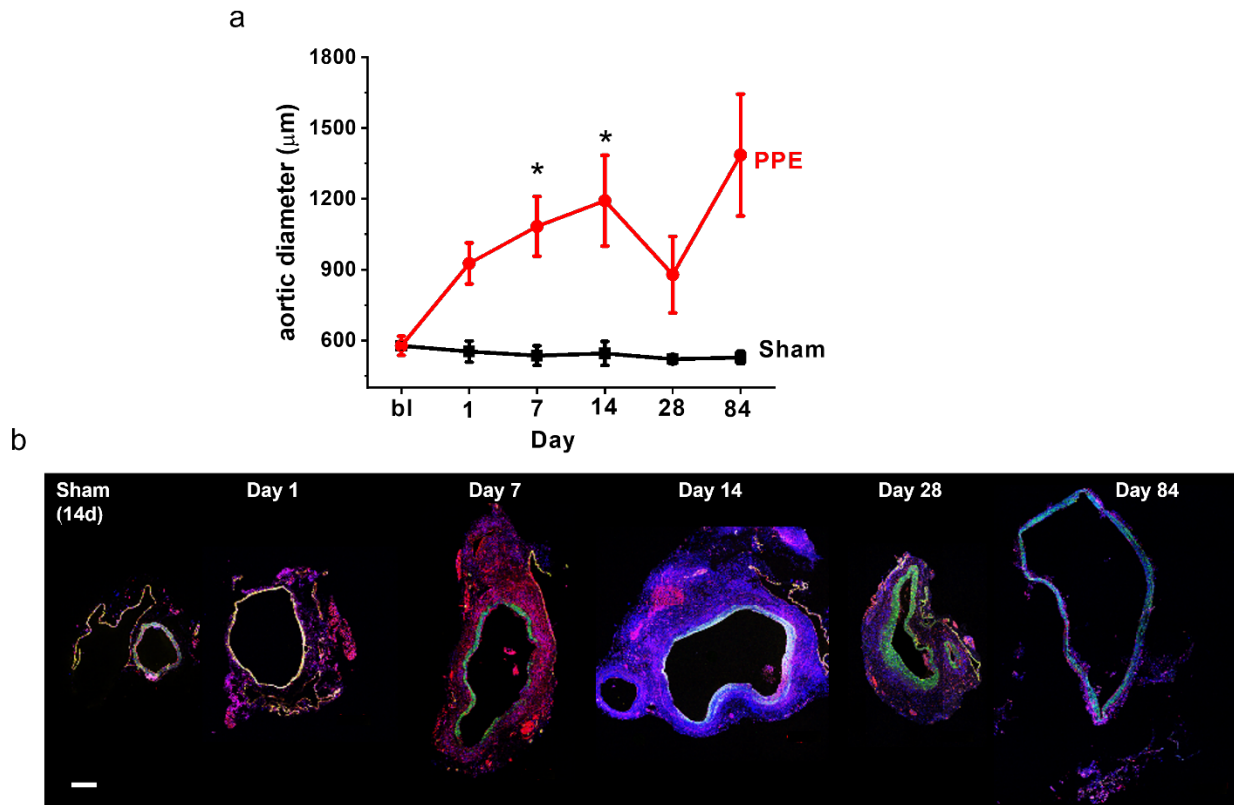


Figure 3.7 Quantification of dimensions of PPE-induced AAA in SM-MHCCreER^{T2}mTmG^{+/+} mice.

(a) Mean aortic diameter of PPE applied and sham operated SM-MHCCreER^{T2}mTmG^{+/+} animals at baseline (bl) and 1, 7, 14, 28 and 84 days post-surgery. **(d)** Representative images of those used for the quantification analysis showing control aorta at 14 days (first image) and PPE applied aorta at 1, 7, 14, 28 and 84 days post-surgery. n=3-6 for each data point; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. Scale bar: 200μm. (part of the work done by Dr Lucy Craggs).

3.3.2 VSMC Lineage tracing studies in PPE AAA

Next, I went on to perform lineage tracing studies in PPE AAA compared to sham operated mice in the SM-MHCCreER^{T2}mTomG^{+/+} line. Six time points were studied - day(s)- 0 (baseline), 1, 7, 14, 28, and 84 following the surgery.

Increase in mGFP and mTom cells by AAA induction

First, the number of DAPI+ (blue), mGFP+ (green) and mTom+ (red) cells were counted per region of interest. In the sham operated controls, from baseline to day 84, the total number of cells (DAPI+) remained constant. Also, there was negligible increase in green cells in some animals and red cells remained at same level (Figure 3.8). In contrast, in the PPE-applied aortae I observed a progressive increase in the total number of cells (DAPI+) until day 28 followed by a decline in total cell number by day 84. There was a significant increase in lineage traced SMC (mGFP+) cells in PPE AAA, peaking at day 14 before plateauing and decreasing back towards baseline by day 84. Non-SMC origin (mTom+) cells also increased gradually in PPE treated aortae until 28 days and then dropped back towards baseline by day 84 (Figure 3.8).

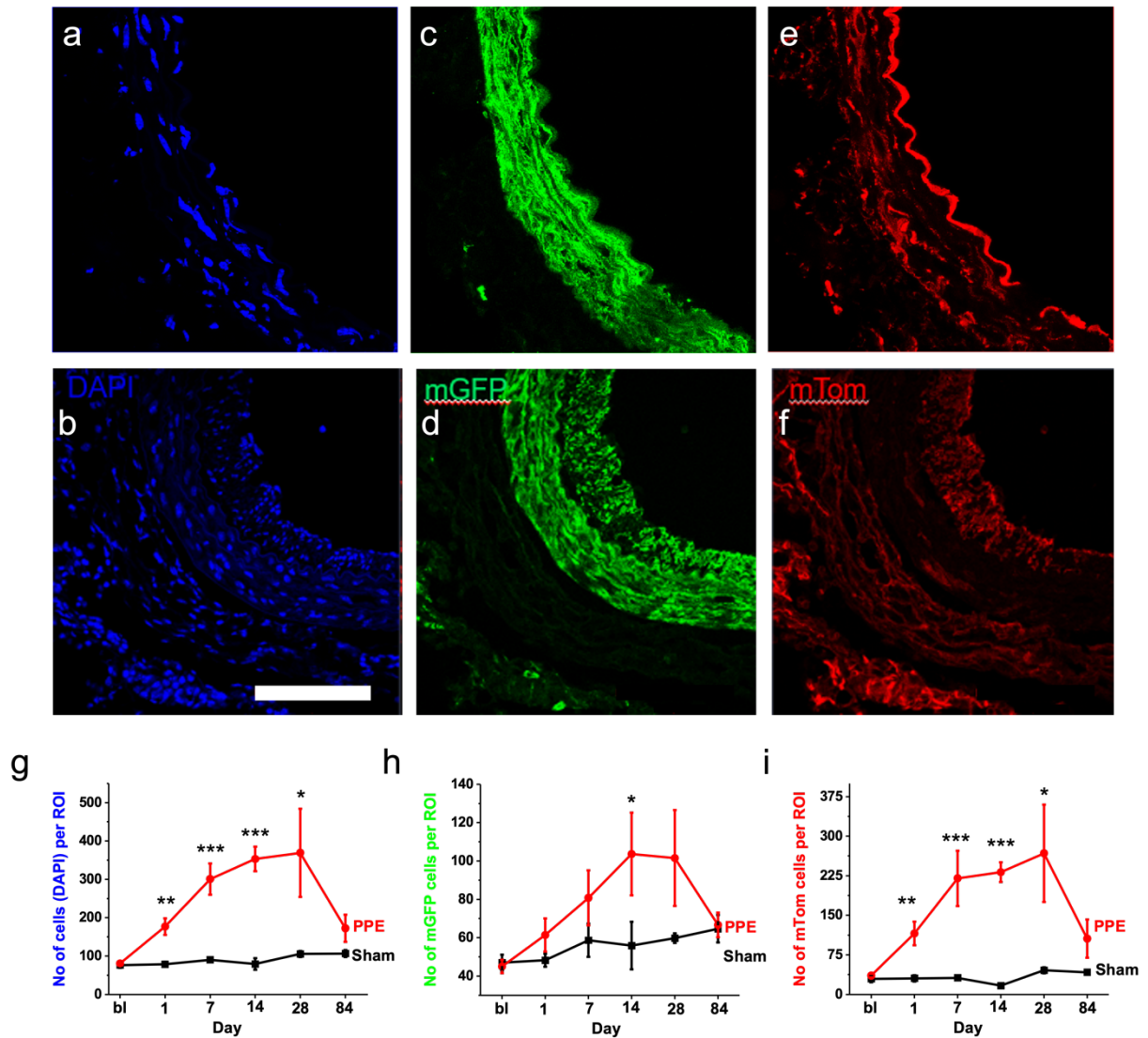


Figure 3.8 Cell numbers in aortae of PPE-induced AAA in SM-MHCCreERT²mTmG^{+/+} mice.

(a-f) Representative images and of those used for the quantification analysis showing all/DAPI (a&b), mGFP (c&d) and mTom (e&f) cells. Nuclei are stained with DAPI (a, c & e are at baseline; b, d & f are on day 14). (g-i) Average count of all (g), mGFP (h; mG) and mTom (i; mT) cells in regions of interest in aorta of PPE applied and sham operated SM-MHCCreERT²mTmG^{+/+} animals at baseline (bl) and 1, 7, 14, 28 and 84 days post-surgery. n=3-6 per group; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. Scale bar: 100 μ m. (part of the work done by Dr Lucy Craggs).

Lineage traced VSMC differentiation status

Next, I sought to determine the differentiation status of the lineage traced VSMC, adoption of a macrophage-like phenotype in the lineage traced VSMC and evaluation of the number of infiltrating macrophages and any non-lineage traced VSMC expressing VSMC identification markers in both sham operated and PPE-induced AAA aortic sections.

In the first set of experiments, I co-stained for SMC marker α -SMA and macrophage marker Mac2; and DAPI was used to counterstain the nuclei. I first analysed the differentiation status of the lineage traced VSMC (mGFP+) based on the co-staining of ratio of α -SMA. mGFP+/SMA+ cells represent lineage traced VSMC that are differentiated. mGFP+/SMA- cells represent lineage traced VSMC that are de-differentiated. While ~100% mGFP+ cells had α -SMA stain in the sham controls, in the PPE-AAA model there was a gradual reduction in α -SMA positive cells (differentiated SMC) until day 14 after which there was evidence of re-differentiation (Figure 3.9; from $82.4 \pm 5.3\%$ on day 1 reducing to $55.5 \pm 12.5\%$ on day 14 and then increasing to $83.5 \pm 11.1\%$ on day 84). The mGFP+ counting showed increase in lineage traced VSMC in these PPE AAA aortae (Figure 3.8). Taken together these data suggest an increase in VSMC number and significant phenotype switching initially that then reverts to a differentiated phenotype later in the timeline.

Non VSMC with VSMC markers

Next, I interrogated the non-VSMC (mTom+ cells) for expression of the classic VSMC identification marker, α -SMA (mTom+/SMA+) likely representing myofibroblasts. These cells were very small in number and remained generally constant in both sham and PPE groups throughout the study period (Figure 3.9b).

Macrophages and Macrophage like cells

Next, I sought to examine lineage traced VSMC adopting a macrophage like phenotype (mGFP+/Mac2+) and infiltrating macrophages of non VSMC origin (mTom+/Mac2+). I observed

a small number (~3%) of mGFP+/Mac2+ cells on day 14 – the maximal time of remodelling seen in the aorta, but this was not significantly different from sham controls. (Figure 3.10). I also observed a very small percentage (~5%) of mTom+/Mac2+ cells in the sham groups (likely patrolling macrophages). In the PPE group, a small (~15%; but not reaching statistical significance) number of mTom+/Mac2+ cells were observed during the early phase of the aneurysm (day 1 and 7) suggesting an early response from infiltrating immune cells (macrophages) that returned to baseline by day 14 (Figure 3.10).

Validation using separate antibodies.

To further validate the above data obtained by lineage tracing studies, further experiments were performed using separate markers of VSMC contractile status (SM-MHC) and macrophages (CD68) at a single time point (14 days post PPE induction). These data confirm the de-differentiation of the lineage traced VSMC at day 14 (Figure 3.11). There was no significant difference observed in CD68 expression in SMC and non-SMC lineage cells of PPE aortae compared to control aortae (Figure 3.11) but overall antibody quality for CD68 was poor.

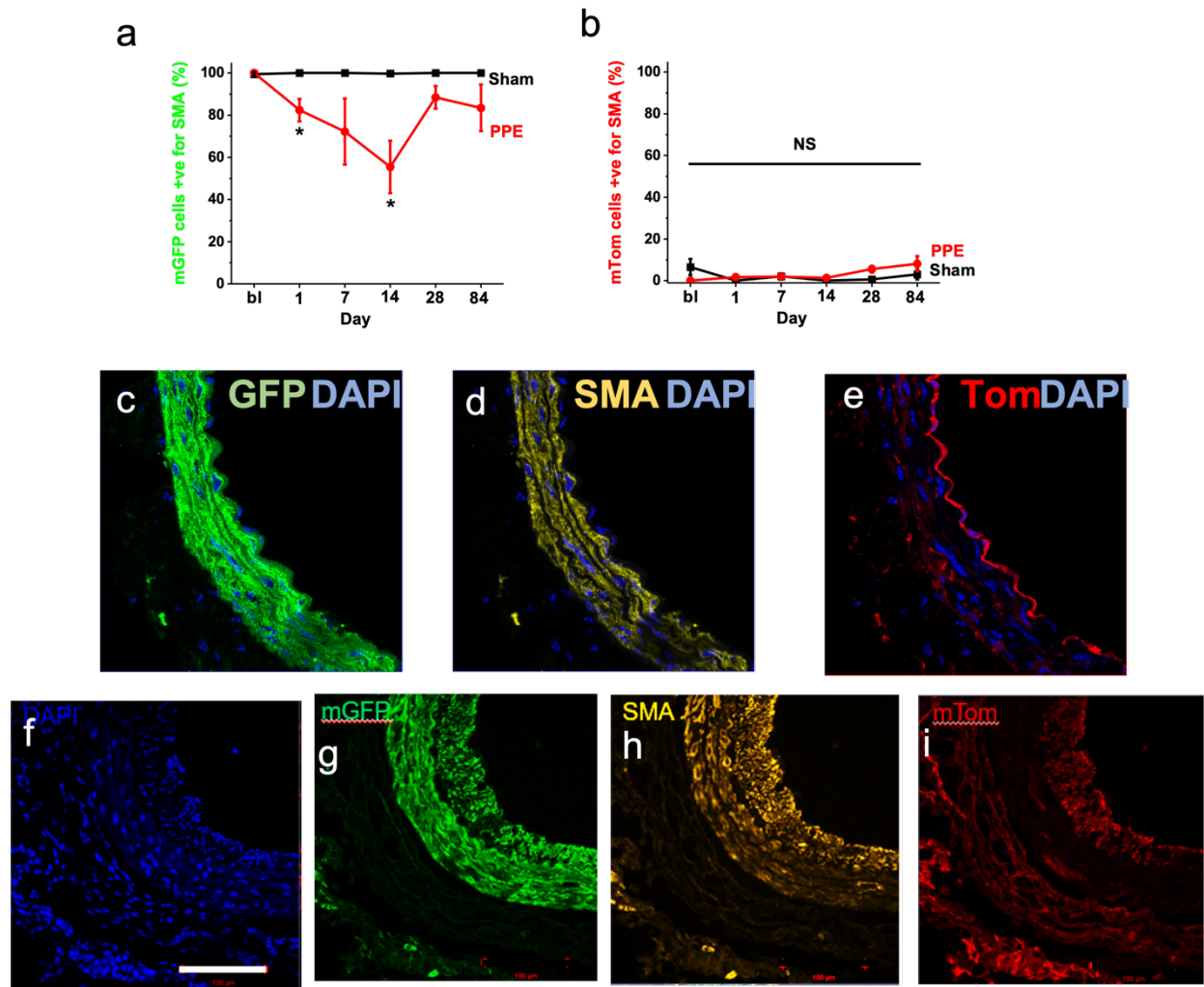


Figure 3.9 Quantification of proportion of α -SMA (α -smooth muscle actin) positive cells in aortae of PPE-induced AAA or sham operated controls in SM-MHCCreERT²mTmG mice.

(a-b) Percentage of α -SMA (yellow) positive mGFP **(a)** and mTom **(b)** cells in regions of interest in aorta of PPE applied and sham operated SM-MHCCreERT²mTmG animals at baseline (bl) and 1,7,14,28 and 84 days post-surgery. **(c-i)** Representative images of those used for the quantification analysis showing all **(f)**, mGFP **(c&g)**, yellow **(d&h)** and mTom **(h&i)** cells **(c-e)** are at baseline; **f-i** are on day 14). n=3-6 per group; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant. Scale bar: 100 μm. (part of the work done by Dr Lucy Craggs).

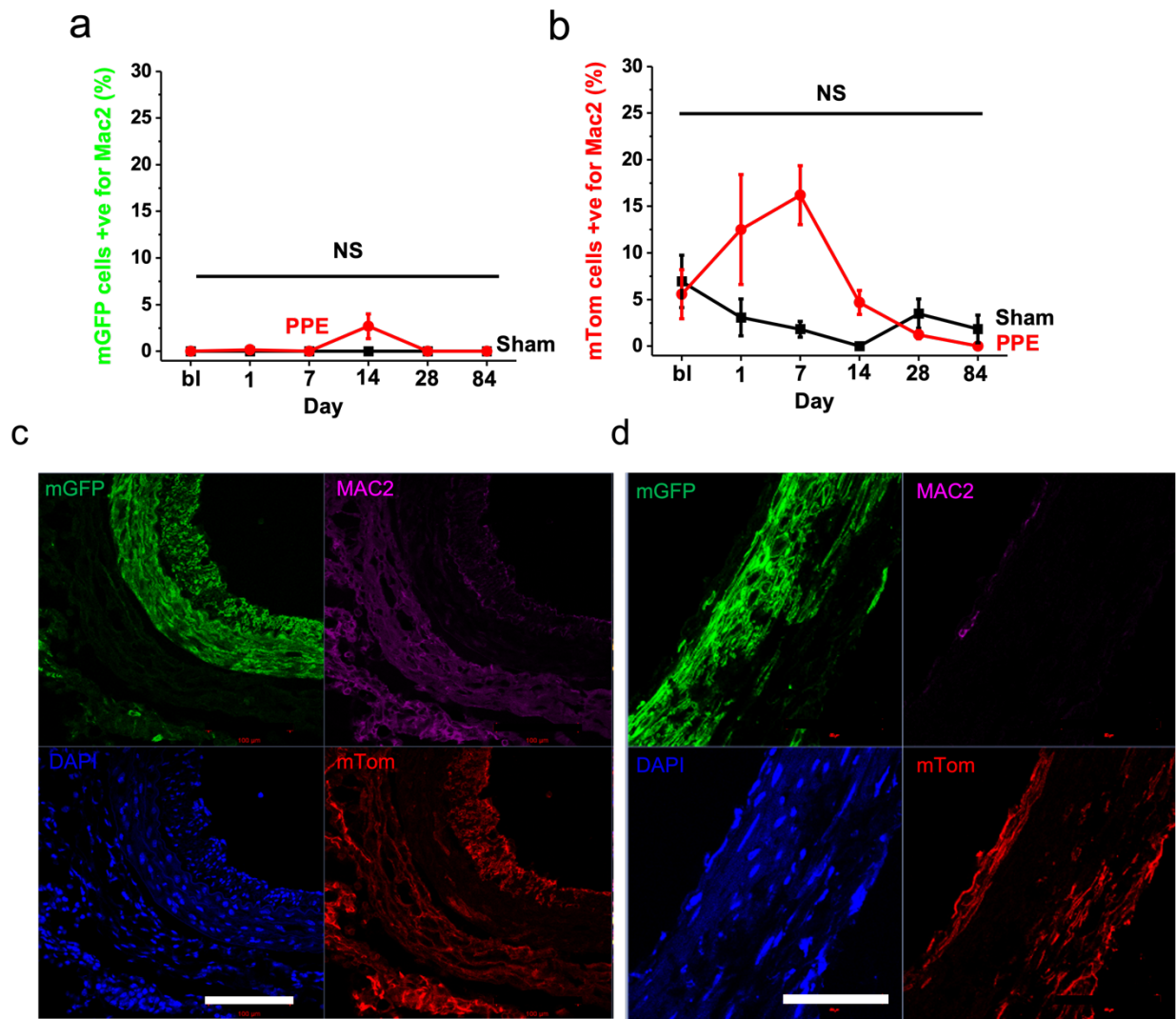


Figure 3.10 Quantification of proportion of Mac-2 positive cells in aortae of PPE-induced AAA in SM-MHCCreER^{T2}mTmG mice.

(a&b) Percentage of Mac2 positive mGFP **(a)** and mTom **(b)** cells in regions of interest in aortae of PPE applied and sham operated SM-MHCCreER^{T2}mTmG animals at baseline (bl) and 1,7,14, 28 and 84 days post-surgery. n=3-6 per group. **(c&d)** Representative images of those used for the quantification analysis showing all (DAPI), green (mGFP), magenta (Mac2) and red (mTom) cells (**c** at baseline; **d** on day 14). NS: not significant. (part of the work done by Dr Lucy Craggs).

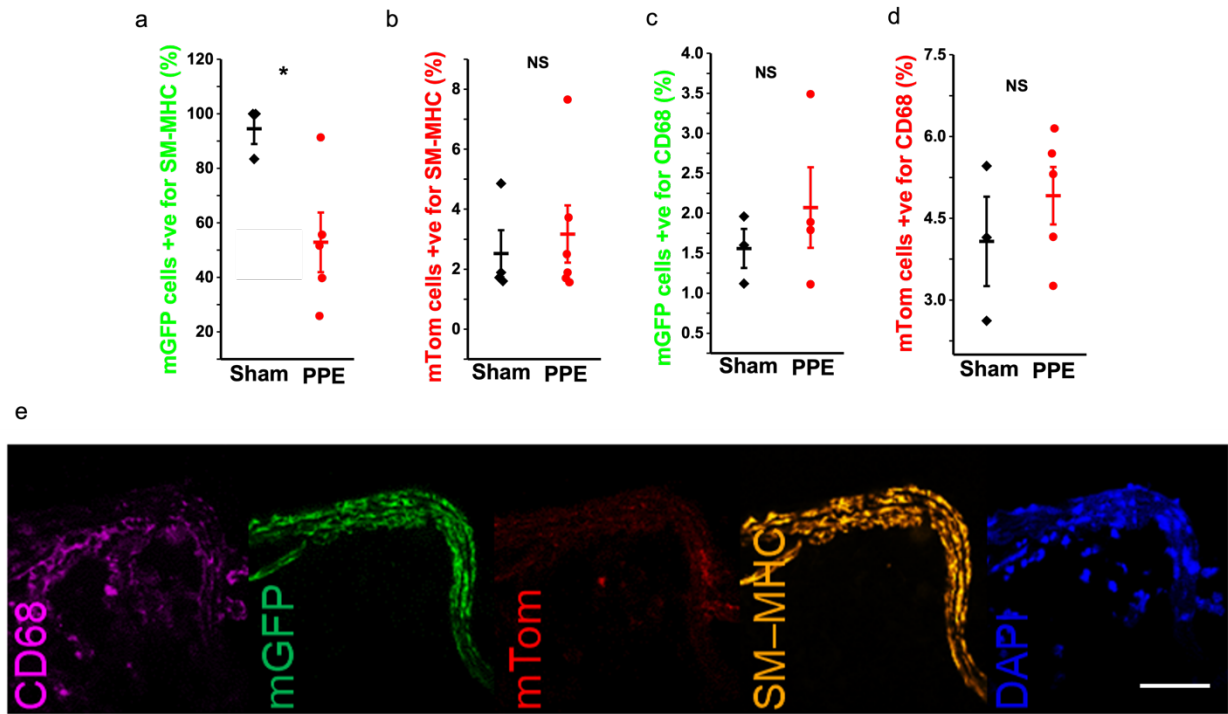


Figure 3.11 Quantification of proportion of SM-MHC positive cells in aortae of PPE-induced AAA in SM-MHCCreERT²mTmG mice.

(a-b) Percentage of SM-MHC (yellow) and CD68 (magenta) positive, mGFP **(a)** and mTom **(b)** cells in regions of interest in aortae of PPE applied and sham operated SM-MHCCreERT²mTmG animals at 14 days post-surgery. **(c)** Representative images of those used for the quantification analysis showing all (DAPI-blue), mGFP, mTom, SM-MHC (yellow) and CD68 (magenta) positive cells. n=3-6 per group; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant. Scale bar: 100 μ m. (part of the work done by Dr Lucy Craggs).

3.3.3 Elevated expression of PDGFR β in AAA VSMC

Given that PDGF signalling is a critical driver of VSMC de-differentiation and remodelling we next performed PDGFR β immunostaining to follow its expression pattern in the lineage tracing experiments. An increase in GFP $^{+}$ /PDGFR β^{+} cells (lineage traced VSMC expressing PDGFR β) was observed in the PPE AAA aortae which reached its peak (and statistical significance) on day 14 ($35.9\pm 7\%$ in PPE Vs $1.5\pm 2\%$ in sham; $p<0.001$), after which it returned to the baseline (Figure 3.12). Interestingly, a similar pattern was observed in Tom $^{+}$ /PDGFR β^{+} cells from day 7 until 28 compared (Figure 3.12; $47.9\pm 11\%$ in PPE Vs $9.5\pm 2.4\%$ in sham on Day 14; $p<0.05$).

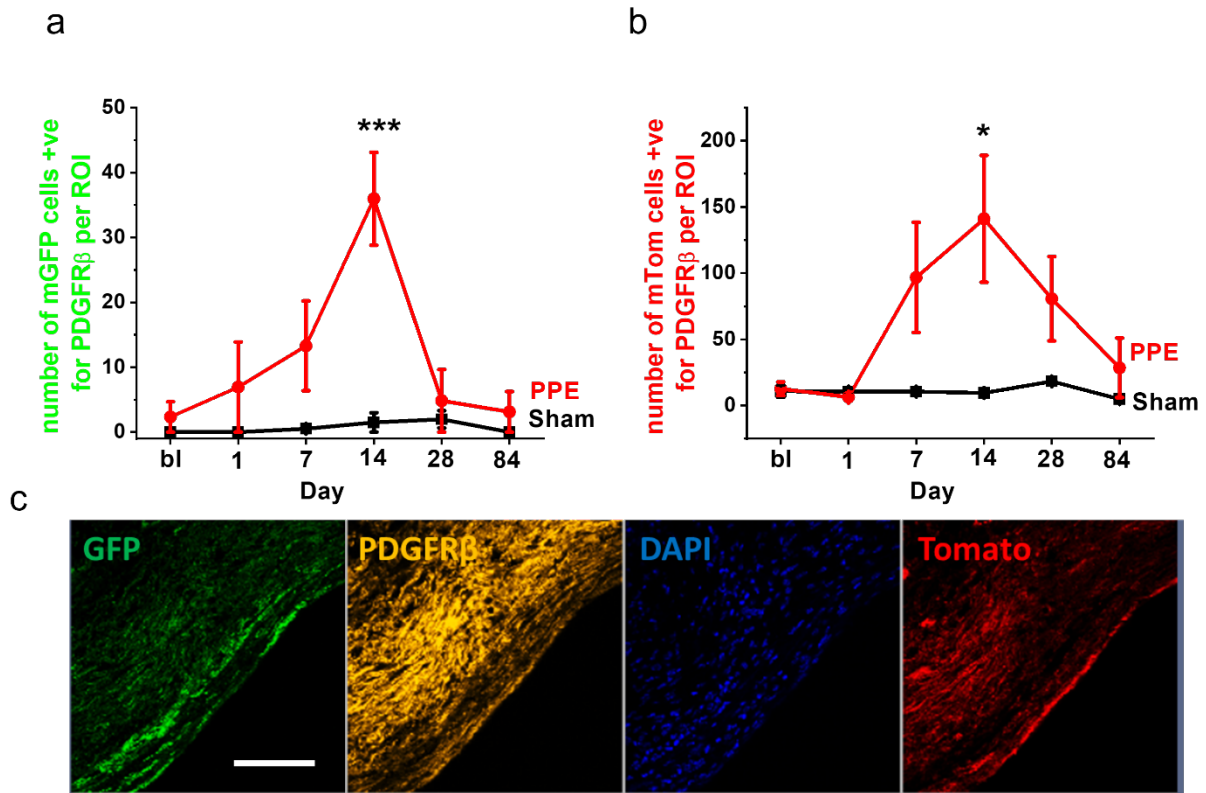


Figure 3.12 Quantification of proportion of PDGFR β positive cells in aortae of PPE-induced AAA in SM-MHCCreER^{T2}mTmG mice.

(a-b) Percentage of PDGFR β (yellow) positive, mGFP **(a)** and mTom **(b)** cells in regions of interest in aortae of PPE applied and sham operated SM-MHCCreER^{T2}mTmG animals at baseline (bl) and 1,7,14,28 and 84 days post-surgery. **(c)** Representative images of those used for the quantification analysis showing all (DAPI-blue), mGFP, mTom and PDGFR β (yellow) positive cells on day 14 post-surgery. n=3-6 per group; *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Scale bar: 100 μ m. (part of the work done by Dr Lucy Craggs).

3.4 Summary of the chapter

In this chapter I have demonstrated successful development of the SM-MHCCreER^{T2}mTmG^{+/+} VSMC lineage tracing line. I induced AAA in this line using peri-adventitial application of PPE which produced AAA as expected. PPE AAA resulted in cellular remodelling in the aorta in both SMC-origin and non-SMC origin cells. I demonstrated the presence of VSMC de-differentiation in response to PPE AAA that coincides with the progressive development of aneurysm in the model before return to baseline. Also, during this time period, there was an accumulation of circulating macrophages but relatively little VSMC differentiation into macrophage like cells or infiltrating immune cells expressing VSMC markers. PDGFR β is highly expressed in lineage traced SMC and non-SMC cells, peaking at the time of maximal aortic dilation.

Chapter 4 : Results II - PDGFR β and downstream signalling in AAA

In the last chapter I demonstrated that in response to vascular injury and AAA formation induced by PPE application to the aorta, the VSMC undergo a phenotypic transition from a contractile to a synthetic phenotype and increase in number during the growth phase of the aneurysm followed by apparent re-differentiation toward a contractile phenotype. The differentiation status of VSMC is influenced by multiple growth factors and signalling pathways. PDGF is one of the major drivers of the synthetic switching and remodelling VSMC through its diverse mechanisms.

Following on from the identification of VSMC de-differentiation and remodelling in the PPE model of AAA using lineage tracing, I next wanted to explore the consequences of enhanced VSMC proliferation on the aortic development / pathology and the impact on AAA formation and progression to determine if the VSMC de-differentiation is functionally relevant to aortic diseases. In order to achieve this, I identified an existing mutant in PDGFR β (PDGFR β^{D849V}) with a single point mutation inducing enhanced PDGFR β phosphorylation under basal conditions and an enhanced response to ligand-induced signalling, resulting in VSMC proliferation in the aorta. A single figure in the manuscript suggested an increase in aortic lumen size in SM22CrePDGFR β^{D849V} pups (Olson and Soriano, 2011).

In this chapter I will describe experiments understanding the consequences of enhanced VSMC de-differentiation and remodelling using two VSMC conditional Cre expressing lines to drive expression of the PDGFR β^{D849V} mutant either constitutively – to study the aortic phenotype, or with temporal control in adulthood with or without application of PPE to induce AAA.

4.1 Constitutively active SM22CrePDGFR β^{D849V} mice

First, we created an SM22CrePDGFR β^{D849V} transgenic line – with constitutively active VSMC targeted Cre driving expression of the transgene (as described in methods). Littermates with SM22CrePDGFR β^{WT} were used as controls. The SM22Cre has already been validated by Jax (<https://www.jax.org/strain/017491>).

4.1.1 Phenotyping of SM22CrePDGFR β^{D849V} transgenic line

Phenotyping from 8 weeks of age showed no significant differences between genotypes in body weight or the individual organ weight/body weight ratios of liver, kidney, and lungs (Figure 4.1). However, the normalised heart and spleen weights of the SM22CrePDGFR β^{D849V} mice were significantly greater (heart: $33\pm 8\%$ and spleen: $45\pm 3\%$) than the SM22CrePDGFR β^{WT} animals (Figure 4.1d). Notably, only 37.5% (3 out of 8) of SM22CrePDGFR β^{D849V} mice survived to 32 weeks of age compared to 75% (3 out of 4) of SM22CrePDGFR β^{WT} mice. Though the exact cause of death was indeterminable, aortic rupture was ruled out by careful post-mortem examination.

Macroscopic observation of the harvested aorta and heart at 32 weeks of age revealed obviously dilated aortae in the SM22CrePDGFR β^{D849V} mice compared to SM22CrePDGFR β^{WT} littermate controls with apparent cardiac enlargement (Figure 4.2a). Next, thoracic, and abdominal aortae were fixed, sectioned, and stained with H & E. Analysis of the H&E images showed significant increase in lumen size and wall thickness in the SM22CrePDGFR β^{D849V} mice compared to SM22CrePDGFR β^{WT} at 4 weeks of age in both the thoracic and abdominal sections (Figure 4.2b-f). Moreover, the VSMC layers in the medial layer appeared disorganised in the SM22CrePDGFR β^{D849V} aorta (4.2d)

There was a progressive increase in aortic volume and anteroposterior (AP) diameter in both suprarenal and infrarenal abdominal aorta of SM22CrePDGFR β^{D849V} animals while that of SM22CrePDGFR β^{WT} was almost static as measured by USS (Figure 4.3 and 4.4). Infrarenal aortic diameter in SM22CrePDGFR β^{D849V} animals was 0.7 ± 0.02 mm at 8 weeks increasing to 0.9 ± 0.06

mm at 32 weeks while that of SM22CrePDGFR β^{WT} was static around 0.6 mm throughout the study period. Suprarenal aortic diameter in SM22CrePDGFR β^{D849V} animals was 1.3 ± 0.05 mm at 8 weeks increasing to 1.7 ± 0.04 mm at 32 weeks while that of SM22CrePDGFR β^{WT} was static around 1.1 mm throughout the study period. Infraarenal aortic volume in SM22CrePDGFR β^{D849V} animals was 5.4 ± 0.3 mm³ at 8 weeks increasing to 8 ± 0.3 mm³ at 32 weeks while that of SM22CrePDGFR β^{WT} was 3.7 ± 0.2 mm³ and 4.3 ± 0.1 mm³ at 8 and 32 weeks respectively. Suprarenal aortic volume in SM22CrePDGFR β^{D849V} animals was 14.7 ± 1 mm³ at 8 weeks increasing to 26.8 ± 2.3 mm³ at 32 weeks while that of SM22CrePDGFR β^{WT} was 9.4 ± 0.2 mm³ and 11.6 ± 0.6 mm³ at 8 and 32 weeks respectively. Alongside vessel dilatation, the distensibility was significantly reduced (i.e., increased vascular stiffness) in SM22CrePDGFR β^{D849V} aortae compared to littermate controls (Figure 4.3c and 4.4c).

Given the more pronounced pathology observed macroscopically in the proximal aorta we also wanted to study this in adult animals. Visualisation with USS was challenging due to the air from the lungs limiting ultrasound penetration. Hence, we developed a whole aorta, micro-computed tomography (μ CT) technique with 3D reconstruction of aorta. COVID-19 disruption limited this experiment, but in the pilot studies, clear dilatation in the arch and thoracic aorta in the SM22CrePDGFR β^{D849V} animals was seen as shown in the representative image (Figure 4.5).

Therefore, in summary, the SM22CrePDGFR β^{D849V} mice develop progressive aortopathy over the period of study with evidence of lumen enlargement and increased cellular content in the medial layer of the vessel wall. The dilation appears most pronounced more proximally in the vessel. Over the period of study, no aortic rupture occurred.

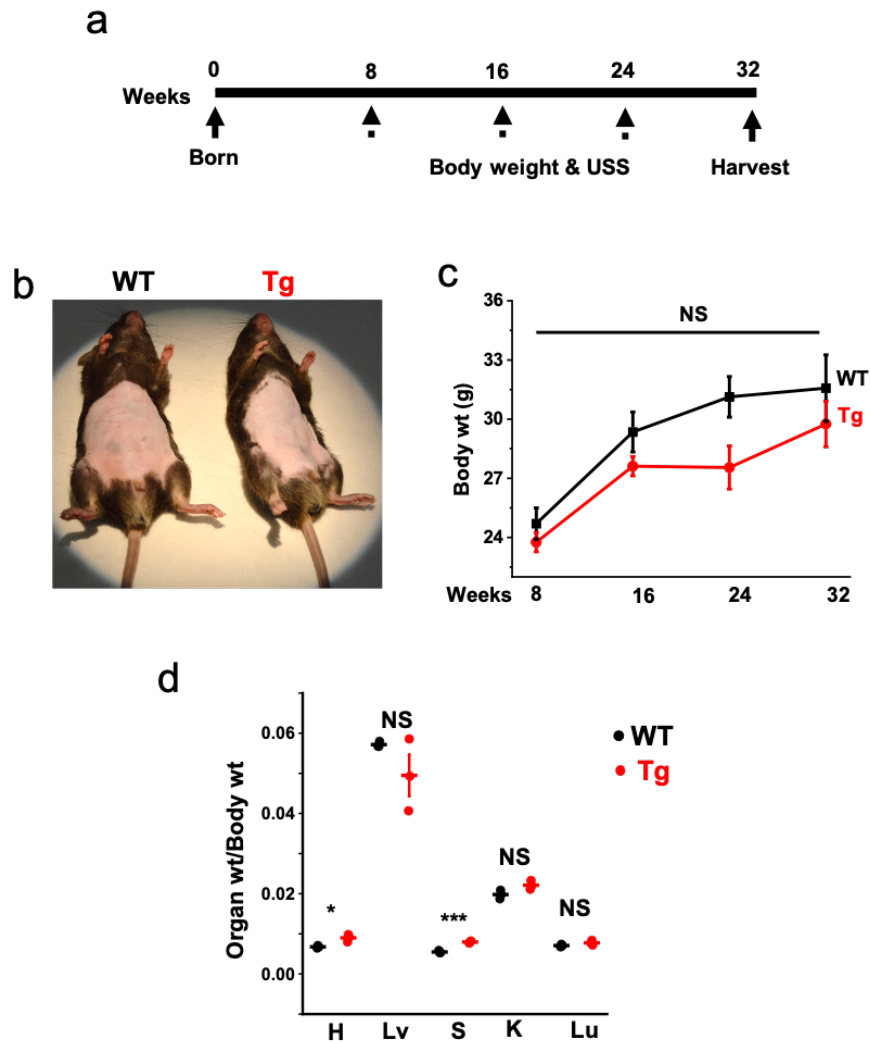


Figure 4.1 Phenotyping of SM22CrePDGFR β^{D849V} mice.

(a) Timeline of experimentation (USS: ultrasound scan). (b) Representative images of viable SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) mice. (c&d) Timeline of average body weight (c) and mean organ/body weight ratio (d) of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) mice at 32 weeks of age (Heart (H), Liver (Lv), Spleen (S), Kidney (K) and Lungs (Lu)). n=3-8 for each data point; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively; NS: not significant.

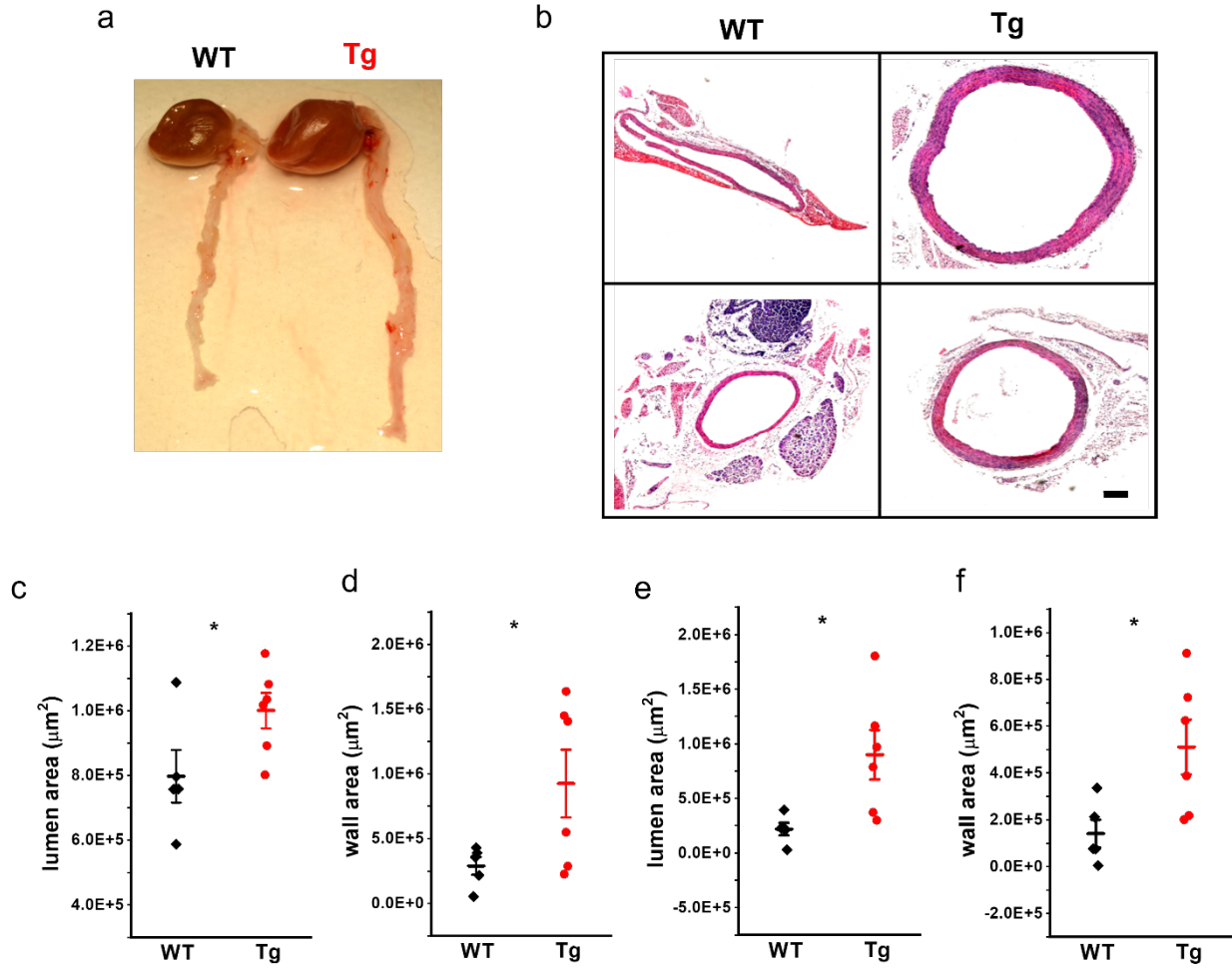


Figure 4.2 H&E staining of SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aorta.

(a) Representative images of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aortae at 32 weeks of age. **(b)** Representative H&E images of thoracic (top panels) and abdominal (bottom panels) aortae sections of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) animals at 4 weeks of age. Scale bar: 300 μm . **(c-f)** Mean lumen area (**c&e**) and mean wall area (**d&f**) of thoracic (**c&d**) and abdominal (**e&f**) aortae of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aortae. n=5-6 for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively.

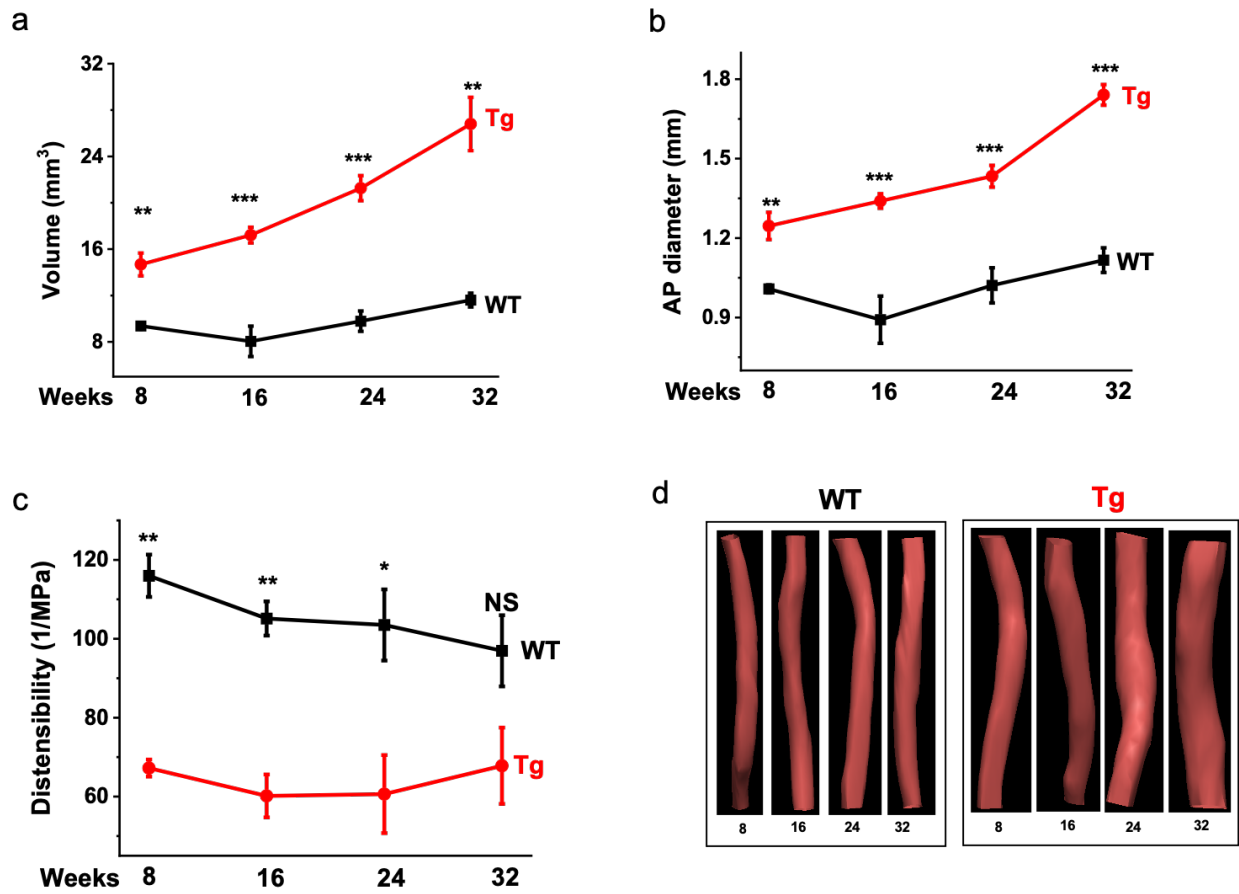


Figure 4.3 USS analysis of SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aorta (suprarenal part).

(a-c) Time course of aortic lumen volume (a), AP diameter (b) and distensibility (c) measured by USS in SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) animals (n=3-8 for each time point). (d) Representative 3D reconstructed images of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aortae. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively; NS: not significant.

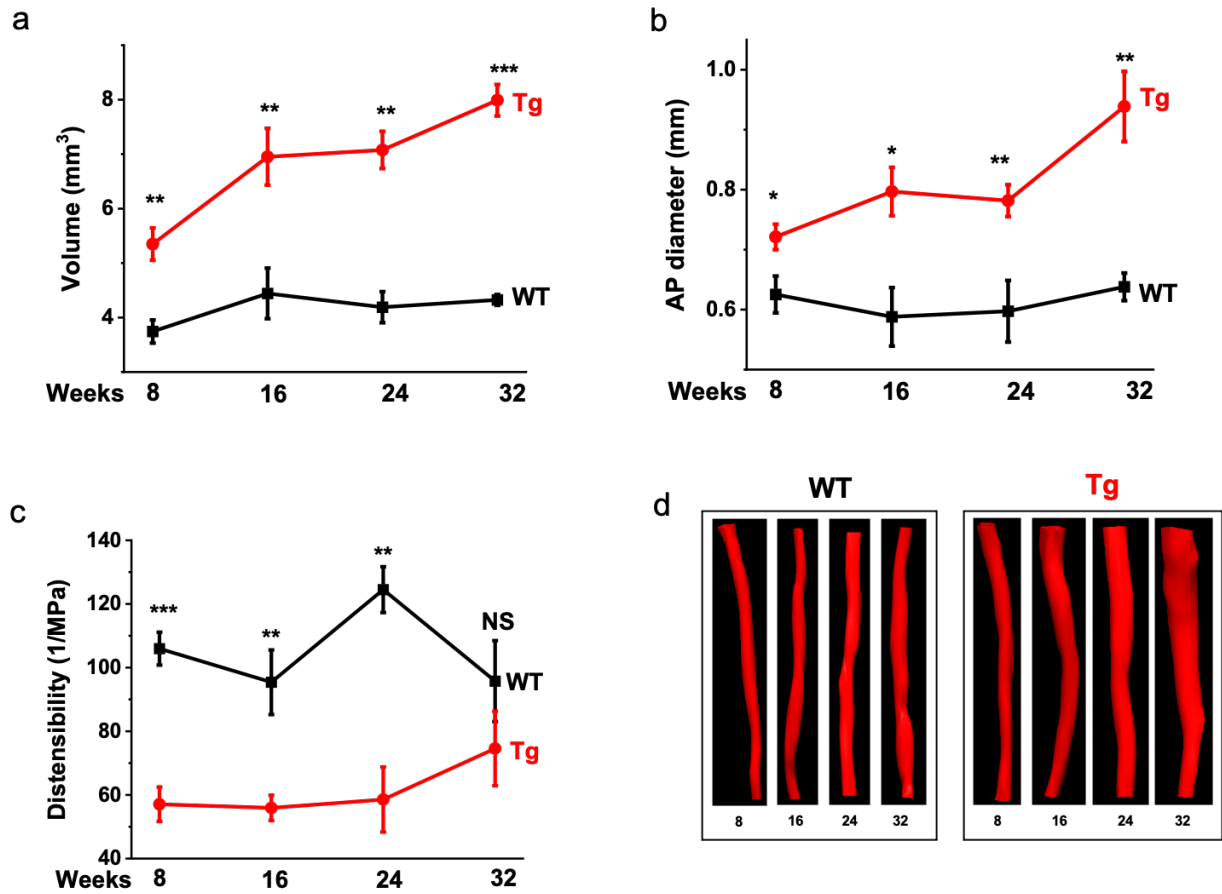


Figure 4.4 USS analysis of SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aorta (infrarenal part).

(a-c) Time course of aortic volume **(a)**, AP diameter **(b)** and distensibility **(c)** measured by USS in SM22CrePDGFR β^{WT} (WT) and PDGFR β^{D849V} (Tg) animals (n=3-8 for each time point). **(d)** Representative 3D reconstructed images of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aorta. *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively; NS: not significant.

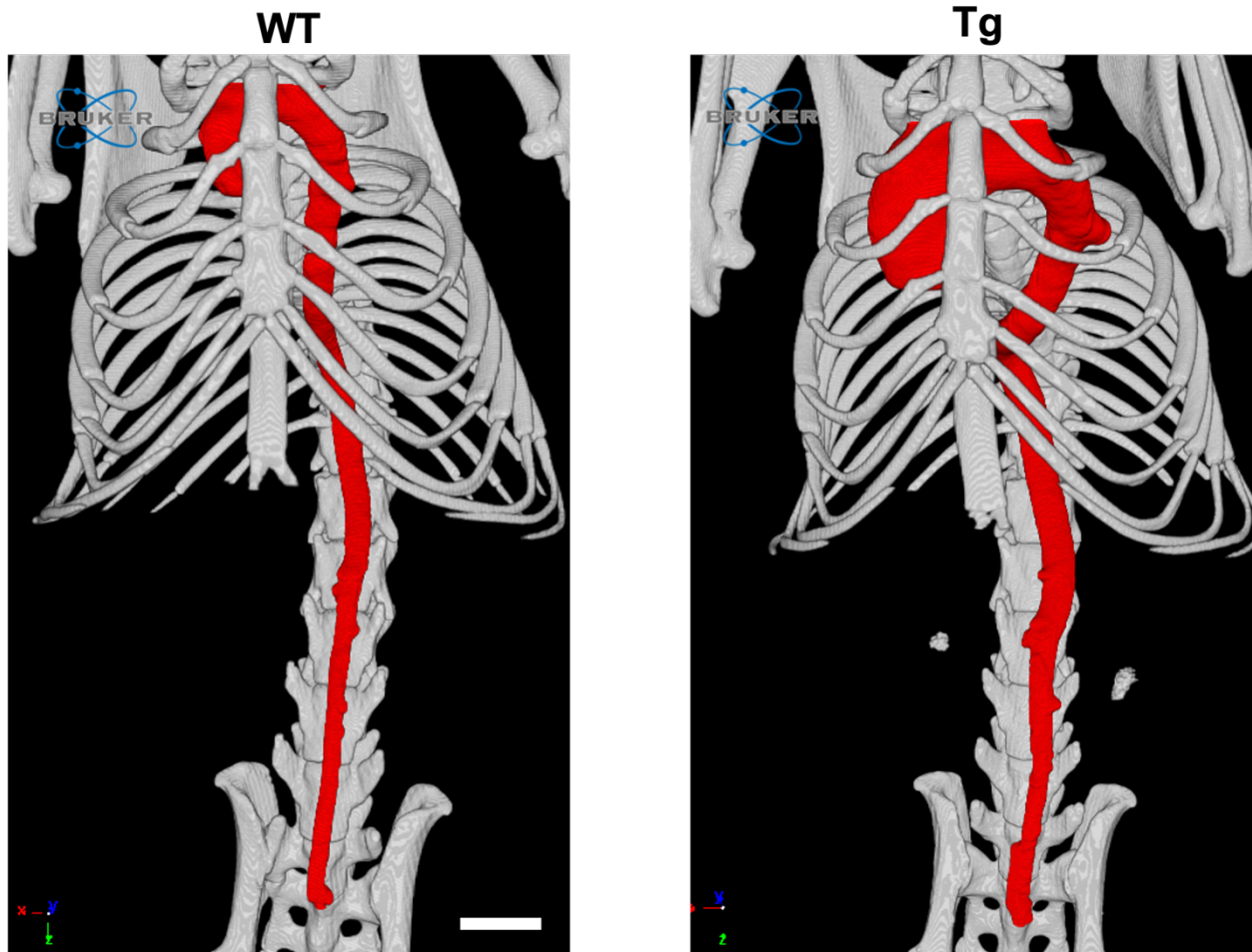


Figure 4.5 μ CT imaging of SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aorta at 8 weeks of age.

Representative 3D reconstructed images of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aorta. Scale bar: 4 mm.

4.1.2 RNA Sequencing analysis

Next, to evaluate the molecular pathways involved in the development of aortopathy involving the whole of aorta in SM22CrePDGFR β^{D849V} vs SM22CrePDGFR β^{WT} mice abdominal aorta was used for non-biased transcriptomic analysis using RNA sequencing (RNA-Seq).

Principal component analysis (PCA)

PCA plots of RNA-seq data show the characteristics/variations of samples according to genome-wide expression levels. The PCA plot was constructed using normalised read counts of 55,417 transcripts. The samples were coloured according to condition; red – SM22CrePDGFR β^{WT} and green – SM22CrePDGFR β^{D849V} . While samples of same genotype clustered together with along the principal component 2 (PC2; y-axis) indicating homogenous expression patterns, loose clustering along x-axis (PC1) suggests variations in expression levels within the groups (Figure 4.6).

Differentially expressed genes (DEGs) in SM22CrePDGFR β^{WT} Vs SM22CrePDGFR β^{D849V}

DEGs are illustrated using a volcano plot (Figure 4.7). There are 2721 significantly expressed genes (adjusted P-value<0.05). Of those, 2217 genes were upregulated in SM22CrePDGFR β^{D849V} and 504 were downregulated in SM22CrePDGFR β^{D849V} .

Functional enrichment analysis

Gene Ontology (GO) analysis shows the putative functions of the DEGs and Kyoto Encyclopaedia of Genes and Genomes (KEGG) and REACTOME enrichment analyses show their signalling pathways. While the REACTOME database covers more pathways including from the computational work in literature, the KEGG database has smaller pathways, based only on experimental results. All three analyses were performed to gain maximum insights into the effect of mutant-constitutively active PDGFR β on aortic pathophysiology. The GO terms for the biological process, cellular components, and molecular functions of DEGs are summarised in

Figure 4.8 & 4.9. While upregulated DEGs were involved in ECM physiology and inflammatory signalling, downregulated ones were involved primarily in electron transport/oxidative phosphorylation. KEGG and REACTOME analysis showed that upregulated DEGs mapped to cytokine interactions, chemokine signalling and ECM regulation pathways, downregulated ones were mapped to neurological pathways and oxidative phosphorylation (Figure 4.10 and 4.11).

Heat map of DEGs

A heatmap was also used to illustrate clustering of the 2,721 DEGs between the genotypes (Figure 4.12). As expected, the horizontal dendrogram separates the samples into two clades, SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} , driven by the large differences in gene expression between genotypes also illustrated by PCA plotting. Out of the large number of DEGs identified, we decided to focus on a subset of genes related to aortic pathophysiology.

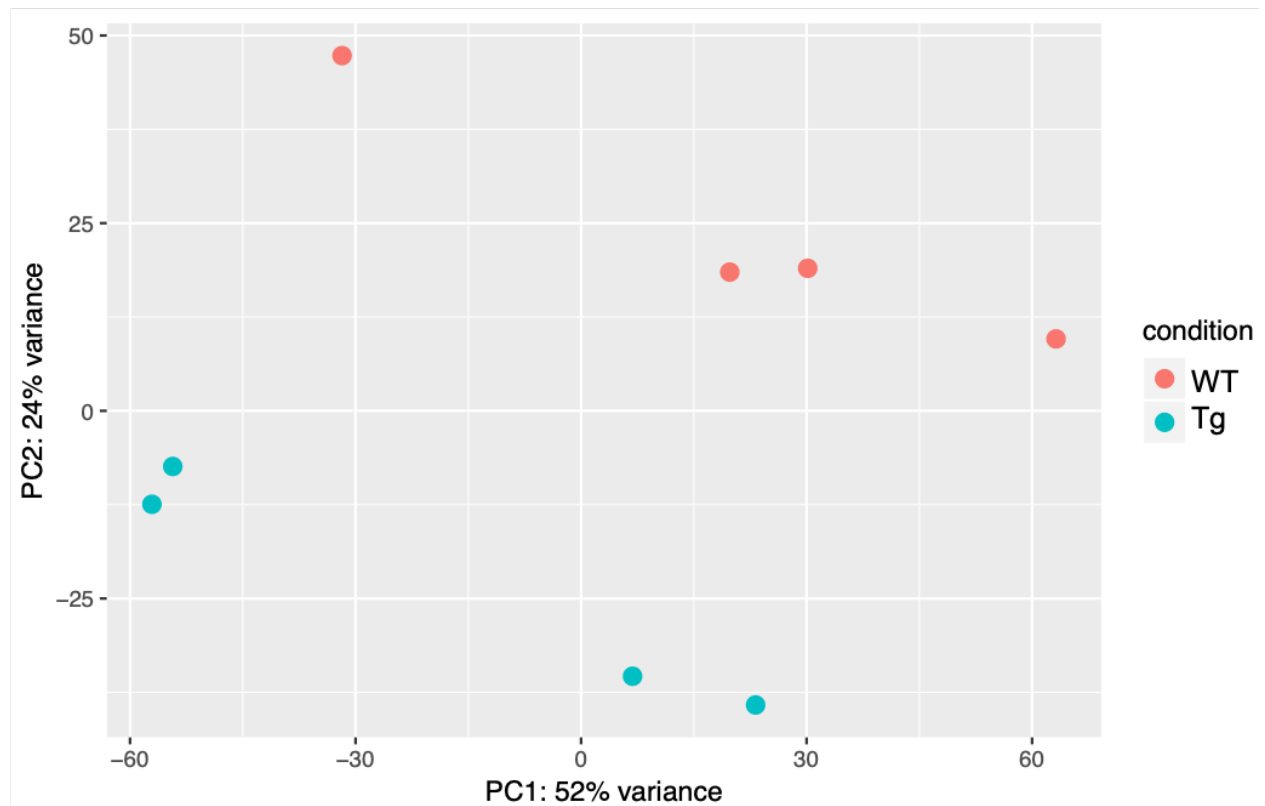


Figure 4.6 PCA of eight samples based on genome-wide expression profile.

Samples were divided into two groups based on the PCA. Each axis represents a principal component (PC1 and PC2) with 52% and 24% of total variance, respectively. The principal components were calculated from normalised read counts implemented in DESeq2. Each dot represents an individual sample with a group, and the colour is assigned according to the groups (SM22CrePDGFR β^{WT} (WT): red, SM22CrePDGFR β^{D849V} (Tg): blue).

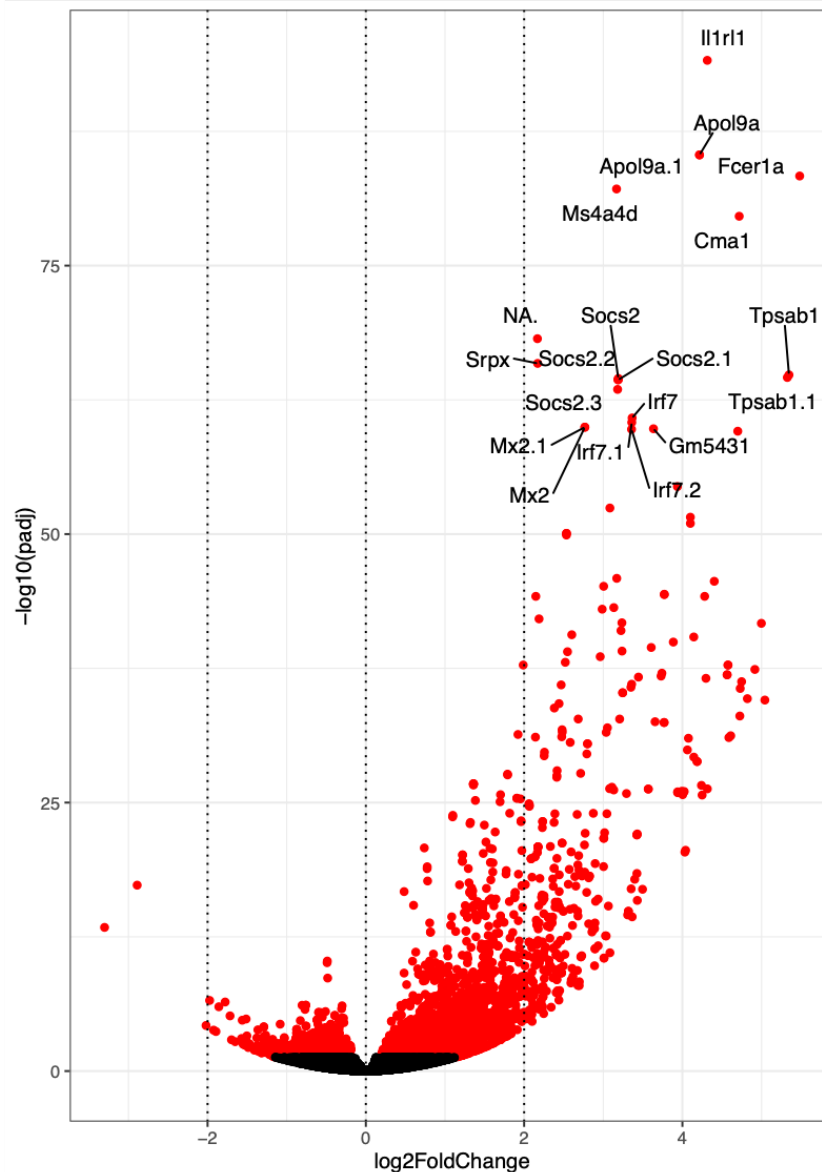


Figure 4.7 DEGs in SM22CrePDGFR β^{WT} Vs SM22CrePDGFR β^{D849V} aortae.

Volcano plot showing statistical significance (p value) versus magnitude of change (fold change). The top 20 DEGs by p value are labelled with their name and all DEGs are coloured in red (versus black- which represent genes other than DEGs).

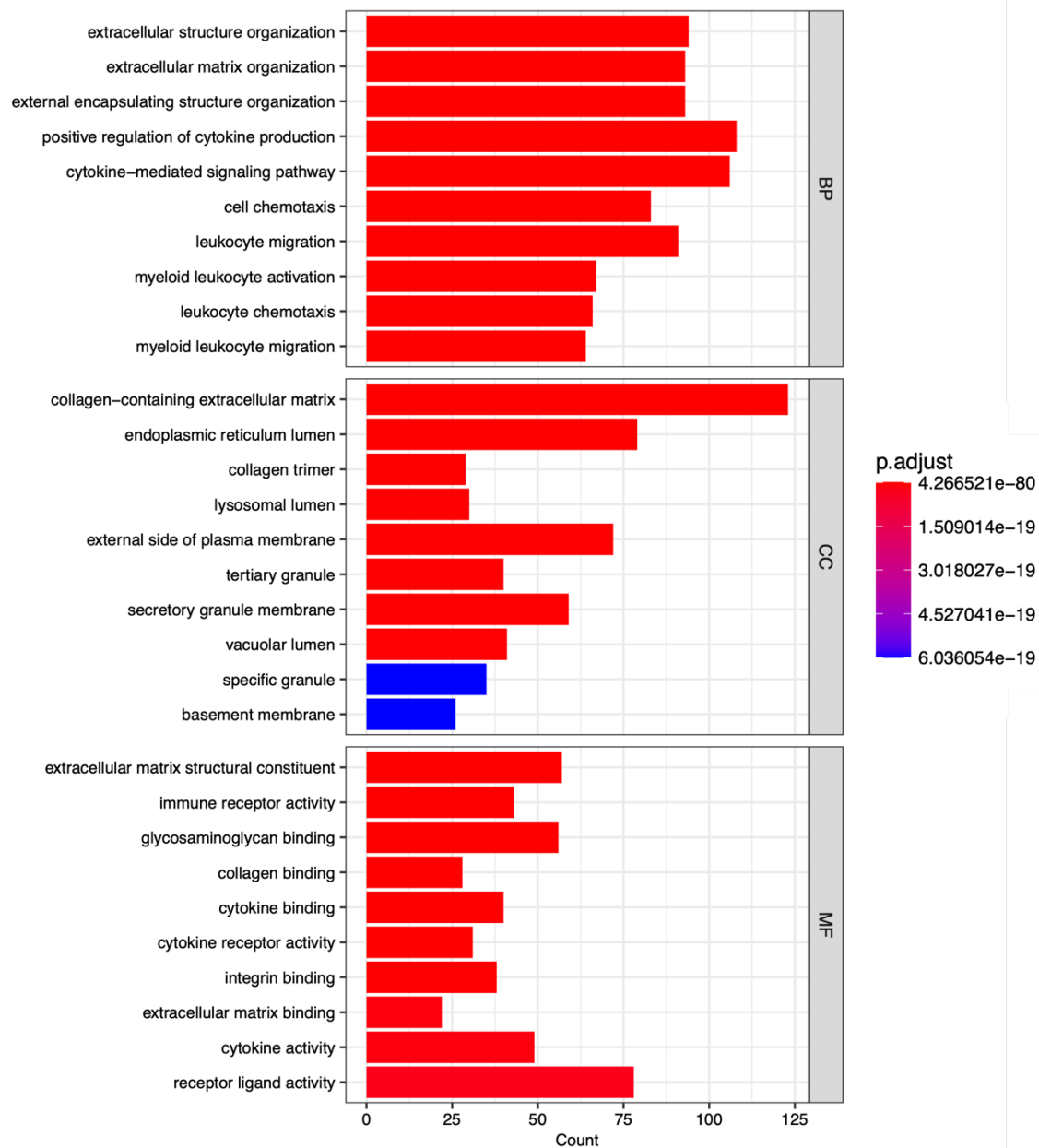


Figure 4.8 Enriched pathways according to GO terms (Upregulated DEGs).

GO terms for the biological process (BP), cellular components (CC) and molecular functions (MF) of upregulated DEGs in SM22CrePDGFR β^{D849V} aortae compared to SM22CrePDGFR β^{WT} . The X axis represents the number of DEGs enriched in the functions. The colour indicates level of statistical significance.

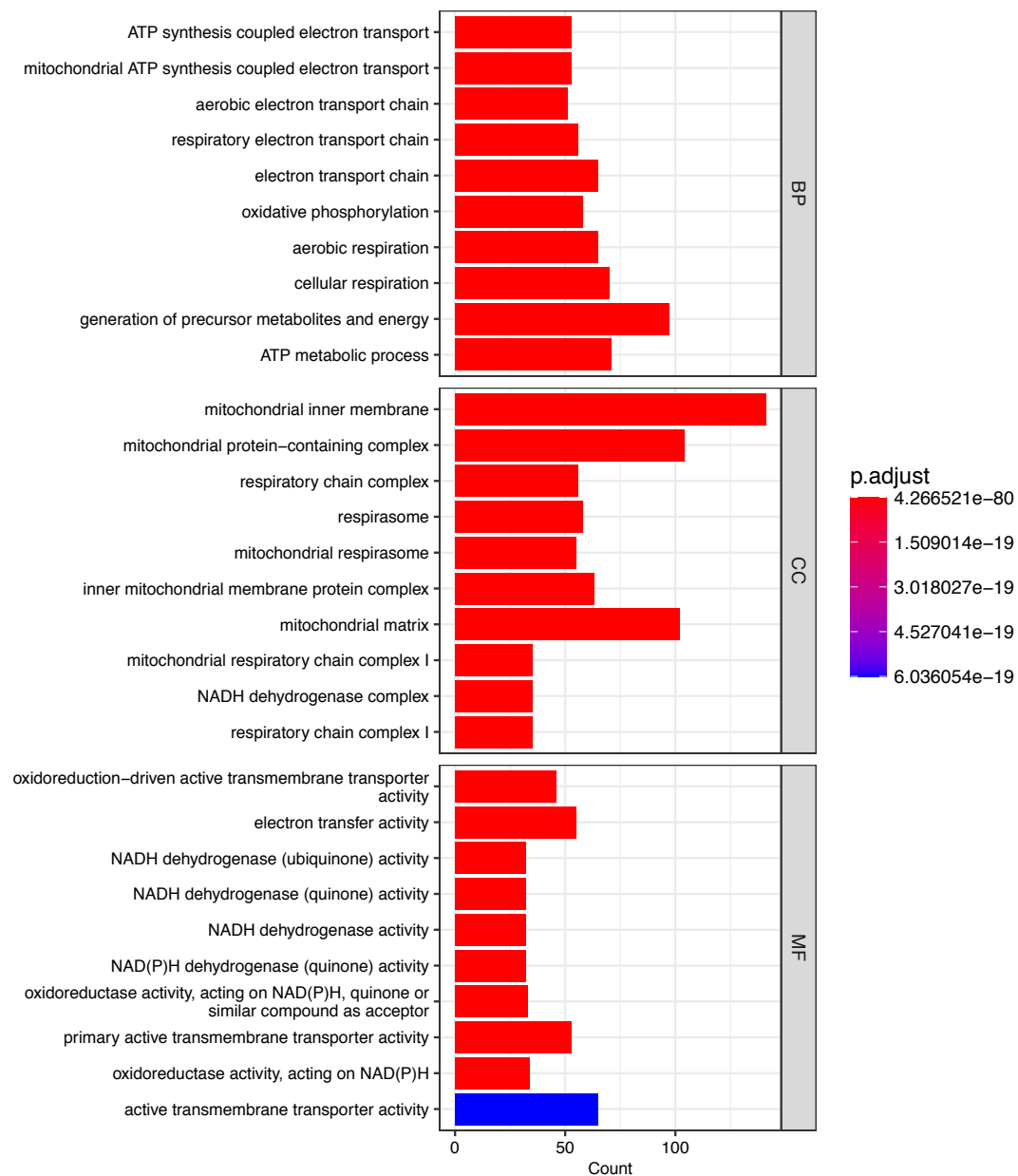


Figure 4.9 Enriched pathways according to GO terms (Downregulated DEGs).

GO terms for the biological process (BP), cellular components (CC) and molecular functions (MF) of downregulated DEGs in SM22CrePDGFR β^{D849V} aortae compared to SM22CrePDGFR β^{WT} . The X axis represents the number of DEGs enriched in the functions. The colour indicates level of statistical significance.

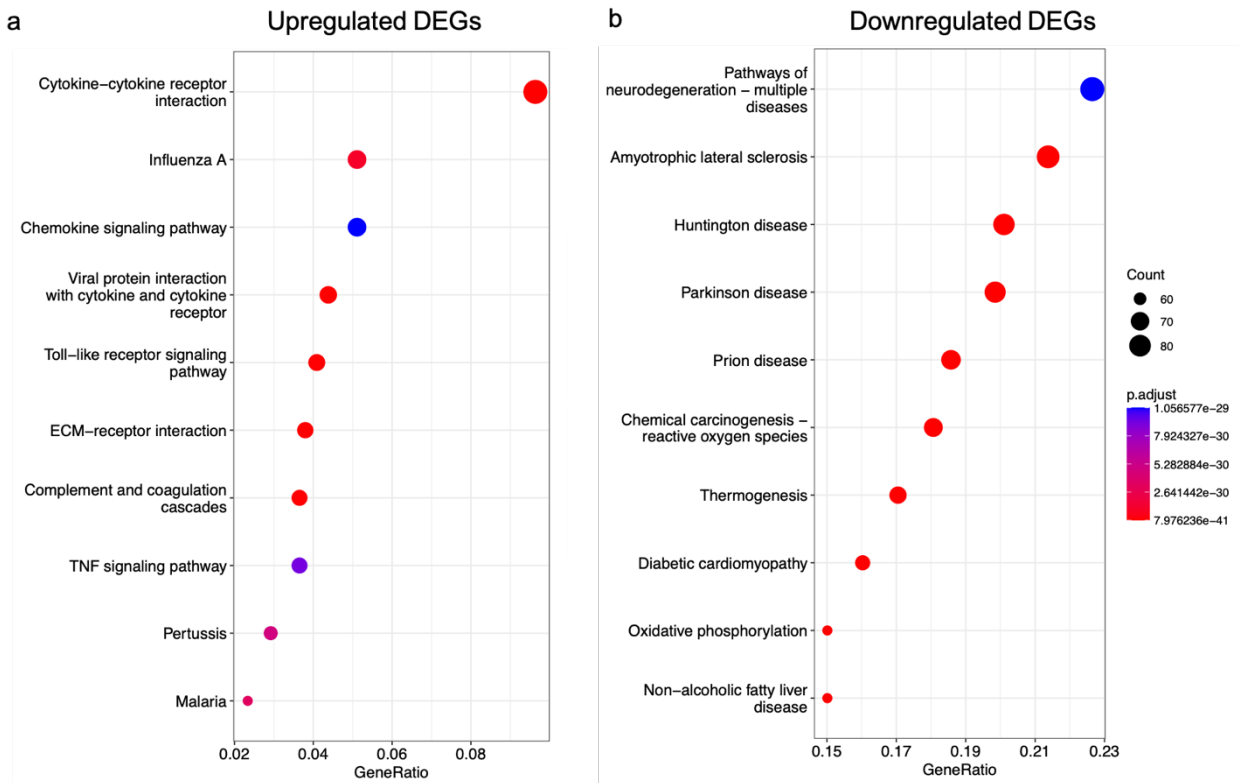


Figure 4.10 KEGG pathway analysis for DEGs.

(a&b) Enriched pathways for upregulated **(a)** and downregulated **(b)** DEGs in SM22CrePDGFR β^{D849V} aortae compared to SM22CrePDGFR β^{WT} according to KEGG terms. The size of the dot represents the number of DEGs enriched in the functions. The colour indicates level of statistical significance and size of the spot indicates gene count (scale in the side panel).

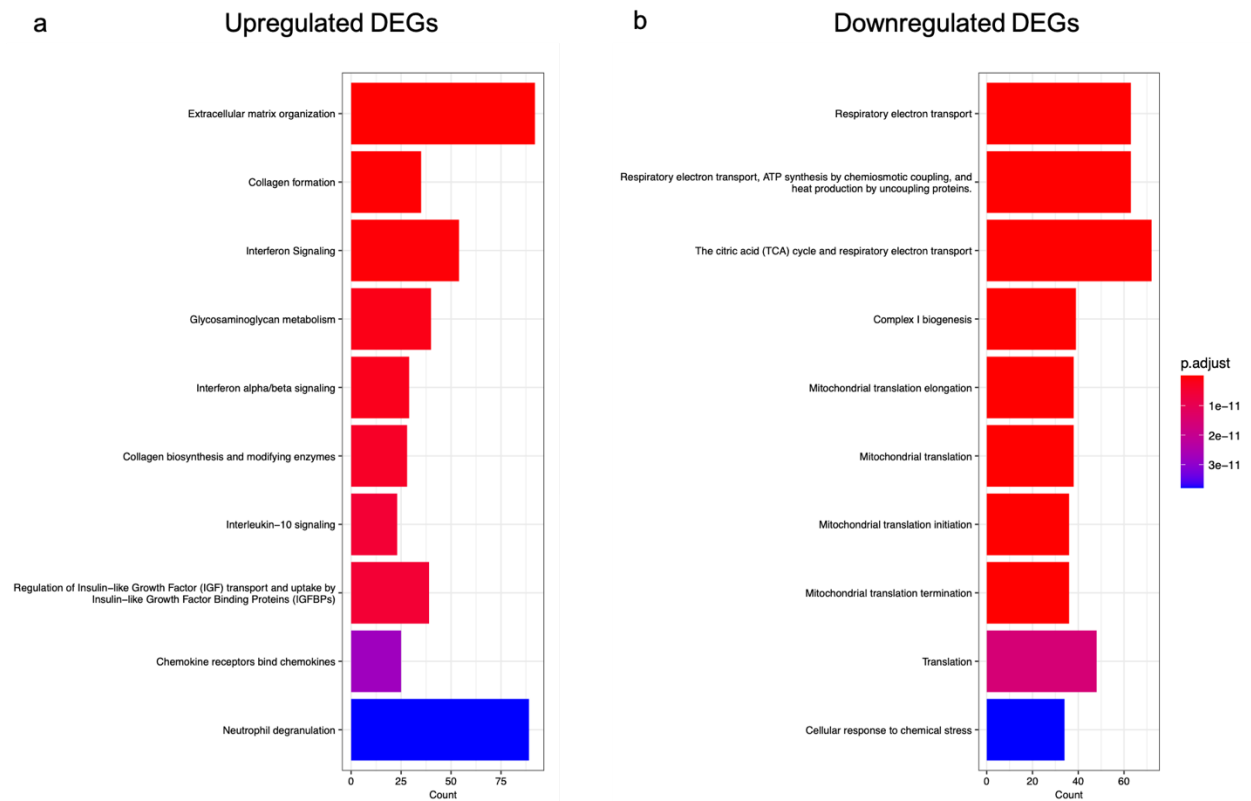


Figure 4.11 REACTOME pathway analysis for DEGs.

(a&b) Enriched pathways for upregulated **(a)** and downregulated **(b)** DEGs in SM22CrePDGFR β^{D849V} aortae compared to SM22CrePDGFR β^{WT} according to REACTOME terms. The X axis represents the number of DEGs enriched in the functions. The colour indicates level of statistical significance.

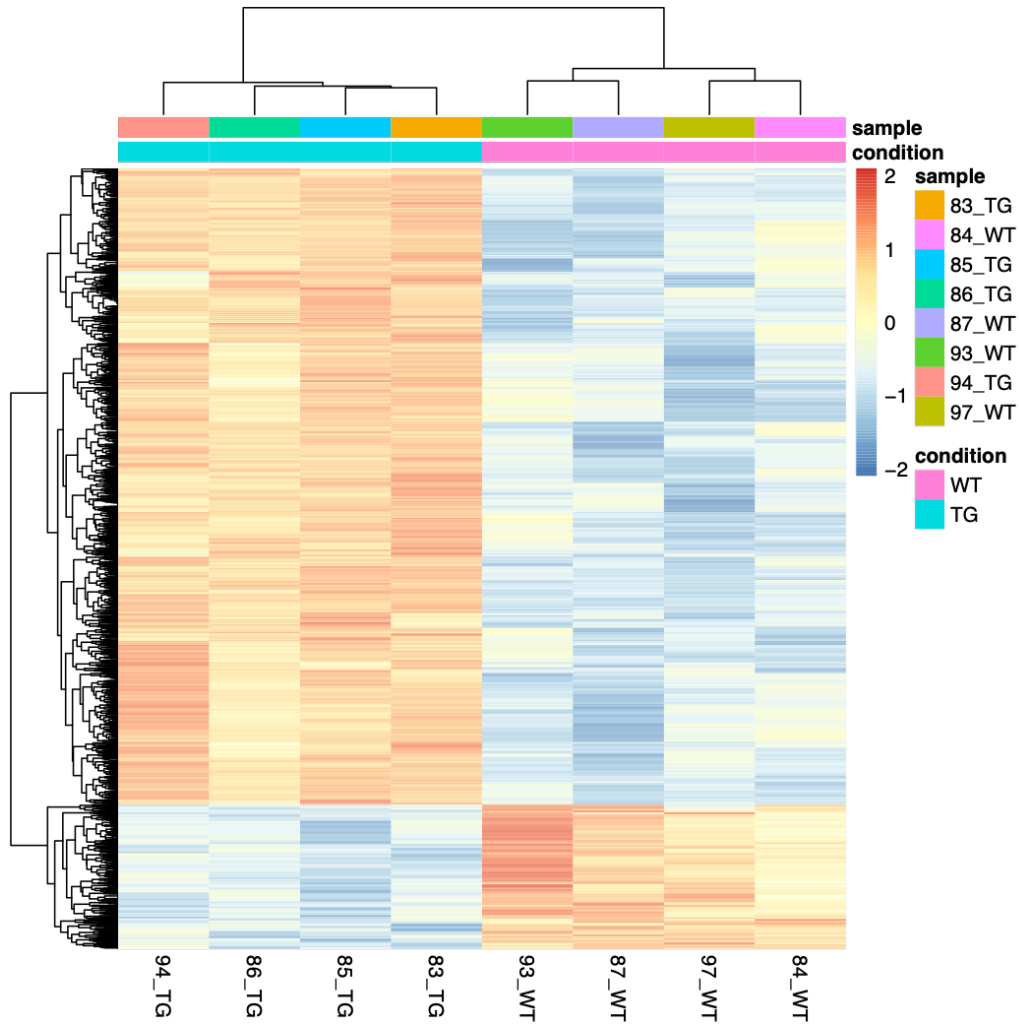


Figure 4.12 Genes differentially expressed between SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aortae.

Heatmap shows the expression levels of 2721 DEGs in SM22CrePDGFR β^{D849V} (TG) aortae compared to SM22CrePDGFR β^{WT} (WT). Using these genes, the horizontal dendrogram separates the samples into two clades, WT and transgenic (TG) suggesting that the levels of expression are different between conditions. The colour scale represents the expression level of DEGs such that, red colour shows higher expression levels in TG aortae whilst blue shows lower expression levels in them. Nos (83, 84 etc indicate the mouse ID numbers)

4.1.3 Validation of RNAseq DEGs by RT-qPCR

In order to validate and further explore some of the specific DEGs identified by RNAseq analysis of SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aortae, the remaining mRNA samples were analysed using RT-qPCR. Within the list of DEGs obtained by transcriptomics analysis, we selected specific genes plausibly related to pathophysiological mechanisms related to aortic disease development by literature survey. Five genes related to VSMC remodelling, *Mmp2*, *Mmp12*, *Timp1*, *Cle3b* and *Spp1* were significantly elevated in the SM22CrePDGFR β^{D849V} aortae compared to that of SM22CrePDGFR β^{WT} (Figure 4.13a-e). However, expression of *Coll9a1*, the gene coding an ECM protein was reduced in SM22CrePDGFR β^{D849V} aortae (Figure 4.13f). Also, mRNA expression of inflammation/chemotaxis related genes, *Il6*, *Bmp2* and *Ccr5* were significantly increased in SM22CrePDGFR β^{D849V} aortae (Figure 4.14a-c). In addition, expression of *Igfbp2* important VSMC signalling molecule was increased. Importantly, expression of *Lrp1*, a known gene candidate that participates in AAA pathogenesis was also increased in SM22CrePDGFR β^{D849V} aorta (Figure 4.14d&e).

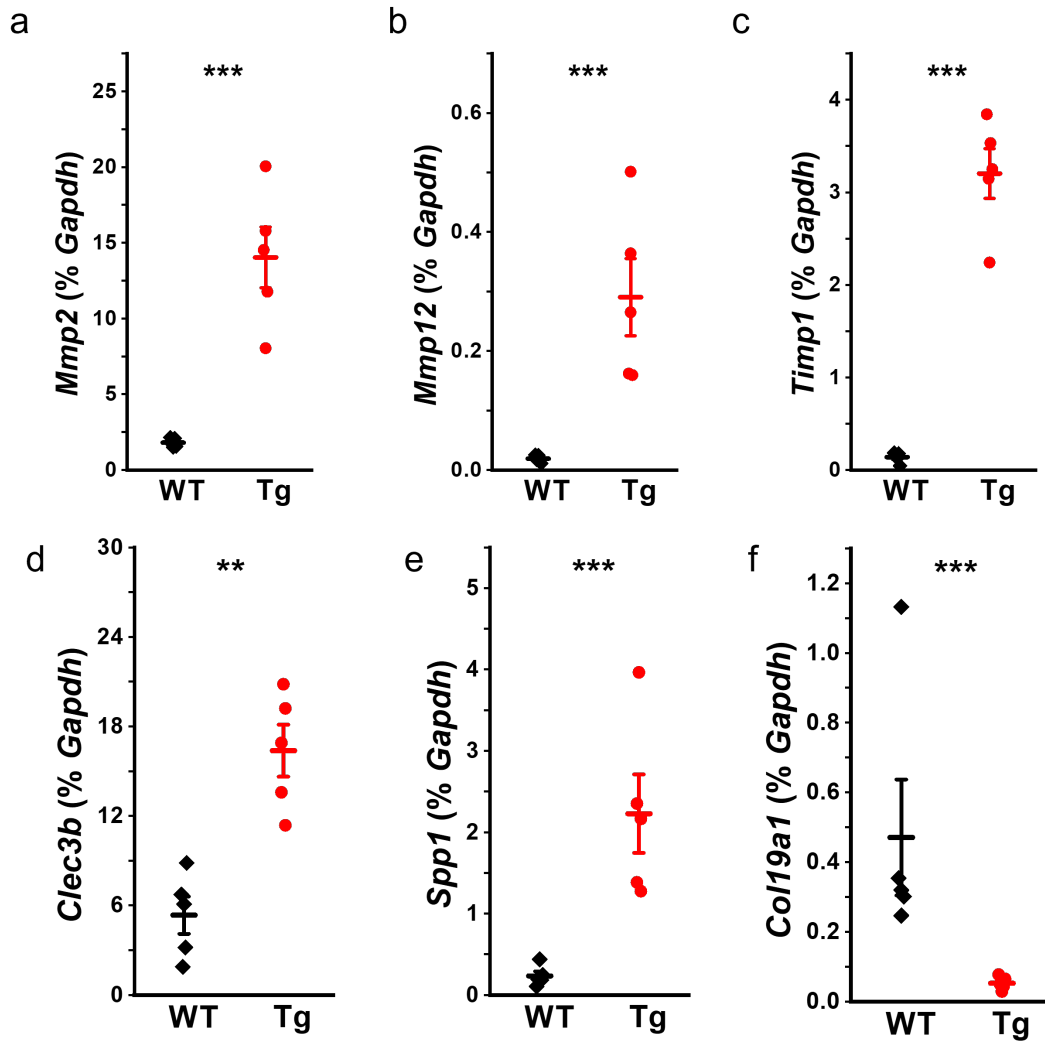


Figure 4.13 qRT-PCR analysis of mRNA expression in SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aortae.

(a-f) Mean mRNA expression of MMP2, MMP12, TIMP1, Clec3b, SPP1 and Col19a1 in aortae of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) mice. Individual mRNA levels were normalized to that of housekeeper (GAPDH). n=5 for each group; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. (Experiments performed by Dr K Hemmings).

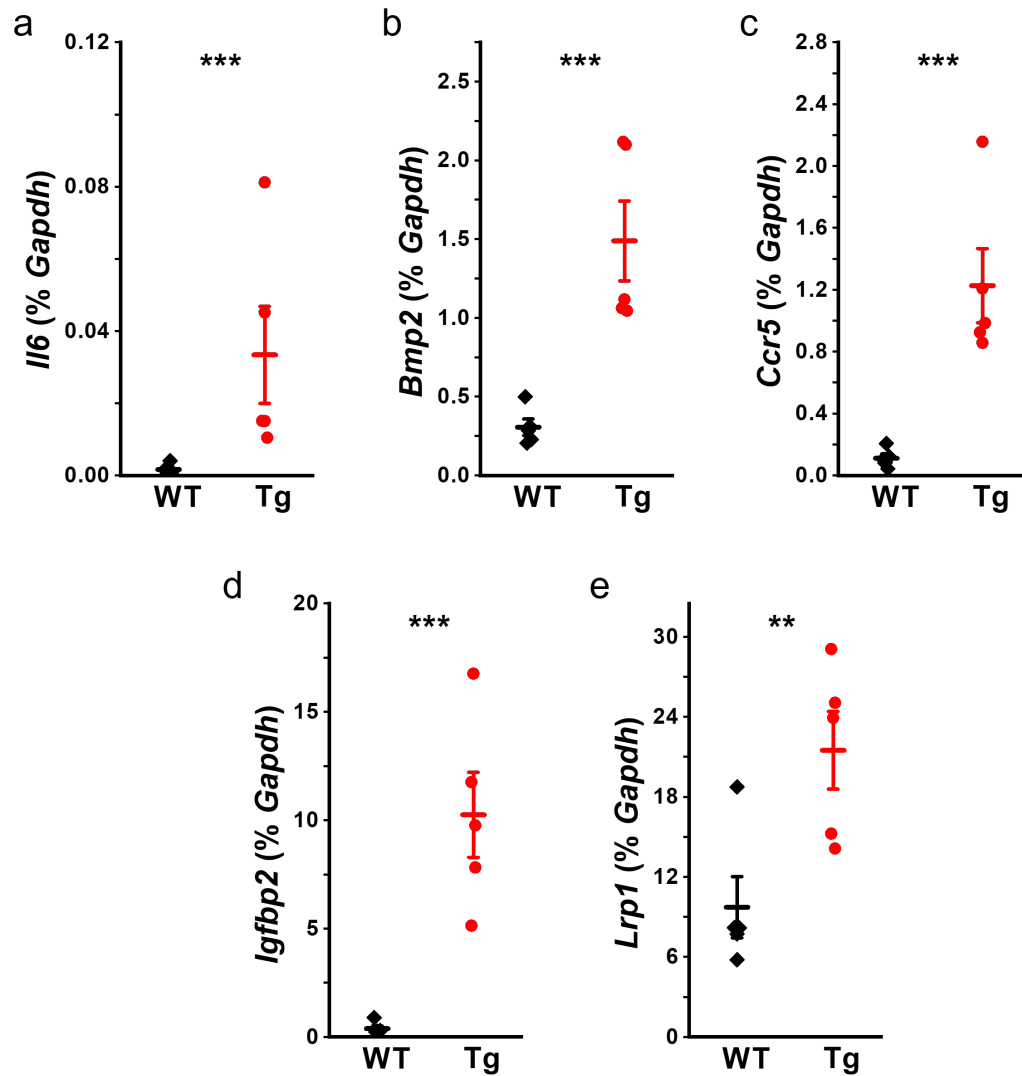


Figure 4.14 qRT-PCR analysis of mRNA expression in SM22CrePDGFR β^{WT} and SM22CrePDGFR β^{D849V} aortae.

(a-e) Mean mRNA expression of IL6, BMP2, CCR5, IGFBP2, and LRP1 in aortae of SM22CrePDGFR β^{WT} (WT) and Tg mice. Individual mRNA levels were normalized to that of housekeeper (GAPDH). n=5 for each group; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. (Experiments performed by Dr K Hemmings)

4.2 Inducible PDGFR β^{D849V} transgenic mice

Next, having observed a phenotype when basal and ligand-induced PDGF signalling is elevated in VSMC throughout embryonic development and post-natal life, we wanted to understand the consequences of increased VSMC-specific PDGFR β^{D849V} expression only after the animals reach adulthood. We therefore crossed the SM-MHCCreER^{T2} with the PDGFR β^{D849V} mutant line to generate SM-MHCCreER^{T2}PDGFR β^{D849V} mice. In this line only upon tamoxifen administration at 8 weeks of age, the Cre- mediated recombination results in expression of mutant PDGFR β^{D849V} and thus increased PDGFR β signalling. Along with regular measurement of body weights and end-point measurements of organ weight, USS was used to measure the aortic size in the supra- and infra-renal abdominal aorta at 12 weeks of age for characterising the transgenic line (Figure 4.15a). Littermates with SM-MHCCreER^{T2}PDGFR β^{WT} genotype were used as controls and denoted as WT in this section.

4.2.1 Phenotyping of SM-MHCCreER^{T2}PDGFR β^{D849V} transgenic line

The animals were viable with no observable phenotype over the 4 weeks following tamoxifen administration (Figure 4.15b). There were no significant differences in body weight (Figure 4.15c) or organ weight/body weight ratios between genotypes (Figure 4.15d). USS measurements at 12 weeks of age showed no dilatation in the suprarenal aorta nor change in distensibility (Figure 4.16). The infra-renal aorta showed a small but statistically significant increase in diameter and lumen area when compared to WT animals, but the effect size was small compared to SM22CrePDGFR β^{D849V} aorta where significant aortopathy was observed. The mean aortic AP diameter of the SM-MHCCreER^{T2}PDGFR β^{D849V} line at 12 weeks of age was 0.67 ± 0.06 mm and that of SM22CrePDGFR β^{D849V} at 16 weeks of age was 0.8 ± 0.04 mm. Also, there was no observed impact on distensibility (Figure 4.16). Histological analysis of H&E-stained sections did not suggest an increase in aortic diameter or structural changes in the aortic wall. (Figure 4.17). However, closer observation showed that cell number (nuclei counts) appeared higher in the transgenic aorta. Hence the number of nuclei in selected ROI of aorta were counted by observers who were blind to the genotype of the aortae (Figure 4.18). The statistical analysis of 10 or more

mice from each group showed that SM-MHCCreER^{T2}PDGFR β ^{D849V} aortic wall has higher cellularity than WT controls (Figure 4.18c).

Next a longer-term study was performed to determine whether a progressive phenotype is revealed over a longer time period in the SM-MHCCreER^{T2}PDGFR β ^{D849V} line. The mice were followed up for up to 64 weeks by regular USS analysis of aorta (Figure 4.19a). During the study, body weight and organ weights were similar between the genotypes (Figure 4.19b&c). At 64 weeks, aortae were harvested, and their appearance were macroscopically similar (Figure 4.19d1). Repeated *in vivo* measurement of aortic volume, AP diameter and distensibility of the infrarenal segment showed no differences until 44 weeks of age when the SM-MHCCreER^{T2}PDGFR β ^{D849V} animals began to develop mild aortic dilation that was progressive by week 64 (Figure 4.20). Even at 64 weeks their average aortic volume was considerably smaller than that reached by SM22PDGFR β ^{D849V} at 32 weeks of age ($5.6 \pm 0.7 \text{ mm}^3$ vs. $7.99 \pm 0.29 \text{ mm}^3$) and AP diameter ($0.73 \pm 0.04 \text{ mm}$ vs. and 0.94 ± 0.06). No aortic rupture was observed throughout the experiment.

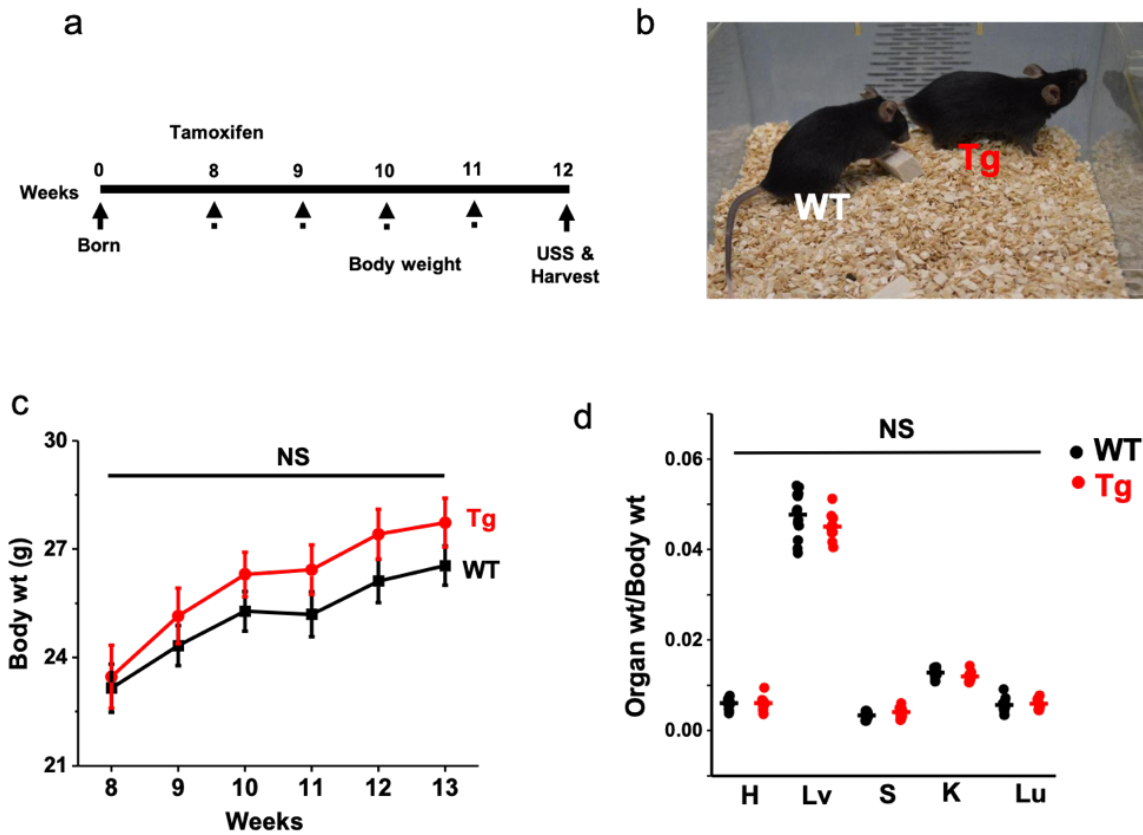


Figure 4.15 Phenotyping of SM-MHCCreER^{T2}PDGFR β ^{D849V} mice.

(a) Timeline of experimentation (USS: ultrasound scan). (b&c) Representative images (b) of viable WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) mice and the timeline of their body weight (c). (d) Individual organ to body weight ratio of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) animals at 12 weeks of age (Heart (H), Liver (Lv), Spleen (S), Kidney (K) and Lungs (Lu)). n=6-13 for each data point. NS: not significant.

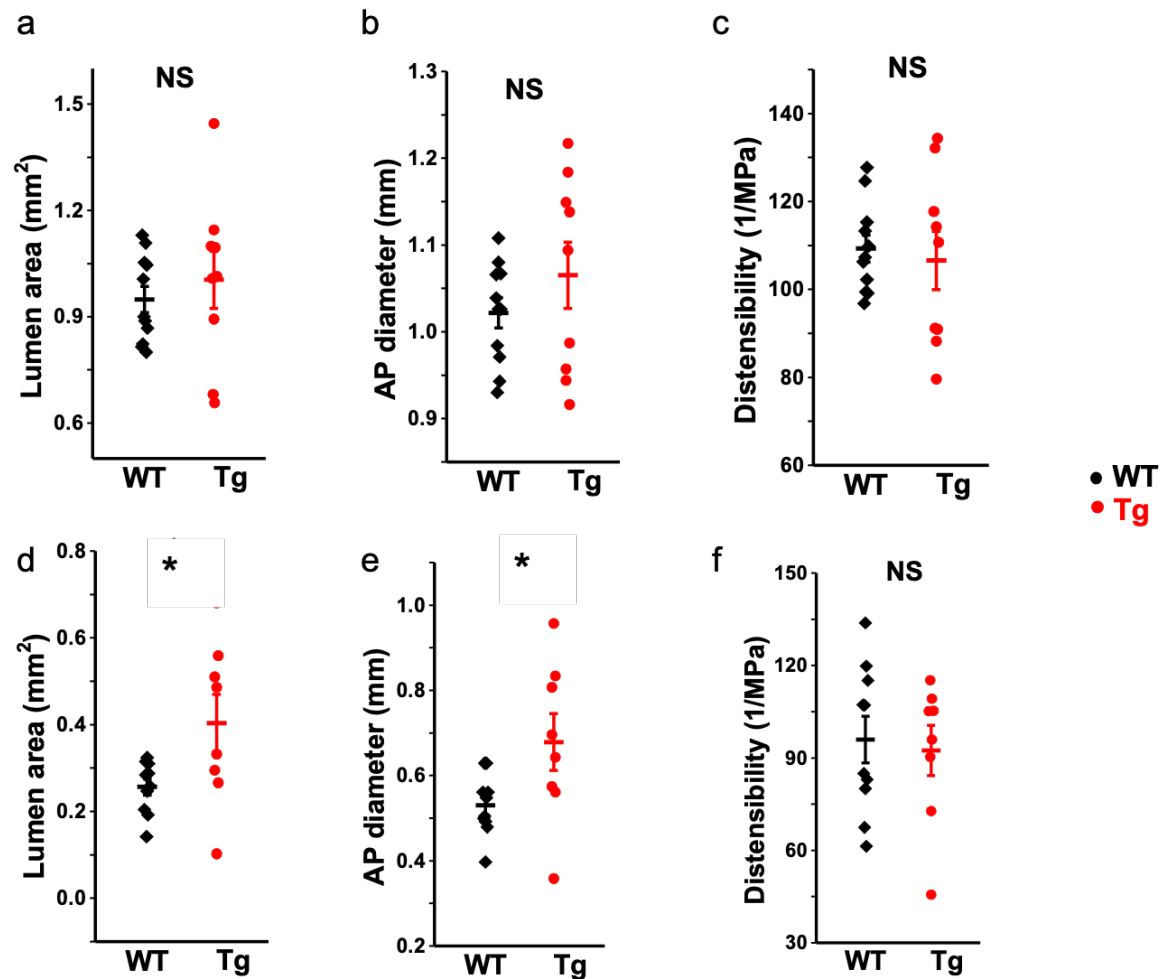


Figure 4.16 USS analysis of supra and infra-renal aortae of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} mice at 12 weeks of age.

(a-c) Mean lumen area (a), AP diameter (b) and distensibility (c) of suprarenal aortae measured by USS in WT and SM-MHCCreER^{T2}PDGFR β (Tg) animals. (d-f) Mean lumen area (d), AP diameter (e) and distensibility (f) of infrarenal aortae measured by USS in WT and SM-MHCCreER^{T2}PDGFR β (Tg) animals. n=8-10 for each data point; *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. NS: not significant.

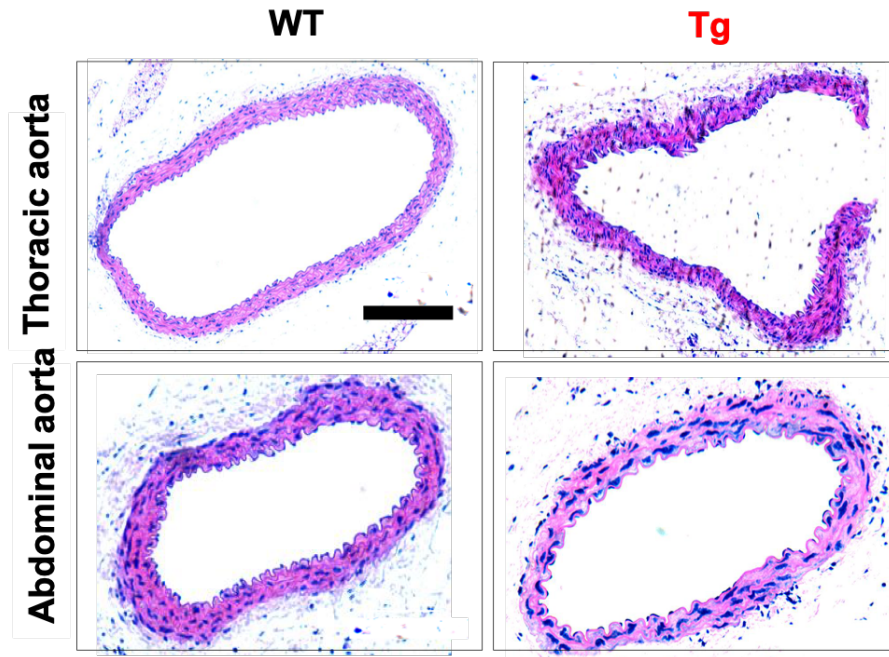


Figure 4.17 H & E staining of thoracic and abdominal aortae of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} mice.

Representative H&E images of WT (left column) and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg; right column) thoracic (top row) and abdominal (bottom row) aorta. Representative of n=10-12 for each group. Scale bar: 200 μ m.

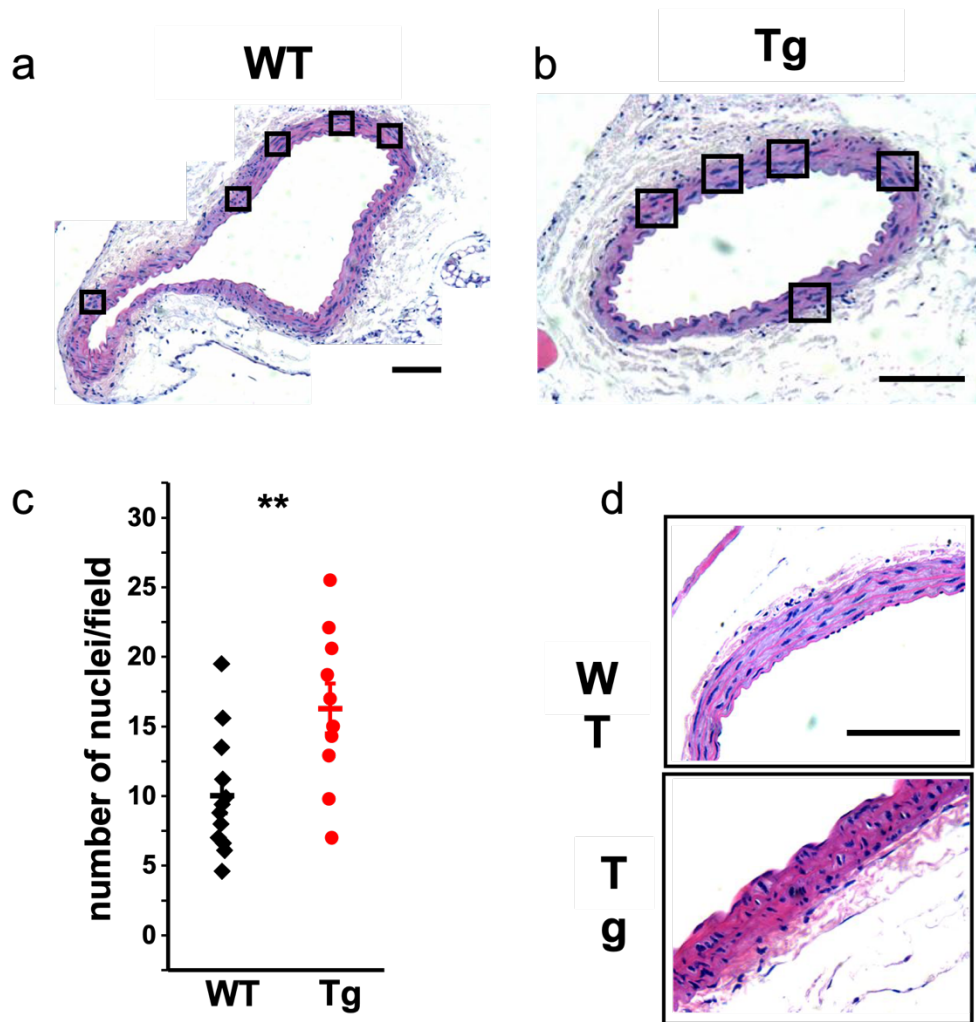


Figure 4.18 Nuclei quantification in SM-MHCCreER^{T2}PDGFR β ^{D849V} abdominal aortae sections.

(a,b&d) Representative H&E images of WT (**a** and top part of **d**) and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) (**b** and bottom part of **d**) of abdominal aortae sections. **(c)** Mean number of nuclei in each high-power field in the H&E images of aorta. n=10-12 for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively; Scale bar: 200 μ m.

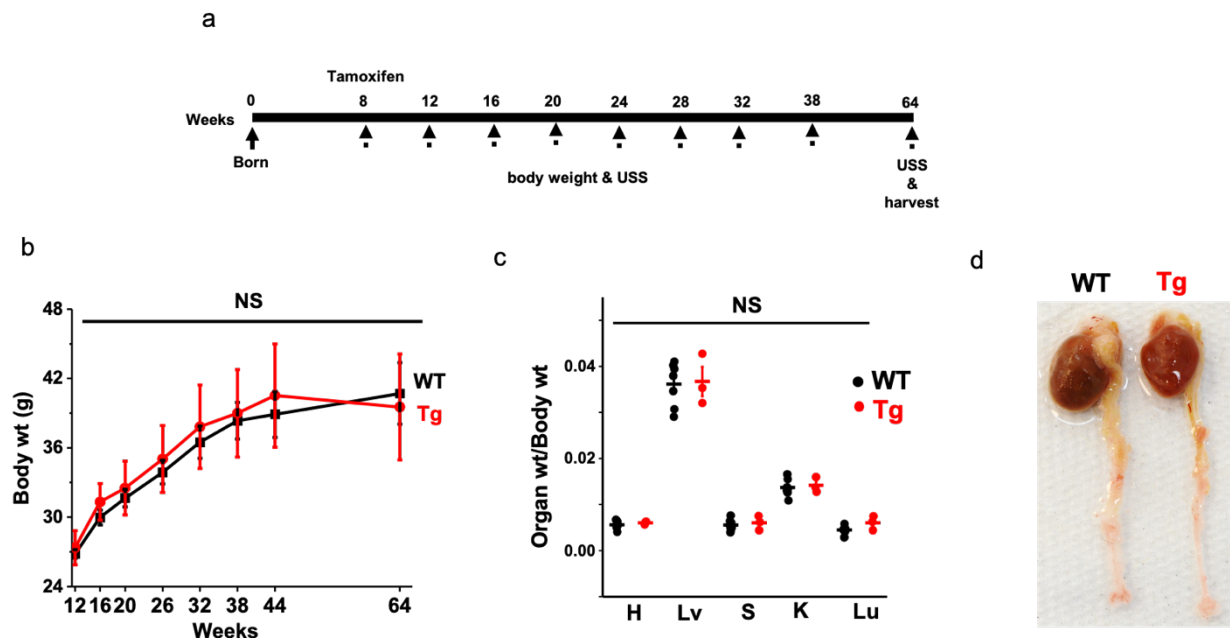


Figure 4.19 Phenotyping of SM-MHCCreER^{T2}PDGFR β ^{D849V} mice- longitudinal study.

(a) Timeline of experimentation (USS: ultrasound scan). (b) Timeline of mean body weight of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) mice. (c) Individual organ to body weight ratio of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) mice at 64 weeks of age (Heart (H), Liver (Lv), Spleen (S), Kidney (K) and Lungs (Lu)). (d) Representative images of the aortae at 64 weeks of age. n=3-7 for each data point; NS: not significant.

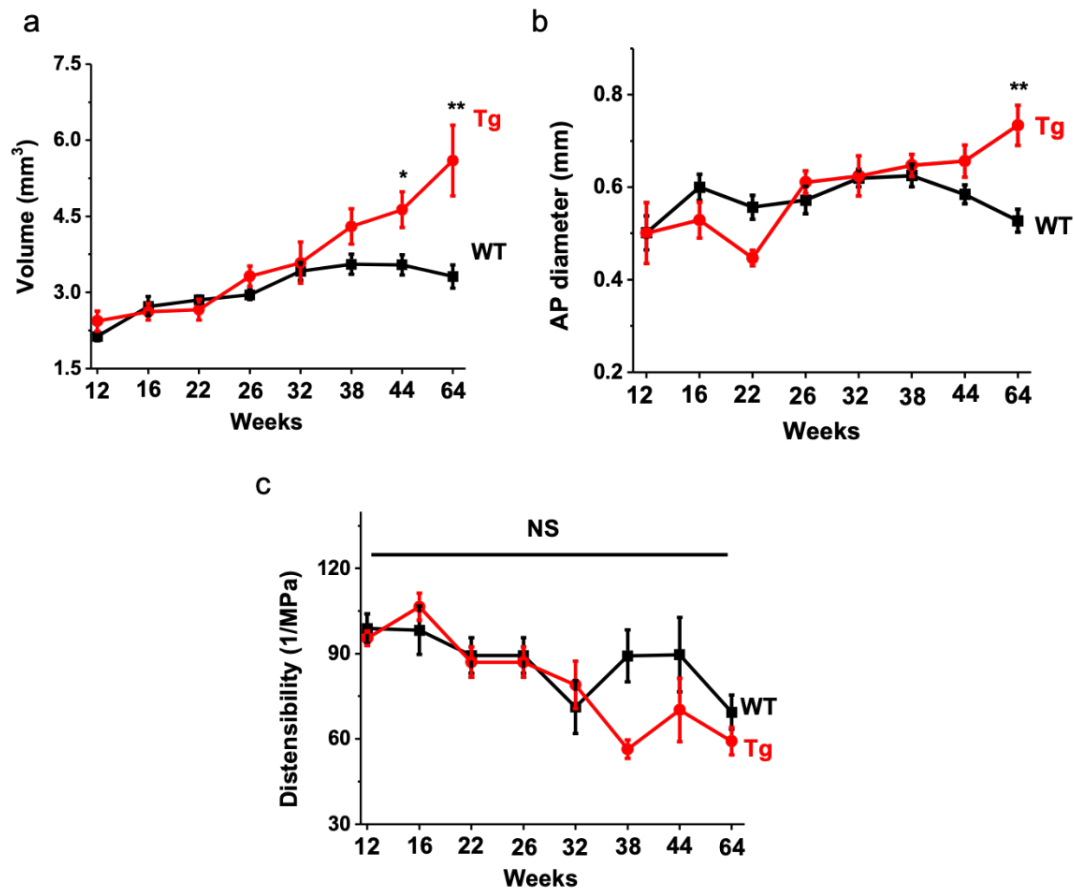


Figure 4.20 USS analysis of infrarenal aortae of SM-MHCCreER^{T2}PDGFR β ^{D849V} mice- longitudinal study.

(a-c) Timeline of mean volume **(a)**, AP diameter **(b)** and distensibility **(c)** of infrarenal aorta of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) mice. n=3-7 for each data point; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

4.2.2 Elastase induced AAA in SM-MHCCreER^{T2}PDGFR β ^{D849V}

Given that only a very mild aortic phenotype was observed at advanced age in the SM-MHCCreER^{T2}PDGFR β ^{D849V} animals in contrast to the stark aortopathy that developed early in the SM22CrePDGFR β ^{D849V} animals I next sought to determine the effect of a pathological insult on the aorta. I utilised the same PPE AAA model as for the lineage tracing experiments. AAA induction was performed at 12 weeks of age, 4 weeks after tamoxifen induced Cre recombination.

The infra-renal aortic size was tracked with USS at 2- and 4-weeks post PPE application (Figure 4.21a). At the standard 2-week time point for the model, PPE application induced AAA formation in both SM-MHCCreER^{T2}PDGFR β ^{D849V} and WT animals (vs. genotype matched sham controls), but with no difference between the genotypes (Figure 4.21). However, USS at 4 weeks post PPE application revealed increased aortic volume in the SM-MHCCreER^{T2}PDGFR β ^{D849V} animals compared to the SM-MHCCreER^{T2}PDGFR β ^{WT} animals, which was statistically significant (Figure 4.22).

Based on these observations, I set out to repeat the study in an independent cohort of mice with a longer follow up post PPE application to determine whether the difference observed at 4 weeks persisted. The AAA was tracked for 12 weeks post-PPE application by USS every 2 weeks. The data confirmed the emerging differences at 4 weeks post-PPE application and revealed ongoing AAA growth in SM-MHCCreER^{T2}PDGFR β ^{D849V} animals (Figure 4.23). In WT aortae PPE application led to initial dilation which was followed by static or even decreasing aortic volume in the infrarenal region over 12 weeks (Figure 4.23b). The mean aortic volume of SM-MHCCreER^{T2}PDGFR β ^{D849V} line 12 weeks post PPE application was 14.9±1.88 mm³ vs. 8.87±2.05 mm³ in WT. Analysis of H&E-stained aorta sections at the end of the study (12 weeks post PPE application) confirmed that SM-MHCCreER^{T2}PDGFR β ^{D849V} animals developed larger AAA compared to WT (Figure 4.24).

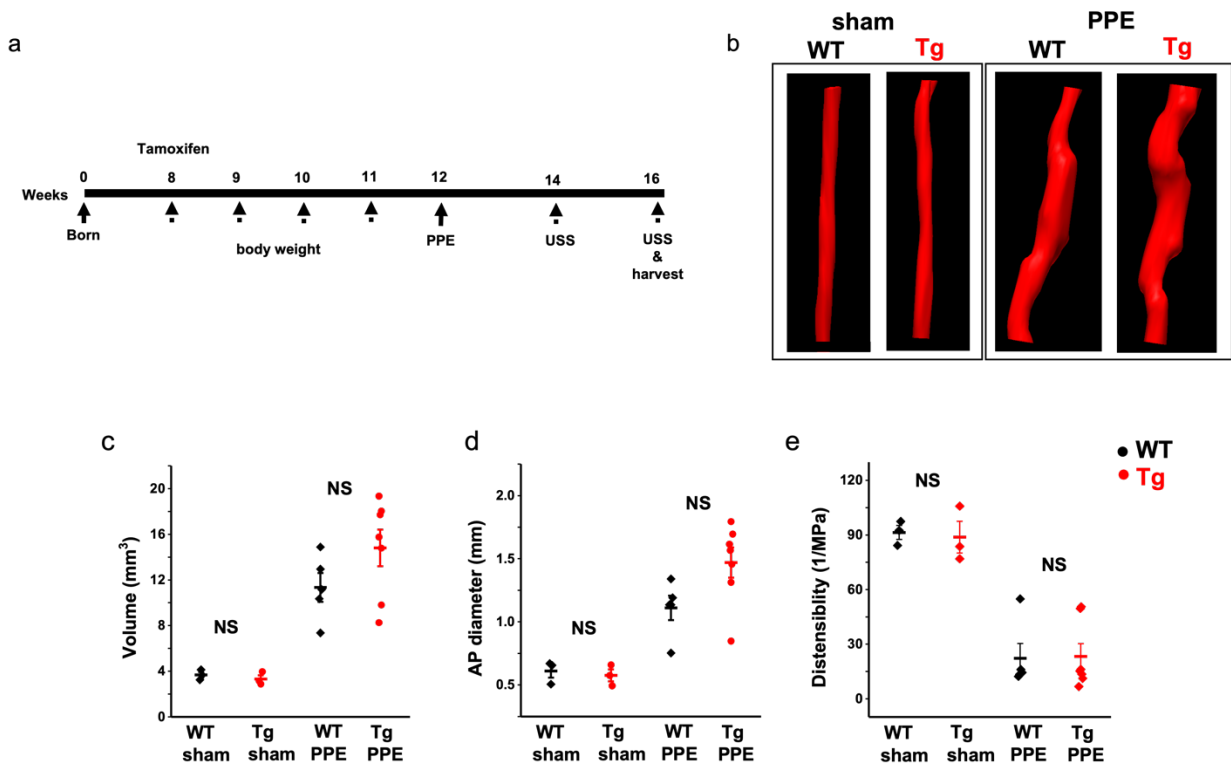


Figure 4.21 USS analysis SM-MHCCreER^{T2}PDGFR β ^{D849V} and WT mice aortae 2 weeks after PPE induction.

(a) Timeline of experimentation (PPE: porcine pancreatic elastase). **(b)** Representative 3D reconstructed images of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) aorta 2 weeks after sham operation or PPE application. **(c-e)** Mean infrarenal aortic volume **(c)**, AP diameter **(d)** and distensibility **(e)** measured in WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) animals. n=3-7 for each time point. NS: not significant.

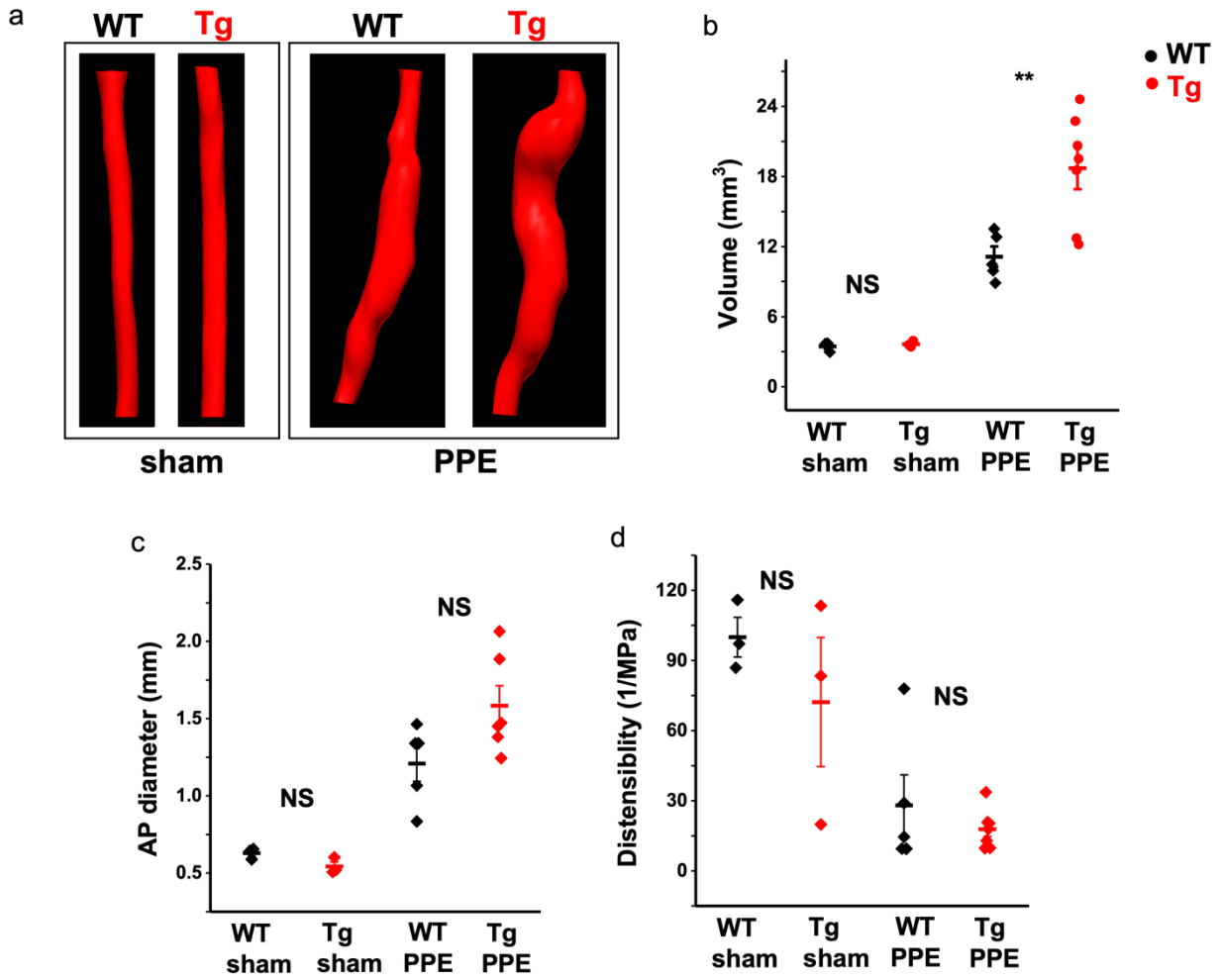


Figure 4.22 USS analysis SM-MHCCreER^{T2}PDGFR β ^{D849V} and WT mice aortae 4 weeks after PPE induction.

(a) Representative 3D reconstructed images of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) aortae 4 weeks after sham operation or PPE application. (b-d) Mean infrarenal aortic volume (b), AP diameter (c) and distensibility (d) measured in WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) animals. n=3-7 for each time point; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

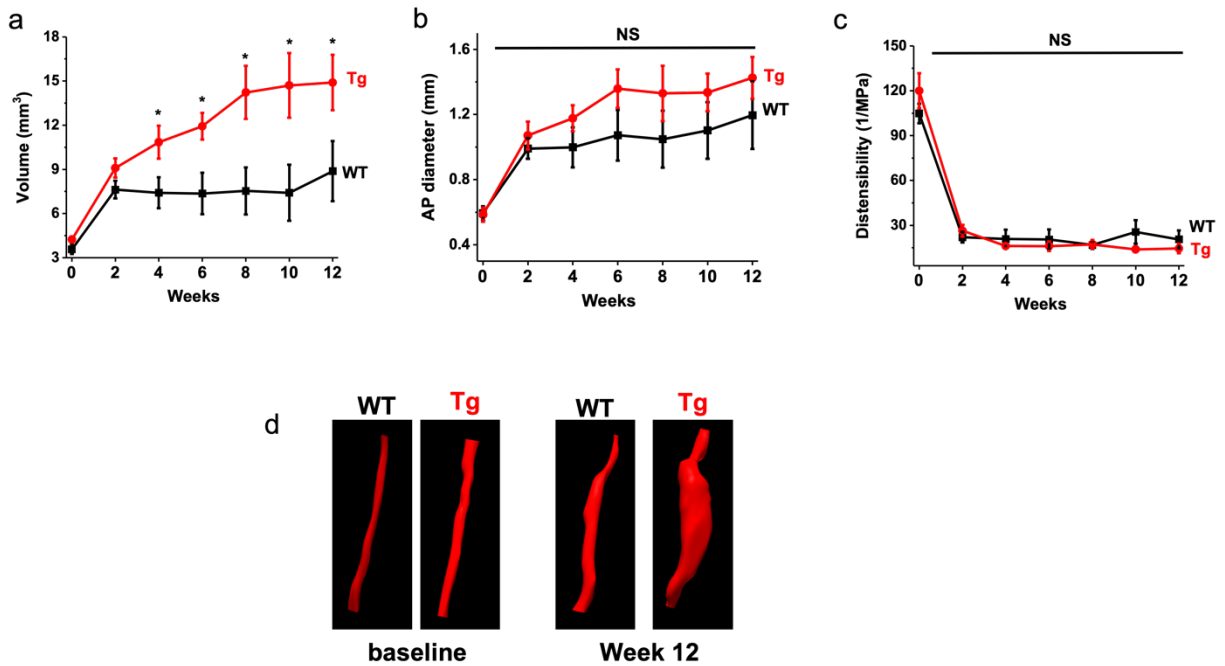


Figure 4.23 USS analysis SM-MHCCreER^{T2}PDGFR β ^{D849V} and WT mice aortae after PPE application- longitudinal study.

(a-c) Timeline of mean infrarenal aortic volume **(a)**, AP diameter **(b)** and distensibility **(c)** of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) infrarenal aortae. **(d)** Representative 3D reconstructed images of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) aortae at baseline and 12 weeks after PPE application. n=3-7 for each time point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

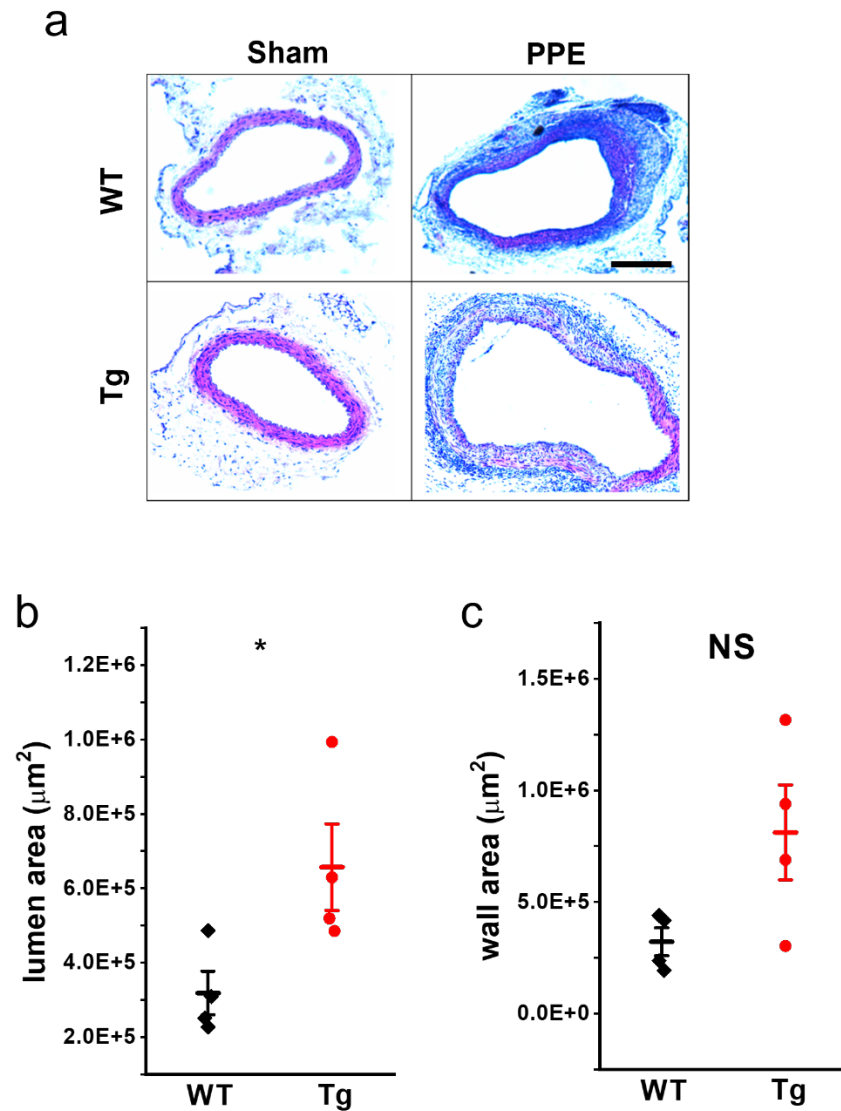


Figure 4.24 H & E staining of infrarenal aortae of SM-MHCCreER^{T2}PDGFR β ^{D849V} and WT mice 12 weeks after PPE application.

(a) Representative H&E images of sham operated (left column) or PPE (right column) applied WT (top row) and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg; bottom row) aortae. Scale bar: 200 μ m. **(b&c)** Mean lumen area **(b)** and mean wall area **(c)** of infrarenal aortae of WT and SM-MHCCreER^{T2}PDGFR β ^{D849V} (Tg) aortae 12 weeks post-PPE application. n=4 for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively; NS: not significant.

4.2.3 SM-MHCCreER^{T2}PDGFR β ^{D849V}mTmG line for lineage tracing studies

Next, we wanted to lineage trace the smooth muscle response to PPE AAA in the SM-MHCCreER^{T2}PDGFR β ^{D849V} transgenic line by crossing it with the mTmG reporter until all animals were mTmG^{+/+}. PPE application was performed at 12 weeks (4 weeks after tamoxifen injection) as per previous studies and aortic USS was used for quality assurance. Unfortunately, the laboratory shutdown during the COVID-19 pandemic significantly impacted the completion of these experiments, so data from one WT and three SM-MHCCreER^{T2}PDGFR β ^{D849V}mTmG animals were generated. The USS data obtained 2 and 4 weeks after PPE application indicates the initiation of AAA development (Figure 4.25 a&b).

Importantly, confocal microscopic imaging of the aortic sections indicated that medial VSMC were green in colour and the rest of the cells are red. However, due to Covid lockdown and difficulties in breeding this line no further experiments were done. Thus, our preliminary analysis showed that this model may be used for further studies for lineage tracing analysis.

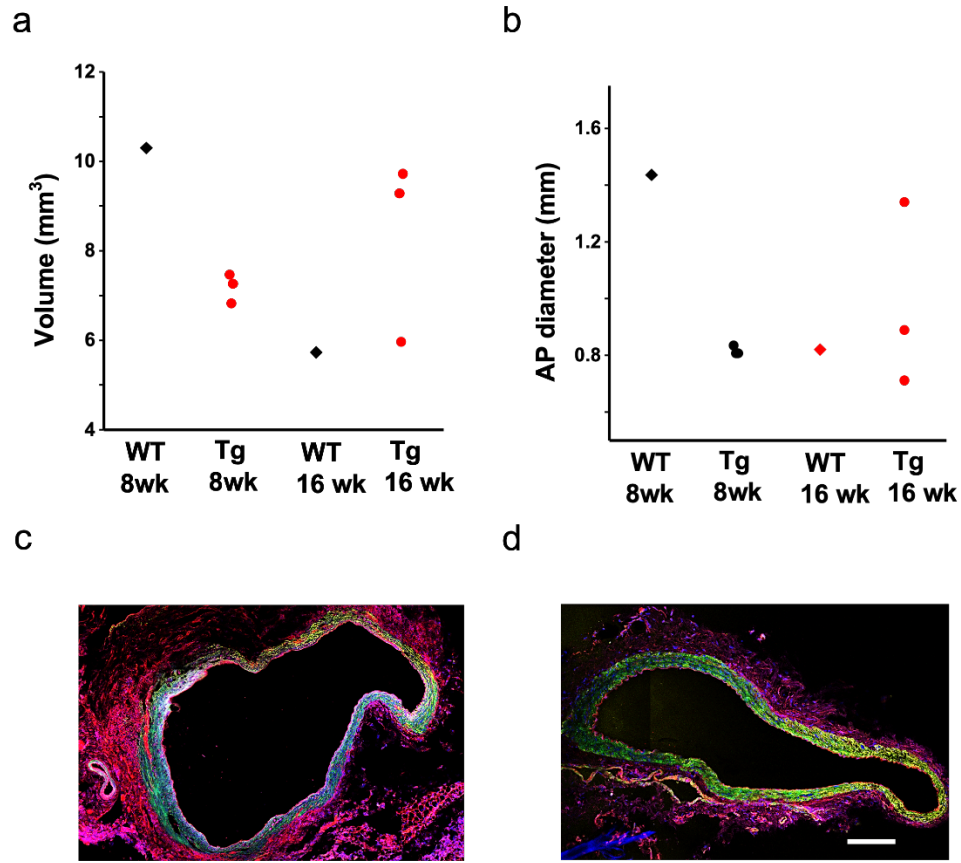


Figure 4.25 Ultrasound and microscopic analysis of infra-renal aortae of SM-MHCCreER^{T2}PDGFRβ^{D849V}mTmG mice after PPE application.

(a&b) Mean volume **(a)** and AP diameter **(b)** of infrarenal aorta of WT and SM-MHCCreER^{T2}PDGFRβ^{D849V}mTmG (Tg) mice at 2 and 4 weeks after PPE application. n=1-3 for each data point. **(c&d)** Representative confocal images of aortic sections of SM-MHCCreER^{T2}PDGFRβ^{D849V}mTmG **(c)** and WT **(d)** aortae 4 weeks post-PPE application. Scale bar: 200 μm.

4.3 Downstream signalling of PDGFR β activation through Orai1

The original study describing the constitutively active PDGFR β^{D849V} mutant demonstrated that downstream signalling is preferentially via phospholipase C- γ (PLC γ) but little other detail is given (Olson and Soriano, 2011). As explained before, store operated calcium entry (SOCE) that occurs upon store depletion induced downstream of PDGF-BB-PLC γ -IP $_3$ R signalling is through Orai1 channels (Feske et al., 2006; Gudlur and Hogan, 2017; Shower et al., 2021). SOCE/Orai1 is implicated in VSMC phenotype switching and pathological vascular remodelling (Martín-Bórnez et al., 2022; Shower et al., 2021). We therefore hypothesised that PLC γ activation was driving enhanced SOCE via Orai1 in the PDGFR β^{D849V} mutant mouse as the mechanism inducing VSMC proliferation and remodelling.

4.3.1 Orai1 expression in SM22CrePDGFR β^{D849V} aorta

First, we sought to determine if Orai1 protein expression was altered in SM22CrePDGFR β^{D849V} aortae. Aortic sections immunostained with anti-Orai1 antibody showed stronger anti-Orai1 antibody staining compared to SM22CrePDGFR β^{WT} littermates (Figure 4.26a). Importantly, Western blotting of aortic protein lysate also suggested increased Orai1 expression in SM22CrePDGFR β^{D849V} aortae (Figure 4.26b), although the intensity of the protein bands in general was low and there are lots of non-specific bands present.

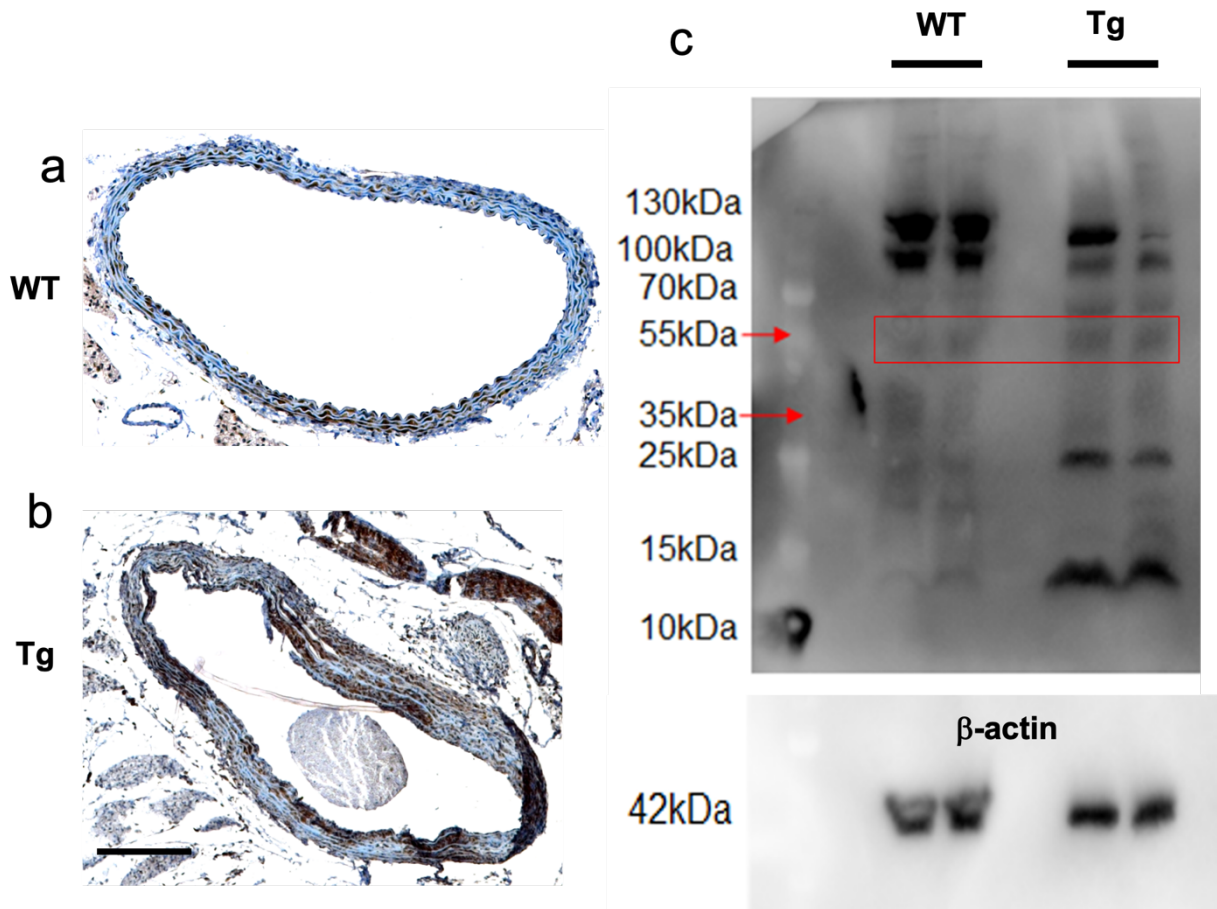


Figure 4.26 Orai1 expression in aortae of SM22CrePDGFR β^{D849V} mice.

(a&b) Orai1 immunostaining of WT **(a)** and SM22CrePDGFR β^{D849V} (Tg; **b**) aortic section. Scale bar: 200 μ M. **(c)** Orai1 Western blot image showing bands corresponding to predicted molecular weight of Orai1 (glycosylated -Orai1 ~50 KDa and non-glycosylated Orai1 ~35 KDa; red box). Two whole aorta lysates obtained from 2 mice of each genotype (WT and SM22CrePDGFR β^{D849V} (Tg)) were probed. Bottom panel shows loading control bands (β -actin; 42 KDa).

4.3.2 SOCE in SM22CrePDGFR β ^{D849V} VSMC and inhibition by JPIII

Next, we sought functional validation by measuring SOCE in the VSMC isolated from SM22CreERPDGFR β ^{D849V} and SM22CreERPDGFR β ^{WT} aortae. Following a previously described method, VSMC were isolated from SM22CreERPDGFR β ^{WT} mice to standardise the technique (Ray et al., 2001). Identity of VSMC was evaluated after 3-4 weeks of culture using the traditional technique of immunofluorescence staining with VSMC, fibroblast and endothelial cell marker antibodies: α -SMA, vimentin and CD-31 respectively. CD-31 was completely negative in all samples. Although many cells stained positive for α -SMC, many were also positive for vimentin, raising concerns contamination with fibroblasts in VSMC culture (Figure 4.27a&b). As antibody markers could give false positive results especially in PDGFR β mutant lines where VSMC phenotype switching could be occurring, it was decided to isolate and check VSMC from those lines using native green (mGFP) fluorescence in mTmG line rather than antibody staining. Using the same VSMC explant technique, cells were isolated from the SM-MHCCreER^{T2}mTmG line and treated *ex vivo* with 4-hydroxy tamoxifen to induce mTom to mGFP switch in VSMC only (4-OHT). Fluorescence imaging showed that initially, majority of cells (92 $\pm$ 3.5% vs 0% in control (solvent) treated cells) were green upon treatment with 4-OHT and in control (vehicle treated) sample, there were no green cells (Figure 4.27c&d). However, after few weeks, many mTom positive cells appeared in the culture. Hence, cells are used as early as possible after isolation.

To determine any changes in SOCE, the cells were exposed to thapsigargin to deplete the ER stores in Ca²⁺ free extracellular solution and then 1.5 mM extracellular Ca²⁺ was added back to the cells. Ca²⁺ was measured using the ratiometric calcium indicator dye Fura-2. We observed a higher baseline intracellular Ca²⁺ in the SM22CrePDGFR β ^{D849V} VSMC compared to controls alongside enhanced SOCE (Figure 4.27a). While there was no difference in store release, the mutant cells showed increased SOCE (Figure 4.27b-d). To demonstrate that this was an Orai1 dependent effect, we used our established selective and potent small molecule SOCE/Orai1 inhibitor 4-(2,5-dimethoxyphenyl)-N-[(pyridin-4-yl)methyl]aniline (known as JPIII, Bartoli et al. 2020). Pre-treatment with 10 mM JPIII did not alter the baseline calcium (Figure 4.28a). While JPIII did not alter store release, it has significantly reduced SOCE in both WT and mutant cells (Figure 4.28a-

d). Moreover, in WT cells JPIII reduced SOCE by $44\pm6\%$ vs. $61\pm2\%$ in SM22CrePDGFR β^{D849V} cells, thus showed more pronounced ($p=0.04$) effect in the latter (Figure 4.28e).

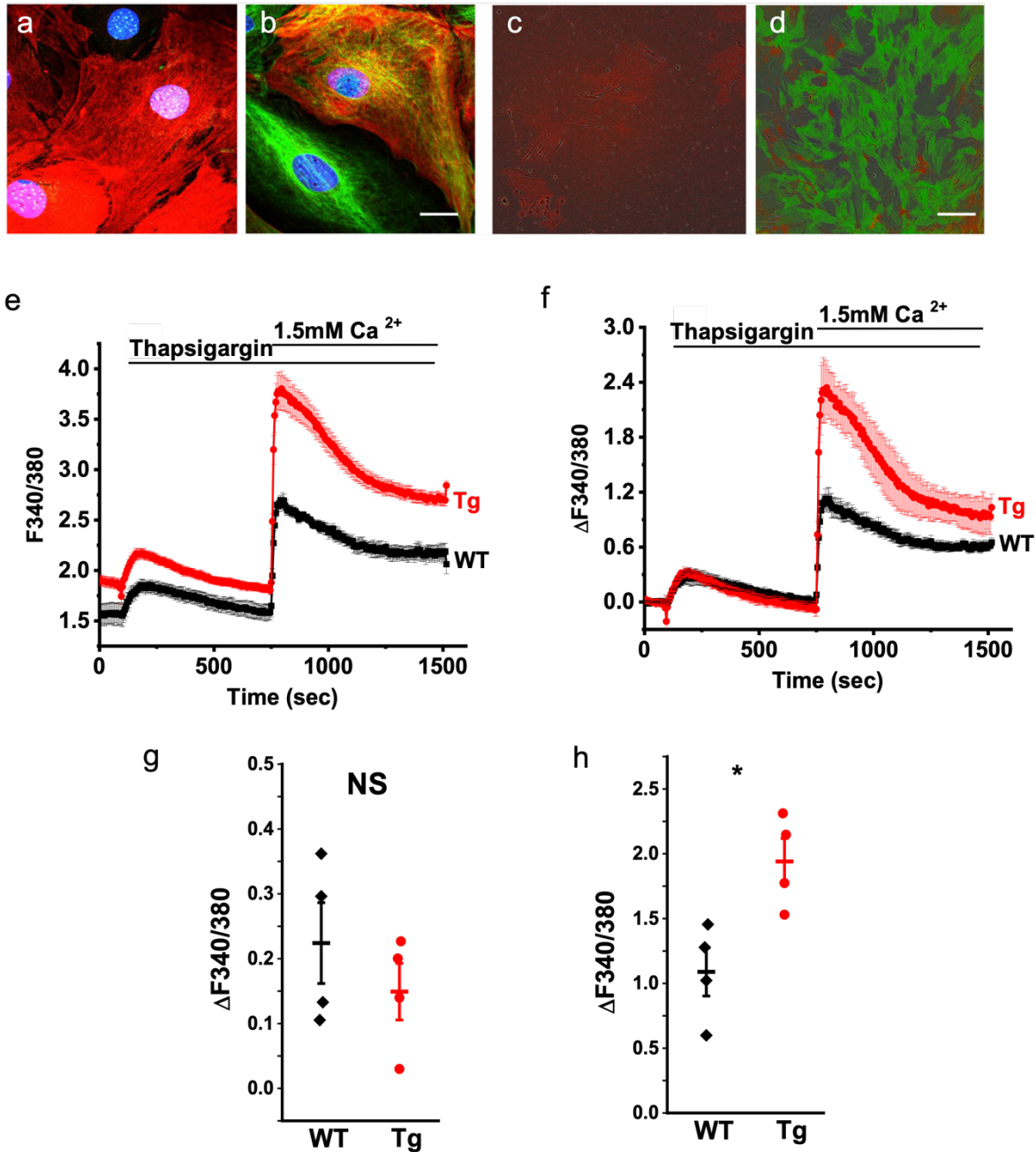


Figure 4.27 SOCE in SM22CrePDGFR β^{D849V} aortic VSMC.

(a&b) Representative fluorescence images of SM22CreERPDGFR β^{WT} VSMC stained with VSMC marker α SMA (red) and fibroblast marker vimentin (green) (blue: nuclear Hoechst staining). Scale bar: 25 μ m. (c&d) Representative fluorescence images of SM22CreERT²mTmG VSMC without (c) and with 4-OHT (d) treatment. Scale bar: 100 μ m. (e&h) Representative traces

showing intracellular Ca^{2+} level in VSMC isolated from SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) aorta upon thapsigargin application followed by addback of 1.5 mM calcium containing solution presented as raw ratio (F(340/380); **e**) and after baseline subtraction ($\Delta\text{F}(340/380)$; **f**). n=4 (technical quadruple) for each data point. Mean peak intracellular Ca^{2+} level after thapsigargin application (store release; **g**) and following Ca^{2+} addback (SOCE; **h**). n=4 (mice) for each data point; *, ** and *** indicates $p<0.05$, $p<0.01$ and $p<0.001$ respectively. NS: not significant.

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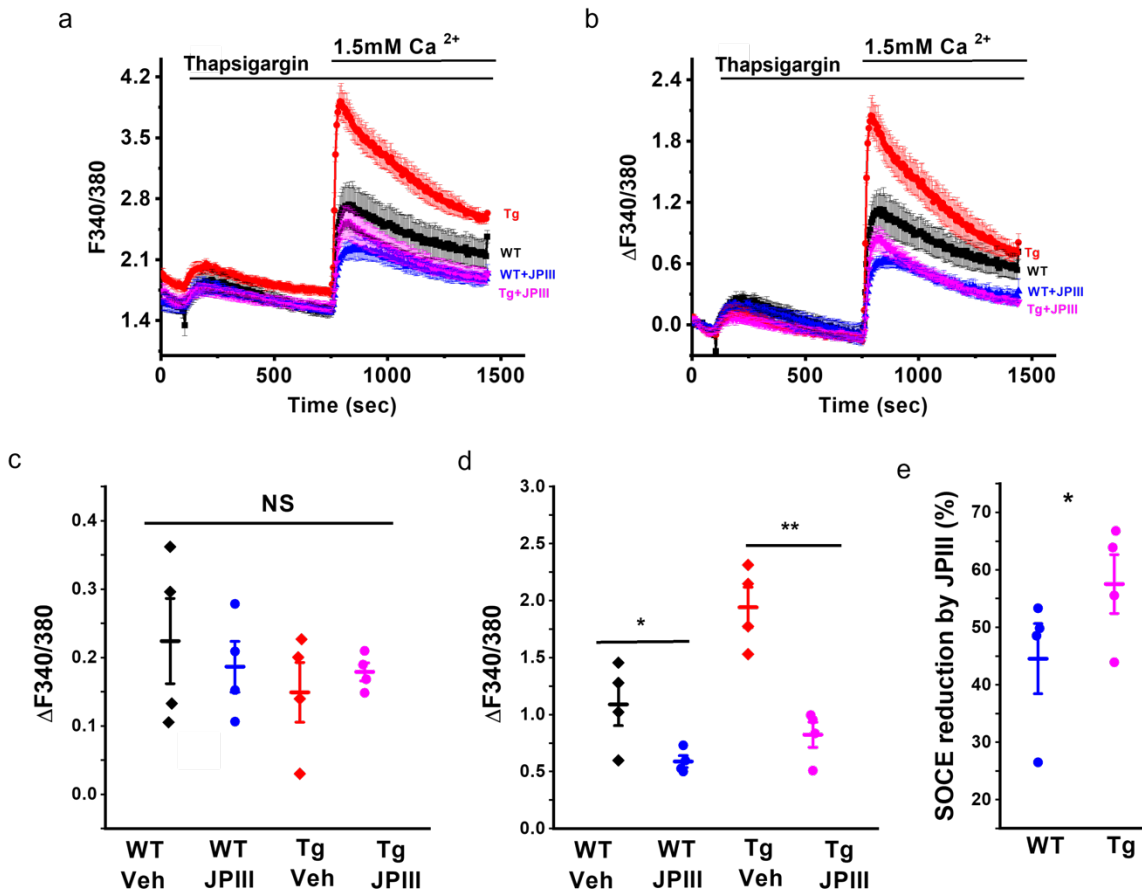


Figure 4.28 JPIII inhibition of SOCE in SM22CrePDGFR β^{D849V} aortic VSMC.

(a&b) Representative traces showing intracellular Ca²⁺ level in VSMC isolated from SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) and pre-treated with JPIII or solvent upon thapsigargin application followed by 1.5 mM calcium containing solution presented as raw ratio (F(340/380); **a**) and after baseline subtraction (Δ F(340/380); **b**). n=4 (technical quadruple) for each data point. **(c&d)** Mean peak intracellular Ca²⁺ level after thapsigargin application (store release; **c**) and following Ca²⁺ addback (**d**). **(e)** Mean percentage reduction in SOCE after JPIII pre-treatment in SM22CrePDGFR β^{WT} (WT) and mutant (Tg) cells. n=4 (mice) for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

4.3.3 Effect of pharmacological inhibition of Orai1 on PPE-induced AAA

Having observed a potent effect of JPIII on SOCE in cells from the SM22CrePDGFR β^{D849V} line *in vitro* we examined the *in vivo* effect of JPIII on PPE-induced AAA in SM-MHCCreER^{T2}PDGFR $\beta^{D849V/WT}$ mice (Figure 4.29a). The infrarenal aorta was measured by USS 2 weeks after PPE application which showed clear AAA development (Figure 4.29b&c). Then JPIII or vehicle was infused using osmotic minipump as described in methods for 14 days. At the end of study period (2 weeks of JPIII infusion and 4 weeks after PPE application), aortae were measured by USS. The data showed no significant change in AAA in response to JPIII infusion either in the WT or SM-MHCCreER^{T2}PDGFR β^{D849V} mice (Figure 4.29d&e). However, the difference in aortic size between WT and transgenic animal was lost after either vehicle or JPIII infusion. Also, there was no difference between WT and SM-MHCCreER^{T2}PDGFR β^{D849V} aortae dimensions after vehicle treatment. These data and along with large variability in the measurements between mice within a group imply we need larger sample size to see the effect of JPIII.

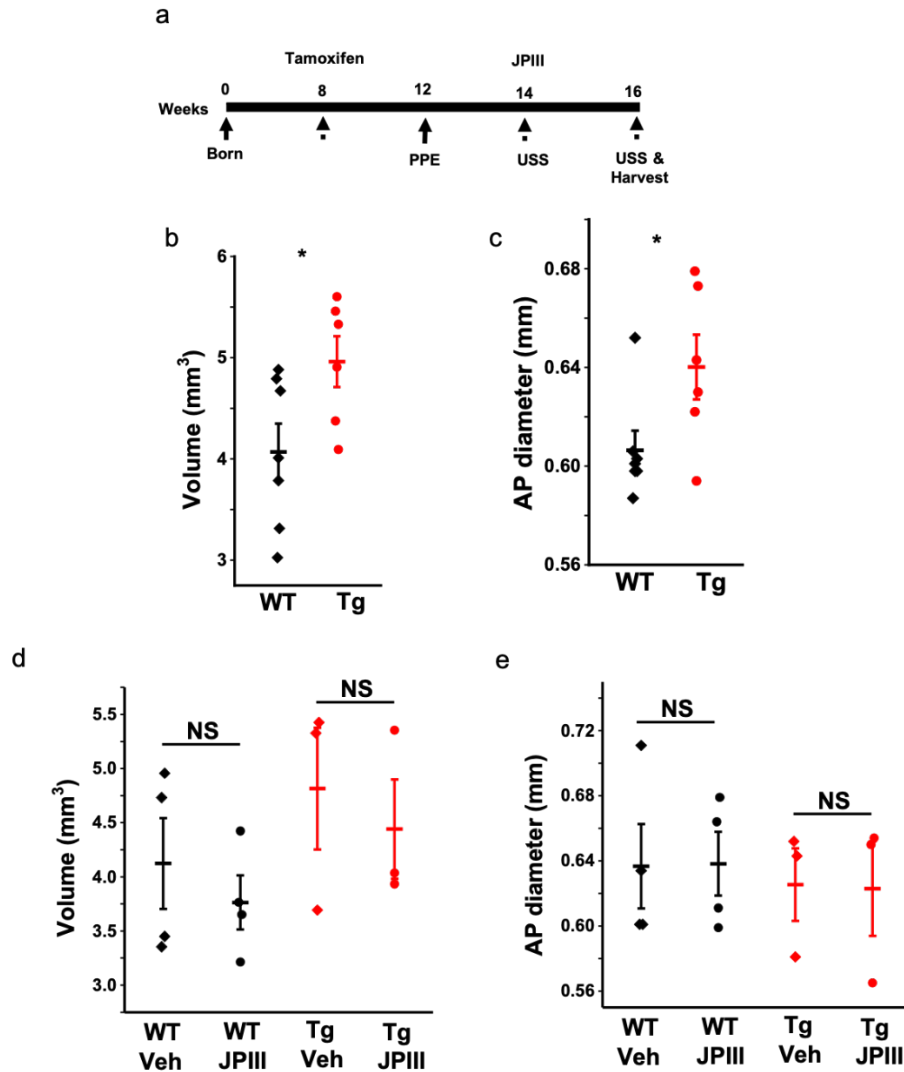


Figure 4.29 Effect of JPIII on PPE-induced AAA in SM-MHCCreERT2PDGFR β ^{D849V} mice.

(a) Timeline of experimentation (PPE: porcine pancreatic elastase). **(b&c)** Mean infrarenal aortic volume **(b)** and AP diameter **(c)** of WT and SM-MHCCreERT²PDGFR β ^{D849V} (Tg) aortae 2 weeks after PPE application. **(d&e)** Mean infrarenal aortic volume **(d)** and AP diameter **(e)** of WT and SM-MHCCreERT²PDGFR β ^{D849V} (Tg) aortae after 2 weeks of JPIII or vehicle (Veh) infusion (4 weeks after PPE application). n=3-4 for each data point; *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

4.3.4 Effect of genetic deletion of Orai1 on AAA

Finally, we sought to determine if deletion of VSMC Orai1 altered the aortic phenotype observed in the SM22CrePDGFR β^{D849V} animals. We crossed Orai1 floxed mice which was previously validated (Bartoli et al., 2020) into the SM22CrePDGFR β^{D849V} line to create a new line (SM22CrePDGFR β^{D849V} Orai1 $^{-/-}$). We studied all Orai1 genotypes (Orai1 $^{+/+}$, Orai1 $^{+/-}$ and Orai1 $^{-/-}$) in the context of PDGFR β^{D849V} and PDGFR β^{WT} littermates. There was no difference in the body weight between all 6 genotypes (Figure 4.30). However, similar to SM22CrePDGFR β^{D849V} line as described in section 4.2, these animals also showed increased heart and spleen weight in PDGFR β^{D849V} genotypes irrespective of Orai1 genotype (Figure 4.30). This work was severely impacted by the COVID-19 pandemic. We therefore pragmatically used USS at 8 weeks of age to measure the supra- and infra-renal aorta. As expected SM22CrePDGFR β^{D849V} line developed aortopathy as shown by increased volume and AP diameter, and reduced distensibility of the supra- and infra-renal aorta (Figure 4.31). Orai1 deletion had no impact on suprarenal or infrarenal aortic dimensions and distensibility of PDGFR β^{WT} animals (Figure 4.32). Also, Orai1 deletion had no impact on suprarenal aortic dimensions and distensibility in PDGFR β^{D849V} animals (Figure 4.33a-c). However, both hetero (Orai1 $^{+/-}$) and homo (Orai1 $^{-/-}$) animals' aortae showed significantly reduced volume (WT: 5.9 ± 0.2 mm 3 ; Orai1 $^{+/-}$: 5.1 ± 0.2 mm 3 ; Orai1 $^{-/-}$: 4.9 ± 0.3 mm 3 ; $p=0.04$; all with PDGFR β^{D849V}) (Figure 4.33d). While no significant difference in aortic diameter was evident between the genotypes, homozygous Orai1 knockout (PDGFR β^{D849V} Orai1 $^{-/-}$) aortae exhibited significantly improved distensibility 62.5 ± 2.8 MPa $^{-1}$ vs. 50.8 ± 1.8 MPa $^{-1}$; $p=0.004$) compared to WT (PDGFR β^{D849V} Orai1 $^{+/+}$) aortae (Figure 4.33d-f)

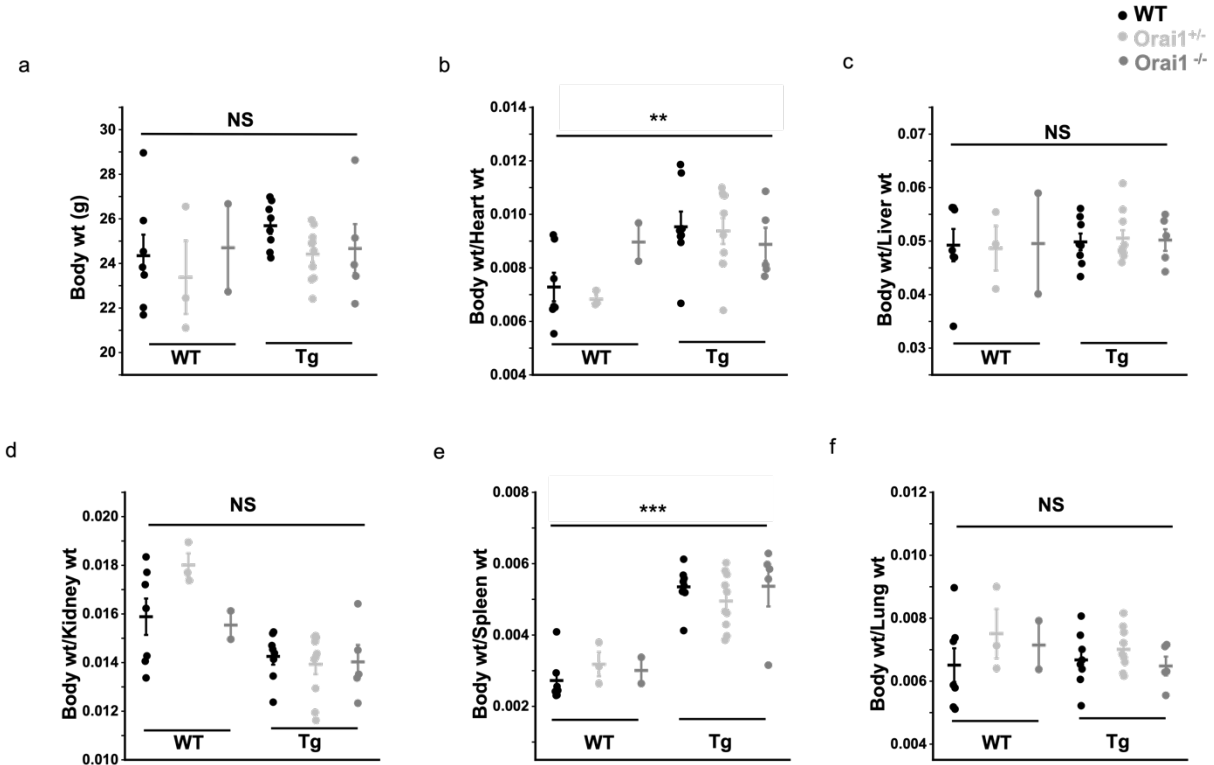


Figure 4.30 Body and organ weights of SM22CrePDGFR β^{D849V} and Orai1 double transgenic mice.

(a-f) Mean body weight **(a)** and individual organ to body weight ratio **(b-f)** of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) mice with no (Orai1^{+/+}; black), heterozygous (Orai1^{+/-}; light grey) or homozygous (Orai1^{-/-}; dark grey) deletion at 8 weeks of age (Heart **(b)**, Liver **(c)**, Kidney **(d)**, Spleen **(e)**, and Lungs **(f)**). n=2-10 for each data point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively (2-way ANOVA). NS: not significant.

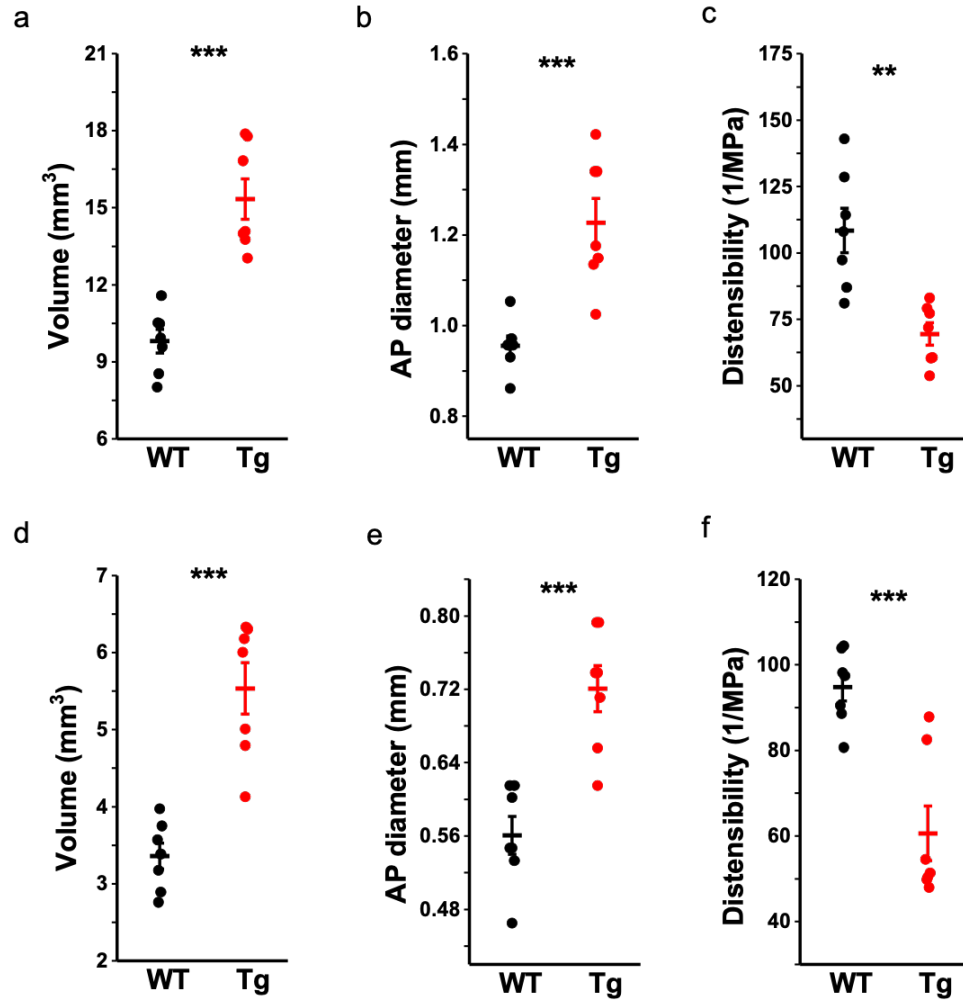


Figure 4.31 USS analysis of supra- and infra-renal aortae of SM22CrePDGFR β^{D849V} and SM22CrePDGFR β^{WT} mice.

(a-c) Mean suprarenal aortic volume (a), AP diameter (b) and distensibility (c) of SM22CrePDGFR β^{WT} (WT) and SM22CrePDGFR β^{D849V} (Tg) transgenic animals at 8 weeks of age. (d-f) Mean infrarenal aortic volume (d), AP diameter (e) and distensibility (f) of SM22CrePDGFR β^{D849V} (Tg) and SM22CrePDGFR β^{WT} (WT) transgenic animals at 8 weeks of age. n=7 for each time point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively. NS: not significant.

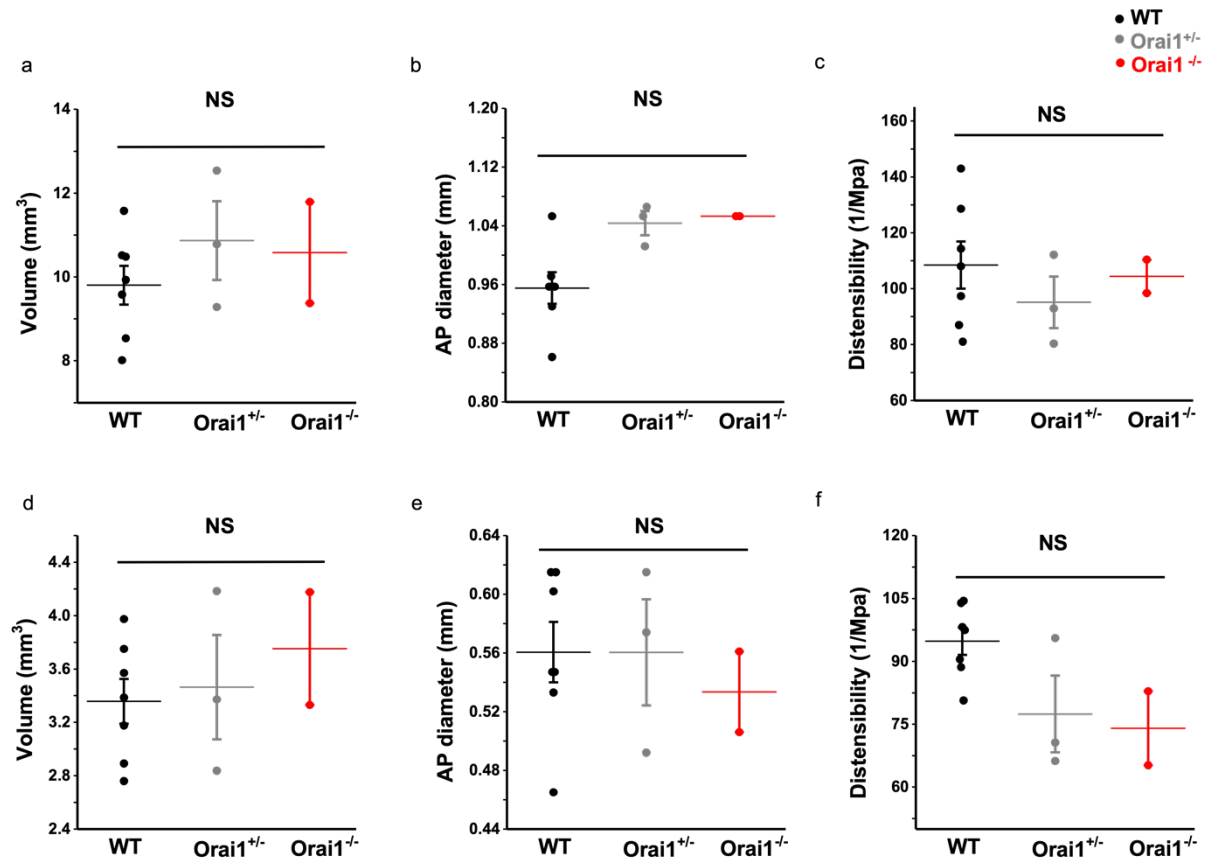


Figure 4.32 USS analysis of supra- and infra-renal aortae of SM22CrePDGFR β^{WT} and Orai1 double transgenic mice.

(a-c) Mean suprarenal aortic volume (a), AP diameter (b) and distensibility (c) of SM22CrePDGFR β^{WT} with no (Orai1 $^{+/+}$), heterozygous (Orai1 $^{+/-}$) or homozygous (Orai1 $^{-/-}$) deletion at 8 weeks of age. (d-f) Mean infrarenal aortic volume (d), AP diameter (e) and distensibility (f) of SM22CrePDGFR β^{WT} with no (Orai1 $^{+/+}$), heterozygous (Orai1 $^{+/-}$) or homozygous (Orai1 $^{-/-}$) deletion at 8 weeks of age. n=2-7 for each time point. NS: not significant.

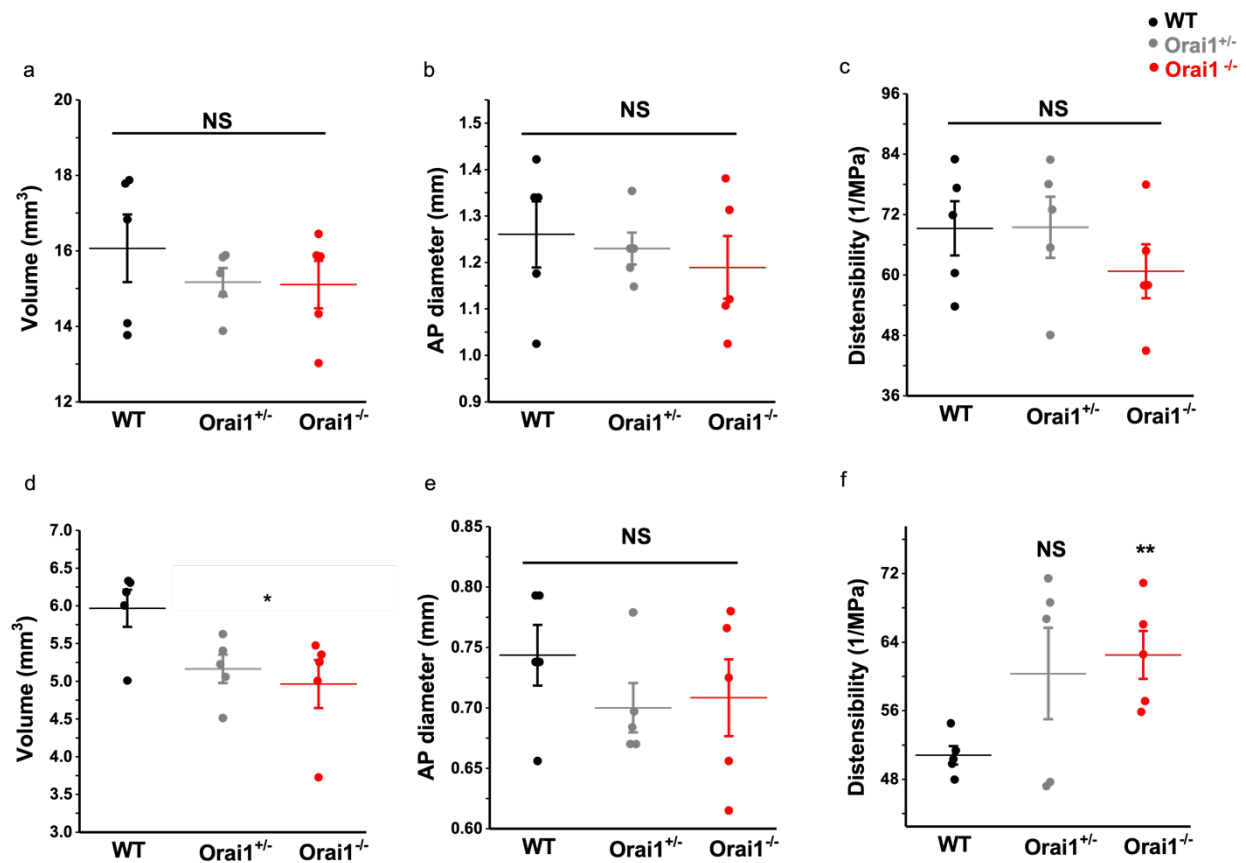


Figure 4.33 USS analysis of supra- and infra-renal aortae of SM22CrePDGFR β^{D849V} and Orai1 double transgenic mice.

(a-c) Mean suprarenal aortic volume (a), AP diameter (b) and distensibility (c) of SM22CrePDGFR β^{D849V} with no (Orai1^{+/+}), heterozygous (Orai1^{+/-}) or homozygous (Orai1^{-/-}) deletion at 8 weeks of age. (d-f) Mean infrarenal aortic volume (d), AP diameter (e) and distensibility (f) of SM22CrePDGFR β^{D849V} with no (Orai1^{+/+}), heterozygous (Orai1^{+/-}) or homozygous (Orai1^{-/-}) deletion at 8 weeks of age. n=5 for each time point. *, ** and *** indicates p<0.05, p<0.01 and p<0.001 respectively (One-way ANOVA). NS: not significant.

4.4 Summary of the chapter

The data obtained from transgenic mice models with perturbations in PDGFR β signalling and *Orai1* expression were described in this chapter. I showed that VSMC specific expression of constitutively active PDGFR β from embryonic stage (SM22CrePDGFR β^{D849V}) induced significant aortopathy from 8 weeks of age. In contrast, VSMC specific overexpression of constitutively active PDGFR β from the adult stage (SM-MHCCreER^{T2}PDGFR β^{D849V}) did not produce any major aortic phenotype until 32-36 weeks of age after which there was a mild dilatation in the infrarenal aorta. Through transcriptomics study and quantitative RT-PCR of SM22CrePDGFR β^{D849V} aorta I identified differential expression of multiple genes that are relevant to AAA pathogenesis. Importantly, expression of LRP1, the first gene associated with AAA pathogenesis in GWAS studies, was elevated in SM22CrePDGFR β^{D849V} aorta. I also identified that *Orai1* expression and SOCE were potentially elevated in PDGFR β^{D849V} aorta. I found that though JPIII can inhibit SOCE in VSMC, it did not significantly alter PPE-induced AAA in SM-MHCCreER^{T2}PDGFR β^{D849V} mice. Moreover, knocking out single or double allele of the *Orai1* gene in SM22CrePDGFR β^{D849V} animals significantly rescued the aortopathy in the infrarenal part of the aorta and therefore modulation of *Orai1* warrants further study as a therapeutic target for aortic diseases.

Chapter 5 : Discussion and Future Directions

5.1 Summary of Results

The purpose of this thesis was to determine the role of VSMC de-differentiation and remodelling in aortic disease, with a particular focus on disease progression. The ultimate goal of the work was to identify novel targets with potential for the medical management of AAA in the future.

First, I generated and validated a VSMC conditional, tamoxifen inducible mTmG lineage tracing mouse model. I then used this mouse line to gain new insights into AAA by surgically inducing AAA using the peri-adventitial PPE application model. PPE application induced cell proliferation leading to an increase in the numbers of cells of both SMC and non-SMC origin within the aorta. I made important observations that there was evidence of increased lineage traced VSMC in the aorta following PPE application, with evidence of de-differentiation at the point of maximal AAA size followed by regression towards normal. PDGFR β was significantly upregulated in the model in cells- of both SMC and non-SMC origin.

Next, I set out to drive VSMC de-differentiation and remodelling using two novel mouse lines with constitutively active PDGFR β signalling. The SM22CrePDGFR β^{D849V} line was used to study the impact of VSMC de-differentiation and remodelling on aortic phenotype from birth and the SM-MHCCreER^{T2}PDGFR β^{D849V} line to induce expression of the transgene only in adults which were then exposed to the PPE AAA model.

Constitutively active PDGFR β (SM22CrePDGFR β^{D849V}) expression throughout embryonic development and subsequent post-natal life led to a clear and dramatic development of an aortopathy throughout the aorta with thickening of the aortic wall which was progressive over time. Transcriptomics of whole aortic tissue from these mice suggested perturbation of genes involved in pathways including extracellular matrix synthesis and maintenance, VSMC remodelling and inflammation/chemotaxis. RNAseq findings were validated using qRT-PCR. Specifically, we identified upregulation of VSMC remodelling genes *Mmp2*, *Mmp12*, *Timpl*,

Clec3b, and *Spp1* and inflammation related genes *Bmp2* and *Ccr5* in SM22CrePDGFR β^{D849V} aorta. *Lrp1*, a known gene involved in AAA pathogenesis was also upregulated.

Next the inducible SM-MHCCreER^{T2}PDGFR β^{D849V} model was studied which showed a modest increase in medial layer cellularity 4 weeks post tamoxifen administration but minimal aortic phenotype. When these animals were challenged with the PPE AAA model, I observed larger and more progressive AAA in SM-MHCCreER^{T2}PDGFR β^{D849V} animals compared with SM-MHCCreER^{T2}PDGFR β^{WT} littermate controls. It is known that the PDGFR β^{D849V} transgene drives signalling predominantly through PLC γ . We therefore studied SOCE and Orai1 which act downstream of this, and we identified increased Orai1 expression and enhanced SOCE in VSMC obtained from SM22CrePDGFR β^{D849V} mice which could be blocked by our novel small molecule inhibitor, JPIII. Inhibition of Orai1/SOCE by JPIII *in vivo* did not show significant effect on PPE-induced AAA in SM-MHCCreER^{T2}PDGFR β^{D849V} line. Knocking out the Orai1 gene in the SM22CrePDGFR β^{D849V} significantly protected animals from the aortic dilatation.

5.2 General Discussion

The AAA screening program is aimed at preventing/reducing deaths in the community from unexpected rupture. However, at the individual level a diagnosis of AAA-unless the size meets the criteria for intervention, means "wait and monitor", which must be a difficult situation for the patient concerned for a long period. So, in spite of recent decline in the incidence of AAA and declining rate of rupture and resultant mortality, human AAA is still a clinical condition that exerts significant impact on individuals' health.

The age at which clinically relevant aneurysms are detected is increasing for many AAA patients meaning they have accumulated more medical co-morbidities and clinically meaningful frailty. They are poor candidates for either open or EVAR surgery. The search for non-surgical medical treatment of AAA is still very much needed. As human samples are often of advanced stage of the disease, animal models remain the mainstay for the research in this area to understand the processes of AAA development and growth, especially for target identification and validation.

Human tissue studies have shown that pathogenesis of AAA involves multiple mechanisms, including extracellular matrix (ECM) degradation, oxidative stress, apoptosis of VSMC and inflammation. As ECM degradation was considered as the most important mechanism causing AAA, numerous past studies explored inhibition of ECM destruction by targeting matrix metalloproteinases (MMPs) as the primary strategy for the treatment of AAA. Though some strategies including inhibition of MMP-9 and inducing tissue inhibitors of MMPs (TIMPs) were successful in the animal models and showed favourable effect on human tissues/cells *ex vivo*, none have been translated to wider clinical application as expected (Lindeman and Matsumura, 2019; Yoshimura et al., 2018). The reasons may include failure to achieve a right balance between endogenous MMP inhibitors, overexpressed MMPs in AAA and the drug, poor *in vivo* pharmacological characteristics of the drugs including low bioavailability and adverse events and importantly improper designing of animal models and clinical trials (Li et al., 2020). Similarly, non-targeted generic anti-inflammatory medications, antibiotics and cardiovascular protective agents including antihypertensives, antianginals and hypoglycaemic drugs also failed to show any benefits in AAA patients (Meijer et al., 2013; Su et al., 2022). Though doxycycline was successful in reducing AAA when administered during the induction of AAA, it was not effective in already formed AAA which makes it not a relevant treatment option for patients (Xie et al., 2012). Hence, the requirement for identifying new targets with high sensitivity and specificity remains an important clinical need in AAA management.

Our work explored targeting PDGF signalling through Orai1/SOCE as a strategy for AAA management as this strategy targets the AAA growth rather than formation and thus have better potential for clinical translation. While first part of the study focused on elucidating PDGF mediated pathophysiological mechanisms in AAA using transgenic models, the second part focused on evaluation of therapeutic benefits.

5.3 SMC-specific Cre models of AAA

SMC exhibit a remarkable plasticity that contributes to vascular growth, repair and also to cardiovascular pathologies. Mouse models have been an important tool for the functional studies

of SMC over the past few decades. Recent development of SMC-specific Cre-driver lines have resulted in identifying the diversity of phenotypes derived from mature SMC transdifferentiation (Liu and Gomez, 2019). The regulatory regions of the three SMC markers SM-MHC, SM22, and ACTA2 are the most frequently used SMC-specific promoters to drive Cre expression (Alexander and Owens, 2012).

SM22 α is an actin-binding protein which regulates SMC contraction and serves as a marker of the differentiated phenotype. However, it is transiently expressed in cardiac myocytes during embryonic development and also in several other cell types including myofibroblasts, circulating neutrophils and monocytes, peritoneal macrophages and immune cells in the spleen. When a 2.8 kb fragment of the mouse SM22 promoter was used to drive Cre expression, it showed robust expression in SMC across the organs and hence is now widely used (Holtwick et al., 2002). SM22 Cre has been previously described and well characterised in an aortic study (Olson and Soriano, 2011). Expression of ACTA2, a key smooth muscle contractile protein, increases with differentiation. While in the synthetic SMC ACTA2 expression is lower, it is also expressed in many other cell types including fibroblasts and myofibroblasts. Hence many investigators use ActaCre lines primarily for the study of myofibroblasts and fibrosis. But with suitable controls they may have utility for SMC studies as well (Chakraborty et al., 2019).

SM-MHC is considered to be the most specific to SMC and hence they are widely used, especially for SMC lineage tracing studies. However, there is recent evidence for non-SMC expression of SM-MHC such as in certain cells in the alveoli of the adult lung and in a subset of pericytes (Murgai et al., 2017; Sheikh et al., 2014). Another important issue is that the SM-MHCCreER^{T2} transgene is incorporated into the Y chromosome which has limited investigations with this transgene to male gender only. However, a colony of SM-MHCCreER^{T2} line in which the transgene is inherited by both male and female achieved by inducing translocation of the transgene to the X chromosome has been reported recently (Liao et al., 2017).

As none of the SMC specific Cre models achieves complete specificity, we decided to use two complementary models for this study- one driven by SM22 α promoter and constitutively active

and another by SM-MHC promoter in which Cre recombination was induced by tamoxifen injection.

Few investigators have also shown that SM-MHCCreER^{T2} may exhibit Cre activity even without tamoxifen injection especially in the aorta (Steffensen et al., 2022). Hence, we have generated SM-MHCCreER^{T2}LacZ line to test the specificity of spatial (SMC) expression and SM-MHCCreER^{T2}mTmG line to evaluate the dependency of expression (temporal specificity) on tamoxifen. LacZ line showed no expression of LacZ (indicated by blue staining) in non-SMC regions of the tissues. However, in SM-MHCCreER^{T2}mTmG line some tissues showed green fluorescence without tamoxifen injection indicating a leak in the Cre activation.

All Cre-driving promoters are prone to the limitations of the Cre-loxP system including the dependency to tamoxifen induced Cre-mediated recombination of floxed genes. Promoters that drive high expression of Cre are more likely to result in tamoxifen-independent recombination compared with weaker Cre-promoters. SM-MHCCreER^{T2} drive high levels of Cre expression in SMC and hence a detectable level of leak is not unexpected (Deaton et al., 2023). However, importantly this tamoxifen-independent nuclear translocation of Cre resulting in recombination is limited to SMC and thus the model is still useful albeit only on male mice due to the location of transgene in Y, for our studies with suitable control. Hence for the functional experiments we used SM-MHCCreER^{T2}PDGFR β ^{WT} as controls rather than SM-MHCCreER^{T2}PDGFR β ^{D849V} without tamoxifen injection.

5.4 Driving PDGFR β signalling in the aorta

Driving the PDGFR β signalling in VSMC consistently induced aortopathy in our models, albeit at different levels, sizes and time periods. Constitutively active Cre model, SM22CrePDGFR β ^{D849V} where PDGFR β signalling was enhanced from embryo developed significant and progressive aortopathy in both supra and infrarenal aorta starting from 8 weeks of age. In SM-MHCCreER^{T2}PDGFR β ^{D849V} line where the signalling was induced in adult mice by tamoxifen-induced Cre recombination, only a small increase in lumen size of infrarenal aorta was observed 4 weeks post-induction. These results clearly signify the role of PDGF signalling in aortic disease.

PDGF signalling has been well established as a driving pathway for SMC proliferation, migration and phenotypic switching (García-Olivas et al., 2007; Lu et al., 2018). All those pathophysiological cellular processes were previously shown to contribute to the pathogenesis of AAA. However, we are the first one to show the direct stimulation of AAA development by driving PDGF signalling *in vivo* murine transgenic model.

Interestingly, the line in which PDGFR β signalling was enhanced from developmental stage, has developed aortopathy from the root to the infrarenal aorta. Transforming growth factor- β (TGF- β) signalling, is considered to be the primary mechanism for inducing thoracic aortic aneurysm (TAA) and the VSMC derived from the neural crest while PDGFR β signalling is the most important factor in the rest of the VSMC derived from mesoderm (Wang et al., 2013). In converse, TGF- β protects against AAA formation, progression and rupture by decreasing inflammatory infiltration, ECM degradation, and VSMC apoptosis (Angelov et al., 2017; Wang et al., 2013). However, there is evidence for cross talk between PDGF and TGF- β signalling pathways (Contreras et al., 2019). In mesenchymal stem cells PDGF signalling was shown to activate TGF- β pathway and in glomerular mesangial cell TGF- β acts through PDGFR β receptor (Maity et al., 2020; Yokota et al., 2014). Hence inducing PDGF pathway through overexpressing constitutively active PDGFR β could have led to enhancing TGF- β signalling and thoracic aortopathy.

The constitutive Cre model animals started to die from the age of 16 weeks. While cardiomegaly and splenomegaly were observed in the line and aortic rupture was ruled out on post-mortem, cause of death could not be determined. PDGFR β signalling in cardiomyocytes was shown to be essential for angiogenesis and cardiac remodelling in response to load-induced stress (Chintalgattu et al., 2010). PDGF-BB signalling has been shown to induce pulmonary hypertension and expression of many proteins associated with the PDGF signalling were increased in cardiovascular tissues of hypertensive patients (Solinc et al., 2022; Stoynev et al., 2014). So, it is plausible that increased blood pressure in these mice could be the cause of the cardiac hypertrophy. Due to technical difficulties, we were not able to measure the blood pressure. Also, PDGFR β signalling also enhanced proliferation of spleen-derived various progenitor cells (H. Wang et al. 2012). In agreement with this many genes involved in inflammatory signalling were perturbed in this line as shown by transcriptomics analysis. Thus, enhancement of PDGFR β signalling in those

organs/cells could explain our observations. Though the VSMC specific promoters (SM22 and SM-MHC) we used, limited the expression of constitutively active PDGFR β signalling to VSMC as per our data showing no X-Gal staining in heart early on (1-2 weeks post-induction), there could have been some leak in the expression later as described above.

Transcriptomics analysis of SM22CrePDGFR β^{D849V} aortae gave valuable insights into various genes and signalling mechanisms which may have significant roles in AAA pathophysiology. The principal component analysis (PCA) plot showed reasonable grouping of the samples and differentially expressed genes (DEGs) analysis highlighted changes in the expression of a huge number of genes. Two standout pathways that were identified by GO/REACTOME/KEGG analysis were ECM regulation and inflammation. The strongest DEGs are also related to the proteins involved in those cellular processes. Both- ECM homeostasis and inflammation have previously been shown to play indispensable roles in AAA pathophysiology (Stepien et al., 2022). In addition, various downregulated DEGs mapped to electron transport chain, ATP synthesis and oxidative phosphorylation. While inflammation can be directly linked to them, there is extensive evidence showing their involvement in VSMC phenotype switching, ECM synthesis and degradation (Tanaka and Laurindo, 2017). In fact, many investigators documented increased local and circulating oxidative stress markers in AAA models and human samples (McCormick et al., 2007; Sánchez-Infantes et al., 2021). Further analysis by qPCR showed that *Mmp2*, *Mmp12*, *Timp1*, *Clec3b*, and *Spp1* were significantly elevated and *Col19a1* was reduced in the transgenic aorta. Proteins encoded by all these genes are involved in vascular remodelling and except *Col19a1*, all of them are involved in ECM degradation. Interestingly the protein encoded by *Col19a*, alpha chain of type XIX collagen was shown to be enriched in basement membrane and its genetic disruption led to ECM disruption (Calvo et al., 2020). Thus, reduction in ECM structural proteins and enhancement in the expression of lytic molecules fits well with the known pathogenic mechanisms of AAA. Also, increase in the mRNA expression of inflammation/chemotaxis related genes *Bmp2*, *Ccr5* and *Il6* showed the importance of inflammation in AAA. BMP2 induces VSMC apoptosis in intracranial aneurysms and enhanced CCR5 expression in the infiltrating macrophages can drive the AAA (Ishida et al., 2020; Ye et al., 2023). Human and animal studies have showed that IL6 has an important but limited role in AAA pathogenesis primarily by regulating cellular migration and infiltration (Nishihara et al., 2017). Moreover, IGFBP2 which is an early marker of

VSMC phenotype switching in thoracic aortic aneurysm showed increased expression (Pedroza et al., 2021). GWAS studies identified association between LRP1 gene polymorphisms and AAA, and our analysis showed increased expression of *Lrp1* in transgenic aorta (Wild et al., 2012).

Despite observing a small increase in lumen size of the infrarenal aorta in the SM-MHCCreER^{T2}PDGFR β ^{D849V} line 4 weeks post-tamoxifen induction, there was no change in distensibility. However, there was increased wall cellularity which implies early AAA development. The results imply that long-term induction of PDGFR β signalling is required for AAA formation. In agreement with this interpretation, longer-term follow-up of the transgenic animals showed that they do develop dilatation of the infrarenal aorta after 44 weeks of age which were progressive until over observation period (64 weeks). The advantages of SM-MHCCreER^{T2}PDGFR β ^{D849V} model are, it excludes the embryonic/developmental impact of enhanced PDGFR β signalling and the anatomical location of AAA developed is infrarenal part as found in human disease. However, the variability in the size and non-linear progression of AAA between the littermates along with very long duration of the experiment are their limitations.

Though during the early period after PPE application, both SM-MHCCreER^{T2}PDGFR β ^{D849V} animals and SM-MHCCreER^{T2}PDGFR β ^{WT} littermates has similar size AAA, the transgenic animals developed larger AAA later. Also, the AAA was progressive in the SM-MHCCreER^{T2}PDGFR β ^{D849V} unlike in the WT mice, where it started to resolve. In literature, there is no compelling evidence for disease progression in PPE model and hence our model can serve as a better one for AAA progression studies. Moreover, it highlights that the PDGF signalling resulting in ongoing VSMC remodelling leads to AAA progression. In AngII AAA model too when the VSMC remodelling was driven by growth factor signalling there was progressive AAA (Daugherty et al., 2011). However, we followed it up only for up to 12 weeks post PPE-induction. Further longer-term time course studies will be required to optimise the experimental protocol for progression studies and to evaluate rupture.

VSMC proliferation and remodelling has been suggested as a less well appreciated pathological feature of AAA (Ailawadi et al., 2009; Salmon et al., 2013; Shankman et al., 2015). PDGFR β signalling has previously been shown to induce such changes in VSMC in various vascular

disorders including atherosclerosis (García-Olivas et al., 2007; Lu et al., 2018; Zhou et al., 2021). Though the link between PDGFR β signalling and VSMC phenotype switching has been previously proposed in other diseases, for the first time we show VSMC de-differentiation and infiltration in AAA *in vivo* using constitutively active PDGFR β mutant models.

Obtaining a pure population of primary VSMC from the aorta of AAA transgenic models to enable characterisation of signalling mechanisms proved challenging. While early-stage cells were homogeneously VSMC, at later stages I found many cells expressed fibroblast markers. Cultured fibroblasts are well known to express α -SMA making this marker unsuitable to prove purity of SMC populations. Plans were made to sort the cells by FACS however, despite my efforts, insufficient mGFP positive cells persisted by the time I have generated enough cells for sorting. The possible explanation for the mixed population of cells in the culture include presence of various factors in the foetal serum used to make the cell culture medium favouring the growth of non-VSMC cells such as fibroblasts. However, batch to batch variations in the components of serum made it extremely difficult to clearly establish it. Hence, we resorted to experimenting on the VSMC which were homogenous and within 10 days of isolation (avoiding subculturing) and plating on a 96 well plate which has significantly limited the number of available cells. Therefore, only the intracellular calcium measurement assays have been presented in this thesis.

To identify the origin of the cells in AAA lesion lineage tracing studies were performed using SM-MHCCreER^{T2}mTmG mice. Though the data showed an increase in the numbers of both SMC and non-SMC origin cells, the number of SMC-origin cells expressing classical VSMC markers such as α SMA and SM-MHC significantly reduced upon AAA development. We believe this document the VSMC de-differentiation process occurring in real-time. We have also shown for the first time that in PPE induced AAA, lineage traced VSMC initially expand in number and undergo de-differentiation. We also showed that by driving VSMC de-differentiation and remodelling through enhanced PDGFR β signalling the phenotype of the PPE induced AAA can be changed from a resolving to a progressive type. Interestingly, in PPE induced AAA, there was an increase in PDGFR β expressing cells- of both SMC and non-SMC origin signifying the importance of PDGFR β signalling in VSMC de-differentiation and AAA pathogenesis. Though the relative

contribution of these two cell types to AAA pathogenesis is unclear, our results signify that this process could be a potential target for medical management of AAA.

5.5 Inhibition of Orai1 on AAA

Ion channels and transporters serve as drug targets for numerous diseases and importantly many of them, especially calcium-permeable channels can be targeted for treating a multitude of cardiovascular disorders (Cheng et al., 2019; Garcia and Kaczorowski, 2016). Small molecule inhibition of Orai1 has recently been shown to beneficially reduce adverse remodelling in the heart following pressure overload heart failure in a transgenic model and in pulmonary arterial SMC obtained from patients and rats with pulmonary hypertension (Bartoli et al., 2020; Masson et al., 2022). Importantly, previous work on the SM22CrePDGFR β^{D849V} line suggested that the downstream signalling was selectively through PLC γ , which leads to SOCE through Orai1. This in turn leads to NFAT activation and gene transcription perturbations (Olson and Soriano, 2011; Spinelli and Trebak, 2016). Here, we evaluated whether Orai1 inhibition can significantly alter the development and progression of PDGFR β signalling driven AAA, using JPIII, a pharmacological inhibitor of SOCE or genetic deletion of the Orai1 gene. Enhanced PDGFR β signalling significantly increased the SOCE which was inhibited by JPIII. The *in vivo* data obtained from our mouse model did not show significant effect of JPIII on aorta size.

Our lab previously found upregulation of Orai1 resulting in enhanced SOCE in VSMC explanted from human AAA and also actively proliferating VSMC of murine AAA (MA Bailey PhD Thesis 2016 and H Shower PhD Thesis 2023). Moreover, it was also shown that Orai1 inhibition reduces VSMC remodelling which may be beneficial in various AAA models including AngII- and CaCl₂-induced ones (MA Bailey PhD Thesis 2016 and H Shower PhD Thesis 2023). In line with those unpublished data, this work shows that in constitutively active PDGFR β murine VSMC, Orai1 was upregulated and SOCE was enhanced. However, unlike the previous *in vivo* data, we did not observe significant change in PPE-induced AAA of inducible PDGFR β^{D849V} and in the AAA of constitutive PDGFR β^{D849V} models. There can be multiple potential reasons for this observation.

For the pharmacological inhibition studies, we administered 500 ng/kg/min dosage of JPIII for 2 weeks starting from 14 weeks of age which was 6 weeks post induction of PDGFR β^{D849V} expression and 2 weeks after peri-adventitial application of PPE. As discussed before the primary mechanisms through which PPE induces AAA are disruption of medial elastic fibres and induction of inflammatory infiltration. Though VSMC proliferation was observed in the aorta, only subtle AAA formation was recorded in the inducible PDGFR β^{D849V} model, and it required PPE application to induce significant AAA. If we discount the issues pertaining to the drug (dosage, timing of administration and quality of the chemical), it can be deduced that disruption of *Orai1*/SOCE may not be a relevant pathological mechanism to target to get beneficial effects in short term in the PPE model (SM-MHCCreER^{T2}PDGFR β^{D849V} with PPE induction 4 weeks post tamoxifen injection). Though we saw significant difference in diameter of AAA induced by PPE between the WT and SM-MHCCreER^{T2}PDGFR β^{D849V} mice, that might have been too small a difference to see the benefit of *Orai1* inhibition by JPIII. Previous studies showed that same dose of JPIII albeit for 28 days successfully reduced aortic diameter and lumen size in the CaCl₂ model and aortic diameter in the PPE model when created in WT background by about 30 to 40% (MA Bailey PhD Thesis 2016). To see this difference, we require at least 10 animals in each group as per power calculation. So, my sample size may be too small (3-4 per group) to see it. In the current study JPIII infusion was only for 14 days which may also explain the lack of effect of JPIII. Interestingly, the difference in AAA size between WT and transgenic animals was lost after vehicle or JPIII infusion indicating that at the time point we measured aorta, the contribution of PDGFR β^{D849V} signalling in AAA pathogenesis in our model was not significant. We observed increased aortic wall cellularity in these mice at 12 weeks of age (4 weeks after tamoxifen induction; Figure 4.17). So, JPIII administration at 14 weeks of age (6 weeks after tamoxifen induction) might have been too late to intervene as active VSMC remodelling could have already occurred. In conclusion, both our model (PPE induction) and the experimental protocol (timeline) have muddled the results and may not be appropriate to answer the research question.

In contrast to the pharmacological inhibition, both homozygous and heterozygous deletion of *Orai1* in the SM22CrePDGFR β^{D849V} line significantly reduced the size of infrarenal aorta demonstrating that *Orai1* plays an essential role in AAA formation/progression thereby confirming our hypothesis. The suprarenal aortic dilatation was not altered by *Orai1* gene deletion. We

planned to extend the observation until the animals reached 16 weeks of age, but due to Covid lockdown we had to sacrifice the animals and stop the work at 10-12 weeks of age. However as both PDGFR β and Orai1 genetic perturbations were induced prenatally in this model caution is needed in generalising these results to the process of AAA development in humans. Thus, further work is needed to ascertain the therapeutic potential of Orai1 perturbation in AAA conclusively.

5.6 Genetic Drift

As both the VSMC specific PDGFR β^{D849V} lines started showing inconsistent phenotype we had to backcross the floxed PDGFR β^{D849V} line with C57BL6/J mice which significantly impacted the later parts of the project. Though not confirmed, we believe genetic drift might have led to changes in the observed phenotype. Genetic drift refers to spontaneous changes in genomic DNA that can arise in any generation of mouse breeding. Drift comes in the form of DNA replication or repair mistakes that get passed on in the germ cells (Benavides et al., 2020; Stevens et al., 2007). Drift can result in no observable/measurable change or create a tremendous impact resulting in significant changes in experimental results.

Most of the transgenic lines are created and maintained through inbreeding of the specific strain. The background strain we used were C57BL6/J. The defining characteristic of an inbred strain is its' genetic homogeneity which ensures consistent and reproducible phenotype across the litters irrespective of investigators and their geographical locations. Though inbred strains are not natural, rather a manmade construction, they are still prone to some natural variation. Any type of spontaneous mutations, such as point mutations, deletions, and duplications, can arise in any inbred strain and have been studied for more than 100 years (Paigen, 2003). Importantly, some types of mutations such as *de novo* mutations arising from the insertion of transposable elements are 100-fold more common in mice than humans (Maksakova et al., 2006). Residual heterozygosity, where both the set of loci that are not yet fixed within an inbreeding population and the proportion of individuals who are still heterozygous at a specific locus at a specific generation within an inbreeding population, is another reason behind genetic drift (Stevens et al., 2007). The third reason is contamination with other strain due to human error in setting up breeding cages.

As elucidating the reasons behind the genetic drift that resulted in phenotype changes in our lines is beyond the scope of our study, it was decided to rederive the line from new founders obtained from Jackson Laboratories. We have successfully achieved that, and further work is ongoing on the new lines.

5.7 Future directions

5.7.1 Transgenic models of AAA and PDGFR β signalling

Though utilising two models has strengthened our results, as discussed above both models are prone for non-specific and/or leaky expression spatially and temporally (Chakraborty 2019). Moreover, all our studies were performed in male animals only, not least because the SMMHCCreER^{T2} is carried on the Y chromosome and will only direct alteration in gene expression in male animals. This was a limitation of the current study, as discussed before despite lower incidence of AAA in women, one third of all ruptures occur in women. Further experimentation with complementary models and in females are required to build confidence in the results. SM22CrePDGFR β ^{D849V} line developed aortopathy throughout the aorta, although most prominently at the root which indicates the molecular mechanisms induced in this model are complex and may involve a cross-play with TGF- β . Hence mechanistic studies should be performed for understanding the molecular pathogenesis. Moreover, although we have identified the aortopathy by μ CT, this was done during the later stage of the project, and we therefore did not do full characterisation and quantification of the diseases. This model warrants further investigation by μ CT angiography for characterisation of the proximal aortic disease.

Also driving the PDGFR β signalling in the adult mice resulted in minimal aortopathy and required PPE induction to develop significant AAA. As it signifies that the duration of PDGFR β signalling is crucial for AAA pathogenesis, future work should focus on studying the time-course of AAA development in the model including lineage tracing, histological analysis, and AAA quantification.

Importantly, we observed deaths in our model where PDGFR β signalling was enhanced from embryonic stage. We could not determine the cause of death, though we found cardiomegaly and splenomegaly. Hence, before utilising this model for mechanistic and therapeutic studies, further studies are needed to fully characterise this line to ascertain all pathological features and understand the mechanisms.

Transcriptomics identified more than 2700 DEGs in aorta from SM22CrePDGFR β^{D849V} mice compared to SM22CrePDGFR β^{WT} littermates and further analysis mapped them into various cellular processes and signalling pathways. While some of them were in agreement with the literature many were completely novel. For e.g., ECM organisation and receptor interaction and cytokine signalling are well-established cellular mechanisms and signalling pathways that are involved in aneurysmal pathology (Toczek et al., 2016). However, our transcriptomics analysis identified that multiple genes involved in mitochondrial membranes, oxidative phosphorylation and respiratory/electron transport chain have changed upon inducing PDGFR β signalling (i.e., AAA induction). No knowledge exists on the importance of those mechanisms on AAA pathogenesis and progression. Further work is needed to validate the transcriptomics data using independent samples and evaluate the potential mechanisms linking them to AAA pathophysiology. For e.g., KEGG analysis showed that multiple DEGs that are downregulated in the transgenic aorta mapped to various neurodegenerative processes. However, caution is required when selecting the candidate genes/pathways for further studies as we used the model in which PDGFR β signalling was driven from embryonic stage and thus some of the gene dysregulations could have been due to developmental defects rather than AAA pathology. Transcriptomics analysis of AAA from SM-MHCCreER^{T2}PDGFR β^{D849V} aorta would avoid those issues and thus may provide better clues towards further studies exploring downstream signalling pathways. Another potential route is utilising pure VSMC isolated from the transgenic aorta for RNAseq rather the whole aortic tissue to get better focus on VSMC pathology as multiple cell types are present in the whole aorta tissue. However, getting sufficient pure VSMC for transcriptomics whilst avoiding the artifacts induced by tissue culture may be a challenge. Single cell transcriptomic studies may be required to overcome this challenge.

As GAPDH was routinely used in our laboratory as house-keeper gene, I too have used it in my qPCR experiments to validate the transcriptomics results. However, the PDGFR mutant aorta (SM22CrePDGFR β^{D849V}) showed changes in various metabolic pathways including citric acid cycle and glycosaminoglycans metabolism. Hence, GAPDH-which is involved in glucose metabolism, may not be the most appropriate housekeeper gene in these experiments. So, the qPCR experimental results should be further verified with other housekeeper genes such as 18S and β -actin.

5.7.2 Targeting PDGFR β /Orai1/SOCE for AAA management

Although the results presented in this thesis support the hypothesis that targeting PDGFR β and Orai1 signalling could be a viable strategy for medical treatment of AAA, due to phenotype drift observed in the PDGFR β mutant line we could not validate it unequivocally. Histopathological analysis of the aortae of our models (AAA induced by PPE in SM-MHCCreER^{T2}PDGFR β^{D849V} and also aortopathy induced in SM22CrePDGFR β^{D849V}) is needed to identify clues on whether and how our Orai1/SOCE inhibition strategies alerted the vascular wall cells.

Pharmacological inhibition of PDGFR β /Orai1/SOCE has potential for translation into therapeutics. Specific work plans are needed to determine ADME (absorption, distribution, metabolism, and excretion) characteristics, dosage, and route of administration. It is also important to note that both PDGFR β and Orai1/SOCE signalling play numerous physiological functions e.g., PDGFR β signalling is essential for vascular development, angiogenesis, and wound healing (Chen 2013) and Orai1 mediated SOCE is essential for acquired and innate immunity, wound healing and exocrine secretion (Chen et al., 2013; Putney et al., 2017). Therapeutics need to be targeted to prevent unnecessary adverse side-effects. For e.g., knocking out Orai1 in zebrafish led to development of cardiomyopathy and skeletal muscle myopathy (Völkers et al., 2012). Also, PDGFR β /Orai1/SOCE mediated vascular remodelling may be beneficial in various pathological states including neo-intimal hyperplasia and delaying the rupture of AAA and atherosclerotic plaques (Shawer et al., 2021).

In vivo studies showing acute changes in aortic VSMC calcium in response to Orai1/SOCE modulation are needed to show the direct effect of SOCE inhibition under physiological condition. Using *in vivo* calcium sensors will be essential for such studies. While various methodologies are available for *in vivo* neuronal calcium imaging, the tools for aorta/VSMC are limited (Che and De Marco García, 2021). However, a fluorescent calcium indicator strain Acta2^{BAC}-GCaMP2 transgenic line in which high calcium-sensor was expressed in SMC has been recently described (Lee et al., 2021). Upon binding to calcium GcaMP2 exhibits increased eGFP fluorescence which is directly proportional to the SMC calcium. Also, longer term studies showing that SOCE modulation can result in inhibition of VSMC proliferation and pathological remodelling will have to be performed. PET-CT functional imaging techniques using 18F-FLT tracer can detect proliferating cells *in vivo* in atherosclerotic model (Ye et al., 2015). We tried identifying proliferating SMC in our AAA model using the same technique. Though the preliminary results were inconsistent, our efforts indicated that it is a feasible method to study SMC behaviour in AAA *in vivo* (Kandavelu & Bailey, unpublished data). These studies will reinforce our hypothesis and strengthen the results and support the progression to clinical studies.

Along with murine studies, complementary work with human tissues and cells should be performed. Utilising alternative/complementary *ex vivo* models of AAA such as the bioreactor containing porcine carotid arteries under flow can aid in further understanding of the pathogenic mechanisms while reducing the limitations of working with human samples such as availability, ethical issues, and variability. VSMC isolated from those models showed similar phenotype characteristic of those cultured from end-stage AAA samples (Riches et al., 2013). Hence using the VSMC from the models and human samples and employing pharmacological and genetic approaches to modulate PDGFR β /Orai1/SOCE pathway, results obtained from murine cells should be validated before progressing to clinical studies.

Though JPIII showed high potency, safety, selectivity and ADME properties in pre-clinical models, lack of understanding of its mechanism of action makes it a less attractive candidate for clinical trial (Bartoli et al., 2020; Shower et al., 2021). However, multiple small molecule inhibitors are Orai1/SOCE are available, and a few are under clinical trial for treating various disorders. CM4620, an Orai1 inhibitor developed by CalciMedica is under trial for pancreatitis and two

molecules developed by Rhizen Pharmaceuticals (RP3128 and RP4010) are under trial for cancer therapy (Shawer et al., 2021). Few approved drugs such as leflunomide, teriflunomide, lansoprazole, tolvaptan and roflumilast are found to inhibit Orai1/SOCE and hence are being tried in repurposing clinical trials (Rahman and Rahman, 2017). Hence multiple drug candidates are available for future translational studies.

Orai1 knockout animals showed significant reduction in the size of infrarenal AAA induced by mutant PDGFR β expression. This model had Orai1 deletion and PDGFR β mutation from embryonic stage which might have had an impact on our observation. To investigate whether the genetic loss of Orai1 specifically in VSMC in adult mice, the experiment should be performed in other novel murine models of inducible *Orai1* deletion so that we can clearly delineate the role of VSMC Orai1 in VSMC phenotypic remodelling and AAA development induced by PDGFR β signalling.

5.8 Conclusions

In conclusion, our project has generated two transgenic models of AAA for *in vivo* studies. Lineage tracing studies showed proliferation of both SMC and non-SMC origin cells in AAA and indicated that VSMC phenotype switching may play an essential role in AAA pathogenesis. Next, we demonstrated that enhanced PDGFR β signalling in VSMC leads to VSMC de-differentiation and infiltration which contribute to spontaneous aortopathy. When this enhanced PDGFR β signalling was initiated in adulthood, though the development of AAA induced by PPE application was not altered, it made them progressive. Enhancement of PDGFR β signalling led to increased SOCE in VSMC as measured *in vitro* and genetic deletion of *Orai1* *in vivo* led to attenuation of aortopathy. Thus, our results suggest that inhibiting the PDGFR β /Orai1/SOCE signalling pathway can be a potential strategy for medical management of AAA.

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