# Thymosin β4 – A novel regulator of Low density lipoprotein receptor related protein 1(LRP1) in vascular disease




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# Abstract

Cardiovascular diseases, including atherosclerosis and abdominal aortic aneurysm (AAA), are amongst the foremost causes of global morbidity. Despite decades of research, the mechanisms underlying disease progression remain unresolved, resulting in a lack of efficient pharmacological therapies to prevent disease progression. Maintaining vascular stability is fundamental to protect against disease pathogenesis. Contractile smooth muscle cells (VSMCs) and elastin-rich extracellular matrix (ECM) are major determinants of vascular stability and tone. Injury-induced growth factors stimulate phenotypic modulation of VSMCs, from their quiescent contractile state to a more active, synthetic phenotype, that proliferate, migrate and accelerate progression of atherosclerosis and AAA. Inhibiting VSMC phenotypic transformation has thus been shown to attenuate disease progression. Thymosin β4 (Tβ4), a small actin-binding peptide, promotes VSMC differentiation during development and preserves VSMC differentiation postnatally in the healthy aorta. Tβ4 interacts with Low density lipoprotein receptor related protein 1 (LRP1), an endocytic regulator of PDGFRβ signalling in VSMCs, and LRP1 variants have been identified by GWAS as major risk loci for AAA and coronary artery disease. We hypothesised that Tβ4 may function to protect against vascular disease via LRP1 mediated growth factor signalling. Using well-characterised models of atherosclerosis and AAA, increased susceptibility to disease was confirmed in mice lacking Tβ4. Tβ4-null mice displayed increased susceptibility to AAA, ranging from aortic dilation to overt medial dissection and rupture in <5 days. Similarly, in the aortic root and descending aorta, mice lacking Tβ4 on $ApoE^{-/-}$ background develop more unstable atherosclerotic plaques, with large necrotic cores. Diseased vessels were characterised by enhanced contractile-synthetic VSMC switching and increased elastin degradation, underpinned by augmented LRP1-PDGFRβ signalling. Primary aortic VSMCs lacking Tβ4 show enhanced sensitivity to PDGF-BB, coinciding with

dysregulated intra-cellular trafficking, leading to increased recycling of LRP1-PDGFRβ and reduced lysosomal targeting. Given that exogenous Tβ4 can regulate endocytosis, and due to the known anti-fibrotic, anti-inflammatory and anti-apoptotic properties of Tβ4, the therapeutic potential of exogenously administered Tβ4 was evaluated in the AAA model. Simultaneous administration of Tβ4 and AngII in C57BL/6J mice reduced aneurysms and the associated elastin degradation, preserved contractile VSMC phenotype, and attenuated activation of PDGFRβ. This research identifies endogenous Tβ4 as a novel regulator of LRP1 in the aorta to protect against atherosclerosis and AAA and reveals the potential for using Tβ4 as a therapeutic agent to protect against vascular disease. However, further studies are required to validate these findings and to elucidate the exact molecular mechanisms through which protection is achieved.

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# List of Abbreviations

| | |
|---|---|
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| AAA | abdominal aortic aneurysm |
| AAV | adeno-associated virus |
| ApoE | apolipoprotein E |
| AT1R | angiotensin type 1 receptor |
| AT2R | angiotensin type 2 receptor |
| $CaCl_2$ | calcium chloride |
| CETP | cholesteryl ester transfer protein |
| CTGF | connective tissue growth factor |
| CVD | cardiovascular disease |
| Dcn | decorin |
| DAPI | 4',6-diamidino-2-phenylindole |
| ECM | extracellular matrix |
| ECs | endothelial cells |
| EEA1 | early endosome antigen 1 |
| EDP | elastin-derived peptides |
| EdU | 5-ethynyl-2'-deoxyuridine |
| ELISA | enzyme-linked immunosorbent assay |
| EPDCs | epicardium-derived cells |
| ET1 | endothelin-1 |
| F- actin | filamentous actin |
| G-actin | globular actin |
| GSK-3 | glycogen synthase kinase |
| GWAS | genome wide association studies |
| HDL | high-density lipoprotein |

| | |
|---|---|
| Hand1 | heart and neural crest-derived transcript-1 |
| HTRA1 | high-temperature requirement factor A1 |
| HUVECs | human vein endothelial cells |
| ICD | intracellular domain |
| ILK | integrin-linked protein kinase |
| iNOS | inducible nitric oxide synthase |
| LDL | low-density lipoprotein |
| LDLR | low-density lipoprotein receptor |
| LRP1 | low-density lipoprotein related receptor protein 1 |
| MAPK | mitogen-activated protein kinase |
| MCP-1 | monocyte chemoattractant protein 1 |
| MI | myocardial infarction |
| MIP | macrophage inflammatory proteins |
| MOVAS | mouse aortic smooth muscle cells |
| MMPs | matrix metalloproteinases |
| MYOCD | myocardin |
| PAI-1 | plasminogen activator inhibitor type 1 |
| Pcsk9 | protein convertase subtilisin/Kexin type 9 |
| PDGF-BB | platelet-derived growth factor subunit BB |
| PDGFRβ | platelet-derived growth factor receptor β |
| PLA | proximity ligation assay |
| PFA | paraformaldehyde |
| RAP | receptor-associated protein |
| RIP | regulated intramembrane proteolysis |
| ROS | reactive oxygen species |
| RTK | receptor tyrosine kinase |
| scRNA-seq | single-cell RNA sequencing |

| | |
|---|---|
| siRNA | small interfering RNA |
| SRF | serum responsive factor |
| SM22α | smooth muscle protein 22 α |
| SM-MHC | smooth muscle myosin heavy chain |
| TAA | thoracic aortic aneurysms |
| Tβ4 | thymosin β4 |
| TIMPs | tissue inhibitors of MMPs |
| TNFα | tumour necrosis factor α |
| TfR | transferrin receptor |
| TGFβ | transforming growth factor β |
| tPA | tissue plasminogen activator |
| Trop | tropomyosin IV |
| UMAP | Uniform Manifold Approximation and Projection |
| uPA | urokinase plasminogen activator |
| VEGF | vascular endothelial growth factor |
| Vim | vimentin |
| VSMCs | vascular smooth muscle cells |
| WD | western diet |

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# Chapter I

# Introduction

## Vascular stability and protection against disease

Atherosclerosis and abdominal aortic aneurysm (AAA) are progressive, degenerative diseases of the aortic vessel, and a major burden to cardiovascular disease (CVD)[1] that remain the leading cause of morbidity and mortality worldwide[2, 3]. Despite decades of research, poor understanding of the underlying mechanisms limits development of effective pharmacological therapies. Although there are differences in aetiology of these disease, chronic vascular remodelling remains the common denominator contributing to compromised vascular stability that can lead to acute life-threatening events, such as aortic rupture, in the aortic aneurysm or myocardial infarction (MI), stroke, or end-organ failure in atherosclerosis [4-6]. Vascular stability and tone are principally determined by the amount and organisation of contractile vascular smooth muscle cells (VSMCs) and elastin-rich extracellular matrix (ECM)[7], localised to the medial layer of the vessel.

## 1.1 Vascular system

The cardiovascular system comprises the heart and the blood vessels. The heart pumps blood, which flows through a carefully orchestrated network of interconnecting vessels that are divided into two sub-systems: a) The arterial system comprises the arteries and arterioles that carry oxygenated blood from the heart to the rest of the body, except the pulmonary artery that carries deoxygenated blood from the heart to lungs for oxygenation, and b) The venous system, made up of venules and veins, that return blood to the heart, except for the pulmonary vein that returns oxygenated blood to the heart.

Together they play a crucial role in transporting oxygen and nutrients and removing waste from the system. Therefore, maintaining the structural and functional integrity of the vessels is important to sustain life.

### 1.1.1 Structure of the aorta

The aorta is the largest elastic artery that carries oxygenated blood from the heart to the rest of the body. Being the first segment of the systemic circulation, the aortic wall is continuously subjected to high tensile stress. To withstand wall tension, the arterial wall is structured into three spatially organized layers, from inside to outside: tunica intima, tunica media, and tunica adventitia [8], as shown in **Figure 1.1**.

The tunica intima is the innermost layer that lines the lumen of the blood vessel. It consists of a single layer of endothelial cells (ECs) and subendothelial connective tissue that accounts for <1% of the total wall thickness and remains uniform along the aorta[9]. The ECs form the interface between the blood and the tissue to allow exchange of metabolites and gases. ECs are known to control vascular function by responding to various hormones, neurotransmitters and vasoactive factors[10, 11]. A thin sheet of elastin, called the internal elastic lamina, separates the intima from the media.

The tunica media is the middle layer, which accounts for the bulk of the wall. It is composed of several layers of circumferentially arranged VSMCs that alternate with elastin lamellae and collagen bundles[12] Together, they provide key structural support and endow the vessel with elasticity and contractility. A dense layer of elastin tissue, called the external elastic lamina, separates the media from the adventitia.

The tunica adventitia or externa is the outermost layer, made of fibroblasts, embedded in a collagen-rich matrix and supplied by a network of small blood vessels called the vasa vasorum [13].

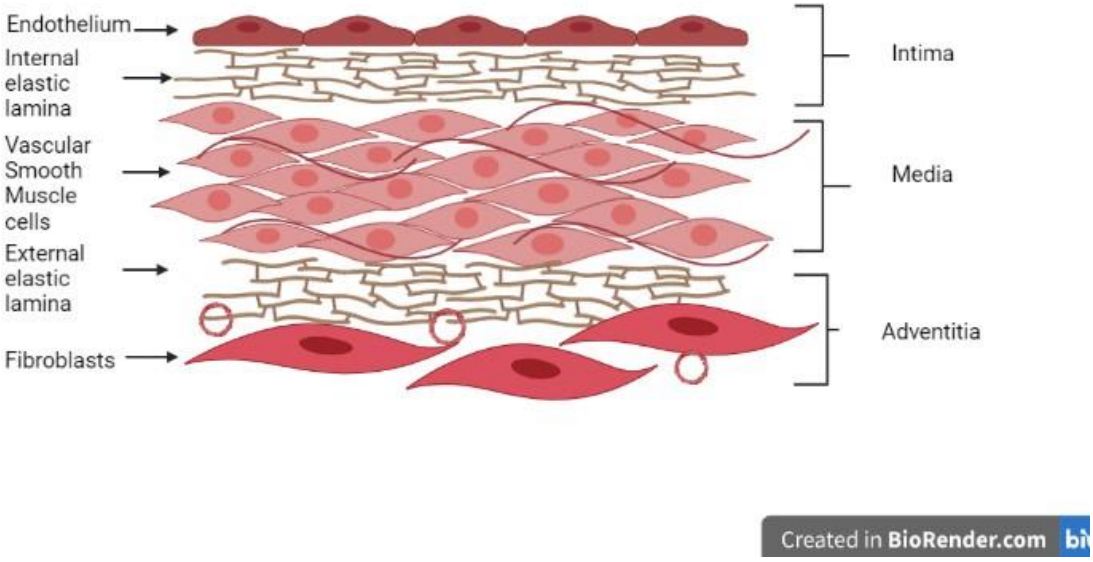



***Figure 1.1: Morphology of the aortic wall.*** *Image of a healthy aorta, showing three distinct layers: tunica intima, tunica media, and tunica adventitia[8]. The tunica intima is made up of a single layer of endothelial cells and sandwiched between the intima and the tunica media is the internal elastic lamina. The tunica media is the middle and largest layer of the aortic wall, made up of multiple layers of VSMCs, elastin, and collagen. It must be noted that while VSMCs are primarily located in the media in murine arteries, a subpopulation of intimal VSMCs is found in human arteries[14]. The external elastic lamina separates the tunica media from the tunica adventitia. The adventitial layer is made up of fibroblasts and supplied by the nutritive vasa vasorum. Image created using BioRender.com*

### 1.1.2 Anatomical division of the aorta

Anatomically, the aorta is divided into four sections: ascending**,** aortic arch**,** thoracic and abdominal (**Figure 1.2**). The aorta initiates at the aortic valve in the left ventricle. The ascending aorta lies within the pericardium and continues to form the aortic arch. The brachiocephalic left common carotid, and left subclavian arteries, supplying the head,

neck, and shoulders, branch from the aortic arch. As the aortic arch descends downwards in the thorax, it is called the thoracic aorta. Multiple visceral and parietal vessels sprout from the thoracic aorta. At the aortic hiatus, a hole in the diaphragm, the aorta becomes the abdominal aorta. The region of the abdominal aorta above the renal arteries is called the supra-renal abdominal aorta, while the region below the renal arteries is called the infra-renal abdominal aorta. The infra-renal abdominal aorta bifurcates into the iliac arteries and supplies the lower body[15].

It is important to note that aortic diameter is not uniform; in humans, the ascending aorta is ~3 cm in diameter at its origin, and, as it descends downwards, the diameter gradually decreases to ~2 cm in the abdominal region[16]. This structural heterogeneity is attributed to a difference in the number of lamellar units (concentric bands of elastin filaments with associated collagen fibres and VSMCs) in different regions of the aorta[17].

### 1.1.3 Region-specific vascular smooth muscle cell (VSMC) heterogeneity

Alongside structural heterogeneity, compelling evidence from multiple lineage tracing studies[18, 19], and more recently single-cell RNA sequencing (scRNA-seq) experiments confirm cellular heterogeneity in the aortic media[20, 21]. Previous studies have established that VSMCs in different segments of the aorta originate from multipotent precursors from several developmental origins[19], including the neural crest, lateral plate mesoderm, somatic or paraxial mesoderm, and splanchnic mesoderm[18]. The embryonic origins of VSMCs located in different parts of the aorta are illustrated in **Figure 1.2**. It must also be noted that the aortic VSMCs derived from distinct progenitors exhibit differential ECM order and protein expression[22]. Furthermore, different regions experience different shear stress and together these

factors contribute to region-specific susceptibility to aortic aneurysm and atherosclerosis, both in mice and humans [23].

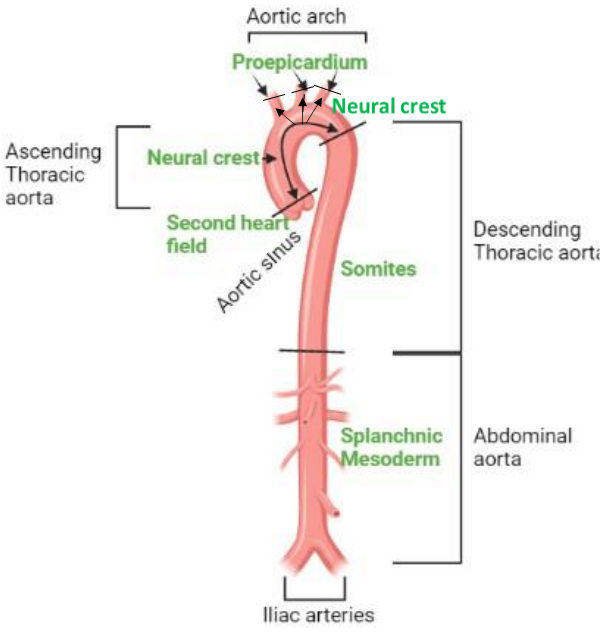



***Figure 1.2: Anatomical segmentation of the aorta based on embryonic heterogeneity.*** *Text in black indicates the anatomical division of the aorta into the ascending thoracic aorta, the aortic arch, descending thoracic aorta and the abdominal aorta that bifurcates to the left and the right iliac artery. The text in green represents different progenitor populations from which VSMCs originate. Image adapted from[19]. Image created using BioRender.com*

## 1.2 VSMCs in vascular stability

### 1.2.1 VSMC plasticity

VSMCs are the major constitutive cells of the vascular wall, which assume a variety of structural and physiological functions, including maintaining vascular tone, ECM production, and arterial repair after injury[24]. VSMCs of the medial layer are not terminally differentiated and can switch between quiescent contractile phenotype and an

active so-called ‘synthetic’ phenotype, by a process called phenotypic modulation. Phenotypic modulation of VSMCs can occur naturally during embryonic vascular development, pregnancy, and exercise[25, 26], or in response to vascular injuries, such as atherosclerosis, AAA, hypertension, and Marfan syndrome[27-31]. Although beneficial in the initial stages of disease, promoting repair [32], as the disease progresses, chronic phenotypic modulation contributes to vascular stiffness, exacerbates inflammation, which affects vascular contractility and stability to accelerate disease progression[33]. The functional properties of VSMCs are attributed to the phenotype they acquire. It is therefore important to understand the difference between the two phenotypes and factors affecting their modulation.

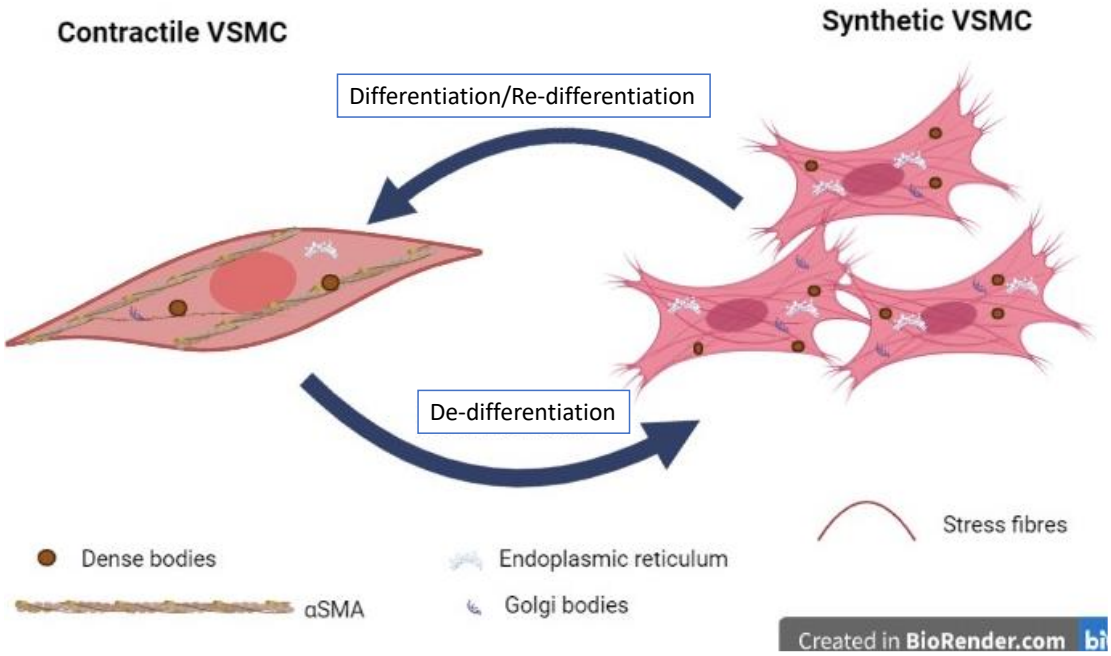



***Figure 1.3: Phenotypic modulation between contractile and synthetic phenotype.*** *VSMCs switch between contractile and synthetic phenotype by a process known as phenotypic modulation. Contractile VSMCs convert to synthetic phenotype by de-differentiation, while synthetic VSMCs can convert to contractile phenotype by differentiation during development or re-differentiation during vascular remodelling.*

*Contractile VSMCs are elongated, spindle-shaped cells with increased density of myofilaments and few cellular organelles. Contractile VSMCs express contractile proteins, such as α smooth muscle actinin (αSMA) and smooth muscle myosin heavy chain (SM-MHC). Contractile VSMCs can de-differentiate to less differentiated VSMCs called 'synthetic' VSMCs. These cells are smaller, cobblestone shaped with abundant intermediate filaments (Vimentin), cellular organelles, and few myofilaments. Image created using BioRender.com*

### 1.2.2 Comparison between contractile and synthetic VSMCs

#### 1.2.2.1 Contractile VSMCs

VSMCs of a healthy adult aorta are mostly contractile, differentiated cells with a characteristic elongated, spindle shape morphology[34, 35]. The elongated morphology increases the dynamic contractile range of VSMC and regulates vessel diameter and tone[36]. Ultra-structural studies confirm the presence of tightly bundled myofilaments with few organelles, such as endoplasmic reticulum and Golgi, in the cytoplasm[37]. Additionally, these cells are known as quiescent cells, as they proliferate and migrate at an extremely low rate[38, 39]. The contractile function of these cells is regulated by the expression of canonical contractile markers such as α smooth muscle actinin (αSMA)[40, 41], smooth muscle protein 22 α (SM22α)[42], Calponin[42, 43], h-Caldesmon (high molecular weight isoforms of Caldesmon)[43] and smooth muscle myosin heavy chain (SM-MHC)[44, 45]. An important molecular regulator of VSMC contractile phenotype is myocardin (MYOCD), a transcription factor, that interacts with serum responsive factor (SRF); the SRF-MYOCD complex binds to consensus CArG box elements [$CC(A/T)_6GG$] within the promoter of contractile genes[46] to promote their expression[47].

#### 1.2.2.2 Synthetic VSMCs

Synthetic VSMCs, on the other hand, are observed during embryogenesis, disease, wound repair, and tissue engineering[25]. These cells are smaller than the contractile VSMC and are recognised by their cobblestone morphology[39, 40], and high proliferative and migrative index[48]. Ultra-structural studies on human foetal VSMCs (14-20 weeks of gestation) using transmission electron microscopy revealed that VSMCs in the early stages of vasculogenesis are similar to those seen in vascular disease, with fewer myofilaments, and cytoplasm rich in rough endoplasmic reticulum and Golgi bodies[49]. Since most of the characterisation of synthetic VSMCs comes from studies in disease[50], a lot of ambiguity exists with regards to the phenotype of synthetic VSMCs and protein markers that define the phenotype. Classically, synthetic VSMCs are recognised by reduced expression of canonical contractile markers[26, 51]. However, studies have identified upregulation of specific proteins in de-differentiated VSMCs, such as l-Caldesmon (low molecular weight isoforms of Caldesmon)[52, 53], Vimentin (Vim)[54], Tropomyosin IV (Trop)[55], Lgals3 (macrophage-like phenotype)[56], Decorin (Dcn) and Lumican (Lum) (fibroblast-like phenotype)[21]. It should be noted that the term ‘synthetic’ is likely an over-simplification and emerging scRNA-seq studies demonstrate that modulated VSMCs are heterogeneous, with sub-populations that share properties of fibroblasts, macrophages, and osteoblasts[21, 57, 58]. These studies should identify more selective markers for the characterisation of discrete populations for use in future studies.

**Table 1.1 Differences between contractile and synthetic VSMCs**

| Features | Contractile VSMCs | Synthetic VSMCs |
|---|---|---|
| Shape | Elongated, spindle shape | Cobblestone shape |
| Cell organelles and myofilaments | Increased myofilaments and decreased cellular organelles | Decrease myofilament and increased cellular organelles |
| Expression of Contractile proteins | Increased expression of αSMA, SM22α, SM-MHC, Calponin, h-Caldesmon | Decreased expression of contractile markers |
| Expression of Synthetic proteins | Decreased expression | Increased expression of l-Caldesmon, Vimentin, Decorin, Lumican, Tropomyosin, Osteopontin |
| Proliferation and migration | Very low | High |

### 1.2.3 Phenotypic modulation of VSMCs affects ECM composition

ECM is the non-cellular component of the media, primarily responsible for providing structural support to the aortic wall[9]. ECM is additionally known to regulate the availability of growth factors and cytokines that influence physiological or pathological vascular remodelling. Vascular compliance is primarily a function of two major scaffolding proteins: collagen and elastin, the synthesis and degradation of which are tightly regulated in vascular homeostasis[59]. VSMCs are responsible for the synthesis and degradation of ECM proteins, and changes in the VSMC phenotype are accompanied by parallel changes in their adhesion to ECM proteins[60]. VSMCs synthesise ECM proteins both during embryogenesis and pathological vascular

remodelling. However, the ECM produced by adult synthetic VSMCs during vascular injury[61], favours production of monomeric collagen type 1 that promotes phenotypic modulation of VSMCs[62], over fibrillary collagen type 1, that provides mechanical strength to the vessel.

In addition to the involvement in specific ECM protein synthesis, VSMCs modulate ECM structure by the production of proteolytic enzymes called matrix metalloproteinases (MMPs)[63] and endogenous tissue inhibitors of MMPs (TIMPs), proteases like urokinase plasminogen activator (uPA), and tissue plasminogen activator (tPA), along with crosslinking enzymes like lysyl oxidase [64]. Interestingly, the relationship between VSMCs and the ECM is bi-directional and they regulate the structure and function of each other, for example, MMPs released by VSMC degrade elastin to produce elastin-derived peptides (EDP), which act as a chemotactic signal and promote inflammatory cell migration[65]; migrating inflammatory cells release cytokines that promote phenotypic switching of VSMCs, setting a vicious cycle that leads to loss of vascular stability.

The process of phenotypic modulation is well accepted and is extensively studied, however, the mechanisms that underpin VSMCs phenotypic switching remain elusive[34]. Multiple factors, secreted proteins, and signalling molecules are reported to regulate VSMC phenotype[34], including, but not limited to, those shown in **Figure 1.4**. Endothelin-1 (ET1), synthesised by ECs, promotes VSMC phenotypic modulation by binding to endothelin type A ($ET_A$) and $ET_B$ receptor present on VSMCs [66]. Studies using loss- or gain-of-function of Notch suggest the importance of Notch signalling in phenotypic modulation of VSMC during development and disease[67, 68]. In fact, Notch pathway components including Notch1, Notch3, Jagged-1, Jagged-2, Hey1, and Hey2 are temporally regulated in experimentally induced vascular injury[69-71]. *In vitro* Wnt1

and Wnt3a are capable of inducing β-catenin signalling and cyclin D1 expression to promote VSMC proliferation[72, 73].

Transforming growth factor $\beta_1$ (TGF-$\beta_1$) is another important factor that may function via Smad-dependent or Smad-independent signalling to regulate VSMC phenotype. TGFβ mediated Smad signalling activates the expression of early contractile marker expression by interacting with transcription factors. Besides, Qui *et al* have shown that Smad3 physically interacts with SRF to facilitate VSMC-specific gene expression[74] and can recruit myocardin to the SM22 promoter in a CArG box independent manner to promote SM22α transcription *in vivo*[75]. Using *in vitro* studies, Zhu *et al* proposed that TGF-$\beta_1$ induced human aortic VSMCs phenotypic switch via the phosphoinositol 3 kinase (PI3K)/AKT/ inhibitor of DNA binding 2 (ID2) signalling pathway[76]. It is well accepted that PI3K/AKT is an important pathway that regulates the VSMC phenotype. As well as being activated downstream of TGF$\beta_1$ AKT is additionally a downstream effector of the Platelet-derived growth factor (PDGF) signalling pathway. The importance of PDGF signalling in VSMC phenotypic modulation is explained in detail in Section 1.3.

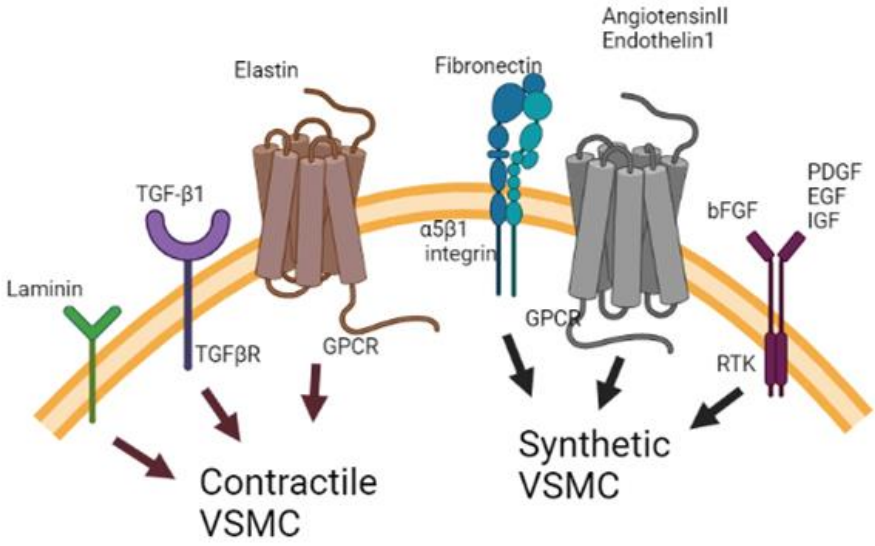


***Figure 1.4: Multiple factors and signalling pathways affect VSMC phenotype.*** *VSMC phenotype is regulated by many different proteins and signalling pathways including integrin, endothelin-1, Transforming growth factor β (TGFβ) family[77, 78], Platelet-derived growth factors (PDGF) family[79, 80], Epidermal growth factor (EGF), Insulin-like growth factor-1(IGF) family receptor tyrosine kinase, etc. Image created using BioRender.com*

## 1.3 Platelet derived growth factor-BB (PDGF-BB) modulates VSMC phenotype

Platelet-derived growth factors (PDGF) are a family of homo- and hetero-dimeric disulfide-bonded growth factors, and based on the type of polypeptide chain, five different PDGFs (PDGF-AA, -AB, -BB, -CC, and -DD) are recognised[81]. A number of different cell types, including platelets, macrophages, vascular ECs, fibroblasts and keratinocytes are known to produce these growth factors[82]. PDGFs act via one of the two transmembrane receptors (PDGFRα and PDGFRβ). The PDGF-BB/PDGFRβ axis has been extensively studied in the context of VSMC phenotypic modulation and vascular stability in health and disease. Activation of PDGFRβ, a type III tyrosine kinase receptor[83], upon PDGF-BB/-DD binding activates intracellular signalling via mitogen-activated protein kinases (MAPKs) and PI3K/Akt[84], to mediate cell proliferation, migration, and angiogenesis.

It is well established that PDGF/PDGFR signalling is crucial during vascular development, since genetic ablation of either PDGF-BB or PDGFRβ is associated with perinatal death from vascular defects in multiple organs[85, 86]. *In vitro,* PDGF signalling is shown to inhibit expression of MYOCD and disrupt SRF-MYOCD interaction via Krüppel-like factor 4 (KLF4), downregulating expression of contractile genes via the CArG box within the promoter[79, 87, 88]. In keeping with this, studies in

*PDGFRβ*$^{Sm22D849V}$ mice (Sm22α-Cre driver initiate D849V point mutation in the endogenous PDGFRβ gene to destabilize the autoinhibited conformation of the receptor) in which both basal and ligand-mediated PDGFRβ signaling are increased due to point mutation in PDGFRβ, promote advanced plaques in plaque-prone sites and also initiates plaque formation in plaque-resistant sites[89]. Furthermore, inhibiting PDGFRβ signalling using antibodies[90, 91] or PDGFR-β siRNA[92] in animal models inhibits intimal hyperplasia after balloon injury and preserves contractile VSMC phenotype. These studies indicate an essential role for the PDGFRβ pathway in phenotypic modulation of VSMCs, making PDGFRβ a promising target for therapeutic intervention.

## 1.4 VSMCs in arterial diseases

### 1.4.1 Aortic Aneurysm

An aneurysm is defined as a localized dilation (≥ 3.0 cm) of the arterial wall that results in progressive loss of the wall's ability to withstand the wall tension generated by high intraluminal pressure which, if left untreated, results in fatal rupture[8]. Current treatment strategies are restricted to late-stage invasive surgical procedures such as endovascular stent graft and open repair, however, these present complications[93], suggesting an urgent need to develop efficient pharmacological therapeutics to protect against the disease.

#### 1.4.1.1 Types of aortic aneurysm

Aortic aneurysms can be classified based on aetiology, shape, and location of dilatation. Aortic aneurysm is identified as a multifactorial disease and several factors including physical trauma, genetic factors, inflammatory disease, and atherosclerosis are shown to contribute to the pathology. Smoking is identified as the most significant epidemiologic risk factor that contributes to aneurysm development and expansion [94].

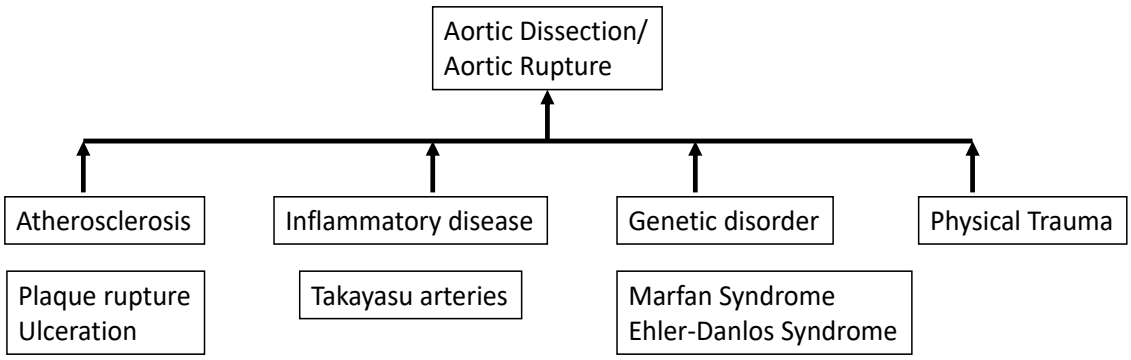



***Figure 1.5: Etiological factors that contribute to aortic aneurysm.*** *Aortic aneurysm is a multifactorial disease and several etiological factors, such as physical trauma, genetic diseases such as Marfan and Ehler-Danlos syndrome, inflammatory diseases like Takayasu and atherosclerosis, are identified to contribute to aneurysmal dilatation, dissection and rupture.*

Based on the shape of the aortic dilatation, aneurysms are classified as saccular and non-saccular/fusiform aneurysms. A saccular aneurysm is an asymmetric dilation, identified by a typical sac-like bulge on one side of the wall. However, the ballooning in the fusiform aneurysm is spindle-shaped, axisymmetric and occurs on all sides of the blood vessel. Despite the common consensus that saccular aneurysm is more prone to rupture compared to fusiform dilation[95], the true rupture risk remains unknown[96]. Moreover, once the aortic diameter exceeds 4.5cm, it is difficult to maintain a fusiform axisymmetric dilation and the aneurysmal dilation becomes asymmetric. The asymmetry to some extent is also determined by the surrounding organs in the region, as aneurysms do not develop in empty peripheral spaces.

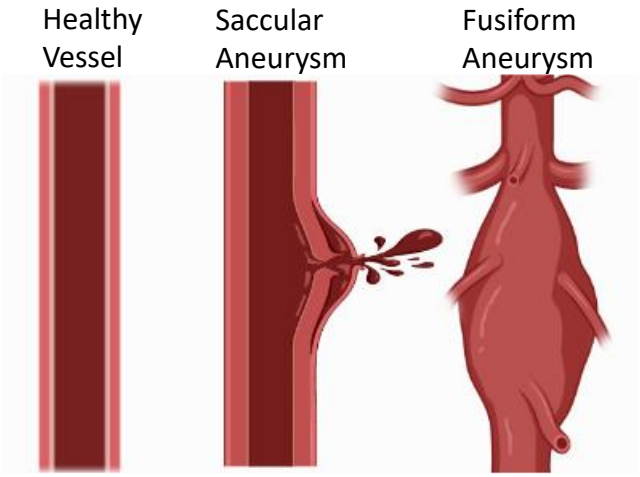



***Figure 1.6: Classification of aortic aneurysms based on the type of dilation**. Based on the shape of the bulge, aortic aneurysm can be classified as saccular or fusiform. Saccular aneurysms are recognised by their sac-like dilation on one side of the wall, while fusiform aneurysms are characterised by dilation on all the sides of the wall.*

Depending on the region where the dilatation occurs, aortic aneurysms are classified as 1) thoracic aortic aneurysms (TAA) occurring in the thoracic region, affecting either the ascending aorta, aortic arch, or descending thoracic aorta and 2) abdominal aortic aneurysms (AAA), affecting the infra-renal or supra-renal region of the aorta. Clinically, the propensity of infra-renal AAA is higher and the leading cause of mortality in males above 65 years of age[97], compared to TAA that affects younger ages without overt gender propensity[19]. Therefore, a considerable amount of effort has been invested to understand the pathophysiology and the underlying mechanism in AAA.

#### 1.4.1.2 Pathological changes in the abdominal aortic aneurysm (AAA)

The pathogenesis of AAA is highly complex and several risk factors such as smoking, genetic predisposition, hypertension, age, sex, and atherosclerosis have been identified to contribute to vascular injury[98, 99]. In response to vascular injury, expression of

inducible nitric oxide synthase (iNOS) in VSMCs, macrophages, and lymphocytes increases[100] and promotes the formation of reactive oxygen species (ROS). An increase in ROS provokes VSMC hypertrophy and production of MMPs that degrade medial elastin. The resulting increase in EDPs invokes a localised inflammatory response and release of growth factors and cytokines at the site of injury that promotes phenotypic modulation of VSMCs[101]. Upregulation of p53 and p21, proteins associated with programmed cell death, are thought to be responsible for increased VSMC apoptosis in AAA tissue. It is indicated that depletion of VSMCs due to apoptosis[102] may stimulate calcification[103] and provoke proinflammatory macrophage infiltration[104], further accelerating disease progression. These studies indicate that VSMCs are a major player in medial integrity, loss of which is associated with medial weakness and degeneration in aortic aneurysm.

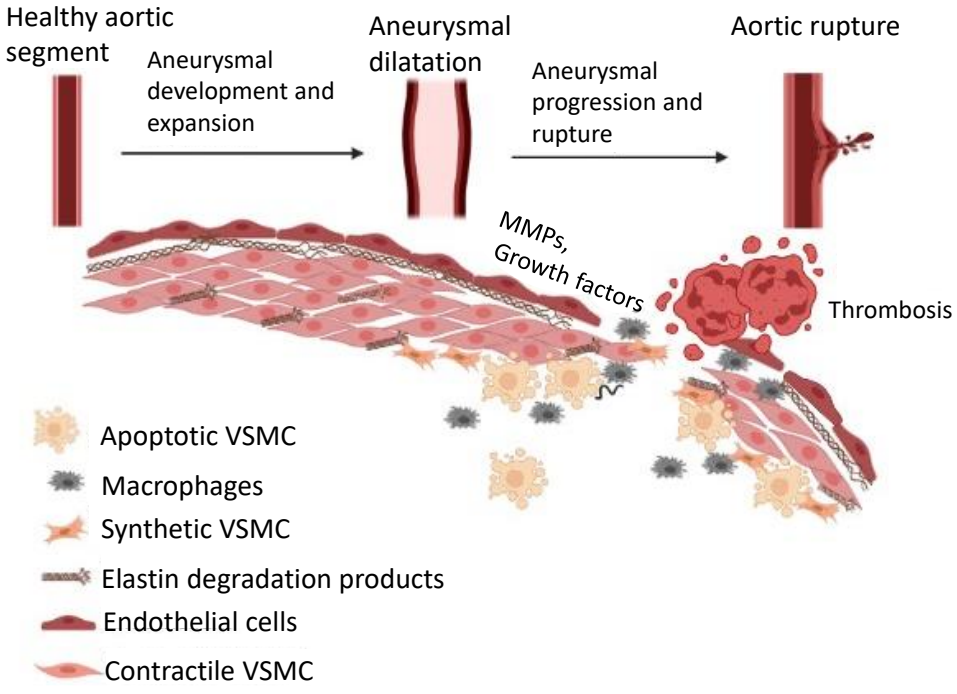



***Figure 1.7: Pathological changes in aortic aneurysm.*** *Oxidative stress and injury-induced release of growth factors and cytokines promote the release of MMPs and serine proteases that cause elastin degradation, increase VSMC phenotypic modulation and*

*aggravate inflammation that leads to loss of vascular stability and disease progression in aortic aneurysm. Image created using BioRender.com*

### 1.4.2 Atherosclerosis

#### 1.4.2.1 Pathological changes in atherosclerosis

Atherosclerosis is a chronic condition that initiates early in life[105] and remains asymptomatic until a plaque becomes large enough to obstruct the lumen to cause ischemic pain or ruptures to result in MI or stroke[50, 106]. Plaques are predominantly made up of cholesterol, fatty substances, cellular waste products, calcium, and fibrin[107]. Several studies have demonstrated that certain regions within a vessel experiencing low shear stress or turbulent blood flow, commonly distributed around branch points, are more susceptible to plaque development and are recognised as atheroprone [108-111], while the less susceptible regions are coined athero-resistant. Besides, atherosclerosis is a multifactorial disease, and several environmental and genetic stimuli including diabetes, obesity, hypertension, family history, and lifestyle choices (lack of exercise, high-fat diet, smoking) are identified as major risk factors associated with the disease[50].

Endothelial dysfunction is identified as the earliest marker of atherosclerosis, which increases endothelial permeability and adhesiveness to platelets and leukocytes. Accumulation of circulating lipoprotein in the sub-endothelial layer of damaged endothelium promotes monocyte infiltration and differentiation to macrophages[112]. Endothelial damage attenuates the release of nitric oxide[113] and triggers the release of growth factors and cytokines such as PDGF-BB that promote phenotypic modulation of medial VSMCs. These phenotypically modulated VSMCs, along with macrophages, accumulate cytoplasmic lipid droplets to form foam cells[114]. Oxidation of trapped lipid

favours accumulation of cholesterol esters and contributes to the formation of the fatty streak[115]. Acting in concert, all these events culminate in the formation of mature fibro-fatty lesions[116].

VSMC proliferation and neo-intimal formation increase plaque size, which gradually causes occlusion of the vessel. In the advanced stages, medial VSMCs migrate to form a cap over the atherosclerotic plaque[56, 117, 118]. VSMCs in the cap synthesise ECM proteins, such as collagen, to form a fibrous cap that promotes plaque stability, suggesting an atheroprotective role for synthetic VSMCs of the cap in the advanced stages of disease. However, the release of proteases and pro-inflammatory factors by macrophages provoke elastolysis and VSMC apoptosis that leads to plaque rupture and thrombosis, resulting in major clinical complications[50, 106].

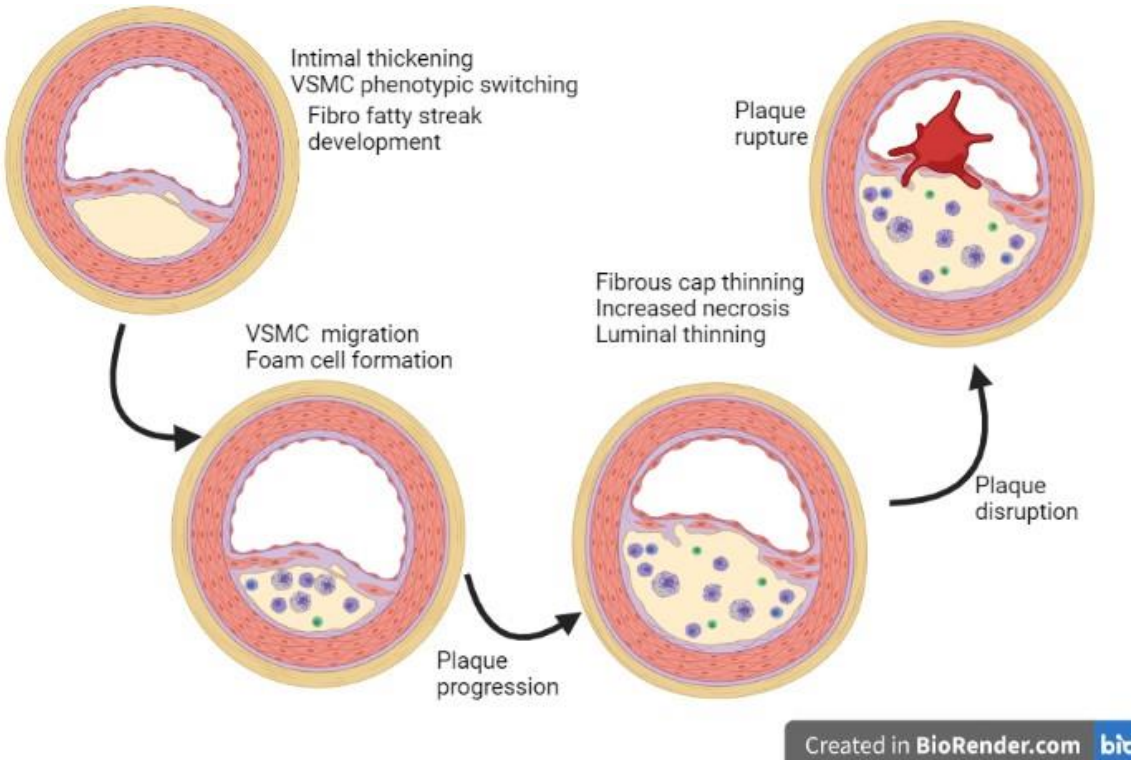



***Figure 1.8: Pathological changes that result in plaque formation.*** *Endothelial damage triggers the subendothelial accumulation of lipoproteins, monocyte-macrophage*

*infiltration that releases growth factors and cytokines which promote VSMC phenotypic switching to a less differentiated phenotype. These cells proliferate and migrate to form a cap over the fat deposition forming a fibrofatty plaque. Cap cells eventually undergo phenotypic modulation and migrate into the plaque centre. VSMC and macrophages take up fat deposits, which converts them to foam cells. As the plaque progresses, plaque cells either undergo apoptosis or erode, thereby thinning the cap; together with an increase in the necrotic core size, this may provoke plaque rupture. Therefore, maintaining plaque stability is important to protect against life-threatening, detrimental consequences. Figure created in BioRender.com.*

## 1.5 Murine models used to study arterial disease

### 1.5.1 Advantages and limitations of using mouse models

Over the last century, several different animal models have been developed to unravel the complex pathologies of human vascular disease. The use of small animals such as mice has been favoured, due to the ease of handling and housing, short gestation time, and cost effectiveness[119]. Despite significant differences between species, mouse shares 95% of protein-coding regions of the genome identical to human[120]. Moreover, the ability to manipulate the mouse genome makes them an excellent genetic and molecular toolbox to model human pathologies[121]. While murine models have been widely used to model CVD, consideration must be given while designing the study to overcome potential limitations, such as selection bias due to candidate gene approach, difficulty in recapitulating the exact human environment in mice[120].

### 1.5.2 Murine models of aortic aneurysm

An aortic aneurysm can be induced in mice either by genetic modification or by chemical induction. The most commonly used are chemically induced AAA models, which

reproduce major determinants of AAA such as aortic dilation, ECM destruction, VSMC apoptosis, calcification, and inflammation.

#### 1.5.2.1 Elastase perfusion model

Anidjar *et al* first demonstrated that infusion of elastase in rats resulted in inflammatory cell infiltration similar to that seen in humans[122]. This model requires isolating a segment of the aorta using a distal suture, infusion of porcine elastase, an enzyme that extensively degrades medial elastin for a short period, followed by restoration of blood flow[123]. Infrarenal aortic dilatation is reported 14 days post-intervention, along with enhanced elastin lamellae degradation that provokes inflammation and loss of medial stability[124]. Although this model has been extensively used to study AAA, it is invasive, technically challenging and aneurysms produced are highly variable[125].

#### 1.5.2.2 Calcium chloride model

Periaortic application of calcium chloride ($CaCl_2$), either directly or using gauze soaked in a concentrated solution of $CaCl_2$[126, 127], is shown to promote aortic dilatation, structural disruption of the medial layer and enhanced inflammatory responses. AAA developed using $CaCl_2$ shares characteristics similar to human AAA, such as elastin degradation and calcification, however, intraluminal thrombus and aortic rupture that are common in human AAA are not seen[128].

#### 1.5.2.3 Angiotensin II (AngII) infusion model

Chronic administration of AngII, a vasoactive peptide[129], for up to 28 days, at the rate of 500-1000ng/kg/min via subcutaneously implanted osmotic mini pumps, has been shown to produce aneurysm in mice. AngII can bind with high affinity to both angiotensin type 1(AT1R) and type 2 (AT2R) receptors [130]. *In vivo*, administration of Losartan, an

AT1R antagonist, in AngII-induced mice, inhibits aneurysm formation[131], indicating that AngII initiates aneurysm formation via signalling through the AT1R. Furthermore, administration of hydralazine in AngII-induced apolipoprotein E knockout ApoE$^{-/-}$ mice, lowered systolic blood pressure but did not prevent AAA formation, suggesting that AngII induction promoted AAA formation independent of its effect on blood pressure[132]. Systemic infusion of AngII in mice is shown to promote ROS production that contributes to VSMC phenotypic modulation, hypertrophy, and MMP production that leads to medial elastin degradation and chronic macrophage infiltration[133]. AngII infusion stimulates AAA development in hypercholesterolemic ApoE$^{-/-}$ or low-density lipoprotein receptor knockout LDLR$^{-/-}$ mice and C57BL/6 mice on a normal chow diet[134]. However, the frequency of AAA development in C57BL/6 mice is much lower (37%) compared to ApoE$^{-/-}$ or LDLR$^{-/-}$ mice suggesting that hyperlipidaemia is not required, but beneficial for AngII induced AAA development[135].

### 1.5.3 Murine models of atherosclerosis

Impaired clearance of plasma lipoproteins initiates the complex pathology of atherosclerosis. Unlike humans where most of the cholesterol is transported as low-density lipoprotein (LDL), mice transport most of the cholesterol as high-density lipoprotein (HDL)[136]. Furthermore, the absence of cholesteryl ester transfer protein (CETP) in mice makes them naturally resistant to atherosclerosis[137]. To bypass this limitation, genetically modified mice are generated by targeting genes that alter the lipoprotein profile upon dietary modification[106], the most commonly used being ApoE$^{-/-}$[138] and LDLR$^{-/-}$[139]. It has recently been shown that the ectopic introduction of a mutant *protein convertase subtilisin/Kexin type 9* (*Pcsk9*) gene can also serve to model changes in atherosclerosis. Although this model avoids genetic crosses, as single adeno-associated virus (AAV) injection is sufficient to generate the mouse model in a

much quicker time frame compared to the ApoE$^{-/-}$ or LDLR$^{-/-}$ models, PCSK9 mice are not hyperlipidemic from birth and their plaques are smaller than LDLR$^{-/-}$ or ApoE$^{-/-}$ mice of the same age [106]. Additionally, there is a possibility of an anti-viral host immune response due to AVV vectors [140]. While regular chow diet usually contains 0.02–0.03% of cholesterol and 5–6% fats[141]; mice are fed atherogenic diets also termed 'humanised diet' or 'Western diet' (WD) that contain a much higher percentage of fat and cholesterol (21% fat and 0.15%-1% cholesterol)[106].

ApoE is a structural component of all lipoproteins and a ligand that mediates lipoprotein clearance via LDLR, while LDLR is a membrane receptor that mediates endocytosis of circulating LDL[139]. Consequently, genetic ablation of *Apoe* or *Ldlr* leads to increased accumulation of plasma cholesterol in mice fed on the WD to accelerate atherosclerosis[142, 143]. Lack of endogenous *Apoe,* but not *Ldlr,* leads to an imbalance of cholesterol loading, as a result, ApoE$^{-/-}$ mice show higher plasma total cholesterol levels (400–600 mg/dl) and develop atherosclerotic lesions even on regular chow[143]. Similarly, LDLR deficient mice are prone to obesity and insulin resistance, making them a useful tool to examine the relationship between diabetes and atherosclerosis[142].

## 1.6 Genome-wide association studies

Gene profiling is a powerful, unbiased approach to map traits of complex multifactorial diseases at the molecular level[144-146]. Genome wide association studies (GWAS) provide valuable information on susceptibility genes regulating biological processes that, when disrupted, cause disease. Recently, GWAS has identified several genetic loci associated with the risk of CVD. One such candidate gene that is of particular interest is Low-density lipoprotein related receptor protein 1 (LRP1), associated with a high risk of

vascular diseases, including AAA[147], acute aortic dissection[148], carotid[149], and coronary artery disease[150].

### 1.6.1 Structure of Low density lipoprotein related protein 1 (LRP1)

LRP1 (also known as CD91 or α2macroglobulin) is a large multifunctional endocytic receptor that belongs to the LDL receptor family, regulates receptor-mediated endocytosis of several ligands, cell signalling and lipoprotein transport[151]. LRP1 is highly expressed in various tissues and cell types including VSMCs[152, 153]. Structurally, LRP1 consists of three domains: a) a large extracellular domain (515 kDa) that can bind at least 40 different ligands[154], including proteases and protease-inhibitor complexes, b) a single-pass transmembrane domain and c) an 85 kDa cytoplasmic domain which consists of two NPxY motifs and a YxxL sequence. While phosphorylation of the terminal NPxY domain is important for intracellular signalling, YxxL along with two di-leucine motifs provides a signal for endocytosis[155]. The intracellular signalling activity of LRP1 is mediated via its intracellular domain (ICD). LRP1 mediates its action by one of three ways: i) directly regulating endocytosis of its ligands, ii) regulating growth factor signalling [156, 157], iii) translocation of cleaved LRP1-ICD into the nucleus to regulate nuclear signalling[158] by a mechanism called regulated intramembrane proteolysis (RIP).

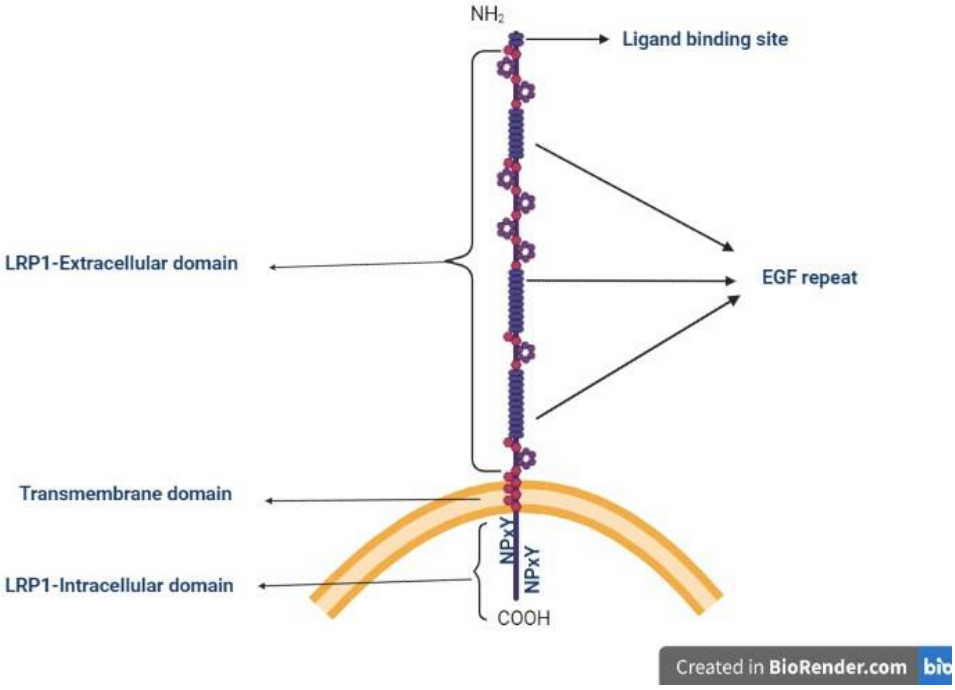



***Figure 1.9: Structure of Low density lipoprotein receptor related protein-1 (LRP1).***
*This large endocytic receptor consists of a 515-kDa extracellular domain that allows ligand binding at the -$NH_2$ terminus, a transmembrane domain and an 85 kDa cytoplasmic domain that harbours the two NPxY repeats. The intracellular domain plays an important role in regulating signalling pathways. Image created in BioRender.*

### 1.6.2 LRP1 in vascular stability and homeostasis

Studies have established a crucial role for LRP1 in vascular development, remodelling, inflammation, differentiation, and cell migration[150, 159]. Mice lacking smooth muscle specific LRP1 (smLRP1$^{-/-}$) show increased accumulation of matricellular proteins and proteases that increase ECM degradation due to dysregulated endocytosis and catabolism of these proteins[160], suggesting an important role for LRP1 in ECM homeostasis. Isometric contraction assay on LRP1 deficient *ex vivo* aortic rings demonstrated attenuated vasocontraction, indicating an essential role for LRP1 in VSMC contraction[161]. Plasminogen activator inhibitor type 1 (PAI-1) a matricellular

protein[162] regulated via LRP1 mediated endocytosis[151] is shown to inhibit tPA-regulated vascular tone through LRP1[163].

Multiple studies have demonstrated that LRP1 mediates vascular protection by regulating endocytosis and signalling of PDGFRβ[164] which is explained in detail in future. VSMC specific deletion of *Lrp1* increased activation of PDGFRβ and susceptibility to atherosclerosis, restenosis, and AAA[150, 165]. In animal models of AAA and atherosclerosis, administration of pharmacological inhibitor-Gleevec (Imatinib), suppressed activation of PDGFRβ and transactivation of LRP1 to preserve VSMC contractile phenotype and reduce disease pathology[164, 166, 167]. Deletion of the *Lrp1* gene during development affects recruitment of VSMCs and pericytes to the developing vessels, resulting in vascular haemorrhage and embryonic lethality[159].

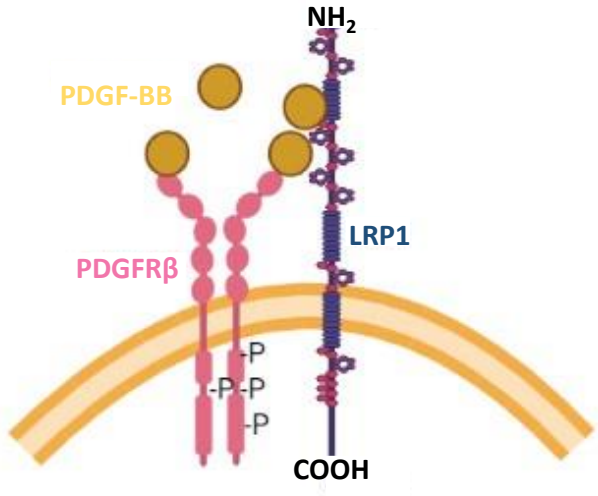



***Figure 1.10: LRP1-PDGFRβ receptor complex on the cell membrane**. LRP1 is an endocytic co-receptor of PDGFRβ[168, 169], that regulates its internalisation and downstream signal transduction. Phosphorylation of LRP1 at the terminal NPxY motif by phospho-PDGFRβ is essential for intracellular signalling of the receptor[168]. Image created in BioRender.com.*

Although LRP1-PDGFRβ endocytosis is extensively studied, yet the mechanisms controlling intracellular trafficking and recycling of the receptor complex remain unexplored. Interestingly, a yeast two-hybrid screen of an embryonic cDNA library performed by a previous student in the Smart group identified a putative interaction between LRP1 and Thymosin β4 (Tβ4)[170].

## 1.7 Thymosin β4: structure and function

Thymosin β4 (Tβ4) is a small globular (G-) actin sequestering peptide[171, 172] that belongs to the family of β thymosins. It is ubiquitously expressed in all cells except erythrocytes[173, 174] at concentrations of 0.3–0.4 mM[175]. The expression of Tβ4 is highly conserved from mammals to echinoderms[176].

### 1.7.1 Molecular structure of Tβ4

#### 1.7.1.1 The gene for Tβ4

Tβ4 is a naturally occurring peptide made up of 43 amino acids[177]. In humans, the gene encoding Tβ4, *TMSB4X* is located on the X chromosome, at locus Xq21.33-q22. In mouse, *Tmsb4x* is located on the distal arm of the X chromosome[178] and is annotated as having three exons and two introns; however, exon 1 encodes the bulk of the 5'-untranslated sequence and the start codon AUG for ubiquitous Tβ4 mRNA is located in exon 2[179], which encodes for 33 amino acids, with exon 3 encoding the remaining amino acids of the peptide[179].

### 1.7.1.2 Amino acid sequence and biologically active sites of Tβ4

Tβ4 is an unstructured peptide, however, the peptide folds to form N- and C-terminal helices upon actin binding to the $^{17}$LKKTET$^{22}$ sequence[180]. Enzymatic cleavage of Tβ4 at the N-terminus can generate a biologically active tetrapeptide, Ac-SDKP [181], while oxidation of methionine at the 6$^{th}$ position produces Tβ4-sulfoxide (Tβ4-SO), another active form of the peptide[182].

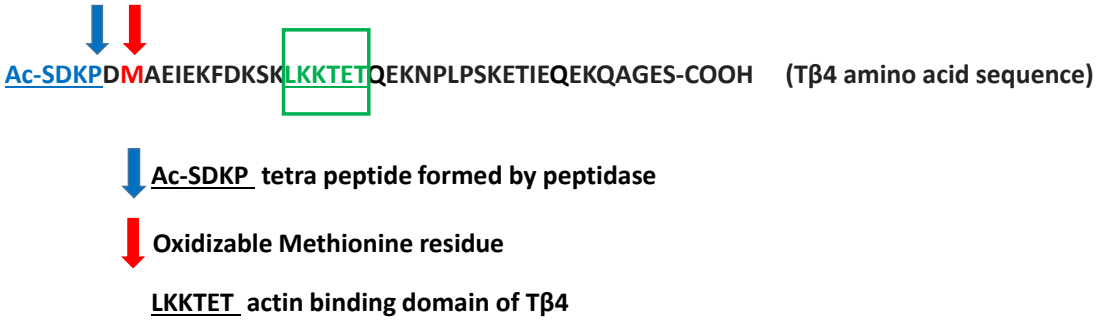



***Figure 1.11: Amino acid sequence of Tβ4.*** *Tβ4 is made up of 43 amino acids. The N-terminal Ac-SDKP, methionine at 6$^{th}$ position, and LKKTET are biologically active sites in the protein.*

## 1.7.2 Intracellular and extracellular roles of Tβ4

### 1.7.2.1 Tβ4 and actin interaction

An important function of Tβ4 is sequestering globular (G)- actin monomers to maintain the cellular pool of monomeric actin molecules. The majority of actin in VSMCs is polymerised and is termed filamentous (F) actin, however, VSMCs also have a small pool of monomeric actin termed G actin. The proportion of G actin further decreases upon stimulation to contraction[64, 183]. There are 3 major actin isoforms in VSMCs, of which αSMA is ~65%, β-actin ~20%, and γ-actin ~15%[184]. α-actin is a component of

the actomyosin contractile units, while, β-actin and γ-actin are part of the monomeric actin pool that polymerizes with stimulation to contract[185, 186].

The $^{17}$LKKTET$^{22}$ sequence on Tβ4 is recognised as an actin-binding site that favours Tβ4:G-actin 1:1 complex formation. The association of G-actin with ATP is a primary step in actin polymerisation. Lowering cellular Tβ4 disrupts Tβ4-(ATP-G-Actin) complex and releases the ATP-G-actin to increase the rate of actin polymerisation, suggesting that intracellular Tβ4 regulates cytoskeletal dynamics by controlling polymerisation and depolymerisation of actin filaments[187, 188].

In addition to its distribution in the cytoplasm, Tβ4 is also found in the nucleus[189], however, its nuclear roles are unclear. About 20% of nuclear actin is shown to have polymeric properties[190], as such, it is likely that Tβ4 may participate in controlling nuclear actin dynamics.

### 1.7.3 Tβ4 functions independent of actin-binding

Until recently, it was assumed that Tβ4 mediates all its functions solely via actin-binding, however, there is compelling evidence to highlight the multifunctional intracellular and extracellular roles of Tβ4 independent of actin-binding.

#### 1.7.3.1 Intracellular binding partners and signalling functions of Tβ4

Tβ4 forms a functional complex with particularly interesting new Cys-His protein 1 (PINCH) and integrin-linked protein kinase (ILK), activating AKT to promote cardiomyocyte migration, survival, and repair after coronary artery ligation in mice[191]. In ECs, Tβ4 activates c-Jun N-terminal kinase (JNK) signalling when partnered with Ku80

and accentuates expression of PAI-1 [192, 193]. Tβ4 is involved in stabilin-2 mediated engulfment and clearance of aged RBCs and apoptotic cells[194].

Glycogen synthase kinase (GSK-3) is a key molecule in the Wnt signaling pathway; Tβ4 regulates GSK-3 expression via ERK phosphorylation [195]. Additionally, Tβ4 is upregulated by hypoxia inducible factor-1α (HIF1α)[196]. More recently, Tβ4 was shown to modulate Notch signalling by increasing Notch1 and Notch 4 expression[197]. Another binding partner of Tβ4 is human mutL homolog 1 (hMLH1), an enzyme primarily involved in DNA repair[198]. The association of Tβ4 and hMLH1 in the cytoplasm facilitates the nuclear translocation of these proteins [198].

#### 1.7.3.2 Extracellular signalling of Tβ4

Tβ4 is a secreted peptide found in human serum[199], wound [200], and oral fluids[201]. While the mechanism of Tβ4 release into extracellular space remains unknown, it must be noted that Tβ4 lacks a signal sequence/leader sequence[202], which rules out the possibility of it being actively secreted via the conventional route that involves linking of the ribosome to the ER and translocation of the peptide[203], however, due to the small size of Tβ4 there is a possibility that the peptide transport occurs via other means such as extracellular vesicles, or may passively diffuse out of the cell.

Moreover, the addition of exogenous Tβ4 has been shown to increase intracellular Tβ4 levels, yet the mechanism of internalisation remains unknown[204]. A paracrine cardioprotective role of Tβ4 has been demonstrated for embryonic endothelial progenitor cells in the heart post-MI [205]. Moreover, endothelial Tβ4 was shown to act in a paracrine fashion to stimulate mural cell differentiation from the mesoderm in the aorta by enhancing the activity of TGFβ signalling[206]. Recently, Tβ4 was shown to interact

with $F_1$-$F_0$ ATP synthase, a cell surface receptor to induce endothelial cell migration in human vein endothelial cells (HUVECs) [207]. Thus, multiple putative mechanisms may underlie the exogenous effects of Tβ4 although their relative importance in different biological contexts remains to be determined.

### 1.7.4 Tβ4: A multifunctional protein in health and disease

#### 1.7.4.1 Cell migration

Cell migration is an important step in wound healing, angiogenesis and tumour metastasis; Tβ4 has been shown to play an important role in these processes, in part by regulating cell migration. *In vitro*, Tβ4 acts as a chemoattract and stimulates directional migration of HUVECs[208]. In addition to increasing re-epithelization post-wounding, Tβ4 stimulates attachment and spreading of keratinocytes to induce wound healing[209, 210]. In keeping with this, siRNA-mediated knockdown of Tβ4 in tumour cells inhibits their ability to migrate in a scratch wound assay[211].

In contrast, the introduction of photoactivated caged (inactive) Tβ4 in fish scale keratocytes reduced directional cell migration and loading exogenous Tβ4 in these cells reduced cellular contractility by causing disassembly of actin filaments[212]. Similarly, Oregon green labelled Tβ4, when injected into HeLa (human cervical cancer) cells, inhibited their migration and prevented wound healing[213]. These contradictory findings suggest that intracellular Tβ4 may regulate cell migration via different mechanisms in different cells and requires further investigation.

### 1.7.4.2 Anti-apoptotic effects

The anti-apoptotic properties of Tβ4 has been reported in a wide range of cells including human corneal cells where Tβ4 treatment reduces activation of caspase- 2, 3, 8 and 9 to diminish apoptosis [214]. Tβ4 is also shown to inhibit caspase -3 and 9 in human endothelial progenitor cells, by reducing the expression of pro-apoptotic protein Bax, which plays an essential role in regulating apoptosis by controlling mitochondrial permeability and release of cytochrome c[215]. Systemic administration of Tβ4 post-MI inhibits myocardial and endothelial apoptosis and activates cell survival signalling in cardiomyocytes [205], independent of its actin-sequestering activity[216].

### 1.7.4.3 Anti-inflammatory properties

The anti-inflammatory properties of Tβ4 have been explored recently in relation to wound healing. Tβ4 decreases the expression of chemokines and chemotactic agents like macrophage inflammatory protein-2 (MIP-2)[217], to inhibit migration of macrophages and reduce inflammation[218]. Topical application of Tβ4 reduced levels of pro-inflammatory cytokines such as interleukin (IL-1α and β), macrophage inflammatory proteins MIP1 (α and β) and monocyte chemoattractant protein-1 (MCP-1) in the experimentally injured cornea in rats[217]. Reduced levels of proinflammatory cytokines-tumour necrosis factor α (TNFα) and IL-1α were demonstrated in mice pre-treated with Tβ4 in lipopolysaccharide-induced septic shock models[219]. Tβ4 prevents nuclear translocation of NFκB and prevents activation of promoter regions of genes for pro-inflammatory cytokines in human corneal epithelial cells[217]. Tβ4-SO attenuated inflammatory cell infiltration and promotes cardiac wound healing in zebrafish[220].

#### 1.7.4.4 Anti-fibrotic properties

Tβ4 is a precursor of Ac-SDKP, a potent antifibrotic agent; *in vitro,* it inhibits collagen production by cardiac fibroblasts, while *in vivo* it limits collagen deposition resulting from hypertension and post-MI[221]. The anti-fibrotic properties of Tβ4 have also been demonstrated in rat dermal wounds[222]. Intraperitoneally administered Tβ4 in monocrotaline-induced pulmonary hypertensive mice reduced expression of connective tissue growth factor (CTGF) and Col3a[223], hallmark proteins for fibrosis.

#### 1.7.4.5 Tumour metastasis

Although cell migration and angiogenesis are considered beneficial in wound healing and tissue regeneration, they are undesirable in tumour growth and metastasis[209, 224]. Overexpression of thymosins (Tβ4, Tβ10, Tβ15) are reported in various tumours (thyroid[225], renal[226] and breast), melanoma[227] and fibrosarcoma[228]. *In vitro*, overexpression of Tβ4 in colon carcinoma cells increased their invasiveness via increased activation of AKT suggesting a role for Tβ4 in tumour metastasis[229]. Tβ4 promotes cell migration in mouse lung tumour cells but did not affect cell invasion, proliferation, and MMP activity[230].

#### 1.7.4.6 Cardiac protection

Expression of Tβ4 begins from embryonic day (E) 8.0 in the ventricular myocardium[231]. Cardiac-specific knockdown of Tβ4 resulted in death of embryos in more severe cases by E16.5 and those that survived had non-compacted myocardium, detached epicardium, and a failure in coronary vessel development, due to trapping of VSMC progenitors in the epicardium. This study was the first to implicate endogenous Tβ4 in

vascular development, specifically a role in the formation of VSMC-coated vessels and the importance of this process for cardiac development[210].

#### 1.7.4.7 Exogenous Tβ4 as a therapeutic agent

Exogenous Tβ4 is shown to stimulate wound healing upon topical application or intraperitoneal injection in a rat punch wound, with benefits evident as early as 4 days post wounding. Exogenous Tβ4 induces differentiation and survival of rat brain progenitor cells in a model of cerebral artery occlusion[232]. The beneficial effects of Tβ4 treatment are also seen in a model of spinal cord injury[233]. Similarly, Tβ4 treatment accelerates re-epithelialization of corneal wounds and promotes wound healing in alkali-burn animal models[217]. Topically administered Tβ4 in patients with venous stasis ulcers suggests a potential to accelerate wound healing[234].

*In vivo*, Tβ4 treatment improved cardiac function post-infarction by reducing the death of cardiomyocytes and scarring[191, 235]. Tβ4 activates adult murine epicardial cells, promoting cardiac regeneration through the differentiation of a small portion of reactivated epicardial cells into *de novo* cardiomyocytes and promoting neovascularisation, likely by paracrine stimulation from the epicardium[236-238]. Tβ4 activates ILK/AKT signalling in cardiomyocytes and preserves function in the ischaemic adult heart[191]. Ac-SDKP, derived from Tβ4, inhibits inflammation and fibrosis in a rat model of MI-induced heart failure[239].

## 1.8 Tβ4 in the vasculature

### 1.8.1 Endothelial tube formation in angiogenesis and vasculogenesis

Angiogenesis is defined as the formation of new vessels from pre-existing vessels through sprouting or intussusception[240], while, formation of functional *de novo* vessels from progenitor cells (e.g. from embryonic mesoderm during development) is termed vasculogenesis[241]. Tβ4 has been shown to promote both angiogenesis and vasculogenesis, as discussed below.

#### 1.8.1.1 Angiogenesis

During angiogenesis, certain ECs on the vessel specialise to become tip cells, and start to invaginate into the surrounding media, a process that is initiated by a hypoxic stimulus and driven by vascular endothelial growth factor (VEGF)-A, a pro-angiogenic growth factor. Nitric oxide dependent HIF1α is shown to upregulate the expression of Tβ4 via the Tβ4 promoter[196]. Tβ4 treatment was shown to cause up-regulated expression of VEGF-A[230], however, it was unclear if this was mediated by HIFα. Both endogenous and exogenous Tβ4 promote EC migration *in vitro*[208, 209]. To facilitate EC migration during angiogenesis, Tβ4 induces an increase in the production of MMPs to degrade the basement membrane[208].

#### 1.8.1.2 Vasculogenesis and arteriogenesis

Vessels are formed by vasculogenesis[242] and Tβ4 has been shown to promote vasculogenesis in the yolk sac, systemic and coronary vasculature. Tβ4 was identified as a transcriptional target of Heart and neural crest-derived transcript-1 (Hand1) and this axis was found to be crucial in regulating yolk sac vasculogenesis[243]. During development, multipotent progenitors from the epicardium (epicardium-derived cells;

EPDCs) migrate into the myocardium and differentiate into VSMCs to support the developing coronary vasculature. Tβ4 and its derivative AcSDKP tetrapeptide are essential factors secreted from the developing myocardium that act in a paracrine manner to induce the migration and differentiation of EPDCs in cardiac-specific *Tmsb4x* knockdown embryos, EPDCs were trapped in the epicardium and coronary vessels regressed due to the lack of VSMC support, defining a crucial role for Tβ4 in this process[236]. Tβ4 is also shown to play an important role in recruitment of mesodermal progenitors to the developing aorta and differentiated into mural cells (pericytes, and VSMCs) to form stable vessels[244].

#### 1.8.1.3 Recruitment of VSMCs to stabilize newly formed vessels

Once endothelial tubes have formed by vasculogenesis, a number of factors are recruited, including TGFβ, Notch1 and PDGF-BB contribute to the recruitment and differentiation VSMCs to the developing vessels[77, 78]. Rossdeutsch *et al* demonstrated that EC-secreted Tβ4 stimulates mural cell differentiation in the developing aorta by enhancing TGFβ signalling. Global and EC-specific knockdown of Tβ4 resulted in vascular haemorrhage, coincident with a reduction in VSMC coverage[206]. This study confirms an essential role for Tβ4 both in VSMC recruitment and differentiation in the developing embryo.

Two key studies have demonstrated that Tβ4 recapitulates its developmental role to promote coronary vessel formation post-MI in adult mice. In the first, exogenous Tβ4 was shown to enhance epicardial activation, expansion of the capillary network, and the extent of VSMC support (arteriogenesis); although epicardial activation and modest capillary growth occur as an endogenous repair mechanism following injury, Tβ4 significantly augmented the proportion of stable, perfused vessels[237].

The endogenous vascular response (expansion of the capillary network into the infarcted region and throughout the expanded epicardium) was further investigated by Dubé *et al*. In Tβ4KO mice, significantly reduced epicardial activation was accompanied by severely diminished vascular growth and the few vessels that formed lacked VSMCs, suggesting that Tβ4 is required for epicardial activation and VSMC recruitment to the newly formed vessels, recapitulating its embryonic roles in the ischaemic adult heart to promote repair[245].

## 1.9 Work prior to my DPhil

Given that Tβ4 promotes embryonic vascular development and, specifically, is required for VSMC differentiation and vascular stability during development[206, 236, 237], we reasoned that Tβ4 might play a role in maintaining VSMCs in a fully differentiated contractile state postnatally. Therefore, the role of Tβ4 in adult vasculature was investigated in knockout and control mice at baseline[170].

### 1.9.1 Tβ4 maintains medial layer stability in the adult aorta

To investigate the role of Tβ4 in the adult vasculature, aortas from 12-16 weeks old male global Tβ4 knockout ($T\beta4^{-/Y}$) and control ($T\beta4^{+/Y}$) mice were compared, due to the higher mortality in male *Tmsb4x*/Tβ4 null embryos[206]. Histological analysis (**Figure 1.12 A**) confirmed significant aortic dilation (**Figure 1.12 B**) and aberrant elastin deposition in $T\beta4^{-/Y}$ (**Figure 1.12 C&D**). This was accompanied by highly disorganised VSMC morphology and a shift to a synthetic phenotype both at the mRNA and protein level (**Figure 1.12 F-G**). Medial layer defects in $T\beta4^{-/Y}$ mice are similar to those observed in

patients with AAA[246] suggesting compromised vessel wall stability and predisposition to vascular disease.

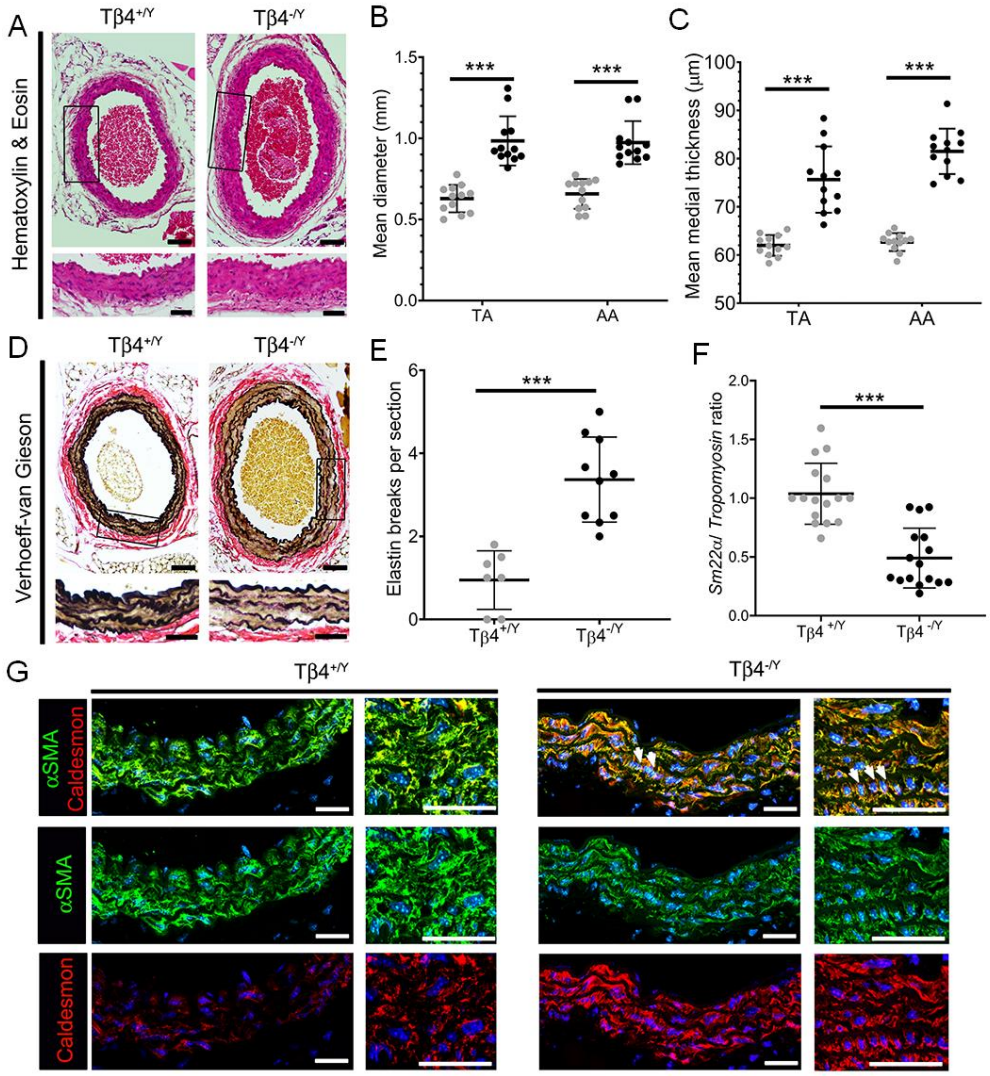



***Figure 1.12: Mice lacking Tβ4 show aortic defects at baseline in adulthood**. **A**) Hematoxylin & Eosin staining to assess morphology, **B**) quantification of aortic dilatation; medial thickness **C**). Verhoeff-van Gieson staining (**D**) assessed elastin integrity, quantified by the number of breaks per section (**E**). Contractile/synthetic smooth muscle markers were assessed by qRT-PCR (**F**) and protein levels by Immunofluorescence; Altered contractile-synthetic marker profile was accompanied by altered morphology, with dense clusters of cells with a cuboidal, rather than elongated appearance (white arrowheads) (**G**). Data are presented as mean ± SD, with each data point representing*

*an individual animal (12-16 week old male mice, n=10 in **B**, **C and E**, n=16 in **F**), were calculated using Mann-Witney non-parametric tests (**B**, **D**, and **F**), with Holm-Sidak correction for multiple comparisons in **B**. *** P ≤ 0.001 for Tβ4$^{+/Y}$ vs Tβ4$^{-/Y}$. Scale bars: **A**, **C**, and **E**: all 50μm. Data published in [170], © J.Clin.Invest.*

***Acknowledgement of Contribution**:*

*Histological analysis was performed by Dr. Nicola Smart*

*Some of the qRT-PCR was performed by Giles Neal (FHS student), under my supervision*

### 1.9.2 VSMC-specific requirement for Tβ4 in vascular stability

To determine the role of Tβ4 specifically in VSMCs, conditional Tβ4 knockdown mice were assessed, in which expression of Tβ4 was depleted at 3 weeks of age in Myh11-expressing cells upon tamoxifen induction, as described in detail in Section 2.2.2, Knockdown of *Tmsb4x* in VSMC was confirmed in aortic sections using RNAScope (**Figure 1.13 A-B**). Aortas from VSMC-specific Tβ4 knockdown (Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$) and control (Myh11$^{CreERT2}$; Hprt$^{+/+}$) mice were harvested at 12 weeks of age and systematically analysed using histology and immunofluorescence. Significantly dialated aorta (**Figure 1.13 D**), accompanied with medial elastin disruption (**Figure 1.13 F**) and an increase in synthetic VSMC was observed in VSMC-specific Tβ4 knockdown mice, compared to controls (**Figure 1.13 G**). These results confirm that VSMC-specific Tβ4 knockdown mice recapitulate the global knockout phenotype and additionally show that Tβ4 is not only required during development, rather there is a lifelong requirement for Tβ4 to maintain healthy vessels.

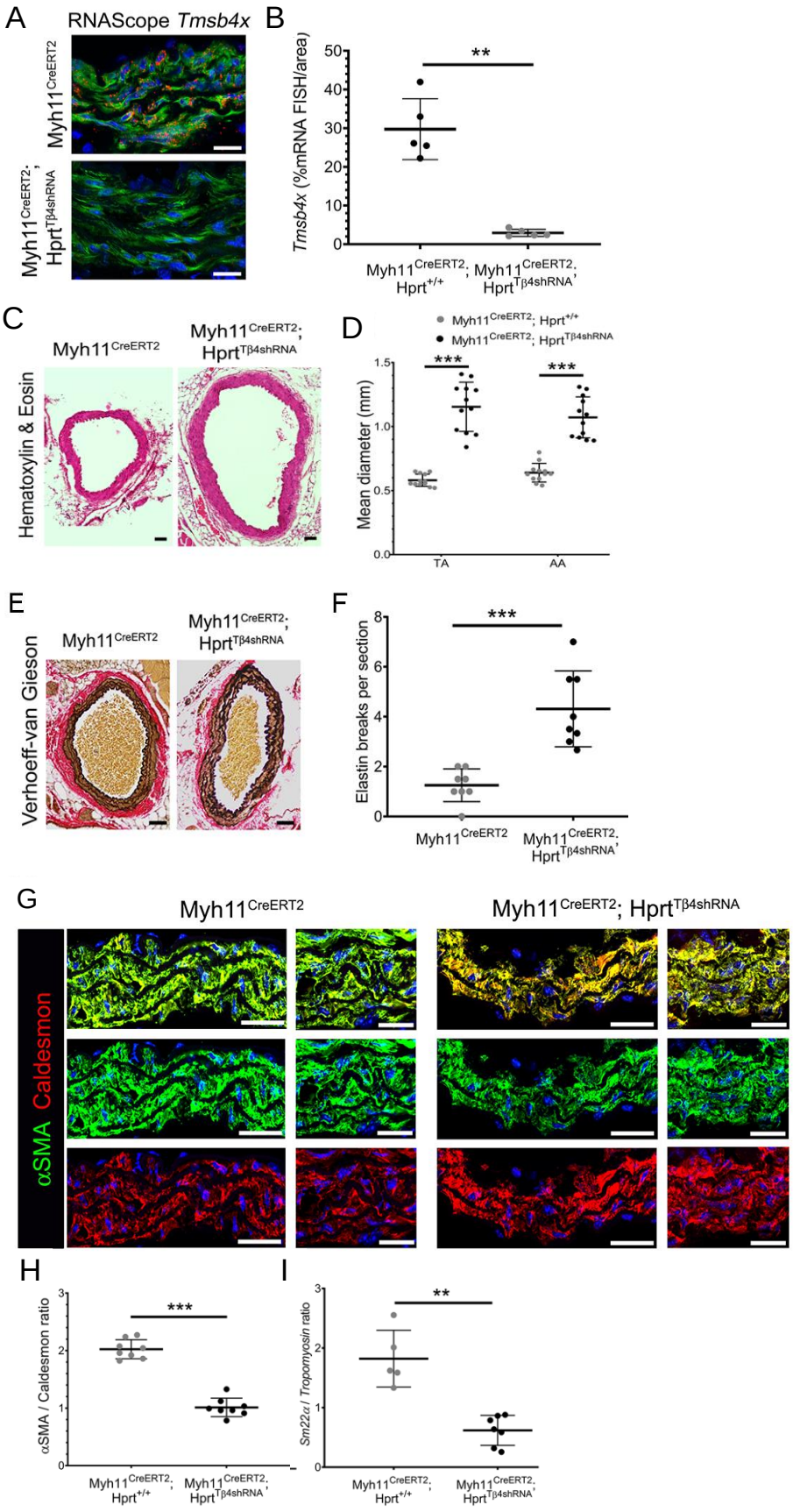

A
RNAScope Tmsb4x
Myh11CreERT2
Myh11CreERT2; HprtTβ4shRNA
B
Tmsb4x (%mRNA FISH/area)
**
Myh11CreERT2; Hprt+/+
Myh11CreERT2; HprtTβ4shRNA
C
Myh11CreERT2
Myh11CreERT2; HprtTβ4shRNA
Hematoxylin & Eosin
D
Myh11CreERT2; Hprt+/+
Myh11CreERT2; HprtTβ4shRNA
Mean diameter (mm)
***
***
TA
AA
E
Myh11CreERT2
Myh11CreERT2; HprtTβ4shRNA
Verhoeff-van Gieson
F
Elastin breaks per section
***
Myh11CreERT2
Myh11CreERT2; HprtTβ4shRNA
G
Myh11CreERT2
Myh11CreERT2; HprtTβ4shRNA
αSMA Caldesmon
H
αSMA / Caldesmon ratio
***
Myh11CreERT2; Hprt+/+
Myh11CreERT2; HprtTβ4shRNA
I
Sm22α / Tropomyosin ratio
**
Myh11CreERT2; Hprt+/+
Myh11CreERT2; HprtTβ4shRNA

***Figure 1.13: VSMC-specific requirement for Tβ4 for maintenance of healthy aorta.***

***A**) RNAscope showing deletion of Tmsb4x from medial VSMCs of 3 week old Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ KD mice; compared with Myh11$^{CreERT2}$; Hprt$^{+/+}$ control mice n=6, (**B**) quantification. Hematoxylin & Eosin staining n=16 (**C**) assessed aortic dilatation and medial thickness in n=8 12 week old mice, quantified in (**D**).Verhoeff van Gieson staining (**E**) to visualise elastin integrity, quantified by the number of breaks per section in n=8 (**F**). Immunofluorescence to assess smooth muscle phenotype in Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$, compared with Myh11$^{CreERT2}$; Hprt$^{+/+}$ aortas (**G**) and quantification in from n=8 (**H**). Contractile:synthetic gene expression by qRT-PCR in (**I**) from n=8. Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using a Mann-Witney non-parametric test (**B**, **F**, **H** and **I**) or two-tailed unpaired Student's t-tests (**D**; with Holm-Sidak correction for multiple comparisons). ** P ≤ 0.01; *** P ≤ 0.001. Scale bars: **C** and **E**: 100μm; **A**: 20 μm; **G**: 50μm (low); 20μm (high). Data published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution**:*

*Histological analysis was performed by Dr. Nicola Smart*

*Some of the immunofluorescence staining was performed by Rachel Davies (FHS student), under my supervision.*

### 1.9.3 Tβ4 interacts with LRP1 in aortic VSMCs

LRP1 was identified as a putative interactor of Tβ4 from a yeast two-hybrid screen[170] performed by a previous student in the Smart group. To evaluate how Tβ4 may function at a molecular level, proximity ligation assay (PLA) was performed and proximity between Tβ4 and LRP1 was confirmed in murine (**Figure 1.14A**) and human (**Figure 1.14B**) aortic sections in AAA and healthy omental artery from patients recruited to the Oxford Abdominal Aortic Aneurysm study. Further, combining PLA with immunofluorescence staining with early endosome antigen 1 (EEA1), an early endosome marker, confirms co-localisation of Tβ4-LRP1 complexes in endocytic vesicles (**Figure 1.14C**). While PLA identified proximity and colocalization, it was important to evaluate if there was any direct interaction between the two proteins.

Nuclear magnetic resonance (NMR) spectroscopy was used to map the interaction of Tβ4 to residues near the NPxY motifs in LRP1 that are associated with signal transduction and receptor recycling (**Figure 1.14D**).

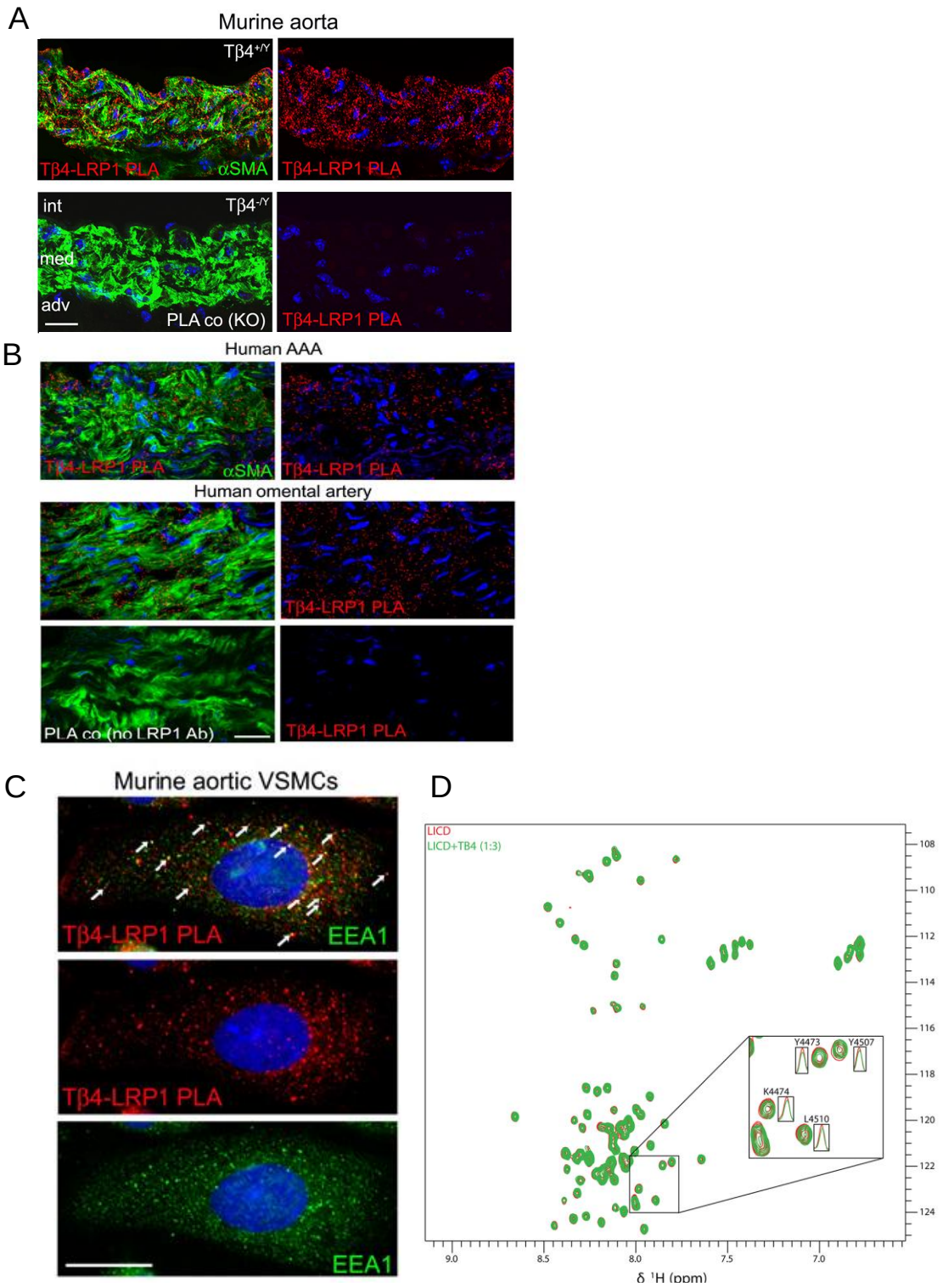


***Figure 1.14: Tβ4 interacts with LRP1 in endocytic vesicles of murine and human arterial smooth muscle cells.*** *Proximity ligation assay (PLA) demonstrated <40nm proximity, suggesting an interaction of Tβ4 with LRP1 in the murine aorta (**A**) representative image of n=3; and (**B**) human aorta from AAA patients and matched omental artery from the same patients (n=10); Immunofluorescence for LRP1 and Tβ4 reveals localization to punctate vesicular structures (**C**) in murine primary aortic VSMCs. PLA for Tβ4 and LRP1, with some signals localising to early endosomes, labelled with EEA1(**C**). (**D**): 1H 15N HSQC NMR spectra of 15N-labeled ICD of LRP1 and 3:1 Tβ4. Scale bars: **A**, **B**: 20μm; **C**: 10μm. AAA: abdominal aortic aneurysm; PLA: proximity ligation assay; int: intima; med: media; adv; adventitia; ICD: intracellular domain (epitope). Data published in[170], © J.Clin.Invest.*

***Acknowledgment of Contribution**:*

*PLA was performed by Dr. Nicola Smart*

*NMR studies were performed by Dr. Alisha Jones and Prof. Michael Sattler, Munich (Collaborators)*

*Human AAA and Omental artery samples from the OxAAA cohort were provided by Mr Regent Lee and Prof Ashok Handa*

## 1.10 DPhil Project

### 1.10.1 Rationale

VSMCs are highly plastic and, in response to disease, they lose their differentiated mature phenotype and de-differentiate to a less mature phenotype which affects vascular stability and integrity[50]. During embryogenesis, Tβ4 is essential for VSMC differentiation in the developing vasculature[206]. In adult mice, loss of Tβ4 at baseline leads to medial layer defects in the aorta, including aberrant elastin deposition, and switch to a more synthetic phenotype[170], suggesting compromised vascular stability Increased VSMC phenotypic switching and compromised vascular stability are characteristics of smLRP1$^{-/-}$ mice, which show increased susceptibility to AAA and atherosclerosis[164].

LRP1 is an endocytic co-receptor of PDGFRβ[247] and correct regulation of this pathway is known to maintain vascular stability in health and disease. Interestingly, Tβ4 has also been shown to regulate endocytosis[204], although the mechanisms have not been defined. Importantly, we demonstrated an interaction of Tβ4 with the intracellular domain of LRP1 which, along with the similar aortic phenotype at baseline[170] supports a common pathway for Tβ4-LRP1 in regulating vascular stability and protection against disease.

### 1.10.2 Hypothesis

Tβ4 functions to maintain a healthy vasculature, limiting VSMC phenotypic switching and ECM degradation, via regulating endocytosis of LRP1 and growth factor receptors, and acts therapeutically to protect against vascular disease.

### 1.10.3 Specific Aims

1 To define the role of endogenous Tβ4 in protection against atherosclerosis and aortic aneurysm.

2 To investigate VSMC phenotypic switching, ECM modulation, and inflammation in Tβ4 mutant mice and compare notable characteristics with LRP1 mutants.

3 To investigate the mechanism underlying VSMC phenotypic switching and ECM modulation in Tβ4 mutant mice.

4 To explore the therapeutic potential of exogenous Tβ4 in vascular protection.

# Chapter II

# Materials and Methods

## 2.1 Animal Husbandry

All animals used for the study were housed in the Biomedical services building and procedures involving the use and care of animals were performed in accordance with the Animals (Scientific Procedures) Act 1986 (Home Office, United Kingdom) and approved by the University of Oxford or University College London Animal Welfare and Ethical Review Boards.

## 2.2 Mouse lines

All mice used for the study were maintained on the C57B6/J background.

### 2.2.1 Global Tβ4 knockout line

Global Tβ4 Knockout ($T\beta4^{-/Y}$) mice were a gift from Martin Turner, Babraham Institute, Cambridge, UK. These mice were created by insertion of a neomycin cassette in place of exon 2 in the *Tmsb4x* locus, as shown in **Figure 2.1**, resulting in the removal of the majority of the coding region and generating a non-functional allele as described in[206]. The gene coding for Tβ4 is located on the X chromosome, therefore, male mice are either wildtype ($T\beta4^{+/Y}$) or hemizygous null, designated as $T\beta4^{-/Y}$. Female mice carrying two X chromosomes can be wildtype ($T\beta4^{+/+}$), heterozygous ($T\beta4^{+/-}$), or homozygous null ($T\beta4^{-/-}$).

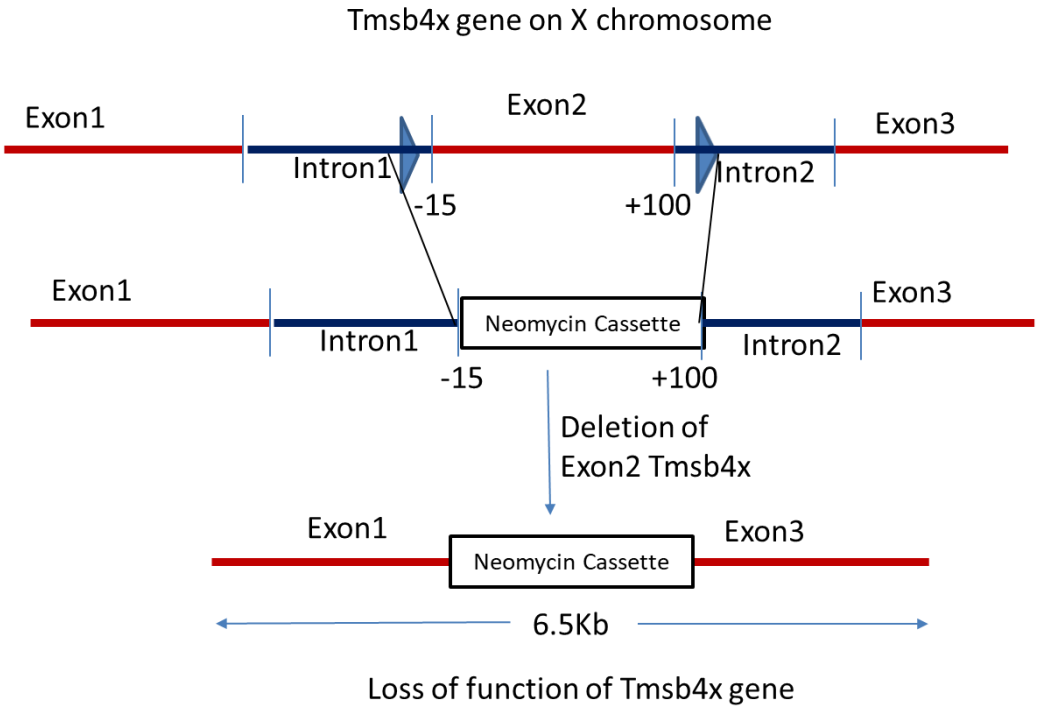



***Figure 2.1: Generation of non-functional Tmsb4x.*** *The Tβ4 gene targeting vector created by ligating 5'arm (exon 1; BamHI-EcoRV) and 3' arm (exon 3; Nhe1-BamH1) flanking a neomycin resistance cassette, thereby deleting exon 2 of the Tmsb4x gene. Figure is drawn based on information in[206].*

### 2.2.2 Conditional Tβ4 knockdown mice

Conditional Tβ4 knockdown mice were generated by crossing Tβ4shRNA mice with inducible VSMC specific-Cre mice, such that expression of Tβ4shRNA is under the control of Cre recombinase[236]. In inducible lines, Cre recombinase catalyses cleavage and ligation of DNA at loxP sites[248] only in the presence of the estrogen analogue, tamoxifen[249].

Tβ4shRNA transgenic mice described in[206, 236] take advantage of RNA interference (RNAi)[238]. Thus in cells expressing both Cre recombinase and the Tβ4shRNA sequence (CTGAGATCGAGAAATTCGATAAG), recombination between the LoxP site

causes excision of a transcription termination sequence, allowing transcription of Tβ4shRNA to silence the expression of the gene by degrading Tβ4 mRNA[250] as shown in **Figure 2.2**.

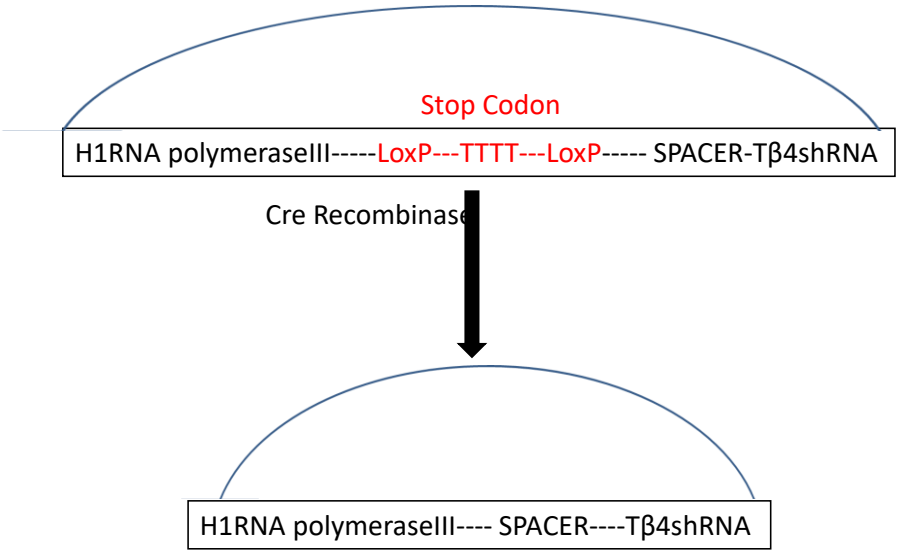



***Figure 2.2: The mechanism of action of Tβ4shRNA***. *The shRNA construct consists of a short 23-base-pair RNA sequence, inserted as a hairpin loop downstream of the H1 RNA polymerase-III promoter. The shRNA sequence is preceded by a 5-thymidine termination sequence flanked by two loxP sequences. Upon Cre recombination, excision of the stop sequence and transcription of the Tβ4 shRNA silences the expression of Tmsb4x. Figure is drawn based on information in [206].*

Myh11$^{CreERT2}$ transgenic mouse line[251] (created by Stefan Offermann, Max-Planck Institute for Heart and Lung) was purchased from Jax (JAX stock #019079). Expression of Cre is under the control of the mouse *Myh11* promoter, and the transgene is inserted on the Y chromosome. Thus, male mice are hemizygous and express a single copy of the transgene, while female mice carry no transgene and are wildtype. For this thesis, male Myh11$^{CreERT2}$ mice were crossed with female *Hprt1*-targeted, floxed Tβ4shRNA[251], to generate Myh11$^{CreERT2}$; HprtTβ4$^{shRNA}$ offspring.

### 2.2.3 Conditional LRP1 knockout mice

Myh11$^{CreERT2}$ transgenic mice were crossed with the previously described floxed *Lrp1* line[164]; obtained from Jax. At 3 weeks of age, LRP1 was knocked out specifically from VSMCs by tamoxifen administration to Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$.

### 2.2.4 Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice

To study the role of Tβ4 in atherosclerosis, ApoE$^{-/-}$ mice[252] purchased from Charles River were crossed with Tβ4$^{+/-}$ female mice to generate littermate control and mutant male mice.

## 2.3 Tamoxifen induction

Tamoxifen (Sigma #T5648) was prepared in peanut oil (Sigma) with 10% ethanol at a final concentration of 10mg/ml. Conditional knockdown of Tβ4 or deletion of *Lrp1* were induced by oral gavage of 3 week-old mice with 3 doses of tamoxifen (80mg/kg) on alternate days.

## 2.4 Genotyping

Ear biopsies were collected at age 12-14 days for genotyping. Tissue was digested in 100µl digestion buffer (50mM Tris-HCl (pH 8.5), 1mM EDTA, 0.5% Tween 20, 50µl/ml of 10mg/ml Proteinase K) for 2 hours at 56ºC. To stop the activity of ProteinaseK the reaction mixture was incubated at 95 ºC for 10 minutes. Illustra PuReTaq Ready-To-Go PCR Beads were reconstituted in nuclease-free water to contain 200µM dNTP, 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl,1.5 mM $MgCl_2$, 1µl genomic DNA and 0.5pmol each of forward and reverse primers (described in **Table 2.1**) per 25µl.

PCR programmes used to genotype the mice are presented in **Table 2.2**. The amplicon was run on a 1.5% (w/v) agarose gel at 125V for 1 hour with 1X orange G/GelRed loading dye per sample, to confirm the genotype.

**Table 2.1: Primers used for genotyping**

| Allele | Forward primer (5'- 3') | Reverse primer (5'- 3') |
|---|---|---|
| *Tmsb4x*$^{tmPrri}$ | CCAGCCCGTTCTGAAAATGG GTCCTTTTGGAACTGGGAGA | CGCCTTCTTGACGAGTTCTT |
| *Hprt*$^{tm(H1-RNAI:Tmsb4x)Prri}$ | TGCATGTCGCTATGTGTTCTG | AAGTCGTGCTGCTTCATGTG |
| *Tg(Myh11*$^{creERT2}$*)1Soff* | TGACCCCATCTCTTCATAGTCCCTCACATCCTCAGGTT | CTAGGCCACAGAATTGAAAGATAT GTAGGTGGAAATTCTAGCATCATCC |
| *Lrp1*$^{tm2Her}$ | CATACCCTCTTCAAACCCCTTCCTG | GCAAGCTCTCCTGCTCAGACCTGGA |
| *Apoe*$^{tm1Unc}$ | TGTGACTTGGGAGCTCTGCAGC | GCCGCCCCGACTGCATCT |

**Table 2.2: PCR programmes used for genotyping**

| Allele | Initial denaturation | Number of cycles | Cycles | | | Final extension |
|---|---|---|---|---|---|---|
| | | | Denaturation | Annealing | Extension | |
| *Tmsb4x*$^{tmPrri}$ | 94°C 2’ | X35 | 94°C 30’’ | 58°C 30’’ | 72°C 45’’ | 72°C 1’ |
| *Hprt*$^{tm(H1-RNAI:Tmsb4x)Prri}$ | 94°C 2’ | X35 | 94°C 30’’ | 60°C 30’’ | 72°C 45’’ | 72°C 7’ |
| *Tg(Myh11-cre/ERT2)1Soff* | 94°C 2’ | X35 | 94°C 30’’ | 60°C 30’’ | 72°C 45’’ | 72°C 7’ |
| *Lrp1*$^{tm2Her}$ | 94°C 3’ | X35 | 94°C 30’’ | 65°C 30’’ | 72°C 30’’ | 72°C 2’ |
| *tdTomato* | 94°C 5’ | X35 | 94°C 30’’ | 61°C 30’’ | 72°C 30’’ | 72°C 7’ |
| *Apoe*$^{tm1Unc}$ | 94°C 15’ | X28 | 94°C 20’’ | 68°C 40’’ | 72°C 2’ | 72°C 7’ |

## 2.5 Vascular injury models

Vascular injury studies were designed and executed in line with the recommendations of the American Heart Association Council on Arteriosclerosis, Thrombosis and Vascular Biology; and Council on Basic Cardiovascular Sciences[253].

### 2.5.1 Atherosclerosis model

Age-matched (8-12 week old) mice, on the ApoE$^{-/-}$ background, were fed on Western diet (21.4% fat; 0.2% cholesterol; Special Diets Services, Essex, UK) for up to 12 weeks. Mice were weighed weekly and, at harvest, serum was collected for cholesterol analysis (performed by Charles River Laboratories). EdU (5-ethynyl-2'-deoxyuridine), prepared in

0.9% saline, was administered at a dose of 50mg/kg by intraperitoneal injection (4 doses over the 10 days prior to harvest).

### 2.5.2 Aneurysm model

Aneurysm formation was induced in 8-12 weeks old adult male mice by subcutaneous implantation of an osmotic minipump (Alzet) that infused 1mg/kg/day Angiotensin II (AngII) (Sigma; A2900) for 10 days. AngII doses were calculated for each mouse based on animal mass. Mice were first anesthetized under 2% isoflurane and prepped for the mini pump implantation. Preparation of AngII filled osmotic minipump and its subcutaneous implantation into the back of the mouse were carried out under asceptic conditions. Mice were closely monitored for weight loss or any adverse conditions over the period of 10 days post mini pump implantation.

### 2.5.3 Imatinib treatment

For the Imatinib rescue experiment, 12 week old male mice, a combination of C57B6/J and Tβ4$^{+/Y}$ were used as controls (a deviation from the AHA recommendation) and Tβ4$^{-/Y}$ mice were randomly assigned into groups to be orally gavaged with either 10mg/kg Imatinib mesylate or sterile water daily for 10 days, starting two days prior to AngII mini pump implantation.

### 2.5.4 Tβ4 treatment

To determine the therapeutic potential of Tβ4, age-matched male C57B6/J mice were simultaneously infused with 1mg/kg/day AngII and either 12mg/kg/day Tβ4 (gift from RegeneRX pharmaceuticals) or 0.9% saline by osmotic minipump implantation for 14 days. All the animals were regularly monitored and weighed post-surgery until harvest.

### 2.5.5. Isolation of the aorta and segmentation

Mice were euthanized using 50µl Pentoject and the external surface was sprayed with 70% ethanol. Once the reflex response was lost, the chest was opened, the mouse was transcardially perfused with phosphate-buffered saline (PBS; pH7.4) to wash and remove peripheral blood before collecting the aorta. Larger organs including liver, stomach, spleen, pancreas, intestines and kidneys were removed to allow isolation and dissection of the aorta using sterile microscissors and forceps under a dissection microscope. The aorta was transferred to ice cold 1X PBS to clean any blood and cleaned of adipose tissue. For the purpose of this study, the aorta harvested from each mouse was divided into smaller segments, approximately similar in size and anatomical region, to be allocated for different types of analysis as shown in **Figure 2.3**. Although this is advantageous from the perspective of the 3R's (reduction, refinement and replacement), the issue with this approach is that different segments of the aorta have different properties and responses to disease. These limitations and a solution to circumvent such limitations is discussed in detail in Chapter III, Section 3.5.

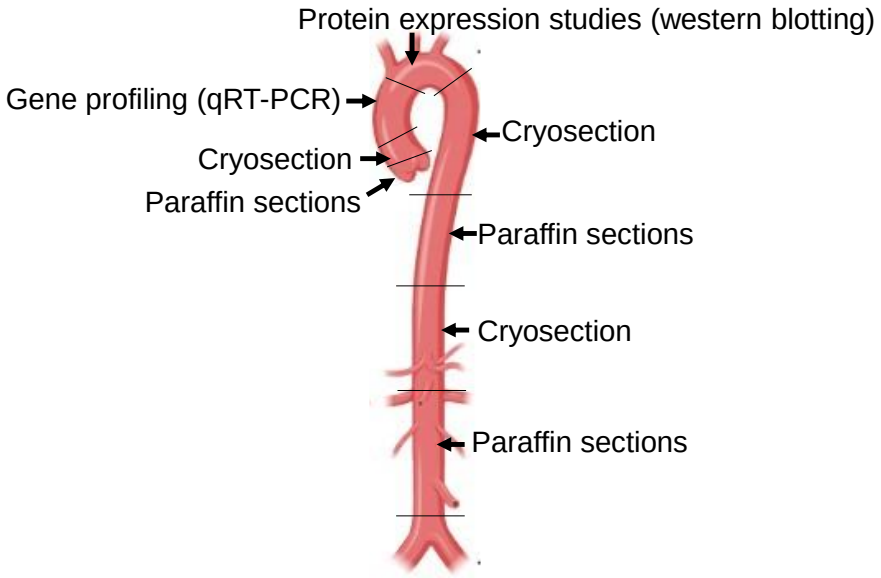



***Figure 2.3: Diagram to show sections of the aorta used for various analyses:*** *After removing the adipose tissue, the aorta from each animal was cut, as shown in the*

*diagram, to allow histological analysis, immunohistochemistry by antibody staining on cryosections, gene profiling by qRT-PCR, and protein expression studies by western blotting.*

## 2.6 Immunohistochemistry

### 2.6.1 Sample Preparation

For histological and immunohistochemical analyses, mouse aortic sinus, aortic arch, and descending aorta were harvested, washed in phosphate-buffered saline (PBS; pH7.4), and fixed in 4% paraformaldehyde (PFA) at room temperature, either for two hours (cryosectioning) or overnight (paraffin embedding).

For histological analysis, samples were dehydrated using a series of graded ethanol concentrations (50-100%, each for at least 2 hours), and cleared in butanol overnight. Samples were incubated at 60°C in 1:1 butanol: molten pastillated fibrowax for 30 minutes, then three times in 100% molten wax, each for 4 hours, before embedding and sectioning (10µm transverse sections).

For immunohistochemical analyses, fixed tissues were rinsed in PBS and equilibrated in 30% sucrose (in PBS) overnight at 4°C. Samples were placed in 50:50 (30% sucrose: Tissue-Tek OCT) for 30 mins, then embedded in OCT and chilled at -80°C. Cryosections of 10µm thickness were collected on Super Frost Plus slides (VWR) and stored at -80°C until further analysis.

### 2.6.2 Histology

Paraffin sections were stained with Oil red O solution, Hematoxylin and Eosin, Elastic stain kit (Verhoeff van Gieson), and Masson's Trichrome kit (all from *Sigma-Aldrich*), each according to the manufacturer's protocol. After imaging, morphology was assessed on sections that had been anonymised and blinded for genotype. Elastin breaks per section were counted manually. Plaque area and aortic diameter were measured using ImageJ. Elastin breaks/score, aortic diameter, plaque size in thoracic/abdominal aorta are all expressed as the mean of 6 sections per aorta. Histological studies were performed on 6-8 sections from each aorta, mean score of which was used for quantification. To avoid bias, aortas were scored whilst blinded to genotype.

### 2.6.3 Immunohistochemistry (IHC)

Frozen sections of descending aorta or aortic sinus were air-dried for 5 minutes and rinsed in PBS. Sections were permeabilized in 0.5% Triton-X100/PBS for 10 minutes, rinsed in PBS and blocked for 1-2 hours (1% BSA, 10% goat serum or donkey serum; 0.1% Triton-X100 in PBS). Sections were incubated with primary antibodies (diluted as shown in **Table 2.3**) at 4°C overnight. Sections were washed 5-6 times in 0.1% Triton-X100 in PBS (PBST) then incubated in secondary antibody (Alexa Fluor conjugated, raised against- rabbit, rat, Armenian hamster IgG (Invitrogen; 1:200) for 1 hour at room temperature. Sections were washed 3 times in PBST and incubated with 300nmol/ul 4',6-diamidino2-phenylindole (DAPI) in PBS for 5 minutes and rinsed a further twice in PBS. Slides were mounted with 50% glycerol: PBS or Vectashield (Vector Laboratories #H-1000). Apoptosis was assessed using the Click-iT TUNEL Alexa Fluor 546 Imaging Assay kit (Thermo fisher) and EdU incorporation by Click-iT EdU Alexa Fluor 546 Imaging Assay kit (Thermo fisher), according to the manufacturers' instructions.

**Table 2.3: List of antibodies used for immunostaining**

| Antibody | Company | Catalogue No | Species | Dilution |
|---|---|---|---|---|
| αSMA | Sigma | F3777, C6198 | Mouse | 1:400 |
| Vimentin | Abcam | Ab45939 | Rabbit | 1:200 |
| PECAM1 | Abcam | Ab119341 | Ar.Hamster | 1:100 |
| Caldesmon | Abcam | Ab32330 | Rabbit | 1:250 |
| CD68 | BioRad | MCA1957 | Rat | 1:200 |
| CTGF | Abcam | Ab6992 | Rabbit | 1:200 |
| PAI-1 | Abcam | Ab66705 | Rabbit | 1:100 |
| HtrA1 | Abcam | Ab38611 | Rabbit | 1:100 |
| P-LRP1 | Santa Crutz biotechnology | sc-33049 | Rabbit | 1:100 |
| P-PDGFRβ | Abcam | Ab134048 | Rabbit | 1:100 |
| LAMP1 | Abcam | Ab67283 | Rabbit | 1:25 |
| Transferrin receptor | Abcam | Ab22391 | Rat | 1:25 |
| EEA1 | Abcam | Ab2900 | Rabbit | 1:50 |
| RAB7 | Abcam | AB198337 | Rabbit | 1:100 |

### 2.6.4 Microscopy and quantification

The samples were imaged using a Leica DM6000 fluorescence microscope or Olympus Fluoview-1000 confocal microscope and processed using Fiji. The expression of a specific marker protein was determined based on the fluorescence intensity in a particular channel. Low power fluorescence images were captured using Leica DM600 of the entire transverse section of the aorta. Channels were split to show individual fluorescence, converted to 8-bit images. The threshold was set to eliminate background fluorescence. Total fluorescence intensity in each channel was measured after carefully outlining the medial VSMC layer using the “Measure” tool. Fluorescence in each channel was then normalised to vessel circumference. To avoid bias, the genotype and treatments were blinded during staining, image capturing and quantification.

## 2.7 Cell culture

### 2.7.1 Primary aortic VSMCs

Primary VSMCs were isolated by enzymatic digestion of murine aortas, as previously described[254], and cultured in DMEM(Gibco) containing 20% fetal bovine serum (FBS) (Gibco #11550356). After the first week, FBS was gradually reduced from 20% to 10% and cells were maintained thereafter in DMEM supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin (Gibco #15140122), in a humidified incubator at 37°C and 5% $CO_2$.

### 2.7.2 Mouse aortic vascular smooth muscle cell line (MOVAS-1)

An immortalised mouse vascular aortic smooth muscle cell line (MOVAS-1)[255, 256] was purchased from ATCC (ATCC® CRL-2797™) and cultured in DMEM (ATCC; 30-

2002) supplemented with 10% FBS, 100U/ml Penicillin-Streptomycin, and 0.2mg/ml G418 (Thermo scientific; G1393) at 37°C and 5% $CO_2$.

### 2.7.3 Migration assay

MOVAS-1 were cultured as a confluent monolayer in 12-well plates in 10% FBS; DMEM media. After overnight serum starvation (1% FBS; DMEM), scratch wounds were introduced with a P200 tip, wells were washed to remove floating cells, reference line was drawn at the bottom of the wells and media containing 1% FBS, 20ng/ml PDGF-BB (PeproTech; 100-14B) and Tβ4(0-10μM) was added. Cell proliferation was inhibited using 25μM hydroxyurea (HU) (Sigma; H8627). Wells were imaged at 0, 6, and 24 hours thereafter, using a Zeiss Axio Imager microscope. Cell migration was calculated between the reference line at 0 and 6 hrs using ImageJ. Wound closure was measured and expressed as % relative to t0, using the formula:

$$\textbf{\% of wound area sealed} = \frac{A_t}{A_0} \text{ X } 100$$

### 2.7.4 Duolink® Proximity Ligation Assay

Primary VSMCs treated with 20ng/ml PDGF-BB were fixed with 4% PFA and permeabilized with 0.1% Tween-20. To assess proximity, the following antibody combinations were used: Mouse anti-LRP1 (1:100; Abcam, ab28320) and Rabbit anti-PDGFRβ (1:100; Abcam, ab32570). Duolink® In Situ PLA Rabbit Plus and Mouse Minus probes were used in combination with Detection Reagent Green (Sigma) following the manufacturer's protocol. Following the Duolink® protocol, cells were co-stained for endocytic compartments using the antibodies described in table 2.3. Colocalisation was determined using the ImageJ plug-in JACoP 2.0[257].

### 2.7.5 Surface biotinylation Assay

siRNA-treated and PDGF-BB stimulated cells were washed twice with cold PBS and surface proteins were biotinylated with 0.5mg/ml Sulfo-NHS-SS-Biotin (Thermo Scientific) for 30min on ice. The reaction was quenched with 15mM glycine (2x 5 min) and cells were lysed in GST lysis buffer (10% glycerol, 50mM Tris-HCl pH7.5, 200mM NaCl, 1% NP-40, 2mM $MgCl_2$, PhosSTOP and cOmplete protease inhibitor cocktail (Roche)). Nunc-Immuno™ MicroWell™ 96 well plates were coated with antibody in 0.05M $Na_2CO_3$ pH9.6 (anti-LRP1 (1:1000, Abcam, ab92544) or anti-PDGFRβ (1:500, Abcam, ab32570)) overnight at 4°C. Plates were washed and blocked for 1 hour in 1% Hammarsten grade casein (Alfa Aesar) with 0.1% Tween-20 in PBS. Lysates were quantified by BCA assay, diluted in blocking buffer, equal amounts added to the plate and incubated overnight. Plates were washed, incubated with streptavidin-HRP (1:1000, Abcam, ab7403) for 1 hour and developed with TMB substrate (BD Biosciences). The reaction was stopped with 1M sulphuric acid and absorbance read at 450nm.

## 2.8 Immunoblotting

Aortic biopsies were lysed in ice cold RIPA buffer (Sigma; R0278) supplemented with 1mM dithiothreitol (DTT), phosphatase and protease inhibitor cocktail (PhosSTOP and cOmplete protease inhibitor cocktail; Roche). Protein concentration was quantified using Pierce BCA Protein Assay kit (ThermoScientific #23227), 15µg of total protein was loaded on a 4-15% Mini Protean TGX Gel (BioRad #4561086) and separated using a standard protocol. Proteins were transferred using the Trans-Blot Turbo Transfer Pack (BioRad #1704456), blocked in 5% BSA/ TBST (20mM Tris, pH 7.5, 150mM NaCl, 0.1% Tween 20), and probed with primary antibodies (listed in the table below) overnight at 4°C. Blots were washed 3 times for 10 min each in TBST and incubation with HRP-conjugated Rabbit secondary antibodies, diluted 1:2500 in blocking buffer. Blots were

rinsed 3 times for 5 min in TBST, then bands were detected using Western Blotting HRP Detection Reagent (Millipore; WBLUR0100).

**Table 2.4: List of antibodies used for immunoblotting studies**

| Antibody | Company | Catalogue No | Species | Dilution |
|---|---|---|---|---|
| phospho-AKT(S473) | Cell Signalling Technology | 4058 | Rabbit | 1:100 |
| phospho-ERK1/2(Thr202/Tyr204) | Cell Signalling Technology | 9101 | Rabbit | 1:100 |
| Calponin1(EP789Y) | Abcam | Ab46794 | Rabbit | 1:400 |
| Caldesmon | Abcam | Ab32330 | Rabbit | 1:100 |
| βTubulin-HRP conjugated(9F3) | Cell Signalling Technology | 5346 | Rabbit | 1:300 |
| AKT | Cell Signalling Technology | 9272 | Rabbit | 1:100 |
| P-44/42 (ERK1/2) (137F5) | Cell Signalling Technology | 4695 | Rabbit | 1:100 |
| eIF4E | Cell Signalling Technology | 2067 | Rabbit | 1:500 |

## 2.9 Gene expression profile

### 2.9.1 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Aortic biopsies, snap-frozen in liquid nitrogen, were homogenised using an automated homogeniser. Total RNA was isolated from aortic lysates using the RNeasy Fibrous Tissue Mini kit (Qiagen), according to the manufacturer's instructions. Absorbance was measured at 260/230 nm using Nanodrop-2000 to record yield and confirm purity.

Following extraction, RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo fisher Scientific #4368814).

Viia 7 Real-Time PCR System ((Life technologies) was used to run the PCR reaction which contained 1X Fast SYBR™ Green Master Mix (Thermo fisher Scientific #4385612), 2ng/μL of cDNA, and 0.2μM forward and reverse primer. All reactions were run in triplicates under the following programme: 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Data were normalised to β-actin expression (endogenous control, after selection of optimal control, in terms of stable expression, from a panel of 6 genes). Fold changes were determined by the $2^{-\Delta\Delta CT}$ method[258].

**Table 2.5: Primers used for qRT-PCR**

| Gene | Forward primer(5'->3') | Reverse primer(5'->3') |
|---|---|---|
| *Acta2* | GTCCCAGACATCAGGGAGTAA | TCGGATACTTCAGCGTCAGGA |
| *Sm22α* | CAACAAGGGTCCATCCTACGG | ATCTGGGCGGGCCTACATCA |
| *Myh11* | AAGCTGCGGCTAGAGGTCA | CCCTCCCTTTGATGGCTGAG |
| *Caldesmon* | GCTCCCAAGCCTTCTGACTT | CCTTAGTGGGGGAAGTGACC |
| *Vimentin* | TCCAGCAGCTTCCTGTAGGT | CCCTCACCTGTGAAGTGGAT |
| *Tropomyosin IV* | CGCAAGTATGAGGAGGTTGC | AGTTTCAGATACCTCCGCCCT |
| *Il1 b* | CAACCAACAAGTGATATTCTCCATG | GATCCACACTCTCCAGCTGCA |
| *tnfα* | CACGTCGTAGCAAACCACCAAGTGGA | TGGGAGTAGACAAGGTACAACCC |
| *ccnd1* | GTTCATTTCCAACCCACCCTC | AGAAAGTGCGTTGTGCGGTAG |
| *β-actin* | GGCTGTATTCCCCTCCATCG | CCAGTTGGTAACAATGCCATGT |

## 2.10 Multiplexed automated cytokine ELISA

Peripheral blood was collected at harvest by cardiac puncture, using pre-filled 0.5M EDTA syringes (ul), placed into chilled microcentrifuge tubes, and kept on ice. Samples were centrifuged at 400 x g for 15 minutes, plasma supernatant was transferred to a fresh tube, stored at -80ºC until further use. Using 25µl serum per assay, cytokine levels were quantified by multianalyte automated ELISA (Ella technology from ProteinSimple) as per manufacturers protocol.

## 2.11 Flow Cytometry

Mice were euthanized using 50µl Pentoject. Once the reflex response was lost, the chest was opened, peripheral blood was collected before dissecting the aorta. The descending aorta, cleaned of adipose tissue, was cut into 4 pieces, then enzymatically dissociated using the Multi Tissue Dissociation kit 2 (Miltenyi Biotec) and gentleMACS Octo Dissociator with heaters according to manufacturer's instructions. Red blood cells were lysed, and cell counts were determined for each aorta. The cell suspension was stained with Zombie Violet Fixable Viability Dye (1:500; Biolegend) for 15 min at RT, followed by pre-treatment with anti-CD16/32 (1:50; Biolegend) for 5 min on ice to block Fcγ receptors, and surface staining with PeCy7 anti-CD45 (1:200; BD Biosciences; 30- F11), FITC anti-CD11b (1:200; BD Biosciences; M1/70), PE anti-CD14 (1:100; Life Technologies; Sa2-8) and APC anti-CCR2 (1:10; R&D systems; 475301) antibody for 30 min on ice. Stained cells were fixed (BD CellFIX) and analysed the next day on BD LSRFortessa X20 cytometer (BD biosciences). Flow cytometry data were analysed using FlowJo software.

## 2.12 Statistics

Randomisation of animals to treatment or genotype groups was introduced at the time of mini-pump implantation (aneurysm); introduction of western diet (atherosclerosis) or harvest (baseline). Pilot studies were initially performed, typically on the basis of which we were able to estimate sample size required to achieve significance. Parametric or non-parametric test were selected based on sample size and normality of data distribution. For small sample sizes, where it was not possible to assess normality, a non-parametric test was preferred. Statistical analyses were performed with GraphPad Prism Software. For the quantitative comparison of two groups, a two-tailed unpaired Student's t-test (parametric) or Mann-Whitney (non-parametric) tests were used, as indicated, to determine any significant differences. For comparison of three groups or more, a one-way ANOVA with Tukey's post hoc test was used. For analyses involving two independent variables, a two-way ANOVA with Bonferroni, Holm-Sidak, or Dunnett's post hoc test was used. Significance is indicated in the figures, as follows: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

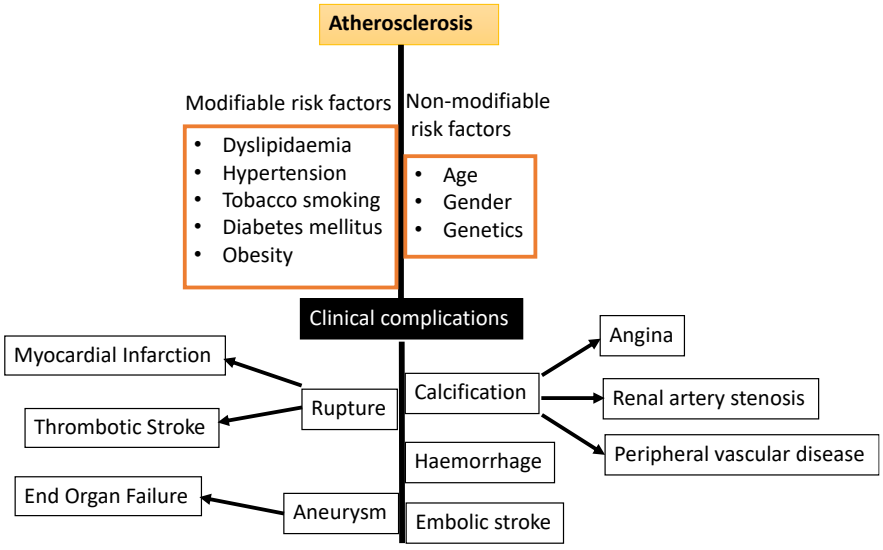
# Chapter III

# Endogenous Tβ4 protects medial smooth muscle cells against atherosclerosis

## 3.1 Introduction

Atherosclerosis is a dynamic arterial disease, initiated by endothelial dysfunction and subendothelial lipid retention, that provokes maladaptive inflammatory responses, resulting in the formation of asymmetric atherosclerotic lesions called plaques on the innermost layer of the artery[116]. Atherosclerotic plaques most often develop at sites that experience turbulent blood flow such as the aortic arch and the abdominal aorta[259].

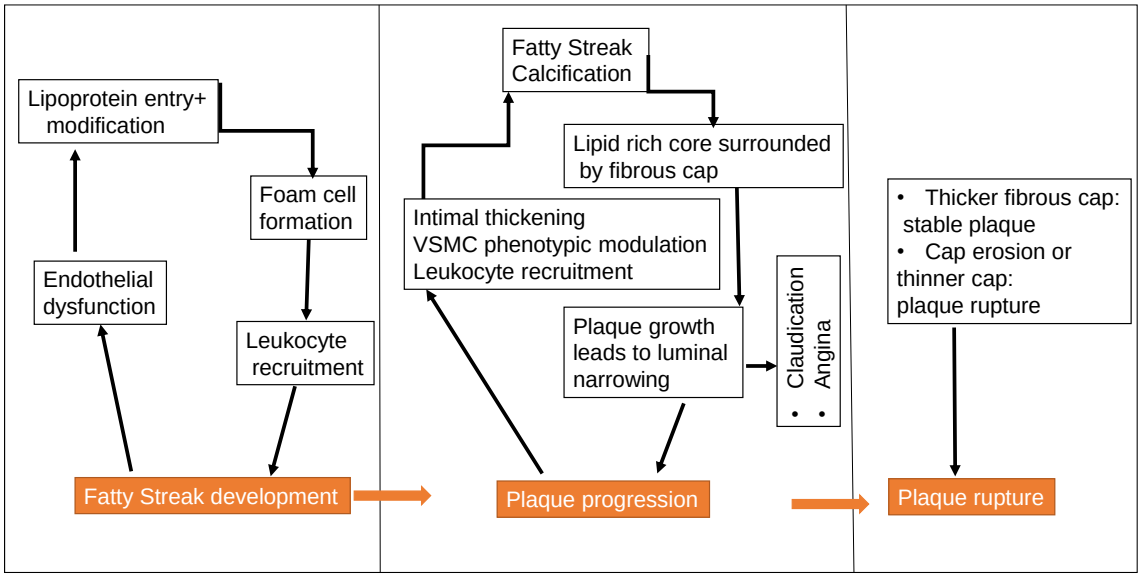
**Flow chart 3.1: Risk factors and clinical complications associated with atherosclerosis.**




The stages and pathological changes that occur during atherosclerosis are explained in detail in Chapter I, section 1.4.2.1 and depicted in **Flow chart 3.2.** Plaque stability is

principally determined by competing degenerative and regenerative mechanisms. The degenerative processes are primarily mediated by the proteolytic actions of macrophages, potentiation of pro-inflammatory signalling, and impaired efferocytosis, while the regenerative processes promote VSMC mediated ECM synthesis to reinstate vascular stability[33]. Maintaining plaque stability is important to prevent rupture and the resultant thrombotic events that lead to complications such as MI and stroke.

**Flow chart 3.2: Pathological changes in atherosclerosis.** This schematic was recreated using information from[260].




Current treatment strategies combine surgical interventions such as balloon angioplasty, with or without stenting, endarterectomy, or surgical bypass grafting[261], along with disease management that demands lifestyle changes, often with cholesterol-lowering drugs such as statins. Despite the benefits, clinical events still occur due to restenosis and neointimal hyperplasia. Therefore, understanding the pathogenesis of the disease

may provide valuable insights into novel targets to control disease initiation and development.

Human specimens provide invaluable information on pathological changes in the advanced stages of the disease. However, a major constraint of using human tissue is the limited availability of tissue at an early stage, due to the asymptomatic nature of the disease. Additionally, researchers are constantly faced with the challenge of obtaining appropriate healthy control tissue biopsies, for example, the availability of healthy coronary arteries or healthy aorta that are prone to develop plaques is scarce, therefore investigators often have to rely on alternative vessels which may differ anatomically, morphologically or functionally. Other issues commonly associated with human studies are the techniques used to collect, label, process, and store the tissue specimens post operation which may affect tissue quality, and the study results[262]. Therefore, experimental murine models are an essential tool to characterise pathological changes at various stages of the disease. The advantages and limitations associated with using murine models are highlighted in **Table 3.1**.

**Table 3.1: Advantages and limitations of using murine models to study atherosclerosis[263].**

| Advantages | Limitations |
|---|---|
| Ease to breed and house. | Mice are naturally resistant to atherosclerosis due to the absence of Cholesteryl ester transfer protein (CETP). |
| Can be genetically modified easily. | Mice transport most of the cholesterol as HDL, instead of LDL as in humans. |
| Relatively low maintenance. | Lesions formed are less complex when compared to human plaques. |
| Development of atherosclerosis over a short period of time. | Lesions most commonly occur in the aortic sinus, in contrast to the coronary artery, carotid and peripheral arteries in humans. |
| Possibility to track cellular and molecular changes at various stages of disease. | Small body size limits frequent blood collection and increases difficulty with post-mortem collection of small arteries. |

The development of ApoE$^{-/-}$ and LDLR$^{-/-}$ single gene deletion, diet-dependent murine models has revolutionised the understanding of atherosclerosis[264, 265]. The key points with regards to the two models have been detailed in Chapter 1, Section 1.5.3. For the purpose of this thesis, the use of ApoE$^{-/-}$ mice was favoured as the sequential events involved in lesion formation are similar to those seen in larger animals and humans, including the development of complex fibrous plaques and lesions, in the aortic root and branch points[266].

### 3.1.1 Role of VSMCs in atherosclerosis

VSMCs are the primary cells of the media, responsible for vasoconstriction and dilation in physiological and pathological conditions[51, 116]. As explained earlier in Section 1.4.2.1, regions within a vessel are classified as athero-prone and athero-resistant

regions; histological studies on human coronary artery autopsy specimens reveal an abundance of VSMCs in athero-prone regions in contrast to fewer VSMCs in athero-resistant regions, suggesting a crucial role for VSMCs in disease pathogenesis[107, 267-269], yet their contribution to atherogenesis remains ambiguous and controversial[38].

In response to atherogenic cues from the endothelium and inflammatory cells, medial VSMCs reprogramme the expression of specific genes and switch to an activated phenotype that is pro-migratory and proliferative. Despite the ongoing debates with regards to the phenotype acquired by VSMCs in atherosclerosis[50], phenotypically modulated VSMCs are commonly designated as 'synthetic' VSMCs[270]. The dogma states that synthetic VSMCs downregulate the expression of contractile markers including αSMA, Calponin, SM22α, and SM-MHC that regulate contractile function, lose their canonical spindle morphology and acquire a smaller cuboidal morphology[34].

Analysis of both transitional and advanced lesions in hypercholesterolemic rabbits confirmed that nearly 45% of all proliferating cells were VSMCs, while only 30% were macrophages[271], suggesting that VSMC proliferation is an important process in atherosclerosis. The proliferation of VSMCs in the transitional stage causes intimal thickening, however, disease progression and an increase in the synthetic VSMC population promotes elastin degradation via the action of proteases (MMPs), which results in arterial stiffness[12] and loss of medial stability[164].

In addition to their role in medial stability, VSMCs in the advanced stages are associated with plaque stability[50]. Plaque caps are made up of a discrete subpopulation of αSMA+ medial smooth muscle cells that clonally expand and migrate to the intima to cover the plaque[40, 272, 273]. However, with disease progression, VSMCs in the plaque cap proliferate, downregulate the expression of contractile markers, and invade the plaque

core[274]. It was previously presumed that foam cells that contribute to the large necrotic core derive solely from macrophages, however, studies in human coronary atherosclerosis demonstrate that VSMCs contribute to >50% of foam cells[275]. In contrast to these findings, Wirka *et al* recently demonstrated that foam cells derived from smooth muscle cells within the lesions are much fewer compared to those derived from macrophages[21]. The difference in these studies could be attributed to 1) difference between the blood vessels assessed (coronary versus aorta); 2) difference in the species (human versus mice); 3) the methodology. The human study recognised smooth muscle-derived foam cells based on αSMA and Oil Red O staining, while the mouse study used atherosclerotic aortic sections from $Tg^{Myh11\text{-}CreERT2}$; $ROSA^{tdT/+}$; $ApoE^{-/-}$ mice, where all cells that originated as smooth muscle cells were labelled with *tdTomato* fluorescence and the fat deposits were labeled using boron-dipyrromethene (BODIPY).

In advanced human atheroma patients, apoptotic VSMCs in the fibrous cap impair plaque stability[276] by decreasing the fibrous cap thickness, which subsequently increases hemodynamic stress acting on stenotic lesions. The apoptotic index of VSMCs in the healthy adult artery is low and a comparative study between VSMCs derived from the normal vessel and coronary atherosclerotic plaque suggests that VSMCs derived from diseased vessels show increased apoptosis[277]. VSMCs undergo multiple processes such as proliferation, necrosis, apoptosis, often simultaneously, at different stages and in different regions of the aorta, therefore, the role of VSMC phenotypic switching and factors that regulate the process in atherosclerosis are still not fully resolved[278].

### 3.1.2 Role of Tβ4 in vascular protection

Tβ4 has been shown to potentiate the recruitment and differentiation of VSMCs during vascular development in coronary[237], yolk sac[243], and systemic[206] vasculature. Loss of Tβ4 leads to reduced VSMC coverage in the developing aorta, resulting in the death of nearly 25% of embryos due to vascular haemorrhage[206]. In addition to its role in regulating VSMC differentiation during development, work carried out by others and myself in the laboratory before my DPhil studies confirm an important role for Tβ4 in maintaining differentiated VSMCs postnatally[170].

Despite the embryonic defects, nearly 75% of global Tβ4 knockout ($T\beta4^{-/Y}$) embryos survive to adulthood, however, as adults their aortas show vascular defects, such as aberrant elastin deposition and shift of VSMCs to a synthetic phenotype[170], similar to those seen in atherosclerotic sections from patients and experimental murine models. Furthermore, baseline defects observed in $T\beta4^{-/Y}$ and smooth muscle-specific Tβ4 knockdown mice ($Myh11^{CreERT2}$; $Hprt^{T\beta4shRNA}$) phenocopy those previously reported in smooth muscle-specific $LRP1^{-/-}$ mice[164, 279, 280]. These observations suggest that Tβ4KO mice may, like LRP1 nulls, be similarly predisposed to develop aortic aneurysm and atherosclerosis[150].

Recently, scRNA-seq technology has facilitated understanding of the functions of discrete cell populations in the context of their microenvironment. Such studies provide information on the regulators of contractile-synthetic switching that allow modulation of VSMC phenotype during various stages of disease that may be potential targets for repair. Taking advantage of publicly available aortic scRNA-seq data derived from $ApoE^{-/-}$ mice with SMC lineage tracing (*Tg*$^{Myh11\text{-}CreERT2}$; *ROSA*$^{tdT/+}$; *ApoE*$^{-/-}$ mice) at baseline, 8- and 16- weeks post high fat diet feeding[21], a preliminary computational analysis was

performed by members of the Smart group to evaluate transcriptomic changes, especially with regards to the expression of *Tmsb4x* in mouse aortic VSMCs at baseline and disease.

The expression of *tdT*omato in these mice was under the control of SMC-specific *Myh11* promoter (*Tg*$^{Myh11\text{-}CreERT2}$), with SMCs labelled by tamoxifen induction before high fat diet feeding and disease onset. FACS allowed sorting of tdTomato$^{+}$ cells (SMC origin) from tdTomato$^{-}$ cells (all the remaining cells) from the aortic root and ascending aorta. Based on transcriptomic characterization, mouse aortic root cells were clustered into 10 different cellular populations as detailed in[21]. As the focus of this thesis was to specifically evaluate the changes in VSMCs in atherosclerosis, the tdTomato$^{+}$ smooth muscle cell cluster was subjected to further analysis.

5 different subsets of VSMCs were annotated, based on the expression of specific marker genes; a heatmap illustrating the top 10 marker genes used to characterise each cluster is shown in **Appendix Figure 1**. Smooth muscle cell clusters were annotated, as shown in **Figure 3.1 A**, as ContSMC_1, ContSMC_2, ContSMC_3 (enriched in contractile VSMC genes), ModSMC (downregulated expression of contractile genes and upregulated genes associated with cell migration and ECM proteins), and Pericytes. UMAP (Uniform Manifold Approximation and Projection) visualisation of the two-dimensional distribution of VSMC populations at baseline, and at 8- and 16- weeks post high-fat diet feeding (21% anhydrous milk fat, 19% casein, 0.15% cholesterol) supports the existing notion that, with disease progression, the number of phenotypically modulated VSMCs, designated here as ModSMC, increases (**Figure 3.1 A**). Interestingly, the ModSMC population in atherogenic mice showed significantly reduced expression of *Tmsb4x* in comparison to the cells in ContSMC_1, ContSMC_2, ContSMC_3, as shown in **Figure 3.1 B** (unpublished data). These changes in expression

of *Tmsb4x* demonstrate strong correlation (high *Tmsb4x* in contractile VSMC populations and low *Tmsb4x* in the modulated cluster) and support our hypothesis that Tβ4 is essential to maintain contractile VSMC phenotype. However, more careful analysis is required to discern if changes in the relative expression of *TMSB4X* are a cause or a consequence of disease.

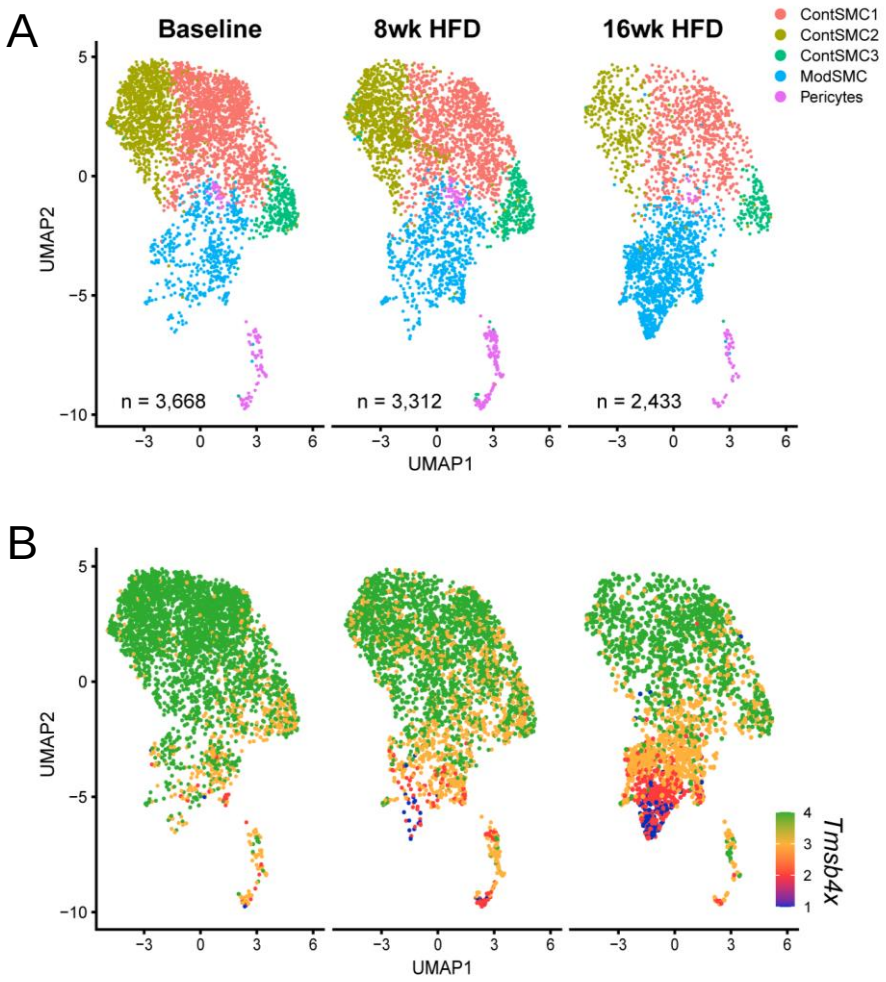



***Figure 3.1: Characterisation of ApoE$^{-/-}$ mouse aortic smooth muscle cells in disease, based on differential gene expression***. *(**A**) UMAP (Uniform Manifold Approximation and Projection) plot demonstrating the changes in the five VSMC subpopulations at baseline and after 8- and 16- weeks of hypercholesterolaemic diet. Cells with the greatest similarity in gene expression colocalise and further segregate into clusters according to more pronounced differences, (**B**) UMAP comparing the changes*

*in expression of Tmsb4x between VSMC subpopulations at baseline, 8- and 16- weeks post-intervention. Each dot is an individual cell and gene expression levels are indicated by the colour spectrum with green representing high, orange-medium and red-low Tmsb4x expression. n=3 for baseline, 8- and 16 weeks.*

***Acknowledgment of Contribution:***

*Previously published data sets were used for analysis[21].*

*Computational analysis of scRNA-seq data was performed by Dr. Andia Redpath and Benjamin Pike (unpublished).*

Despite the crucial role of Tβ4 in maintaining vascular stability in development and during physiological conditions in the adult vasculature, the role of endogenous Tβ4 in vascular protection against chronic vascular disease, such as atherosclerosis, remains unexplored.

## 3.2 Aim

To study the role of endogenous Tβ4 in maintaining medial layer integrity and protection against atherosclerosis.

## 3.3 Results

### 3.3.1 Loss of Tβ4 increases susceptibility to atherosclerosis

The scRNA-seq data shown above supported further investigation into the supposition that endogenous Tβ4 provides vascular protection against atherosclerosis. A well-characterised hypercholesterolemia model was used by crossing $T\beta4^{+/Y}$ and $T\beta4^{-/Y}$ mice onto the $ApoE^{-/-}$ background. Adult (8-12 weeks old) $T\beta4^{+/Y}$; $ApoE^{-/-}$ and $T\beta4^{+/Y}$; $ApoE^{-/-}$ offspring were fed 'Western diet' (WD; 21.4% fat; 0.2% cholesterol) for up to 12 weeks and atherosclerotic plaque formation was assessed in terms of the lesion area, ECM

integrity, VSMC phenotypic modulation, proliferation, apoptosis, necrotic area and inflammation using a combination of histological and immunohistochemical techniques.

### 3.3.1.1 Atherosclerotic mice lacking Tβ4 developed large complex lesions

Accumulation of cholesterol and other fatty rich substances on the inner walls of vessels leads to the development of plaques which are initially detected at the aortic sinus in the murine model of atherosclerosis[281]. Oil Red O staining has been routinely used to visualize and quantify atherosclerotic plaques[282] and hence was initially used to identify the susceptibility to atherosclerosis. Oil Red O stained paraffin sections from the aortic sinus of adult male $T\beta4^{+/Y}$; $ApoE^{-/-}$, $T\beta4^{-/Y}$; $ApoE^{-/-}$ and $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ mice fed on WD for 12 weeks are shown in **Figure 3.2 A.**

The atherosclerotic lesion area was quantified using a modified Paigen method[281] (**Figure 3.2 B**). The mean of 3-4 sections was used for each sample, and the data reveal that mean plaque size was 32% in $T\beta4^{-/Y}$; $ApoE^{-/-}$ in contrast to 22% in $T\beta4^{+/Y}$; $ApoE^{-/-}$ mice and 33% in $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$. Further examination of the plaques indicates the presence of large acellular necrotic regions in $T\beta4^{-/Y}$; $ApoE^{-/-}$ and $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ mice, which were rarely visible in $T\beta4^{+/Y}$; $ApoE^{-/-}$ (**Figure 3.2 A**). It is widely accepted that lesions in the early stages tend to have very little necrosis while the increased necrotic area is indicative of advanced lesions[281]. Moreover, multiple shoulder regions were identified in plaque caps of $T\beta4^{-/Y}$; $ApoE^{-/-}$ and $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ mice, compared to $T\beta4^{+/Y}$; $ApoE^{-/-}$ mice. While plaques were studied in $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ mice, this aspect of the study lacked appropriate controls. However, exacerbated atherosclerosis in sm $Lrp1^{-/-}$ has been demonstrated previously, therefore they served as a positive control for comparison with $T\beta4^{+/Y}$ and $T\beta4^{-/Y}$. Given the hypothesised common mechanism, we sought to compare phenotypes.

Based on these observations, it can be concluded that a) Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice develop larger complex plaques that may be more vulnerable to rupture compared to Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice, b) The plaque size and complexity are comparable between Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Myh11$^{Cre}$; Lrp1$^{fl/fl}$; ApoE$^{-/-}$ mice, suggesting that Tβ4$^{-/Y}$ mice phenocopy VSMC-specific LRP1$^{-/-}$ mice in atherosclerosis.

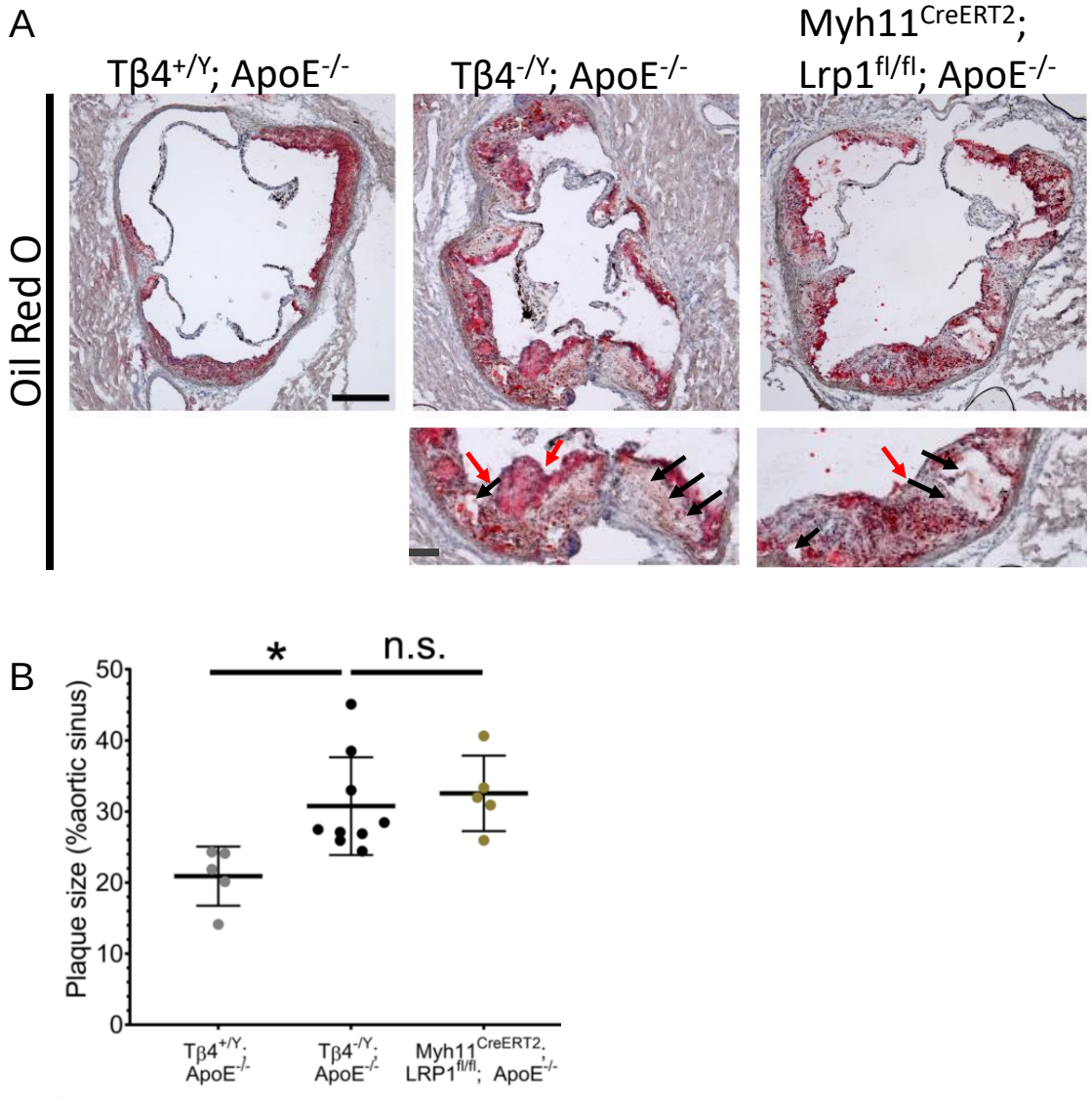



***Figure 3.2: Tβ4-null mice display increased atherosclerotic lesion formation.*** *Oil red O staining of aortic sinus plaques (**A**), black arrow in top and bottom panel highlight*

*the necrotic region devoid of cells, red arrows in lower panel indicate shoulder region in the cap. (**B**) is quantification of total plaque area from Tβ4$^{+/Y}$; ApoE$^{-/-}$ (n=5), Tβ4$^{-/Y}$; ApoE$^{-/-}$ (n=9) and Myh11$^{Cre}$; Lrp1$^{fl/fl}$; ApoE$^{-/-}$(n=5) mice. Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B**). n.s. = not significant; *p ≤ 0.05; **p≤0.01. Scale bars: **A**: Top panel 250μm, Lower panel 50μm.*

***Acknowledgment of Contribution**:*
*Dissection of aortas and oil red o staining was performed in collaboration with Dr Nicola Smart.*

To rule out the possibility that larger plaque size in Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Myh11$^{Cre}$; Lrp1$^{fl/fl}$; ApoE$^{-/-}$ mice was due to increased retention of cholesterol, weight gain was monitored throughout the course of WD feeding and plasma cholesterol levels were measured at various time points. Both Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice gained comparable weights throughout the WD feeding, in contrast to Myh11$^{Cre}$; Lrp1$^{fl/fl}$; ApoE$^{-/-}$ that consistently weighed less, both at baseline and after high-fat feeding (**Figure 3.3 A**). The measurement of plasma cholesterol level showed no significant difference between Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice at all time points (**Figure 3.3 B**). These results indicate that the increase in plaque size did not correlate with the amount of fat ingested or cholesterol levels in these mice.

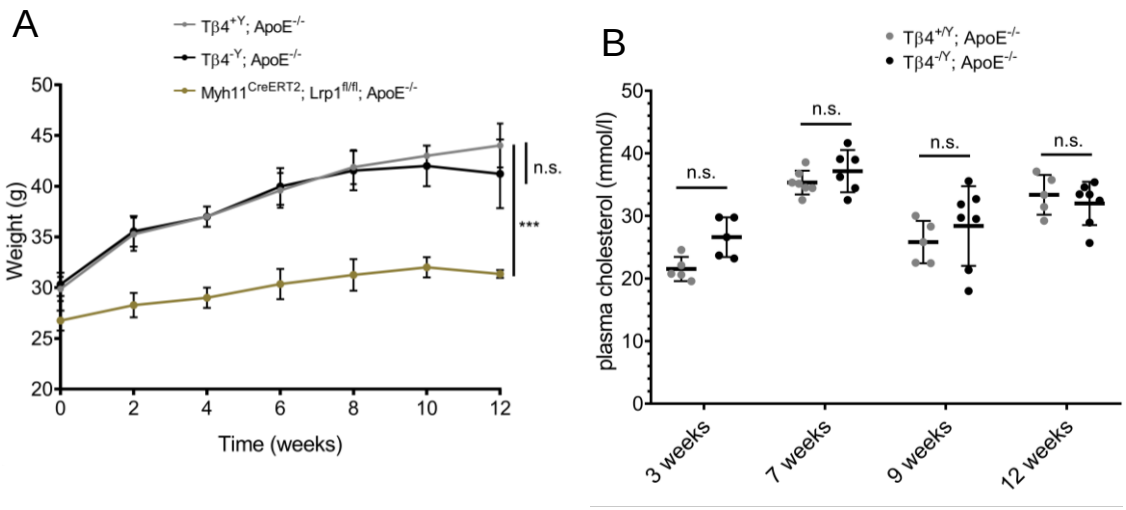



***Figure 3.3: Tβ4$^{+/Y}$; ApoE$^{-/-}$ and Tβ4$^{-/Y}$; ApoE$^{-/-}$ do not show differences in weight gain or cholesterol levels after the western diet.*** *Comparison of weight gain (**A**) and plasma cholesterol levels (**B**) in Tβ4$^{+/Y}$; ApoE$^{-/-}$ and Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice over the time course of western diet feeding regime. Data are presented as mean ± SD, with n=9-11 in **A,** n=5 at 3, 7, 9 and 12 weeks in **B**. Each data point in **B** represents an individual animal. Significance was calculated using two-way ANOVA with Bonferroni correction for multiple comparisons (**A**) and one-Way ANOVA with Tukey's multiple comparison tests (**B**). n.s. = not significant.*

### 3.3.1.2 ApoE$^{-/-}$ mice lacking Tβ4 developed larger plaques even on regular chow

Ablation of ApoE alone is enough to cause atherosclerosis, even without feeding a cholesterol-rich diet[143]; in keeping with this, a female Tβ4$^{-/-}$; ApoE$^{-/-}$ mouse within the maintenance colony, fed on regular chow, was unexpectedly detected with hindlimb paralysis, an indication of possible aortic thrombosis, aneurysm, or rupture. Therefore, further investigation was carried out on a small cohort of regular chow-fed 8 month old female Tβ4$^{-/-}$; ApoE$^{-/-}$ and age-matched Tβ4$^{+/+}$; ApoE$^{-/-}$ mice by others in the Smart group. Histological examination of aortic sections shown in **Figure 3.4 A** demonstrated that, even without a cholesterol-rich diet, aortas of Tβ4$^{-/-}$; ApoE$^{-/-}$ mice were aneurysmal (>1.5 fold dilated). Further examination of the aortic sections revealed severe degeneration of

the underlying elastin layers (**Figure 3.4 C**), with large atherosclerotic plaques in $T\beta4^{-/-}$; $ApoE^{-/-}$ mice compared with $T\beta4^{+/+}$; $ApoE^{-/-}$ (**Figure 3.4 D**).

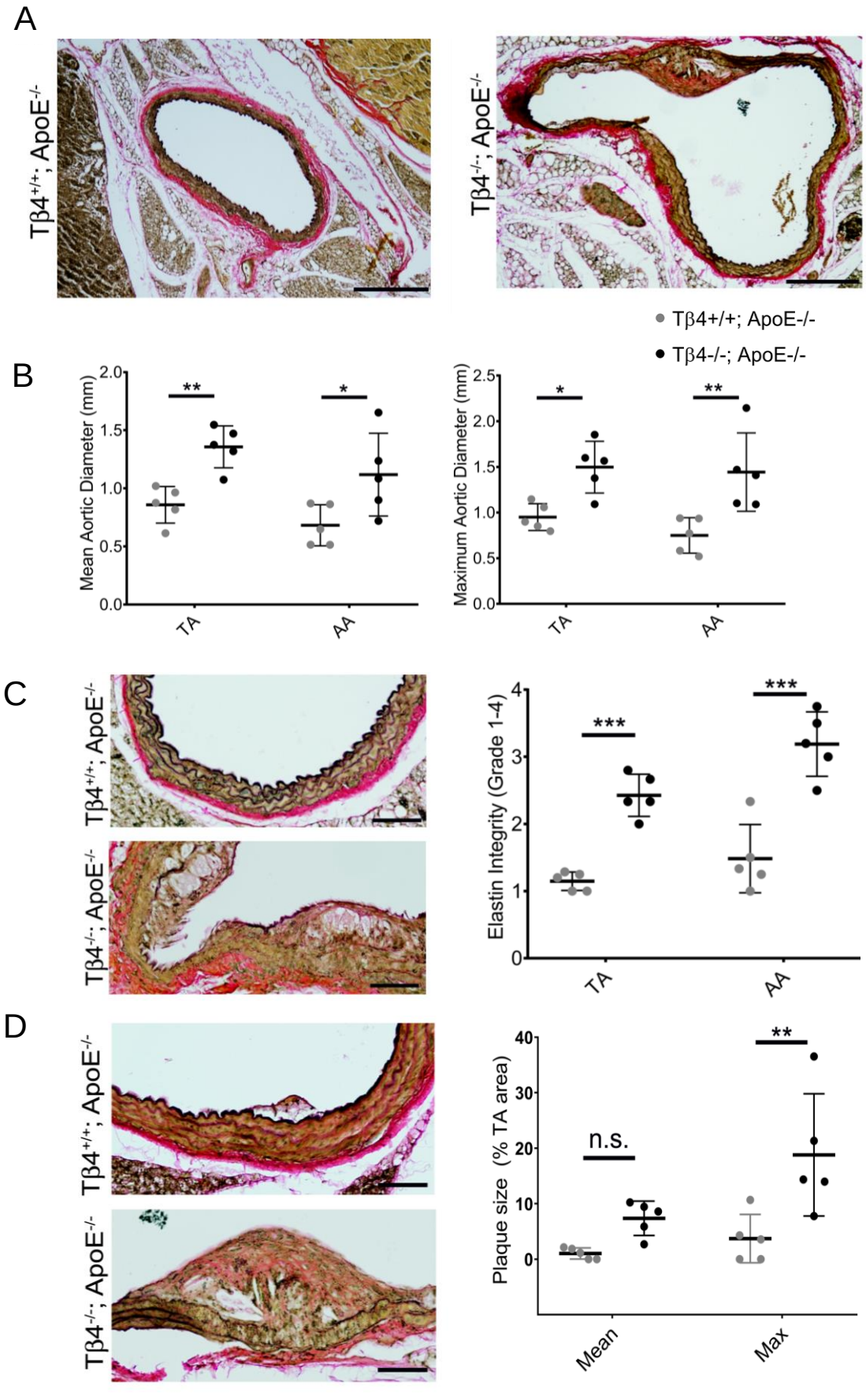

A
Tβ4+/+; ApoE-/-
Tβ4-/-; ApoE-/-
Tβ4+/+; ApoE-/-
Tβ4-/-; ApoE-/-
B
Mean Aortic Diameter (mm)
Maximum Aortic Diameter (mm)
TA
AA
C
Elastin Integrity (Grade 1-4)
D
Plaque size (% TA area)
n.s.
Mean
Max

***Figure 3.4: Tβ4$^{-/-}$; ApoE$^{-/-}$ mice show increased predisposition to aneurysmal dilatation and atherosclerotic plaque formation even on a regular chow diet.*** *Elastin integrity was assessed using Verhoeff van Gieson staining (**A**) and aortic diameter (**B**) in 8 month old Tβ4$^{+/+}$; ApoE$^{-/-}$ and Tβ4$^{-/-}$; ApoE$^{-/-}$ female mice, fed regular laboratory chow. Visualisation of medial elastin and elastin integrity score shown in (**C**). Higher magnification views of plaque to visualise composition and degeneration of underlying elastin lamellae and plaque size measured in **D**. Data are presented as mean ± SD, with each data point representing an individual animal, n=5 in **B**, **C & D**. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B**, **C**, **D**). n.s. = not significant; *p ≤ 0.05; **p≤0.01; ***: p≤0.001. Scale bar: **A**: 500μm; **C**, **D**: 100μm. TA: thoracic aorta; AA: abdominal aorta.*

***Acknowledgment of Contribution:*** *These data were generated by Dr Nicola Smart.*

Propensity to develop atherosclerosis was then assessed in a cohort of female mice, fed on WD for 12 weeks. Enface Oil red O staining of the entire aorta indicates that Tβ4$^{-/-}$; ApoE$^{-/-}$ females developed significantly larger plaques in the aortic arch, compared with Tβ4$^{+/+}$; ApoE$^{-/-}$ mice (**Figure 3.5 A & B**). Interestingly Tβ4$^{-/-}$; ApoE$^{-/-}$ females gained significantly more weight during the period of WD feeding than Tβ4$^{+/+}$; ApoE$^{-/-}$ mice, which may contribute to the increased plaque size in female mice (**Figure 3.5 C**).

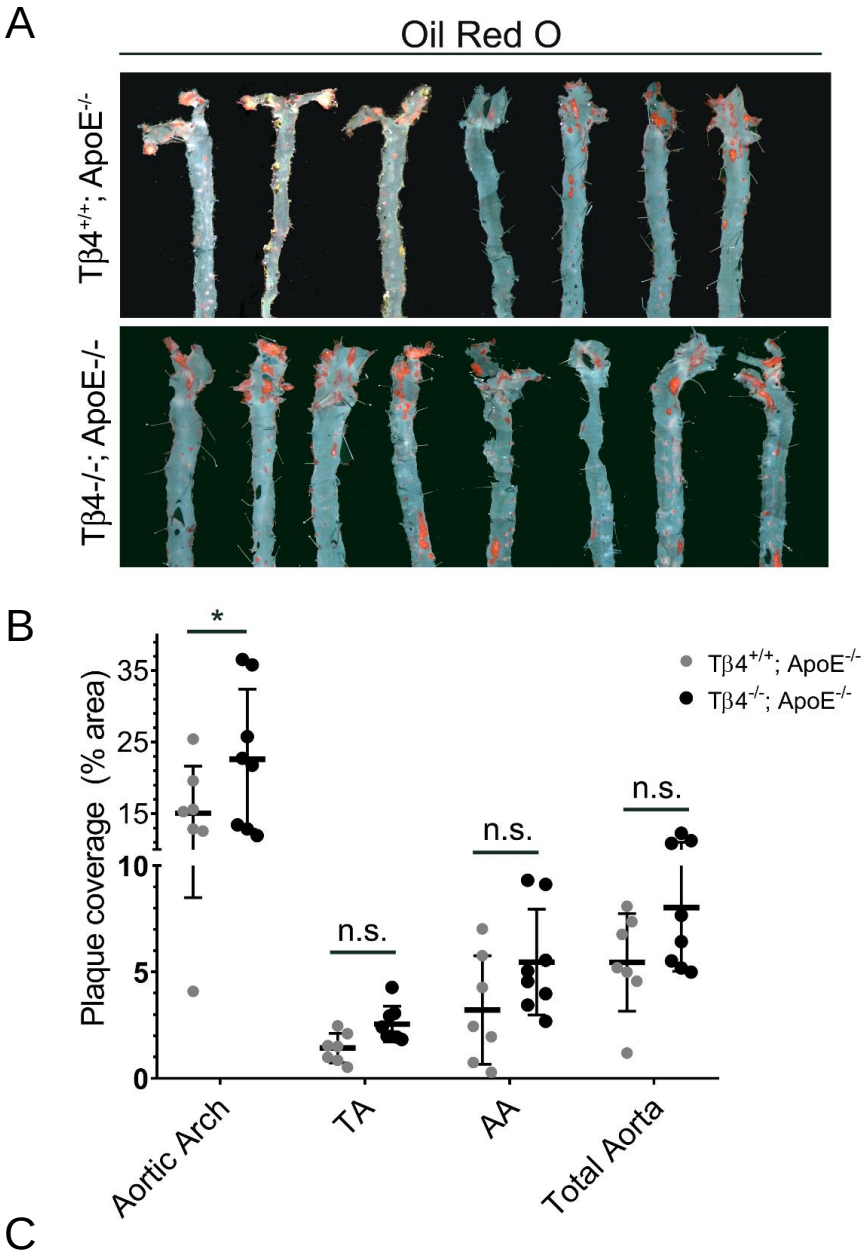

A
Oil Red O
Tβ4+/+; ApoE-/-
Tβ4-/-; ApoE-/-
B
Plaque coverage (% area)
Tβ4+/+; ApoE-/-
Tβ4-/-; ApoE-/-
*
n.s.
n.s.
n.s.
Aortic Arch
TA
AA
Total Aorta
C
Weight (g)
Time (weeks)
n.s.
**
***
*
***
***
***
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***Figure 3.5: Increased predisposition to atherosclerotic plaque formation correlated with weight gain in female Tβ4$^{-/-}$; ApoE$^{-/-}$ mice.*** *En face aorta preparations and oil red o staining to visualize plaques in female mice fed WD for 12 weeks (**A**). Quantification of plaque coverage in the aortic arch, thoracic aorta (TA), abdominal aorta (AA) and total aorta with n=7 for* Tβ4$^{+/+}$; ApoE$^{-/-}$ and n=8 for Tβ4$^{-/-}$; ApoE$^{-/-}$ in *(**B**). Weight gain was significantly increased in female Tβ4$^{-/-}$; ApoE$^{-/-}$, compared with Tβ4$^{+/+}$; ApoE$^{-/-}$ mice from 4-12 weeks' WD feeding (**C**). Data are presented as mean ± SD, with each data point representing an individual animal (**B**) and n=8 in (**C**). Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B**) and two-way ANOVA with Dunnett's post hoc tests (**C**). n.s.= not significant; *p ≤ 0.05; **p≤0.01; ***: p≤0.001.*

## 3.3.2 Tβ4 regulates VSMC phenotype and ECM integrity in atherosclerosis

### 3.3.2.1 Differential gene expression in atherosclerotic Tβ4 null mice

To determine how Tβ4 affects VSMC phenotype in atherosclerosis, changes in the VSMC phenotype were assessed over the course of WD feeding. Pathological phenotypic switching is commonly associated with changes in VSMC morphology and a shift in the ratio of contractile-synthetic markers. Initially, aortic sections from male Tβ4$^{+/Y}$; ApoE$^{-/-}$ and Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice after 7-, 9- and 12- weeks' WD feeding were immunostained to examine morphological and marker expression changes. Medial VSMCs of Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice after 7 weeks of WD displayed a pronounced alteration in morphology, with loss of their characteristic elongated shape and acquisition of a smaller, more rounded appearance (**Figure 3.6 A, top panel**). This was accompanied by a shift in the ratio of synthetic (Caldesmon, increased) over contractile marker (αSMA, decreased). A similar shift was also observed in medial VSMCs in Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice, however, the process was more gradual and became noticeable only from 9 weeks' WD, once plaques were already established (**Figure 3.6 A, middle panel**). Aortic biopsies from 9 weeks' timepoint were subjected to qRT-PCR to evaluate the changes in the

expression of contractile and synthetic genes between the two genotypes. A significant decrease in *sm22α: caldesmon* and *myh11: vimentin* in Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortas confirmed a shift to a synthetic phenotype in these mice (**Figure 3.6 B**). Migration of medial VSMC over the plaque to form a fibrous cap is an important step in atherosclerosis; cap thickness is an important determinant of plaque stability. While the cap thickness relative to lesion thickness was comparable between genotypes at 12 weeks (**Figure 3.6 C**), the caps of Tβ4$^{-/Y}$; ApoE$^{-/-}$ plaques were composed of cells that predominantly expressed synthetic markers, such as Caldesmon (**Figure 3.6 A, bottom panel**). In comparison, fibrous cap cells in control Tβ4$^{+/Y}$; ApoE$^{-/-}$ aortas retained higher levels of contractile markers, such as αSMA (**Figure 3.6 A, bottom panel**).

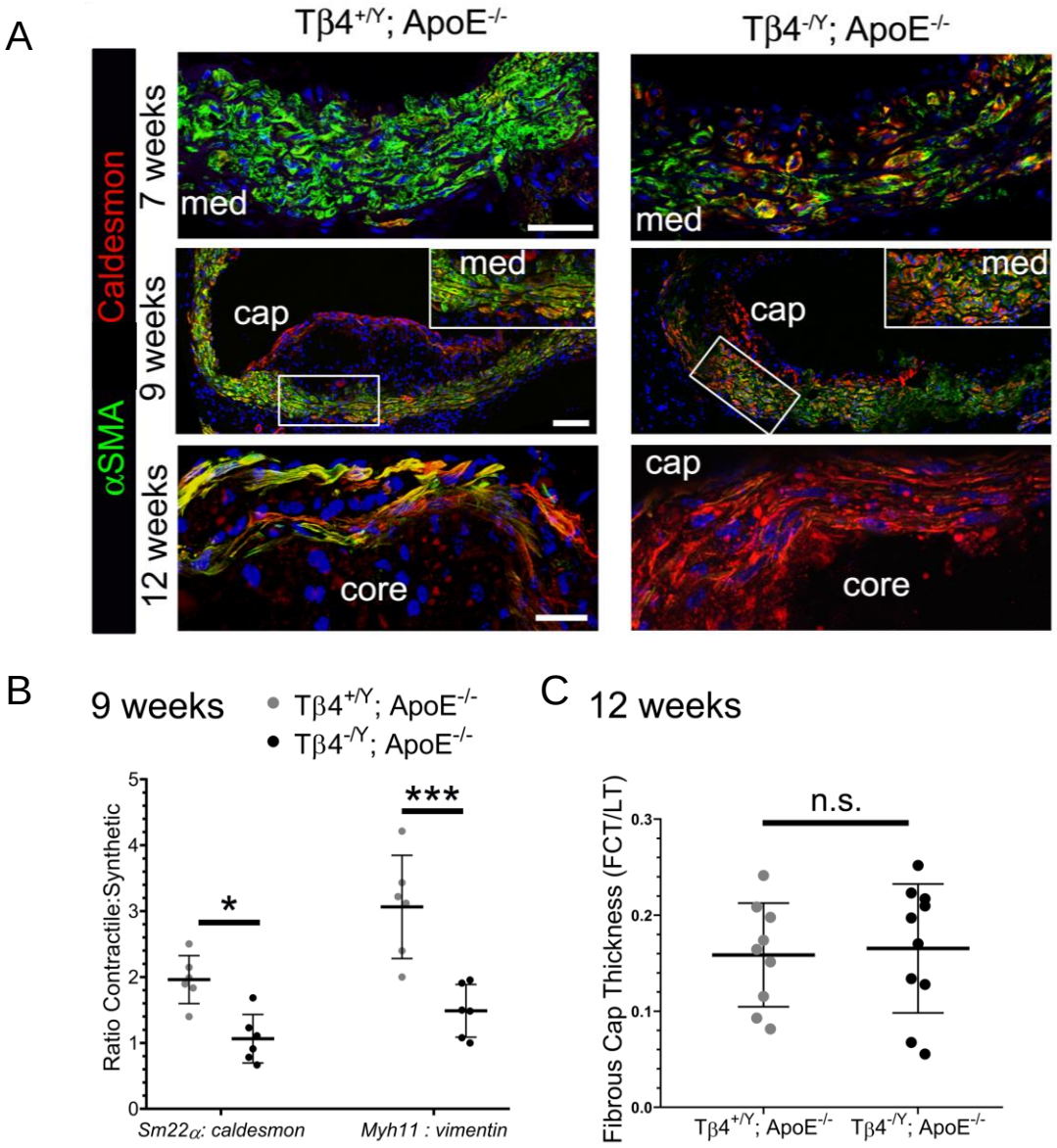



***Figure 3.6: Increased susceptibility to atherosclerotic lesion formation in Tβ4-null mice is associated with accelerated VSMC phenotypic switching.*** *Immunostaining showing shifted contractile-synthetic switching over the course of lesion formation (**A**), quantified by qRT-PCR in **B**. Fibrous cap thickness quantified, relative to lesion thickness (**C**). Data are presented as mean ± SD, with each data point representing an individual animal. n=6 in **B**, in **C** n=10. Mann Witney non-parametric test. n.s. = not significant; *p ≤ 0.05; ***p≤0.001; Scale bars: **A**: 50μm (upper); 100μm (middle); 20μm (lower). Boxed region in middle panel magnified in inset med: media; FCT: fibrous cap thickness; LT: lesion thickness.*

In addition to the assessment of the spatial and temporal distribution of contractile and synthetic VSMCs within the aortic media, the expression level of the contractile- synthetic proteins was quantified using western blotting. This was also advantageous because it allowed differentiation of the high molecular weight Caldesmon (h-Caldesmon; 120-150kDa) expressed by contractile VSMCs and low molecular weight Caldesmon (l-Caldesmon; 60-80kDa) associated with immature VSMC phenotype, which was not possible by immunohistochemistry. However, the antibody readily distinguished the h- and l- isoforms in the aortic lysates by western blotting due to the difference in their molecular weight (**Figure 3.7 A top panel**). It has previously been shown that l-Caldesmon switches to h-form during differentiation, therefore mature VSMCs predominantly express h-Caldesmon, however, the h-form switches to the l-form during de-differentiation[52, 283]. Densitometry of the blot indicated a significant increase in l-Caldesmon to the h-Caldesmon ratio in diseased $T\beta4^{-/Y}$; $ApoE^{-/-}$ aortic lysate compared to $T\beta4^{+/Y}$; $ApoE^{-/-}$ (**Figure 3.7 C**) suggesting a further shift to a synthetic phenotype with the loss of Tβ4. In keeping with this, a significant reduction in expression of Calponin-1, a mature contractile marker, postulated to regulate VSMC contractility[284], was confirmed in the aortic lysates of $T\beta4^{-/Y}$; $ApoE^{-/-}$, compared to $T\beta4^{+/Y}$; $ApoE^{-/-}$, as shown in **Figure 3.7 A middle panel and 3.7 B**.

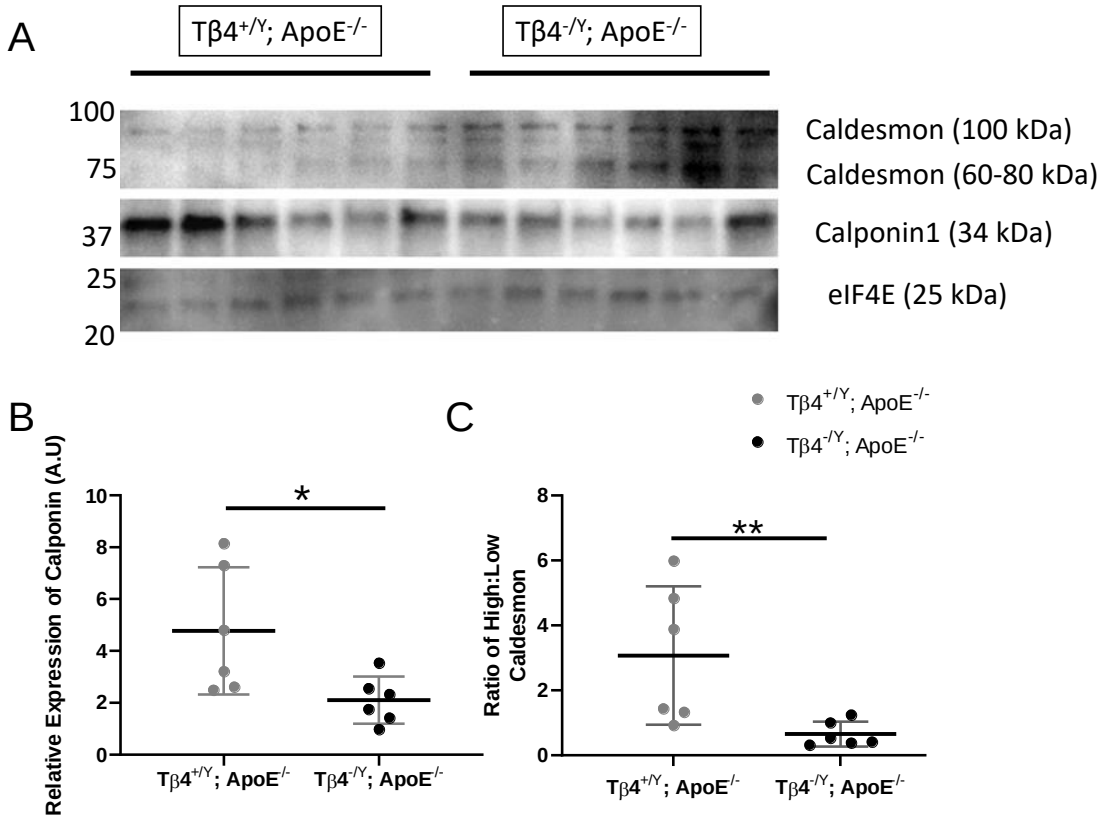



***Figure 3.7: Atherosclerotic Tβ4-null mice show increased VSMC phenotypic switching.*** *Western blot showing expression of contractile (h-Caldesmon, Calponin1) and synthetic (l-Caldesmon) proteins in aortic lysates from Tβ4$^{+/Y}$; ApoE$^{-/-}$ and Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice (**A**), quantification for Calponin1 in (**B**) h-Caldesmon:l-Caldesmon ratio quantified in (**C**). Data are presented as mean ± SD, with each data point representing an individual animal, n=6 in each case. Mann Witney non-parametric test. n.s. = not significant; *p ≤ 0.05; **p≤0.01.*

### 3.3.2.2 Hyperproliferative aortic cells in diseased Tβ4 null mice

Healthy VSMCs exist in a non-proliferative state (G0)[285], however, after injury, activated VSMCs re-enter the cell cycle and proliferate[286]. *In vivo* cell proliferation in atherosclerosis can be determined using EdU (5-ethynyl-2'-deoxyuridine)[287], a nucleoside analogue that incorporates into newly synthesized DNA. EdU is safe and well-tolerated. Additionally, EdU assay monitors accumulative proliferation, rather than

measuring endpoint proliferation[288]. Mice were administered 4 doses of 50mg/kg of EdU by intraperitoneal injection over a period of 10 days between weeks 5 and 7 of WD feeding. Aortas were harvested at 7 weeks and VSMC proliferation was assessed. Consistent with dedifferentiation and acquisition of a synthetic phenotype, medial VSMCs were significantly more proliferative in Tβ4$^{-/Y}$; ApoE$^{-/-}$, compared to the Tβ4$^{+/Y}$; ApoE$^{-/-}$ (**Figure 3.8 A-B**). In addition to VSMCs in the medial layer, EdU labelled cells were also observed within the necrotic core (**Figure 3.8 C**), although there was no significant difference in the % of proliferating cells in the plaque core between genotypes (**Figure 3.8 D**). Since the EdU+ cells did not stain for αSMA, it may be reasonable to conclude that the proliferating cells within the core are macrophages or foam cells as, apart from VSMCs, these cells proliferate the most within the plaque[271]. However, further staining with appropriate markers is required to determine the cell types proliferating within the lesion.

Cyclin D1, encoded by *Ccnd1,* is recognised as the principal regulator of VSMC proliferation[289], and suppression of Cyclin D1 is shown to inhibit VSMC proliferation[290]. Thus, in addition to the EdU assay, aortic biopsies were analysed by qRT-PCR. Significant upregulation of *Ccnd1* in WD fed Tβ4$^{-/Y}$; ApoE$^{-/-}$, compared to the Tβ4$^{+/Y}$; ApoE$^{-/-}$ (**Figure 3.8 E**), indicated increased proliferation in the absence of Tβ4, however with the loss of spatial information and the noted proliferation of non-VSMCs, increased VSMC proliferation cannot be concluded.

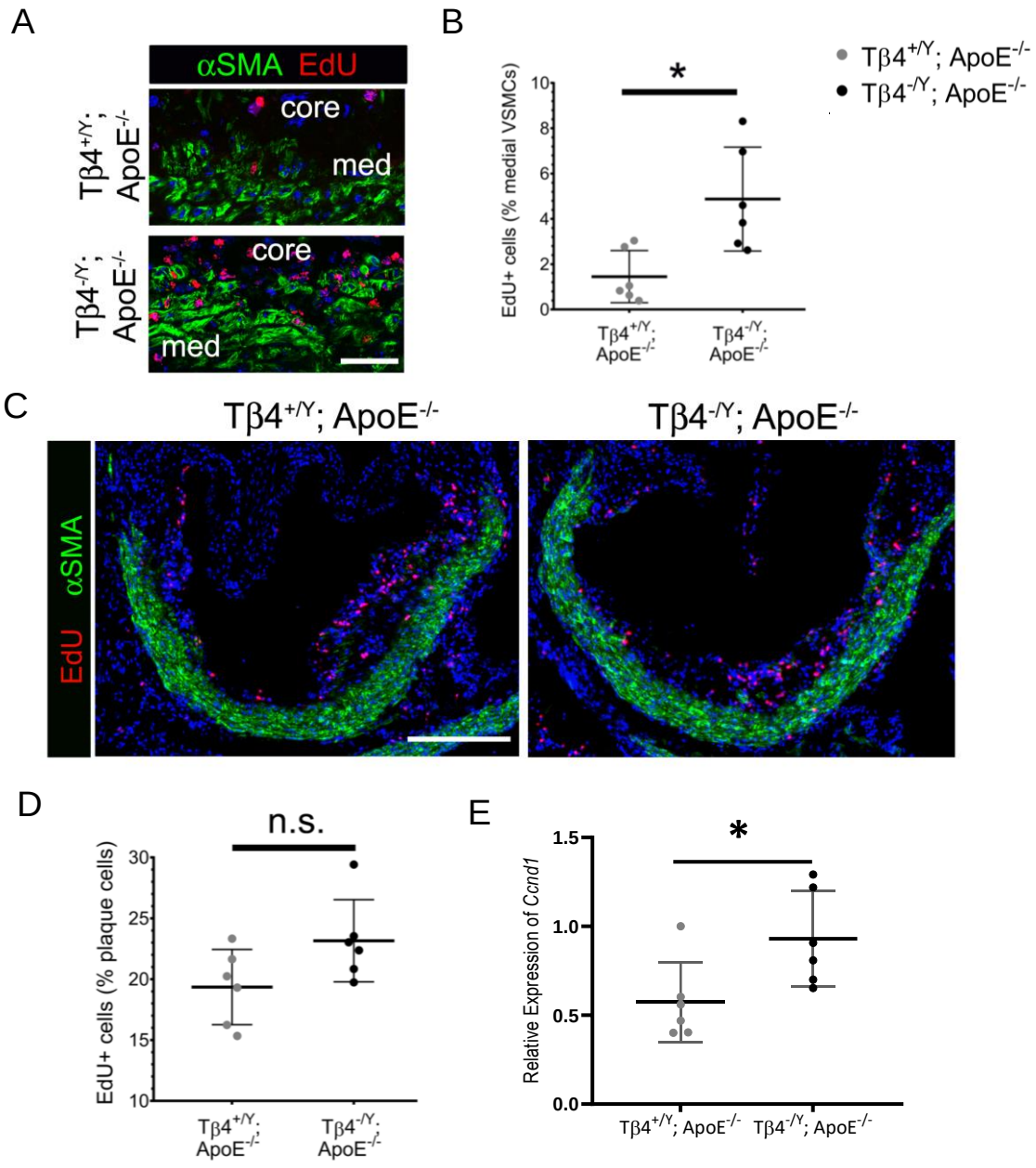



***Figure 3.8: Increased proliferation in Tβ4-null mice in atherosclerosis.*** *Proliferation was assessed by EdU incorporation within the VSMCs (**A**), with quantification (**B**). (**C**) Low power images to demonstrate that most EdU+ cells in the aortic sinus at 7 weeks were present within the plaque core, with quantification of EdU+ cells in the plaque (**D**). qRT-PCR showing increased Ccnd1 expression in atherosclerotic Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortic biopsies. Data are presented as mean ± SD, with each data point representing an individual animal, n=6 in **B**, **D** and **E**. Significance was calculated using Mann Witney non-parametric test (**B**, **D** and **E**), n.s. = not significant; *p ≤ 0.05. Scale bars: **A**: 50μm, **C**: 250μm*

### 3.3.2.3 Increased VSMC apoptosis in early and late atheroma in mice lacking Tβ4

VSMCs of atherosclerotic plaques show increased sensitivity to apoptosis compared to VSMCs from healthy vessels[277]. Moreover, studies in advance human atheroma reveal that apoptosis of vascular cells in fibrous cap affect the stability of the plaques[276]. Endonucleases released in response to disease increase DNA damage[116], creating 3'-hydroxyl (OH) open ends that can be labeled to detect apoptotic cells[291]. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay is identified as a useful technique to label apoptotic VSMCs in situ[291]. **Figure 3.9 A** indicates a significantly higher proportion of apoptotic VSMCs in the aortic media of Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice compared with Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice. TUNEL staining aortic sections at different stages of disease revealed an increase in medial apoptotic VSMCs in Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice compared with Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice fed on WD for 3 weeks' (**Figure 3.9 B**), suggesting that loss of Tβ4 may contribute to medial instability in the early stages of disease. Accelerated disease progression and a likely increase in plaque vulnerability with loss of Tβ4 were further confirmed due to a significantly higher percentage of apoptotic VSMCs in the plaque of Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice (**Figure 3.9 C**).

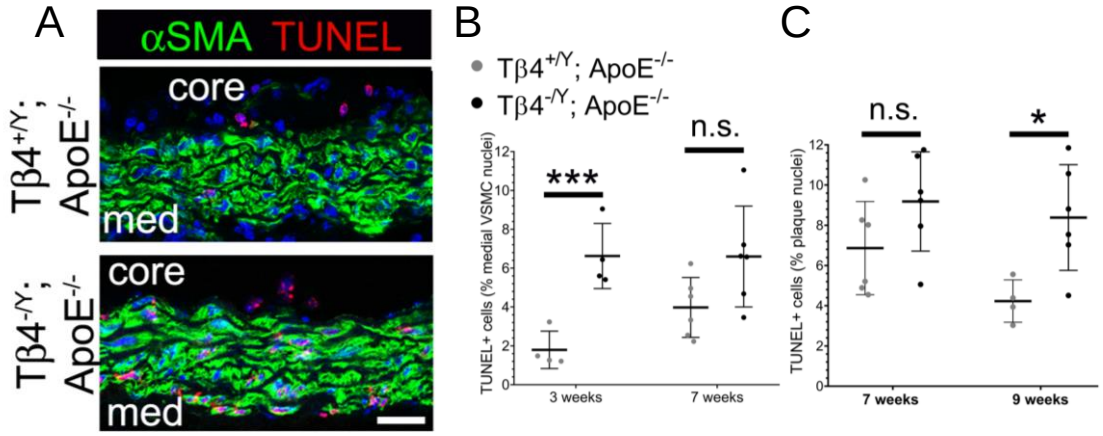


***Figure 3.9: Increase in apoptotic VSMCs in atherosclerotic lesion of Tβ4-null mice.***
*Assessment of apoptotic medial VSMCs and plaque cells using TUNEL staining (**A**), quantification of TUNEL+ VSMCs in media over 3 weeks' (**B**) and in plaque over 9 weeks' western diet feeding (**C**). Data are presented as mean ± SD, with each data point representing an individual animal. n=4 (3 weeks), n=6 (7 weeks), for 9 weeks n=4. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B** and **C**). n.s. = not significant; *p ≤ 0.05; *** p≤0.001. Scale bars: A: 50μm.*

### 3.3.2.4 Tβ4 null mice demonstrated increased elastin degradation

Verhoeff Van-Gieson stained aortic sections from the 12 weeks' WD cohort were carefully analysed to determine elastin integrity. To avoid bias, aortas were scored whilst blinded to genotype. The number of elastin breaks per section was counted for 6-8 sections for each aorta. These values were used to calculate the mean elastin integrity score. A grade 1-4 (1= normal; 2= occasional breaks; 3= frequent breaks, multiple lamellae; 4= severe breakdown) was allotted to each aorta. Compared to $T\beta4^{+/Y}$; $ApoE^{-/-}$ aortas, a striking degeneration of the elastin lamellae was apparent in $T\beta4^{-/Y}$; $ApoE^{-/-}$ aortas (**Figure 3.10 A**) with more elastin breaks per section (**Figure 3.10 B**) giving them a higher elastin damage score than in $T\beta4^{+/Y}$; $ApoE^{-/-}$ aortas (**Figure 3.10 C**) **A** also includes a panel showing elastin degradation in atherosclerotic $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ aorta. Further quantification of elastin breaks per section and elastin damage score suggests that elastin degradation in $Myh11^{Cre}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ aorta are comparable to $T\beta4^{-/Y}$; $ApoE^{-/-}$ aortas(as shown in **Figure 3.10**).

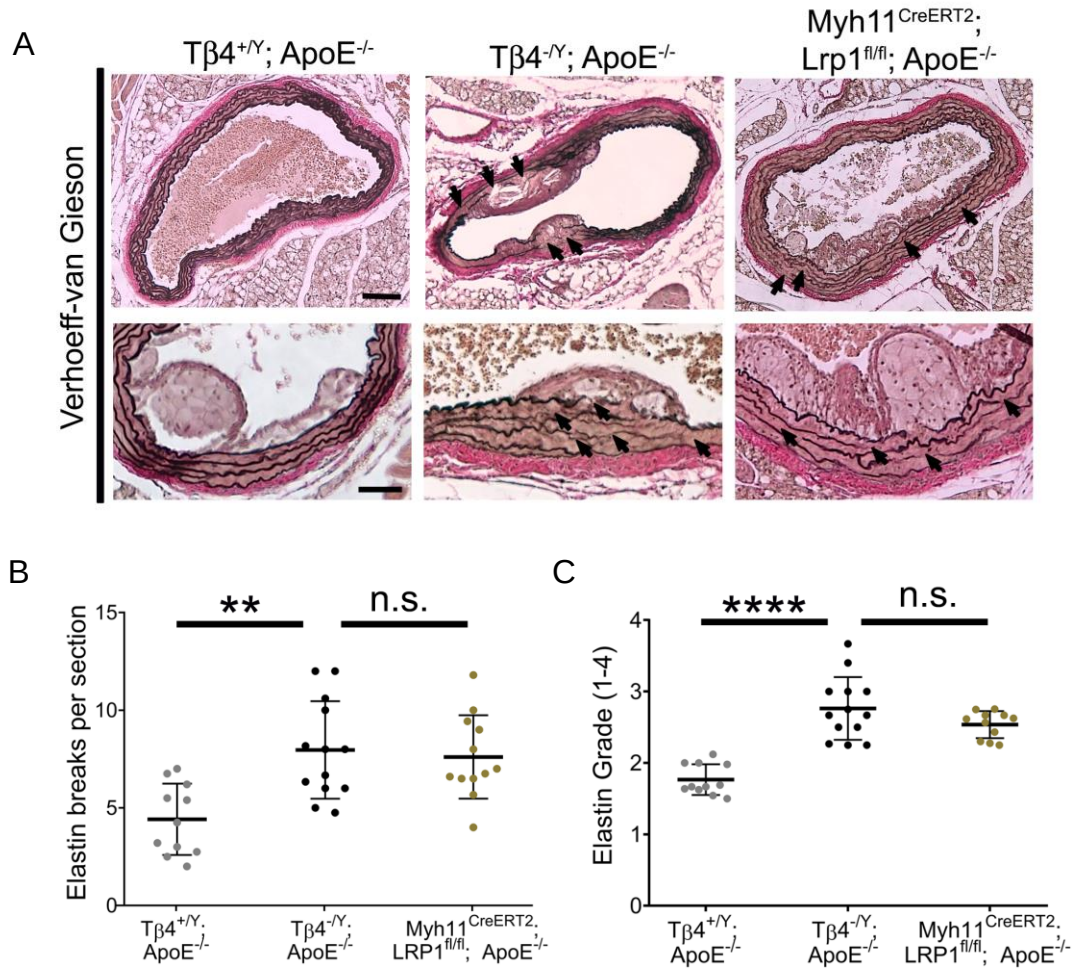



***Figure 3.10: Tβ4-null mice display increased atherosclerotic lesion formation, associated with accelerated elastin degradation****. Verhoeff-van Gieson staining of the abdominal aorta to visualize elastin integrity underlying the plaque (**A**), with quantification of elastin breaks (**B**) and degeneration (increased damage score; (**C**). Data are presented as mean ± SD, with each data point is an average of 6-8 sections for each animal, n=12. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B**, **C**). n.s. = not significant; **p≤0.01; ****p≤ 0.0001. Scale bars: **A**: 200μm (upper); 100μm (lower).*

### 3.3.3 Loss of Tβ4 does not exacerbate inflammation in atherosclerotic mice

Atherosclerosis is recognised as an inflammatory disease and, due to the known anti-inflammatory properties of Tβ4[292], it was imperative to examine inflammation.

Macrophages are the first line of defence[293], and accumulation of macrophages within atherosclerotic plaques has been reported both in murine models and in human patients[294]. Therefore, inflammation was initially assessed based on the expression of CD68, a commonly used tissue macrophage marker[295, 296] in the aortic sinus sections from the 12weeks (advance stage) timepoint (**Figure 3.11 A**). CD68+ macrophage cells were visible either in the plaque or adventitial layer, but not in the media.

Much controversy exists with regards to VSMCs acquiring a macrophage-like phenotype in atherosclerotic lesions. In a study by Andreeva *et al,* cells expressing both αSMA and CD68 were identified on 'en face' preparations from human aortic intima regions with and without lesions[297]. However, a later study challenged this finding and revealed that only some VSMCs expressed macrophage markers after cholesterol loading, it also indicated that VSMCs that expressed macrophage markers were distinct from macrophages despite having phagocytic properties[298]. Therefore, the aortic sections stained with αSMA and CD68 were carefully examined for the presence of cells that stained positively for both αSMA and CD68. Whilst we did not observe any cells either in the plaque or media that were positive for both αSMA and CD68 at an advanced stage in both $T\beta4^{+/Y}$; $ApoE^{-/-}$ and $T\beta4^{-/Y}$; $ApoE^{-/-}$ aorta (**Figure 3.11 A**), genetic lineage tracing of VSMCs would be required to definitively address macrophage contribution.

Next, plaque composition was assessed, and interestingly no significant difference was noted in terms of % area of the necrotic core (black box in **Figure 3.11 B**), % macrophage area (grey box in the **Figure 3.11 B**) and % of the rest of the plaque including VSMC, collagen, etc. (white box in **Figure 3.11 B**) between the two genotypes. It must be noted that the stacked graph in **Figure 3.11 B** shows graph composition as a total of 100% and does not indicate the size. Thus, $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice form larger plaques compared to

Tβ4$^{+/Y}$; ApoE$^{-/-}$ as shown in **Figure 3.2** Section 3.3.1, with large necrotic cores, however, the % composition is unchanged in terms of macrophage content and necrotic core.

Interestingly, although the % of macrophages area within the plaque is indistinguishable between genotypes, the number of CD68 positive cells seemed higher in the adventitial layer in mice lacking Tβ4 and seemed to be infiltrating the medial layer (**Figure 3.11 A & B**). Taken together, these findings suggest that medial layer defects preceded exacerbated inflammation and endogenous Tβ4 may regulate macrophage infiltration in more advanced stages of the disease and a more rigorous assessment is warranted to learn how Tβ4 regulates atherosclerosis at these stages.

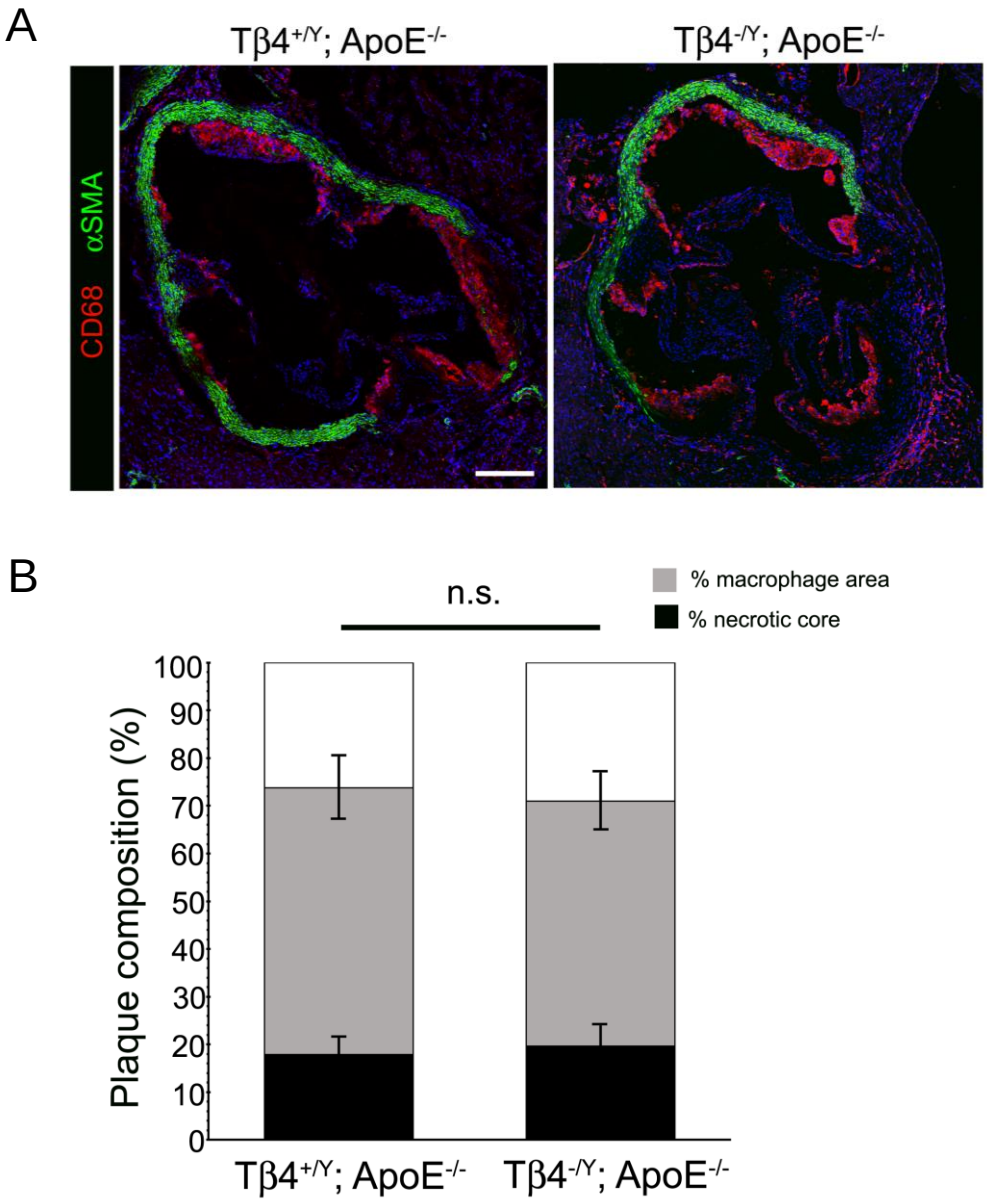



***Figure 3.11: Loss of Tβ4 does not alter infiltration of CD68+ macrophage in the medial layer.*** *Low power immunofluorescence images demonstrate CD68+ macrophage/foam cells in the plaque (**A**), plaque composition, in terms of % occupied by macrophages and % necrotic core (determined from histological staining) (**B**). Data are presented as mean ± SD of n=12. Significance was calculated using Mann Witney non-parametric test (**B**), n.s. = not significant. Scale bar: A: 250μm.*

In addition to immunostaining, a more robust, quantitative approach involving Fluorescent Activated Cell Sorting (FACS) was used to evaluate inflammation. This experiment was performed under the guidance and supervision of Dr Andia Redpath (Smart group). It has been established that leukocyte count increases in atherosclerotic patients[299] and studies have demonstrated a 20-fold increase in macrophages in atherosclerotic mouse aorta[300, 301]. As previously shown in Section 3.3.3, VSMC phenotypic modulation was observed as early as 3 weeks in $T\beta4^{-/Y}$; $ApoE^{-/-}$. To determine whether the inflammatory response is altered in $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice and, if so, whether medial layer defects are a cause or consequence of exacerbated inflammation, inflammatory changes were evaluated in $T\beta4^{+/Y}$; $ApoE^{-/-}$ and $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice at an early stage in the disease (3 weeks' WD).

CD45 is commonly used to label leukocytes both in human and murine atherosclerosis[302]. As aortic lysate is a heterogenous pool of different cells, initially all immune cells expressing CD45 were gated to separate leukocytes from other cells. A comparative assessment reveals a slight increase in leukocyte count in aortic lysates of $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice compared to $T\beta4^{+/Y}$; $ApoE^{-/-}$ mice. However, the difference between genotypes was not significant (P=0.113). It is widely accepted that endothelial damage promotes the recruitment of circulating monocytes that eventually differentiate into macrophages. Although CD14 and CD11b have been used by number of studies to sort monocyte-derived macrophages neither alone is sufficiently specific to distinguish recruited macrophages. It has recently been demonstrated that, in addition to monocyte-derived macrophages, CD14 is expressed by dendritic cells, similarly, CD11b is shown to label several myeloid lineage cells including monocytes, neutrophils, and eosinophils. To overcome this limitation, the use of multiple markers has been recommended. Thus, the leukocyte population was sorted based on the expression of CD11b and CD14. CCR2, a C-C chemokine receptor is implemented in the recruitment of monocyte-derived

macrophages in number of diseases including atherosclerosis[303], therefore, recruited macrophages were further defined based on the expression of CCR2 (**Figure 3.12 A**, bottom panel).

No significant differences in the number of $CD45^+CD11b^+CD14^+CCR2$ macrophages was observed between genotypes (**Figure 3.12 B**), confirming that exacerbated macrophage infiltration is not the primary cause of accelerated disease progression in $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice. However, a more comprehensive analysis to evaluate the involvement of other leukocytes such as T cells, B cells, mast cells, neutrophils, and dendritic cells in the early stages of atherosclerosis is warranted in future.

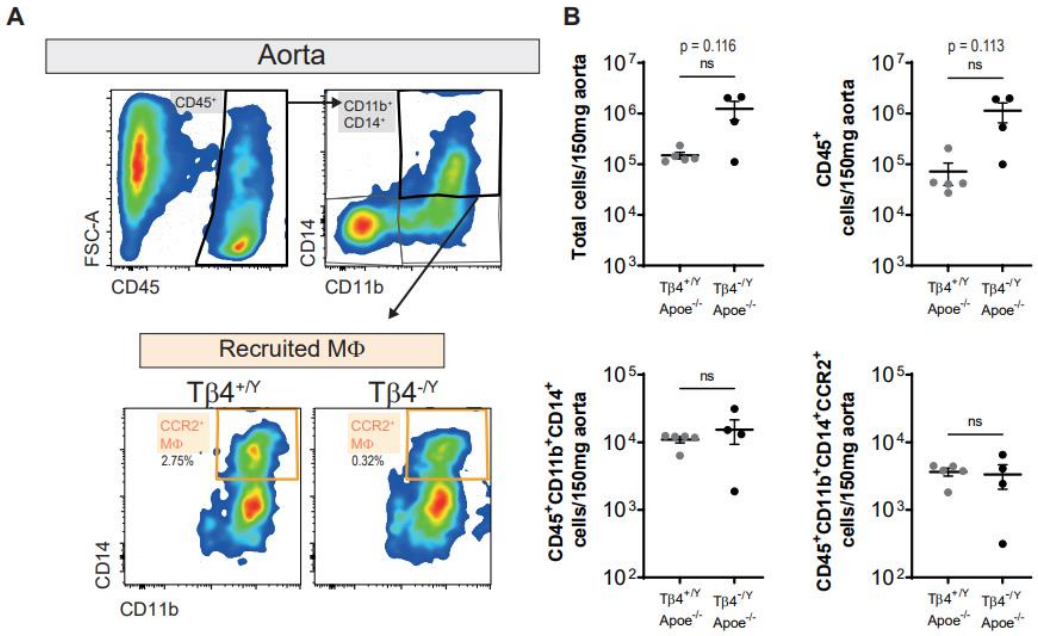



***Figure 3.12: Inflammatory responses to atherosclerosis are not significantly altered with the loss of Tβ4.*** *Flow cytometry was used to quantify macrophages (MΦ) recruited to aortas of $T\beta4^{+/Y}$; $ApoE^{-/-}$ and $T\beta4^{-/Y}$; $ApoE^{-/-}$ mice after 3 weeks of western diet feeding (gating strategy shown in (**A**), with the percentage of $CCR2^+$ macrophages out of total live cells shown). (**B**) Quantification of total $CD45^+$ leukocytes, $CD11b^+CD14^+$ monocytes, and $CCR2^+$ recruited macrophages. Aortas from 5 control and 4 Tβ4-null*

*mice were subjected to FACS analysis. Significance n.s. = not significant was calculated using one-way ANOVA with Bonferroni correction for multiple comparisons.*

### 3.3.3.1 Proinflammatory cytokine levels remain unaltered in diseased mice

Macrophages produce IL-1β and TNFα which increases leukocyte adhesion to the site of injury[116]. Due to the increased sensitivity of qRT-PCR, it has been used in different settings to detect and measure cytokines at the mRNA level [304, 305]. Measurement of *Il1b* and *Tnfα* using qRT-PCR in diseased aortic biopsies collected at various time points confirmed comparable levels of the cytokines between genotypes (**Figure 3.13 A & B**). Cytokines such as IL-6, CCL2, IFNγ, and TNFα are secreted proteins and to more accurately evaluate their levels in peripheral blood from mice fed on WD for 3 weeks, multiplexed ELISA was used. In keeping with the results of the qRT-PCR assay, peripheral blood measurements showed no significant change in the levels of IL-6, IFNγ, and TNFα between Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Tβ4$^{+/Y}$; ApoE$^{-/-}$ (**Figure 3.13 C-D**). Surprisingly, a downregulation (P=0.0159) in peripheral CCL2 levels in Tβ4$^{-/Y}$; ApoE$^{-/-}$ was observed. It must be noted that CCL2 is expressed by a variety of cells, including VSMCs[306]. The significantly higher level of VSMC apoptosis in Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice compared to Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice may be an explanation for downregulation of CCL2 in atherosclerotic Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice.

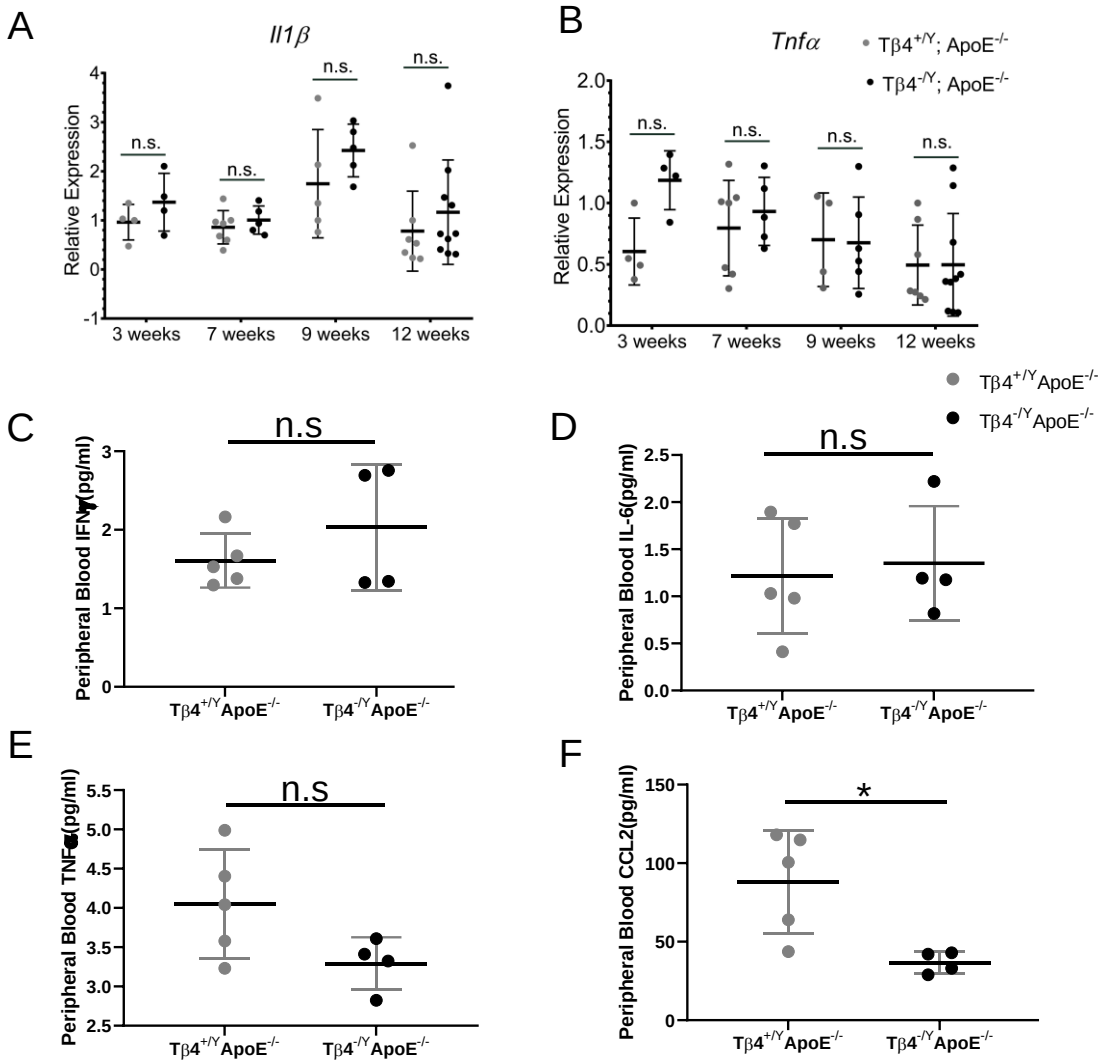



***Figure 3.13: Proinflammatory cytokine levels in atherosclerosis are not significantly altered with loss of Tβ4.*** *qRT-PCR of pro-inflammatory cytokine mRNA levels: Il1β (**A**) and Tnfα (**B**) with n=5 at 3 and 9 weeks, n=6 at 7 weeks and n=8 at 12 weeks. Serum from n=5 mice were assayed by multiplexed automated ELISA for CCL2 (**C**), IFNγ(**D**), TNFα (**E**), and IL-6 (**F**) levels after 3 weeks' western diet feeding. Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**A-B**), Mann Witney non-parametric test (**C-F**). n.s. = not significant, *p ≤ 0.05.*

## 3.4 Discussion

Collectively, the results presented in this chapter demonstrate an atheroprotective role of endogenous Tβ4. Tβ4 null mice on an atherogenic ($ApoE^{-/-}$) background develop large complex plaques, irrespective of gender and diet (regular chow or WD), indicating that loss of Tβ4 exacerbates disease progression. Assessment in both genders was important, due to the increasing reports of sexual dimorphism, both in patients and in experimental studies of CVDs [307]. Moreover, the size of plaques in $Tβ4^{-/Y}$; $ApoE^{-/-}$ mice was similar to the plaque size in $Myh11^{CreERT2}$; $Lrp1^{fl/fl}$; $ApoE^{-/-}$ mice fed on WD for 12 weeks. This is unsurprising considering the studies at baseline indicating that medial layer defects in $Tβ4^{-/Y}$ mice were similar to LRP1 mutants[170], additionally, Tβ4 was identified to interact with LRP1-ICD[170].

Previous studies in mice demonstrated that knocking out the expression of *Lrp1* specifically from VSMCs increases susceptibility to atherosclerosis. Interestingly, the $Myh11^{CreERT2}$; $Lrp1^{fl/fl}$ mice used in this study, induced postnatally at 3 weeks to delete *Lrp1* from VSMCs, recapitulated the phenotype previously described in $SM22Cre^{+}$; $LRP^{fl/fl}$; $LDLR^{-/-}$ mice[164, 308], suggesting that LRP1 has both developmental and maintenance roles in vessels, as shown for Tβ4. These findings further support a common pathway for Tβ4 and LRP1 in vascular protection.

The atheroprotective role of Tβ4 was further confirmed by assessing medial layer integrity over the course of WD feeding. Assessments of data at different stages in disease facilitated the separation of events that occur early and late in disease progression. Combining the results from qRT-PCR, immunostaining, and western blot, it was evident that VSMC phenotypic switching in $Tβ4^{-/Y}$; $ApoE^{-/-}$ initiated earlier, by 7 weeks, in contrast to control aortas, where phenotypic switching was noted but less

advanced, even at 9 weeks. qRT-PCR results were reinforced by protein analysis using immunostaining and western blotting to circumvent any discrepancy between transcriptome and proteome due to post-transcriptional regulation[309]. Moreover, assessment of protein expression using western blotting and immunostaining at different time points provided both quantitative as well as spatial and temporal information. These results were further supported by the scRNA-seq data that demonstrate a strong correlation between *Tmsb4x* expression and VSMC phenotype. Collectively, these findings imply that loss of Tβ4 accelerates disease progression by affecting the differentiated VSMC phenotype.

VSMC phenotypic switching was accompanied by significantly higher levels of apoptosis in Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortas, both in the media (3 weeks' WD) and plaque cap (9 weeks' WD). VSMCs in plaque caps are known to synthesize ECM proteins to stabilize plaque[310], while loss of VSMCs in plaque cap contribute to plaque instability, rupture, and thrombosis[277, 278]. Given that the appearance of apoptotic cells, assessed by light microscopy, is limited to only a few minutes, due to the rapid removal of apoptotic bodies by phagocytosis[311], it is unsurprising that the frequency of visualising the apoptotic cell in this study was low. However, it should be emphasized that even a small proportion of apoptotic cells visualized in tissue sections at any given time may represent a considerable magnitude of chronic cell loss [312].

The occurrence of apoptosis in atherosclerotic plaques may have a double pathophysiological meaning: on the one hand, apoptotic cell death may influence plaque progression (thus being a self-limiting factor in plaque development) and may also quench the inflammatory reaction without affecting plaque volume (Kolodgie et al., 1999); on the other hand, cell death might promote the conversion of a cellular-rich lesion to a fibrous hypocellular (so-called stable) plaque. Plaque stability is commonly determined by the size

of the necrotic core, VSMC: macrophage ratio, cellular composition and thickness of the fibrous cap. Although the cap thickness in Tβ4$^{-/Y}$; ApoE$^{-/-}$ and Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice were comparable, the caps in Tβ4$^{-/Y}$; ApoE$^{-/-}$ were chiefly made up of synthetic VSMCs in contrast to Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice were the caps consist of both contractile and synthetic VSMCs at 12 weeks timepoint. In addition, the EdU data reveal an increase in EdU+SMA+ medial VSMCs at 7 weeks in Tβ4$^{-/Y}$; ApoE$^{-/-}$ but not Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice. These data concur with the previous finding that VSMC phenotypic switching in Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortas begin as early as 7 weeks. Earlier studies by Chappell *et al*. demonstrated that a small subset of mature medial VSMCs gave rise to αSMA+ VSMCs in the fibrous cap[118]. It has also been demonstrated that, as the disease progresses, plaque cap cells proliferate and give rise to progeny that downregulate the expression of VSMC marker (SMA+) and subsequently migrate into the plaque core[274]. Taken together, this suggests that the absence of Tβ4 expedites phenotypic switching of VSMCs both in the media (7 weeks) and plaque cap (12 weeks) accelerating disease progression.

In addition to VSMC changes, Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortas developed significantly exacerbated elastin defects compared to littermate controls. Medial VSMCs are responsible for the synthesis, alignment, and maintenance of elastin to provide effective passive recoil in response to hemodynamic and local tissue deformation[313], while synthetic VSMCs are shown to promote the production of MMPs that encourages elastin degradation in disease[314]. Phenotypic assessments confirmed an increase in the synthetic VSMC population in Tβ4$^{-/Y}$; ApoE$^{-/-}$ aortas, which could probably explain the increased elastin degradation in these mice.

Finally, using a combination of techniques (qRT-PCR, immunostaining, FACS, and ELISA), it was established that exacerbated inflammation was not the primary reason for accelerated disease progression in mice lacking Tβ4. Ruling out inflammation was

important because atherosclerosis is recognised as a chronic inflammatory disease[267] and Tβ4 has been shown to attenuate inflammatory responses in a range of conditions[315-317].

## 3.5 Limitations

For the purpose of this study, the aorta harvested from each mouse was divided into smaller segments, approximately similar in size and anatomical region, to be allocated for different types of analysis. However, the issue with this approach is that different segments of the aorta have different properties. Besides, some segments such as the aortic sinus and branch point in the abdominal aorta may be more prone to developing lesions and may show more profound changes by histology and marker expression compared to the rest of the aorta. To circumvent such limitations, designing techniques that allow RNA extraction from histological sections or laser capture microdissection could be advantageous[318].

It is increasingly recognised that the traditionally used contractile markers- αSMA, SM22α, Calponin, and SM-MHC are expressed by cells other than VSMCs, at least transiently during development, disease, and tissue repair[38]. More recently with the development of genetic engineering, lineage tracing combined with scRNA-seq has been considered the most unambiguous method for tracking VSMC fate[56, 57] and should be employed in future studies. Moreover, the results interpreted using scRNA-seq provide resolution at the level of the single cell, based on cell-specific transcriptomic profiles, whereas the traditional molecular biology techniques are limited due to the amalgamation of cellular populations[319].

## 3.6 Conclusions

Tβ4 regulates VSMC differentiation during development[206, 243] and is involved in maintaining differentiated VSMC postnatally in the non-diseased vessel[170], yet its role in atherosclerosis was previously unexplored. Results presented in this study using a well-established hypercholesterolemia model reveal an important role of endogenous Tβ4 in vascular protection in atherosclerosis. Diseased Tβ4 null aortas phenocopy diseased LRP1 mutant aortas which, along with the demonstrated interaction between Tβ4 and LRP1[170], supports the idea that they may have common pathway in vascular protection.


## Acknowledgement of contribution

Elastin integrity and TUNEL studies presented in this chapter were conducted by Dr Nicola Smart.

Oil red O staining- enface and in sections in regular chow-fed and hypercholesterolemic mice were conducted together with Dr Nicola Smart.

Flow cytometry experiments presented in this chapter were performed under the supervision and guidance of Dr Andia Redpath.

# Chapter IV

# The requirement for Tβ4 in VSMCs to protect against aortic aneurysm

## 4.1 Introduction

An aneurysm is defined as dilation of the arterial wall to over 50% of its original diameter, which if left untreated leads to vessel wall rupture, haemorrhage, and death[320]. It has been reported that nearly 40% of cases with descending aortic diameter of 7cm and 30% of cases with ascending aorta enlargement of greater than 6cm suffer from aortic rupture[321]. The risk of rupture and mortality escalates with a reduction in wall strength and increasing aortic diameter. Although aneurysmal dilatation can develop anywhere along the aorta, aneurysms of the abdominal aorta are most common and affect 2% of men and 1% of women >65 years of age[153]. Several genetic and environmental factors, including atherosclerosis, can initiate aneurysmal dilatation[322, 323], as listed in Section 1.4.1.1.

Much of the current knowledge on the pathology of AAA comes from research in experimental murine models. Aneurysms can be induced experimentally using chemicals, such as $CaCl_2$[127], elastase[324], or Angiotensin (Ang) II[134], as explained in detail in Section 1.5.2. Despite the fact that AngII infusion leads to the development of aneurysm in the ascending thoracic aorta[325, 326] and supra-renal dissecting aortic aneurysm in mice, in contrast to the infra-renal aneurysm seen in humans[327], the AngII model was the preferred choice for this study, due to the technical simplicity and male gender preponderance that resembles human disease[328, 329]. A subcutaneously implanted osmotic mini pump enables the continuous and controlled systemic

administration of AngII. Chronic AngII administration is shown to induce aneurysmal dilatation both in hyperlipidemic mice, e.g. on the $LDLR^{-/-}$ or $ApoE^{-/-}$ background[134, 330] and in normolipidemic mice on the C57BL6 background[135, 331, 332]. The major advantages and limitations of the AngII model, as proposed in [133, 327, 329, 333], are summarised in **Table 4.1.**

**Table 4.1: Advantages and limitations of Angiotensin II induced AAA model**

| Advantages | Limitations |
|---|---|
| Well characterised model | Development of suprarenal saccular aneurysms rather than infrarenal fusiform aneurysms. |
| Recapitulates several aspects of human disease including male gender preponderance, hypertension, and hypercholesterolemia | Aneurysm formation is not consistent, dissections vary in size and severity, spontaneous ruptures post-surgery |
| Technically less challenging surgery and less invasive compared to other models | Small body size limits frequent blood collection and increases the difficulty of dissection of small arteries. |
| Development of AAA over short period of time. | |
| Possibility to track cellular and molecular changes at various stages of disease. | |

Despite extensive research, the sequential pathophysiological events, and the mechanisms underlying AAA, remain to be fully defined[334]. Whilst the inflammatory events that initiate the formation of aneurysm have been extensively studied [335-337], fewer studies have focussed on defining the events that potentiate aneurysm formation. Inflammation-induced dedifferentiation of VSMCs and medial elastin degradation are leading causes of compromised stability and susceptibility to an aneurysm.

Understanding the early events that promote medial degeneration is potentially beneficial to identify high-risk patients who may benefit from early treatment.

Recently, Ailawadi *et al* demonstrated that inflammation-induced VSMC phenotypic switching precedes elastin degradation in aneurysm formation[28]. An increase in the synthetic VSMC population and contractile dysfunction are major events that contribute to aortic dissection[338] that frequently precede the formation of an aneurysm in AngII infused ApoE$^{-/-}$ male mice[133]. Furthermore, the injury-induced release of activated MMPs by inflammatory cells[339] and activated VSMCs[340] provokes elastin degradation and is recognised as an important predictor of TAA[341] and AAA[339]. Elastin fragmentation generates EDP, which promotes inflammatory cell migration and accelerates disease progression[65]. Increased serum EDP levels are reported in AAA patients[101]. Apoptosis is a classical hallmark of aneurysmal tissue; *in vitro*, the epithelioid phenotype, associated with synthetic VSMCs [39, 40], specifically promoted apoptosis over spindle phenotype[342], suggesting that VSMC phenotypic switching may precede apoptosis in an aneurysm. Increased apoptosis results in reduced VSMC density or VSMC rarefaction[343], which may further limit the synthesis of medial elastin and contribute to medial weakness in the advanced stages. Additionally, apoptotic VSMCs release molecules that activate inflammatory macrophages[344] and accelerate disease progression. Taken together, these studies suggest that a possible intervention to prevent VSMC dysfunction may help to prevent disease initiation and progression.

### 4.1.1 Medial layer aberrations accelerate aneurysm formation and progression

Endogenous Tβ4 plays a critical role in maintaining medial layer integrity at baseline[170], and in atherosclerosis, as shown in Chapter III, in which expression of *Tmsb4x* positively correlates with contractile genes and loss of endogenous Tβ4 accelerates VSMC phenotypic switching and elastin degradation. Interestingly, the medial layer defects including aberrant elastin deposition and shift in VSMC phenotype to a synthetic state observed in Tβ4$^{-/Y}$ at baseline[170] are similar to those that underlie the pathogenesis of AAA in patients[246]. Furthermore, *TMSB4X* encoding Tβ4 has previously been reported as the most abundant transcript in the aorta of both healthy individuals and AAA patients[345]. However, a major limitation of the human study was the use of a membrane-based cDNA expression array which allowed screening of only 1176 preselected genes, thus introducing bias, and offering limited sensitivity. Second, the study was performed on lysates from the whole aorta, hence appreciating changes in *Tmsb4x* in different cell types was difficult. As described in Chapter III Section 3.1.3, computational analysis of scRNA-seq data from atherogenic mouse aortas suggests that phenotypically modulated VSMCs downregulate the expression of *Tmsb4x* in atherosclerosis. While a similar decrease might accompany VSMC phenotypic switching in the aneurysmal aorta, it may be masked by increases in other cell types, such as ECs, or a greater abundance of inflammatory cells that also express high levels of Tβ4. Moreover, the direct interaction of Tβ4 with LRP1[170], implicated by GWAS in AAA[147], suggests that Tβ4 and LRP1 may participate in a similar pathway to control VSMC responses and vascular protection against aortic aneurysm.

## 4.2 Aim

Having identified an important role for Tβ4 in vascular homeostasis[170] and protection against atherosclerosis (Chapter III), this chapter aimed to determine if VSMC-autonomous Tβ4 provides protection against aortic aneurysm.

## 4.3 Results

### 4.3.1 Loss of Tβ4 increases susceptibility to aortic aneurysm

Due to the prevalence of AAA in males over females of the same age[346], the increased lethality reported in male *Tmsb4x* null ($T\beta4^{-/Y}$; 36%) compared to female embryos ($T\beta4^{+/Y}$; 16%)[206], and the Y-chromosome locus of $Myh11^{CreERT2}$, the optimal Cre line for inducible targeting of VSMCs, male mice were used for this study. We compared age-matched adult $T\beta4^{-/Y}$, VSMC specific Tβ4 knockdown ($Myh11^{CreERT2}$; $Hprt^{T\beta4shRNA}$) and control ($T\beta4^{+/Y}$ and $Myh11^{CreERT2}$) mice and, additionally, a cohort of VSMC specific LRP1 knockout (KO) ($Myh11^{CreERT2}$; $LRP1^{fl/fl}$) male mice was included to determine any similarities or differences in disease phenotype. AngII, at the dose of 1mg/kg/day, was infused via subcutaneous implantation of an osmotic minipump to induce aneurysm.

#### 4.3.1.1 Loss of Tβ4 increases aneurysmal dissections and rupture

AngII infusion promotes aneurysmal dilatation both in the ascending and descending aorta[325], therefore full-length whole mount aorta from $T\beta4^{+/Y}$, $T\beta4^{-/Y}$, and $Myh11^{CreERT2}$; $Hprt^{T\beta4shRNA}$ mice treated with saline and AngII for 10 days were carefully dissected from the sacrificed animal and cleaned to remove surrounding adipose tissue, as described in[347].

Careful analysis of the whole mount aorta from the base of the heart to below the iliac bifurcation indicates aneurysms in the aortic arch in Tβ4$^{+/Y}$, Tβ4$^{-/Y,}$ and Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ mice, while AAA and ruptures were predominantly observed in Tβ4$^{-/Y}$ and Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ mice (**Figure 4.1**).

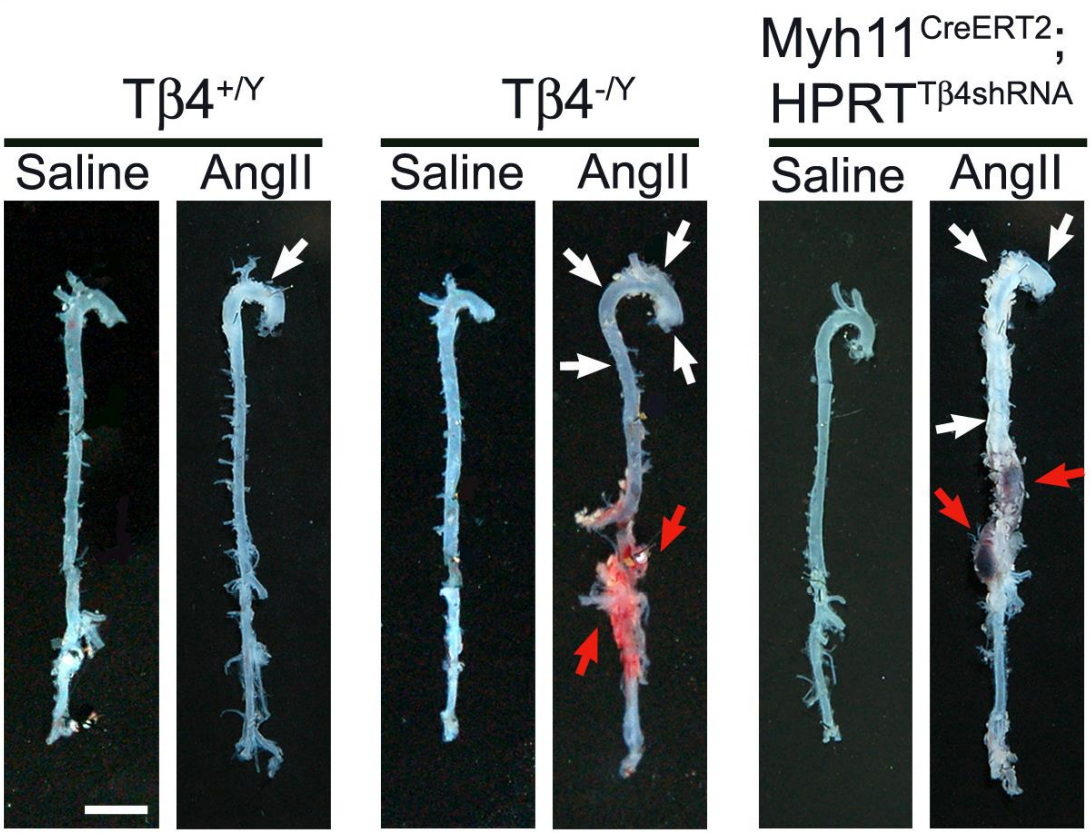



***Figure 4.1: AngII infusion promotes abdominal aneurysms and ruptures in mice lacking Tβ4.*** *Whole-mount aorta from saline- or AngII-infused mice. White arrowheads indicate ascending and descending aortic aneurysms; red arrowheads indicate rupture. n=3 in each group. Scale bar all panels: 200μm. Data published in[170], © J.Clin.Invest.*

Histological analysis confirmed increased incidence of aortic ruptures in aneurysmal Tβ4$^{-/Y}$ and Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ mice. In more severe cases, death in these animals occurred within 5 days of AngII infusion. Infusion of AngII has been shown to form dissecting supra-renal AAA[327]. While aortic dissections were not observed in Tβ4$^{+/Y}$ mice, histological analysis revealed rare (<10%) aortic dissection in Tβ4$^{-/Y}$ and

Myh11$^{CreERT2}$; Hprt$^{T\beta4shRNA}$ mice, ~15% of Myh11$^{CreERT2}$; LRP1$^{fl/fl}$ mice also suffered aortic dissections, characterised by intramural hematoma due to intimal tears that led to blood leak between the tunicae[348]. Aortic sections from a Tβ4$^{-/Y}$ mouse found dead after 8 days of AngII infusion shown in **Figure 4.2 A** revealed circumferentially oriented blood-containing space with intimal discontinuity[349].

An incidence plot to compare the ruptures and dissections in aneurysmal mice shows that nearly 35% Tβ4$^{-/Y}$, 30% Myh11$^{CreERT2}$; Hprt$^{T\beta4shRNA}$ and 20% Myh11$^{CreERT2}$; LRP1$^{fl/fl}$ suffered lethal aortic ruptures, in contrast to 10% of control mice (**Figure 4.2 B**). Interestingly, the incidence of rupture was greater in Tβ4$^{-/Y}$ than Myh11$^{CreERT2}$; LRP1$^{fl/fl}$ mice, however, Myh11$^{CreERT2}$; LRP1$^{fl/fl}$ mice showed a higher incidence of dissection compared to Tβ4$^{-/Y}$ and Myh11$^{CreERT2}$; Hprt$^{T\beta4shRNA}$, although, as stated above, both events were relatively rare, precluding an appropriately powered comparison.

#### 4.3.1.2 Increased aortic dilatation in aneurysmal mice lacking Tβ4

An increase in aortic diameter confers a high risk of aortic rupture and cardiovascular mortality in aneurysm[350]. Histological sections used to calculate aortic diameter revealed a 1.6-fold increase in Tβ4$^{-/Y}$, 1.8-fold in Myh11$^{CreERT2}$; Hprt$^{T\beta4shRNA}$ and 1.5-fold increase in Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$ aortas (**Figure 4.2 C**), suggesting that mice lacking Tβ4 show increased susceptibility to aortic dilatation and this was comparable to Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$ mice in disease. Overall, mice lacking Tβ4 and LRP1 show increased susceptibility in comparison to the respective controls.

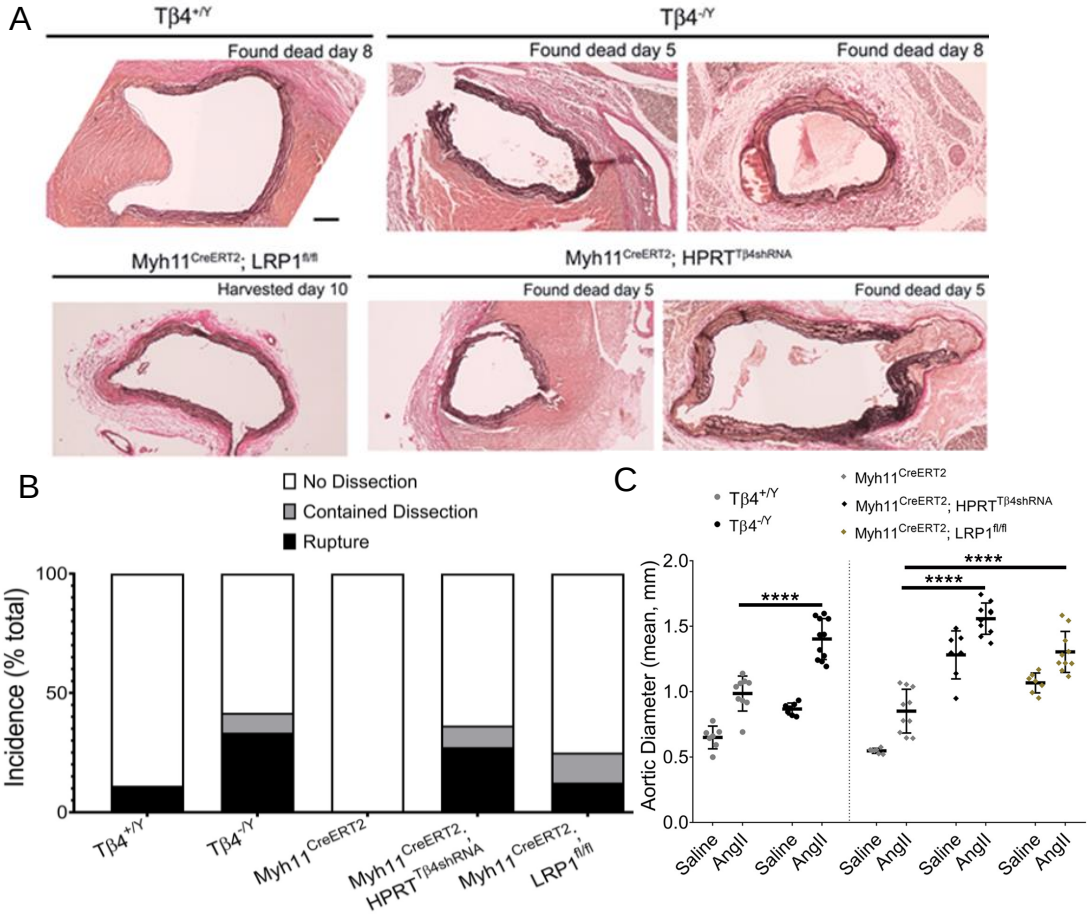



***Figure 4.2: Mice lacking Tβ4 display pronounced aortic rupture and dissections, indicative of more severe aneurysmal phenotypes.*** *(**A**) Histological analysis of aortic sections indicates a higher incidence of aortic rupture and dissection in Tβ4 null mice compared to control, (**B**) quantification of the total number of aortic ruptures and dissection per genotype in response to AngII infusion, **C**) quantification of aortic diameter. Data are presented as mean ± SD, with each data point representing an individual animal, n=8-12. Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**C**). *** P ≤ 0.001; **** P ≤ 0.0001. Scale bar: All panels in **A**: 100μm. Data is published in[170], © J.Clin.Invest.*

### 4.3.2 Enhanced elastin degradation in aneurysmal mice lacking Tβ4

Elastin integrity was assessed in aneurysmal aortic sections from mice that were infused with AngII for 10 days. The number of elastin breaks per section was counted for 6-8 sections for each aorta. To avoid bias, aortas were scored, whilst blinded to genotype. These values were used to calculate the mean elastin integrity score as described in Chapter III, Section 3.3.2.4.

Compared to AngII treated control (Tβ4$^{+/Y}$ and Myh$^{CreERT11}$; Hprt$^{+/+}$) mice, elastin integrity scores of Tβ4$^{-/Y}$, Myh11$^{CreERT2}$; HPRT$^{Tβ4shRNA}$ and Myh11$^{CreERT2}$; LRP$^{fl/fl}$ were significantly higher (shown in **Figure 4.3 A & B**), due to increased elastin fragmentation in multiple layers, suggesting severely breached elastin integrity.

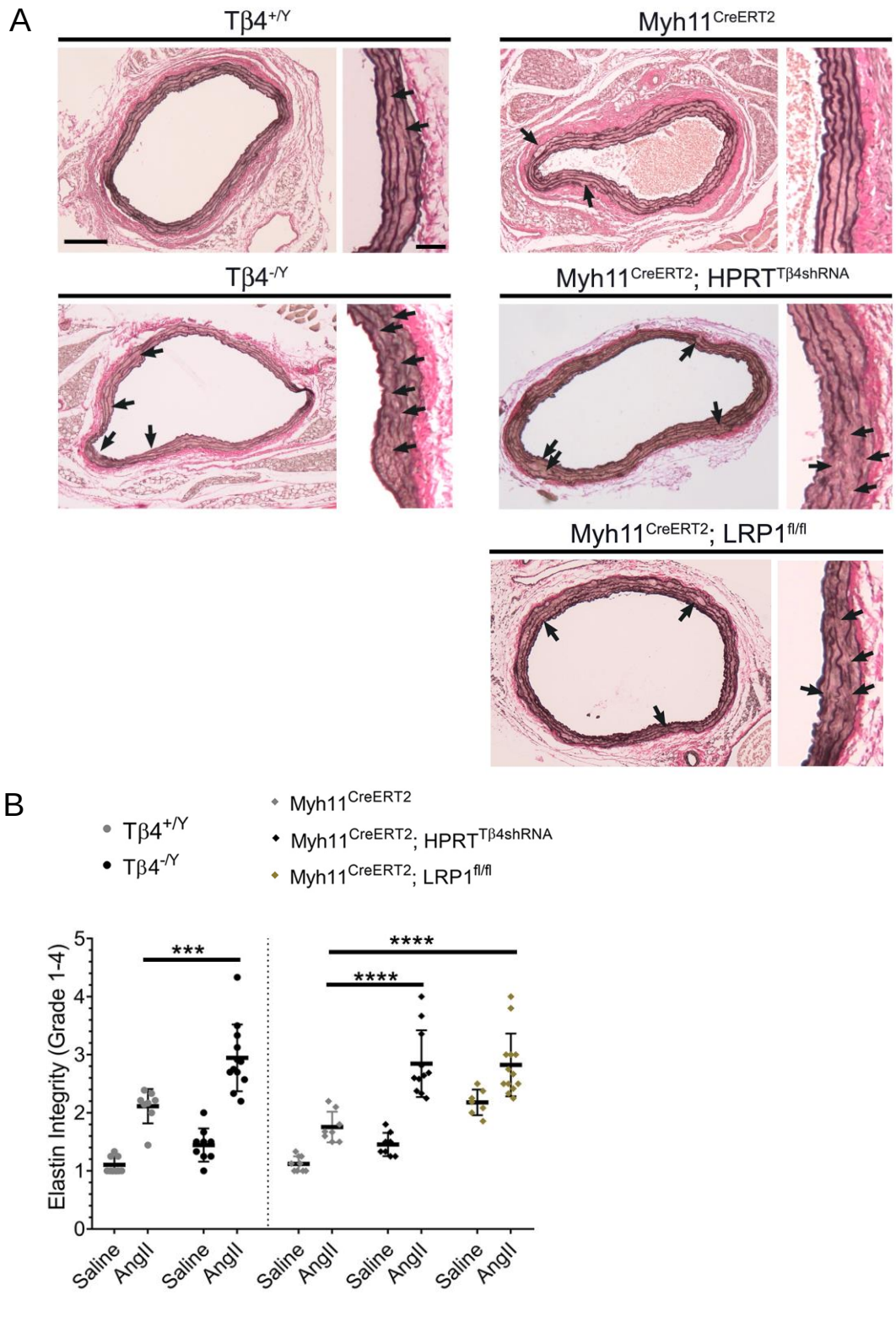



***Figure 4.3: Enhanced medial elastin degradation and aortic dilatation in aneurysmal Tβ4$^{-/Y}$ aortas.*** *Verhoeff-van Gieson staining demonstrates increased elastin degradation in Tβ4 null aortas compared to control, revealed by elastin breaks,*

*indicated with black arrowheads **(A**), (**B**) quantification of elastin integrity based on elastin degradation score. Data are presented as mean ± SD, with each data point representing an individual animal, n=8-12 animals Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests (**B**). *** P ≤ 0.001; **** P ≤ 0.0001. Scale bar: All panels in **A**: 100μm. Data is published in[170], © J.Clin.Invest.*

### 4.3.3 Loss of Tβ4 increase phenotypic modulation of VSMCs in AAA

#### 4.3.3.1 Differential expression of contractile and synthetic markers

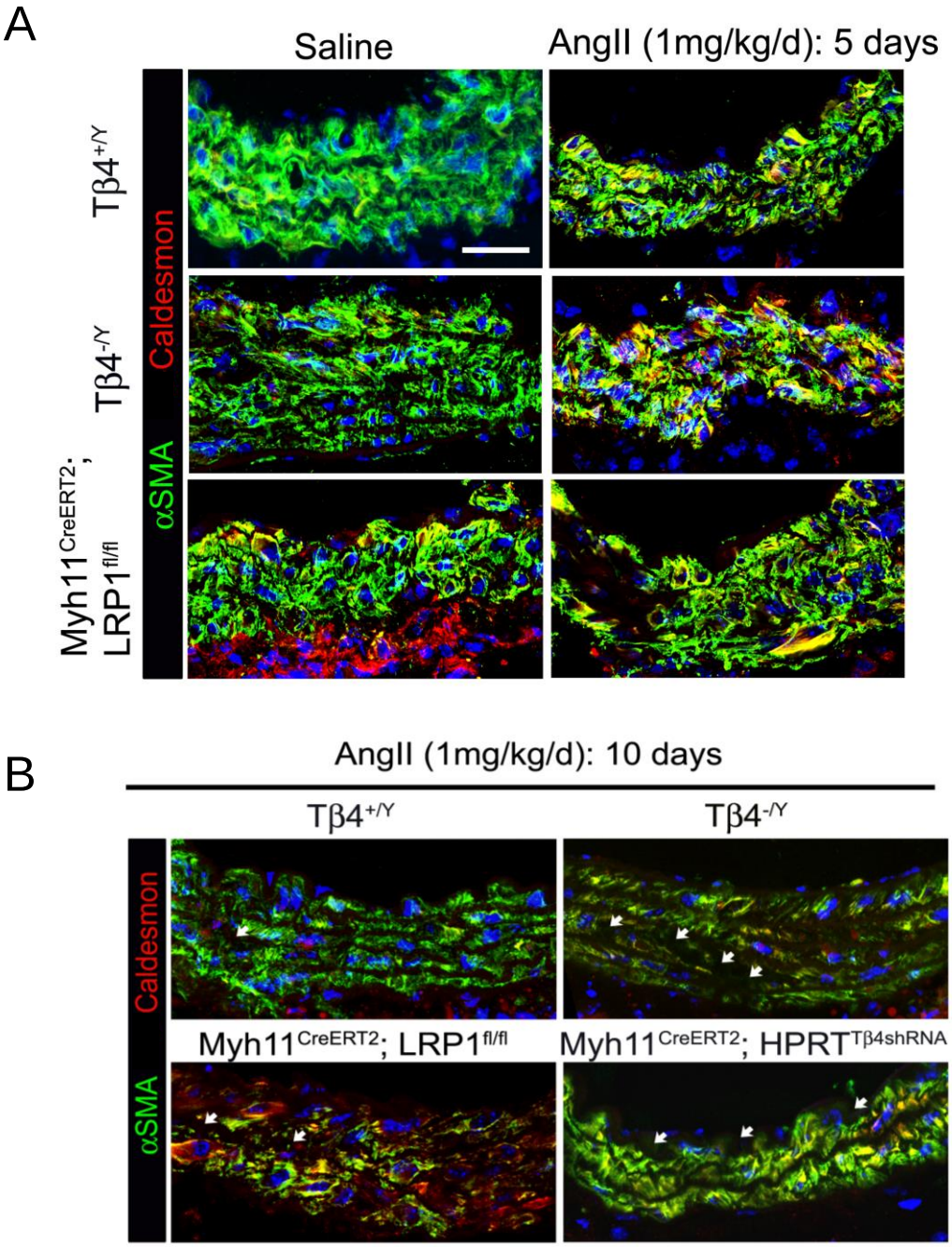
To investigate the role of Tβ4 in the maintenance of VSMC phenotype, functional readouts of VSMC contractile and synthetic markers were obtained using immunostaining and western blotting. Initially, aortic sections from mice that were infused with AngII for 5 and 10 days were stained with αSMA (contractile marker) and Caldesmon (synthetic marker). A shift in phenotype was confirmed based on the decrease in expression of contractile over synthetic marker, accompanied by loss of spindle shape elongated VSMC and increase in cobblestone shaped VSMC. Aortic VSMCs in $T\beta4^{-/Y}$, $Myh11^{CreERT2}$; $Hprt^{T\beta4shRNA}$ KD and $Myh11^{Cre}$; $Lrp1^{fl/fl}$ mice demonstrated an increase in phenotypic modulation, compared to the respective controls. More importantly, VSMC modulation was confirmed as early as 5 days of AngII infusion with a further shift to the synthetic phenotype by 10 days (**Figure 4.4 A-B**).

Upregulation of Vimentin (synthetic marker) in the surgically resected human dilated aorta, compared to a healthy aorta, associated with downregulation of αSMA has been reported previously[29], and due to the inability of Caldesmon antibody to differentiate between the h- and l- isoforms of Caldesmon on immunostained sections, aortic sections were immunostained for Vimentin. As Vimentin is an intermediate filament protein that is normally expressed in aortic VSMCs [351], expression of Vimentin was observed both in

control and Tβ4 null aorta (**Figure 4.4 C**), however, quantification of the immunostained section revealed significant upregulation of Vimentin in Tβ4 null aortas infused with AngII for 5 days, compared to control aortas (**Figure 4.4 D**).

In addition to measuring the expression of contractile (Calponin, h-Caldesmon) and synthetic (l-Caldesmon) proteins by immunostaining, western blotting (**Figure 4.4 E**) was used for more quantitative analysis. The expression of Calponin and Caldesmon (h- and l-) were quantified relative to that of the endogenous control, β-tubulin. This revealed a significant increase in the ratio l-Caldesmon: h-Caldesmon in aneurysmal $T\beta4^{-/Y}$ compared to $T\beta4^{+/Y}$, primarily due to an increase in low molecular weight isoforms of Caldesmon associated with synthetic VSMC phenotype. An increased switch to the synthetic phenotype was further confirmed by a significant decrease in the expression of Calponin, a contractile marker, in aneurysmal $T\beta4^{-/Y}$, compared to $T\beta4^{+/Y}$, aortic lysates, (**Figure 4.4 F & G**, respectively).

Western blotting data reinforced the results obtained from immunofluorescence staining, suggesting that loss of Tβ4 potentiates phenotypic modulation and shift to the synthetic phenotype at a relatively early stage in the development of aortic aneurysm.


A
Saline
AngII (1mg/kg/d): 5 days
Tβ4+/Y
Tβ4-/Y
Myh11CreERT2; LRP1fl/fl
Caldesmon
αSMA
B
AngII (1mg/kg/d): 10 days
Tβ4+/Y
Tβ4-/Y
Caldesmon
αSMA
Myh11CreERT2; LRP1fl/fl
Myh11CreERT2; HPRTTβ4shRNA

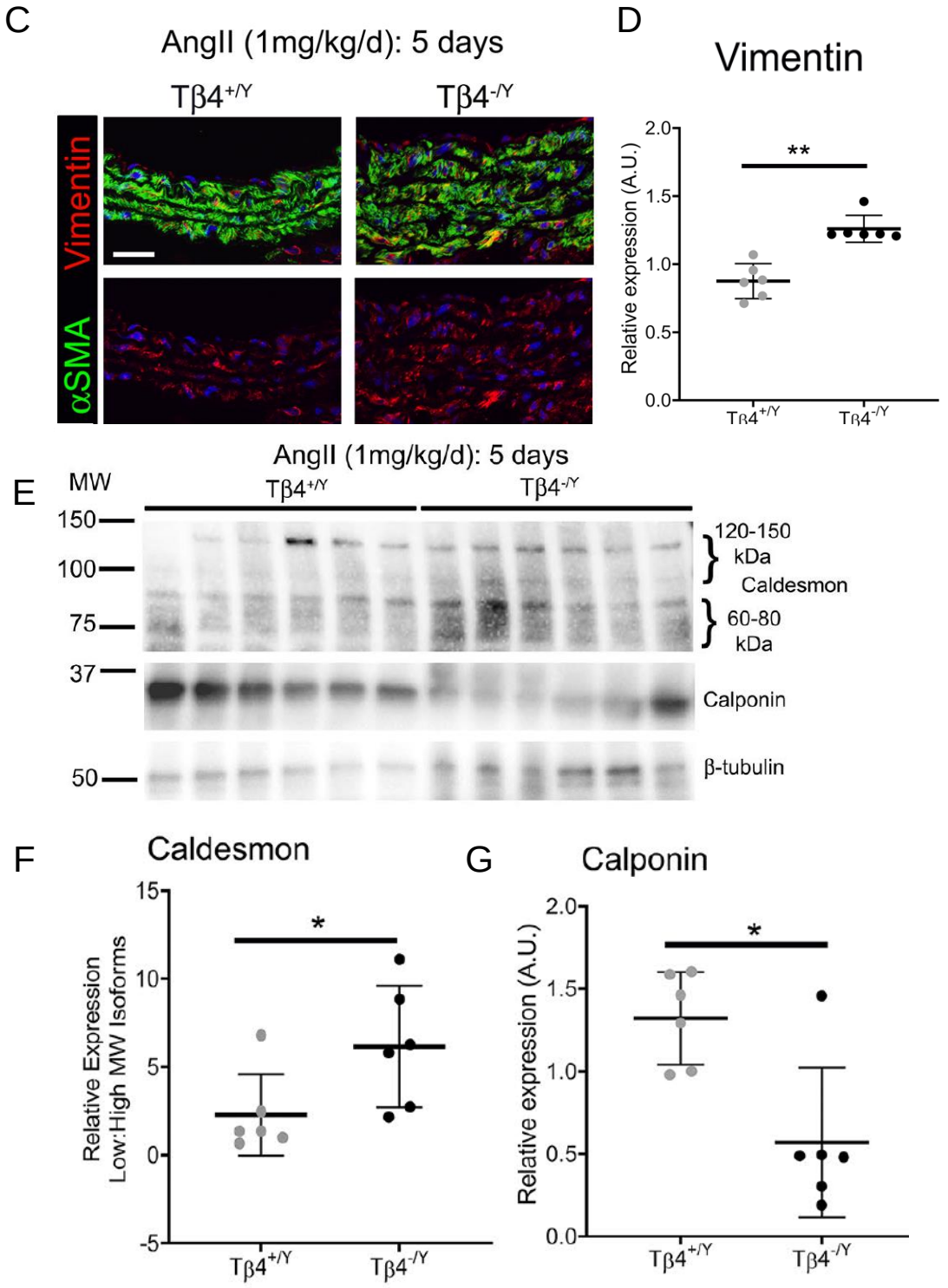



***Figure 4.4: Aneurysm in mice lacking Tβ4 is associated with augmented VSMC phenotypic switching.*** *Immunofluorescence staining to assess medial layer morphology of VSMC phenotype confirms accelerated loss of a canonical contractile marker and increased expression of synthetic markers in aneurysmal Tβ4 null aorta:*

*αSMA and Caldesmon at 5 (**A**) and 10 days (**B**), expression of Vimentin and quantification (**C-D**). Quantification of high and low molecular weight Caldesmon isoforms and contractile marker Calponin by western blotting (**E-G**). Significance was calculated using a Mann-Witney non-parametric test for **D**, **F**, **G**; * P ≤ 0.05; ** P≤0.001. Scale bars: **A**, **B** and **C**: 50μm. n=6 in **B**, **F** and **G**. Data is published in[170], © J.Clin.Invest.*

#### 4.3.3.2 Hyper-proliferative cells in aneurysmal Tβ4 null aorta

Proliferation was determined by measuring the levels of *Ccnd1* in aortic biopsies by qRT-PCR. An increase in *Ccnd1* expression in Tβ4$^{-/Y}$ compared to control confirms an increase in proliferation in Tβ4$^{-/Y}$ in aneurysm (**Figure 4.5**). As stated in the previous chapter, it must be noted that *Ccnd1* is expressed by all proliferating cells, hence a more rigorous analysis using immunostaining with EdU incorporation, flow cytometry or scRNA-seq is required to determine if the cells proliferating are VSMCs and to correlate the phenotype (contractile or synthetic) of VSMCs with those that are proliferating.

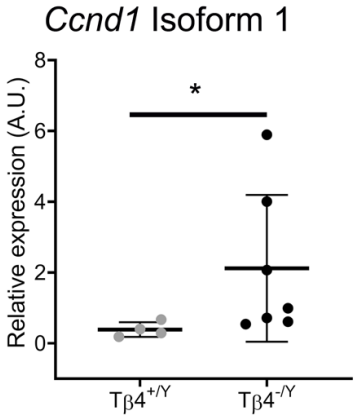



***Figure 4.5: Increased VSMC proliferation in aneurysmal Tβ4$^{-/Y}$ aortas.*** *An increase in VSMC phenotypic modulation was accompanied by increased expression of the proliferation marker, Ccnd1, in some Tβ4$^{-/Y}$ aortas, compared with controls (qRT-PCR) indicating an increase in proliferation. Data are presented as mean ± SD, with each data*

*point representing an individual animal, n=5 for Tβ4$^{+/Y}$ aortas and n=7 for Tβ4$^{-/Y}$ aortas. Significance was calculated using a Mann-Witney non-parametric test. * P ≤ 0.05. Data is published in [170], © J.Clin.Invest.*

### 4.3.4 VSMC apoptosis

VSMC apoptosis is identified as the principal cause of medial layer degeneration in aortic disease[102]. TUNEL assay was used to detect apoptotic cells in sections after 5- and 10- days' AngII infusion. Compared to the control aortas, apoptotic cells were significantly higher in Tβ4$^{-/Y}$ aortas **(Figure 4.6**). While the primary aim was to assess apoptotic VSMCs (white arrows), these were relatively scarce and vastly outnumbered by TUNEL+ ECs (yellow arrows). Moreover, TUNEL+ VSMCs observed in Tβ4$^{-/Y}$ seemed more numerous after 5 days' AngII infusion than after 10 days, consistent with the demonstration that DNA fragmentation precedes removal of apoptotic bodies[352]. The paucity of VSMCs in Tβ4$^{-/Y}$ at day 10 is likely explained by the removal of most of the apoptotic VSMCs via the systemic circulation. As such, quantification of TUNEL+ VSMCs would be inaccurate and misleading, as the more severely affected medial regions, i.e. those completely devoid of VSMC markers in Tβ4$^{-/Y}$, typically contain fewer TUNEL nuclei than regions with less advanced disease. This is exemplified by the comparison of Tβ4$^{-/Y}$ at d5 vs d10 in **Figure 4.7**. While TUNEL may be usefully assessed in chronic injury models, such as atherosclerosis, the rapid VSMC degeneration associated with the AngII model can produce erroneous data, therefore, TUNEL quantification is not shown.

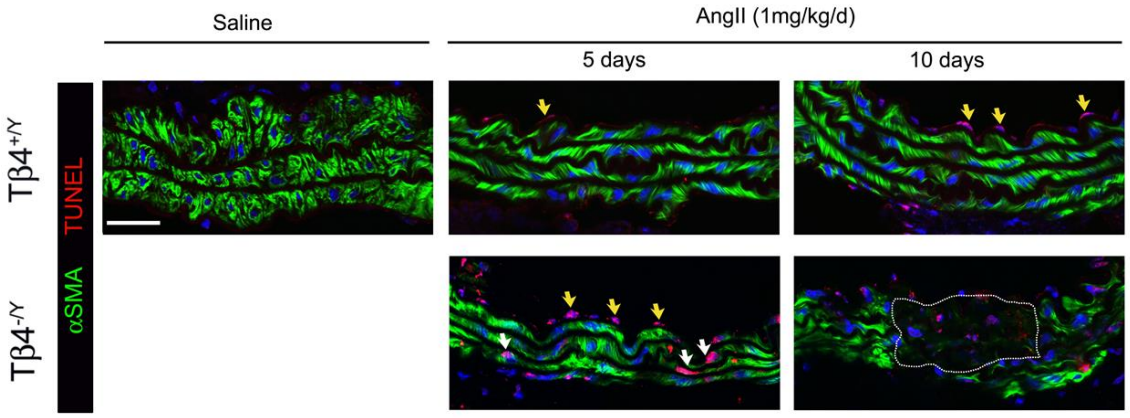



***Figure 4.6: Mice lacking Tβ4 display an apparent increase in apoptosis in aneurysm.*** *TUNEL+ apoptotic cells were rarely detected within the medial layer; most aortas lacked TUNEL+ VSMCs, thus these images could not be described as representative. Endothelial (yellow arrows) and adventitial cells were more frequently TUNEL+ than VSMCs. Quantification was erroneous (not shown) due to the low frequency and rapidity of cell death in the AngII model. Severely affected medial regions devoid of VSMC markers typically contain fewer TUNEL+ nuclei than regions with less advanced disease, Scale bars: 50μm. Figure is published in [170], © J.Clin.Invest.*

### 4.3.5 Loss of Tβ4 does not exacerbate inflammation at an early stage of AAA

AngII-induced aneurysm is recognised to be an inflammation-driven model[330], and due to the known anti-inflammatory properties of Tβ4[219, 317, 353], it was important to evaluate any differences in inflammatory responses between genotypes, which may have led to a differing degree of injury being induced. As previously shown in Section 4.3.3, VSMC phenotypic modulation is underway as early as 5 days of AngII infusion. To determine whether the inflammatory response is altered in $T\beta4^{-/Y}$ mice and, if so, whether medial layer defects are a cause or consequence of exacerbated inflammation, inflammatory changes were evaluated at an early stage (d5) in the disease. Descending aortas from aneurysmal $T\beta4^{+/Y}$ and $T\beta4^{-/Y}$ mice were dissected, enzymatically digested,

and subjected to FACS analysis. This experiment was performed under the guidance and supervision of Dr. Andia Redpath.

Aortic leukocytes ($CD45^+$) and monocyte/macrophages ($CD45^+CD11b^+CD14^+$) were assessed using flow cytometry (**Figure 4.7 A**). CCR2, is implemented in the recruitment of monocyte-derived macrophages to the site of injury in AAA[354], therefore recruited macrophages were defined based on the expression of CCR2. Although no significant difference was recorded in the number of $CD45^+$ leukocytes between the genotypes (**Figure 4.7 B**), two aneurysmal $T\beta4^{+/Y}$ aortae showed a higher number of $CD45^+CD11b^+CD14^+$ monocytes (**Figure 4.7 C**), however, the mean increase was not significant. The number of inflammatory macrophages recruited was comparable between the two genotypes (**Figure 4.7 D**), suggesting that inflammation is not the primary cause of the altered medial layer phenotypes. However, it must be noted that T- and B- lymphocytes[355], mast cells[356], and neutrophils[357] also influence the progression of AAA and a more detailed study is warranted in the future.

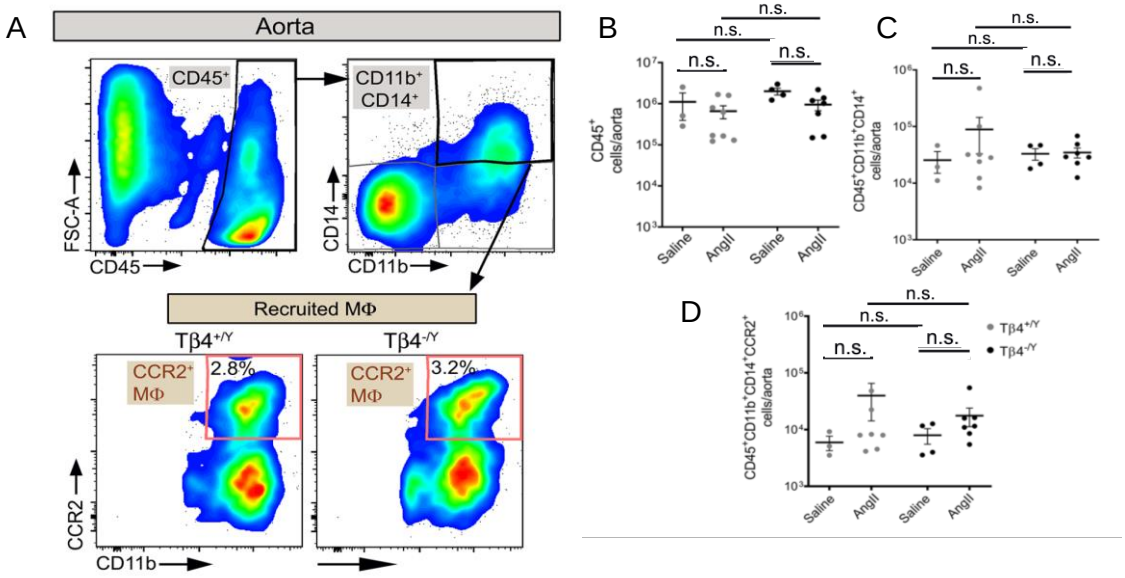


***Figure 4.7: The number of monocyte-derived macrophages is unaltered with the loss of Tβ4 in the early stage of aneurysm formation.*** *Flow cytometry was used to quantify macrophages (MΦ) recruited to aortas of Tβ4$^{+/Y}$ and Tβ4$^{-/Y}$ mice after 5 days' AngII (gating strategy shown in **A**, with the percentage of CCR2$^{+}$ macrophages out of total live cells shown). Quantification of total CD45+ leukocytes (**B**), CD11b$^{+}$CD14$^{+}$ monocytes (**C**) and CCR2$^{+}$ recruited macrophages (**D**). Significance was calculated using one-way ANOVA with Bonferroni correction for multiple comparisons. n=4 animals in Saline treatment group and n=6-8 in AngII treatment. Data is published in[170], © J.Clin.Invest.*

Next, the levels of macrophage-associated pro-inflammatory cytokines, such as TNFα, IL-6, and IFNγ, recognized biomarkers of AAA progression[358, 359], were assessed in peripheral blood. Whilst the levels of TNFα, IFNγ, and CCL2 increased markedly throughout AngII infusion between 5 and 10 days, there was no significant difference between genotypes at any time point; IL-6 levels were unchanged between d5 and d10 and also unaffected by loss of Tβ4 (**Figure 4.8 A-D**). In addition to measuring the levels of pro-inflammatory cytokines in peripheral blood, the levels were measured in aortic lysates using qRT-PCR. Despite considerable variability within each group, there was no significant difference in the expression between Tβ4$^{-/Y}$ and Tβ4$^{+/Y}$ (**Figure 4.8 E**).

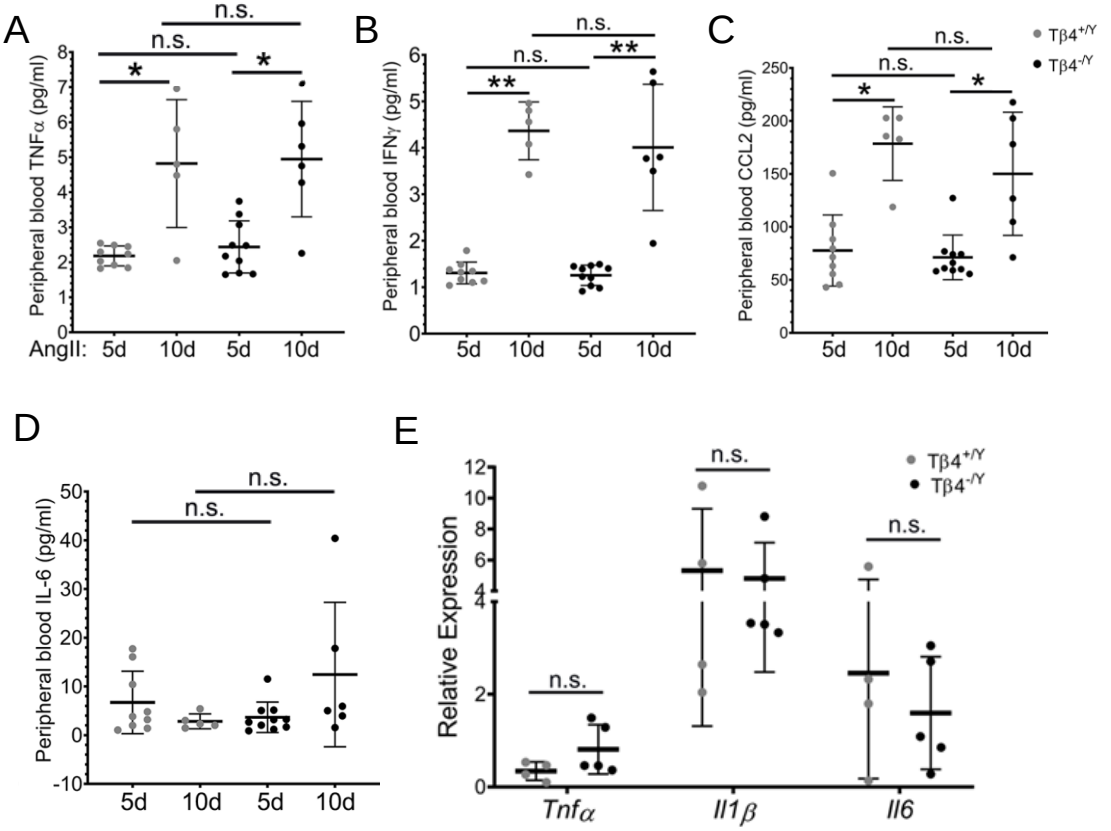



***Figure 4.8: Proinflammatory cytokines were unchanged with loss of Tβ4.*** *Serum from Tβ4$^{+/Y}$ and Tβ4$^{-/Y}$ mice was assayed by multiplexed automated ELISA for TNFα (**A**), IFNγ (**B**) CCL2 (**C**), and IL-6 (**D**) levels after 5- and 10-days' AngII infusion (Peripheral blood from 7-8 animals at 5 days and 4-6 animals at 10 days was assessed). Similar results were observed on measuring mRNA levels Tnfα, Il1β, and Il-6 in aneurysmal aorta after 5 days' AngII infusion (n=4-5). Significance was calculated using one-way ANOVA with Tukey's multiple comparison tests. n.s.= not significant. Data are presented as mean ± SD, with each data point representing an individual animal. Data is published in[170], © J.Clin.Invest.*

## 4.4 Discussion

It is important to tease out the events that culminate in aneurysm formation and progression, to facilitate the identification of new therapeutic targets for the treatment of disease. Data presented in this chapter establish an essential role for VSMC-

autonomous Tβ4 to protect against aortic aneurysms. In the current study, both global Tβ4$^{-/Y}$ knockout and VSMC-specific Tβ4 KD mice on a C57BL/6J background demonstrated accelerated VSMC phenotypic switching and medial elastin degeneration, accompanied by an increased incidence of rupture and mortality. Whole mount imaging of the full-length aorta revealed that AngII infusion promoted aneurysmal development both in the arch and abdominal aorta in Tβ4$^{-/Y}$ and Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ mice, aneurysms were less conspicuous in Tβ4$^{+/Y}$ mice.

Aortic dilatation of C57BL/6J mice in response to AngII infusion has been reported by several studies, however, such aneurysmal dilations are typically demonstrated after 28 days of AngII infusion in most of these studies[360]. An increase in aortic dilation >1.5-fold accompanied by aortic dissections and rupture in aneurysmal Tβ4$^{-/Y}$ and VSMC specific Tβ4 KD mice, on the same genetic background, within 10 days of AngII infusion is indicative of accelerated disease progression. Surprisingly, however, even our control mice showed more aneurysm than the published studies and while it is difficult to pinpoint the exact reason, it has previously been shown in other disease models that environmental factors, such as housing condition, diet, underlying bacterial or viral infection can influence disease progression in mice[361, 362].

Previous studies have implicated VSMC phenotypic switching as a major contributor to aortic dissection and rupture[28, 363]. Aneurysmal Tβ4$^{-/Y}$ and VSMC specific Tβ4 KD aortas display increased VSMC phenotypic switching from their differentiated contractile state to a more synthetic state as early as 5 days of AngII infusion, suggesting that VSMC phenotypic switching is an early event in aneurysm formation. These results are, to some extent, similar to those from a recent study that demonstrated VSMC phenotypic switching 7 days post-surgical intervention in C57BL/6 mice [28]. Accelerated VSMC phenotypic switching in aneurysmal Tβ4$^{-/Y}$ and Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$ mice could be

due to compromised vascular stability even at baseline (non-disease), suggesting that Tβ4 is indispensable in vascular homeostasis and protection against disease[170]. Proliferation is a hallmark of synthetic VSMCs and it is well established that growth factors secreted by inflammatory cells and platelets in response to injury induce VSMC proliferation. An increased shift to a synthetic state was accompanied by an increase in proliferation, indicated by upregulation of *Ccnd1* in $T\beta4^{-/Y}$ aortic lysates. Increased VSMC proliferation is also reported in cultured human AAA explant[364], and in $CaCl_2$ induced aneurysmal rat aortic VSMCs[365]. Clonal expansion of a subpopulation of medial VSMC and their outgrowth into the adventitia and false channel borders has also been reported in AngII-induced mice between 7 and 28 days [366].

Augmented VSMC phenotypic switching observed in aneurysmal $T\beta4^{-/Y}$ and $Myh11^{CreERT2}$; $Hprt^{T\beta4shRNA}$ mice were comparable to $Myh11^{Cre}$; $Lrp1^{fl/fl}$ mice used in this study and similar to those reported previously in LRP1 mutant mice[308], suggesting that Tβ4 null mice phenocopy LRP1 mutants. $Myh11^{CreERT2}$; $Lrp1^{fl/fl}$ mice used in this study, recapitulated the published LRP1 phenotype that used constitutive sm22αCre active in embryonic VSMC progenitors to knockout LRP1[164, 308]. The similarities between the phenotypes observed in Tβ4 null and LRP1 mutant mice at baseline[170] and disease and the direct interaction between Tβ4 and LRP1-ICD[170] strongly suggest a common pathway in maintaining vascular health and protection against disease.

Further data in this chapter confirms enhanced elastin fragmentation in aneurysmal $T\beta4^{-/Y}$ aorta. Pronounced VSMC disorganisation accompanied with elastin fragmentation are major contributors to AAA and aortic dissection, both in humans and mice [367]. However, contradictory views have been reported in the past regarding the importance of elastin in preserving vascular integrity, some studies for example report unchanged elastin content in dissecting aortic aneurysms compared to control[368], while the other

reported a decrease in elastin concentration at the site of dissection in dissected TAA[369]. It must be noted that maximum elastin fragmentation occurs in areas of maximum stretch/ dilatation, while regions away from aneurysmal dilation are histologically more normal in terms of cellular and elastin integrity and this could be a probable explanation for the difference in results. Analysis of inflammation at an early stage of disease (after 5 days of AngII infusion) revealed no significant difference in the level of pro-inflammatory cytokines and the number of recruited macrophages between the genotypes. These results are similar to those observed in Chapter III, Section 3.3.3. confirming that altered inflammatory responses to the AngII insult are not the primary cause of the exacerbated disease phenotype in our model.

## 4.5 Limitations

The study is limited in a number of ways:

1. That the requirement for Tβ4 in protecting against aortic aneurysm has been assessed only in male mice. Age-matched, littermate male mice were primarily used, for two reasons. Firstly, Tβ4 is an X-linked allele, thus while littermate $T\beta4^{+/Y}$ and $T\beta4^{-/Y}$ males can be bred, $T\beta4^{+/+}$ and $T\beta4^{-/-}$ females cannot be generated in the same litter. Secondly, our conditional studies used the $Myh11^{CreERT2}$ line, which is targeted to an allele on the Y chromosome. Early studies used the constitutive sm22αCre[164, 308], however, the major limitations of this line are that *Sm22α* is transiently expressed in a broad range of non-VSCM cell types e.g. cardiomyocytes, epicardium-derived cells during development or disease, rendering the gene targeting non-specific, potentially embryonic-lethal and/or prone to compensation. Subsequently, the inducible $Myh11^{CreERT2}$ became the Cre line of choice due to the high recombination efficiency and specificity of *Myh11* to mature VSMCs[366] The $Myh11^{CreERT2}$ allele was originally inserted into the Y chromosome,

limiting its use to male mice, however, the Myh11$^{CreERT2}$ allele was recently shown to translocate from the Y to the X chromosome, producing a colony of Myh11$^{CreERT2}$ females that could be used to study VSMC phenotype[370]. Owing to the prevalence of AAA in males, this study was performed only on adult male mice, however, recent studies have identified an increase in the number of female patients. Tβ4 is an X linked gene and hence heterozygous males are not possible, however, females carry two X chromosomes, therefore assessing the disease phenotype in female mice (Tβ4$^{+/+}$, Tβ4$^{+/-}$ and Tβ4$^{-/-}$) may provide valuable insights with regards to differences and similarities in disease pathogenesis in females carrying a single copy of the gene.

2. Assessment of aortic diameter based on histology is less accurate relative to alternatives such as Computed tomography (CT) and MRI, direct whole-mount imaging is limited due to poor laser penetration[371]. Also, our measurements could only be made at the experimental endpoint and lacked time-serial diameter measurement and the time course for AAA onset and mortality. Using advanced imaging methods would allow understanding of whether the onset and progression of AAAs in the various mouse strains underwent similar or different temporal changes.

3. Although the data reveal an increase in proliferation, measurement using qRT-PCR on whole aortic lysate is limited in the sense it cannot define which type of cells are undergoing proliferation. EdU/flow cytometry is more sensitive to accurately assess cumulative proliferation and can be combined with cell-type specific markers.

4. An increase in the synthetic VSMC population increases MMPs that provoke ECM degradation, and increase EDP levels, which act as a chemotactic signal to promote inflammatory cell migration[65]. Additionally, apoptotic VSMCs may stimulate calcification[103] and promote proinflammatory macrophage infiltration[104],

accelerating disease progression. Thus, a more comprehensive investigation of inflammation, both with regard to different immune cells and different time points is warranted in the future.

## 4.6 Conclusions

Using the well-established AngII-induced AAA model, this chapter defines a vasculoprotective role for VSMC-autonomous Tβ4 to protect against aortic aneurysm. The result confirms that Tβ4 null mice, like $Myh11^{CreERT2}$; $Lrp1^{fl/fl}$ mice, show increased susceptibility to AAA[2]. Combining results from Chapter III and Chapter IV, it can be concluded that diseased Tβ4 null mice phenocopy LRP1 mutants in terms of medial layer degradation with extensive elastin fragmentation and enhanced VSMC phenotypic switching to a synthetic phenotype. This is an important finding because LRP1 was identified as a GWAS gene in AAA[147], and atherosclerosis[372]. Additionally, the demonstration of direct interaction of Tβ4 with LRP1[170] suggests that they may participate in a common pathway in vascular protection in disease. Thus, identifying the mechanism by which Tβ4 regulates LRP1 may provide important insight into vascular protection to control disease pathogenesis.

## Acknowledgment of contribution

The AngII osmotic mini-pump implantation surgeries were performed by Dr Jyoti Patel and Dr Andia Redpath.

The elastin integrity analysis presented in this chapter was conducted by Dr Nicola Smart.

Flow cytometry experiments presented in this chapter were conducted under the guidance and supervision of and analysed by Dr Andia Redpath.

# Chapter V

# Mechanistic insights into the role of Thymosin β4 in vascular protection

## 5.1 Introduction

The aortic wall has a remarkable capacity to remodel structurally and functionally in response to mechanical stress, injury, and aging. Whilst vascular remodelling is vital during embryogenesis, considerable controversy exists over pathological vascular remodelling in disease; although it begins as an adaptive response to promote repair, chronic remodelling exacerbates inflammation, which leads to maladaptive alterations of the blood vessel and ultimately impaired vascular function[373]. Phenotypic switching of VSMCs has been identified as a prerequisite for both physiological and pathological remodelling, and inhibiting VSMC phenotypic transformation was shown to attenuate disease progression[374]. Multiple factors and signalling pathways, including but not limited to integrin, TGFβ[77, 78], PDGF-BB[79, 80], LRP1 and NOTCH[375] have been identified to play a role in VSMC phenotypic modulation. However, the molecular mechanisms underlying this process are not entirely understood[376].

### 5.1.1 LRP1 in vascular remodelling

GWAS have associated the *LRP1* gene locus with elevated plasma lipids[377], AAA[147], and coronary heart disease[378], suggesting a key role of the receptor in regulating vascular integrity. In the vascular wall, LRP1 plays a key role in controlling VSMC proliferation and migration via TGFβ and PDGF-BB signalling pathways to protect

against disease[308]. However, the precise mechanism by which LRP1 modulates the development and progression of CVDs is currently not well understood[379]. Primary VSMCs isolated from smLRP1$^{-/-}$ mice are highly proliferative and display decreased expression of differentiated VSMC markers, such as αSMA and Calponin, associated with sustained and elevated PDGFRβ activation[165]. LRP1 is required for PDGF-BB stimulated migration of VSMCs[156] and *in vivo* loss of LRP1 specifically in VSMCs leads to hyperactivation of PDGFRβ, resulting in increased VSMC phenotypic switching and susceptibility to AAA and atherosclerosis[150].

PDGFRβ and LRP1 form a complex on the VSMC membrane and incorporate either into caveolae or clathrin-coated pits[164]. Vascular injury triggers the release of PDGF-BB, which in turn binds to its cognate receptor PDGFRβ present on VSMCs and pericytes [380]. The internalisation of PDGFRβ, which is necessary for signal transduction to the nucleus, is regulated via LRP1-mediated endocytosis[247, 381]. Phospho-PDGFRβ trans-phosphorylates the intracellular domain (ICD) of LRP1 at Tyr 4507 at the second NPxY motif[168], whilst either on the cell membrane or within the early endosomes[156, 168]. LRP1-ICD phosphorylation induces conformational changes to facilitate binding of adaptor molecules, such as Shc[382], activating Ras, c-Myc, MAPK, and Akt/PI3K intracellular signalling[383], to induce proliferation and migration of VSMCs.

The established view is that, following signal transduction, a large proportion of the PDGFRβ receptors are degraded, either within the lysosome or proteasome, to prevent continuous signalling [384, 385]. The balance between endocytic uptake, recycling and degradation is a tightly regulated process required to control diverse cellular functions, including migration, cytokinesis, polarity and differentiation[386]. Differential sorting of the receptor to recycling endosomes, rather than lysosomes, may lead to deregulated

signalling and needs an understanding of the precise molecular mechanism that underscores this process.

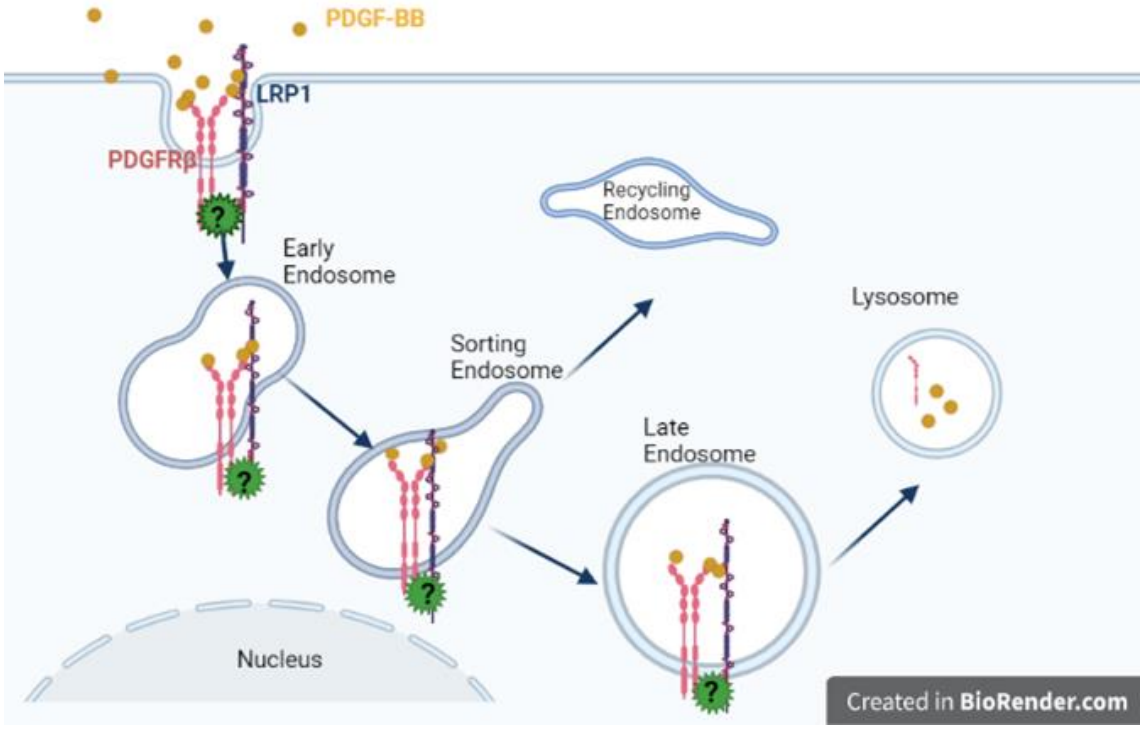



***Figure 5.1: Intracellular trafficking of LRP1-PDGFRβ receptor complex in VSMCs.*** *LRP1 is a transmembrane endocytic co-receptor of PDGFRβ. The binding of PDGF-BB leads to tyrosine phosphorylation of PDGFRβ which transphosphorylates LRP1 at the NPxY domain to facilitate binding of adaptor molecules to activate downstream signalling. Post signalling the receptors can either be degraded in lysosomes or recycled to the cell surface via recycling endosomes. Endocytic sorting of the signalling receptor is a tightly regulated process, and any dysregulation may hamper intracellular signalling and the resulting cellular process. However, the mechanisms controlling LRP1-PDGFRβ intracellular trafficking are not well understood. Image created using BioRender.com.*

Administration of Imatinib ($C_{29}H_{31}N_7O$), a pharmacological inhibitor of receptor tyrosine kinases (RTK), blocks activation of PDGFRβ in $LDLR^{-/-}$; $smLRP1^{-}$

$^{/-}$ hypercholesterolaemic mice and reduces the size of atherosclerotic lesions[164]; moreover, in mice with AngII-induced aneurysm, Imatinib preserves VSMC contractile phenotype and reduces inflammation [166]. Imatinib binds to the ATP-binding site of the receptor and inhibits auto-phosphorylation[387], and thereby docking of signal transduction complexes[388, 389]. Attenuation of signalling in VSMCs attenuates vascular disease.

In addition to PDGFRβ, LRP1 regulates endocytic turnover of several other matricellular proteins, such as connective tissue growth factor protein (CTGF), high-temperature requirement factor A1 (HTRA1), an elastolytic serine protease[390] and PAI-1, a serine protease inhibitor[391], that together dynamically remodel components of the ECM to maintain VSMC phenotype. Increased elastin fragmentation, aortic dilatation, and medial thickening in non-atherogenic smLRP1$^{-/-}$ mice were attributed to increased accumulation of HTRA1 and CTGF [379]. Similarly, it has been shown that disruption of LRP1 mediated endocytosis in the presence of the antagonist receptor-associated protein (RAP), results in reduced degradation and accumulation of CTGF[392]. In the murine model of diabetes-associated atherosclerosis, Imatinib administration inhibited PDGFRβ-dependent signalling pathways, with decreased expression of the CTGF[393]. However, whether PDGFRβ regulates CTGF expression directly or indirectly remains elusive. Interestingly LRP1 is known to regulate both CTGF and PDGFRβ via endocytosis. Collectively, these studies indicate that dysregulated intracellular trafficking of LRP1 and its ligands may be a key cause of increased VSMC phenotypic modulation and ECM degradation in vascular disease.

### 5.1.2 LRP1-Tβ4 interaction in endocytic vesicles of VSMCs

Extensive research has been undertaken to elucidate the molecular and biochemical role of Tβ4 in cellular signalling pathways. Intracellular Tβ4 has previously been shown to regulate cell migration, survival and cardiac protection by forming complexes with integrin linked kinase (ILK), which recruit and activate AKT2[191, 394]. Tβ4 is also shown to act through indirectly activating ATP-responsive purinergic receptors, P2X and P2Y, to regulate HUVEC migration[207]. The identification of a direct interaction of Tβ4 with LRP1-ICD[170], along with results demonstrating that $T\beta4^{-/Y}$ mice phenocopy $smLRP1^{-/-}$ mice both in health and disease supports the notion that Tβ4 and LRP1 may function within a common pathway in vascular protection. However, this does not explain the molecular mechanism(s) involved. The vascular protective functions of LRP1 are largely ascribed to its role in regulating the endocytosis of PDGFRβ[156, 164]. However, there is a paucity of information regarding the mechanisms that control PDGFRβ trafficking and endosomal signalling[395].

Interestingly, Tβ4 has previously been shown to regulate endocytosis, following exogenous application[204]. Besides, our data confirms co-localization of Tβ4 and LRP1 within the endocytic compartments of VSMC of both mouse and human aorta. Taken together, these studies suggest that Tβ4 may be involved in the intracellular trafficking of the LRP1-PDGFRβ receptor complex to orchestrate vascular protection.

## 5.2 Aim

To seek insight into the mechanisms underlying vascular defects in AAA and atherosclerosis and to identify the role of Tβ4 in controlling LRP1-mediated growth factor signalling for vascular protection.

## 5.3 Results

### 5.3.1 Tβ4 regulates LRP1- PDFRβ signalling in vascular injury

To evaluate the role of Tβ4 in regulating growth factor signalling, activation of LRP1, PDGFRβ, and their downstream signalling kinases AKT and MAPK were assessed in murine models of atherosclerosis and aortic aneurysm. Studies have established that transphosphorylation of Tyr4507 in the NPxY domain of LRP1 by PDGFRβ at Tyr1021 [247] mediate interactions with the internalization machinery crucial for receptor mediated endocytosis[155]. Moreover, phosphorylation of PDGFRβ at Tyr1021 is essential to initiate mitogenic response[396]. Therefore, aortic sections from aneurysm mice after 5-days of AngII infusion and hypercholesterolaemic mice after 3 weeks of WD were immunostained for phospho-PDGFRβ (Tyr1021) and phospho-LRP1 (Tyr4507). Quantification of the immunostaining reveals an approximately 1.6- and 1.5-fold increase in phospho-LRP1 and phospho-PDGFRβ, respectively, in aneurysmal $T\beta4^{-/Y}$ aortas compared to $T\beta4^{+/Y}$ aortas (**Figure 5.2 A, B**). Similarly, phospho-PDGFRβ and phospho-LRP1 levels were elevated 2.4- and 1.8-fold, respectively, after 3 weeks of WD in $T\beta4^{-/Y}$; $ApoE^{-/-}$ aortas, compared with $T\beta4^{+/Y}$; $ApoE^{-/-}$ aortas (**Figure 5.3 A, B**).

To further confirm if enhanced receptor activation is relayed downstream, the readouts of effector kinases were evaluated by western blotting. Despite the high biological variability between samples, significantly higher activation (phosphorylation) of p42/p44 MAPK and AKT was observed in aneurysmal $T\beta4^{-/Y}$ aortas compared to $T\beta4^{+/Y}$ aortas after 5 days' of AngII infusion (**Figure 5.2, C & D**). Similarly, western blotting analysis on aortic lysates from mice after 7 weeks of WD confirms augmented activation of both AKT and MAPK in $T\beta4^{-/Y}$; $ApoE^{-/-}$ aortas, compared with $T\beta4^{+/Y}$; $ApoE^{-/-}$ as shown in **Figure 5.3, C & D**. Enhanced LRP1-PDGFRβ signalling may potentially contribute towards the

accelerated VSMC phenotypic modulation and proliferation observed with loss of endogenous Tβ4.

## Aortic Aneurysm

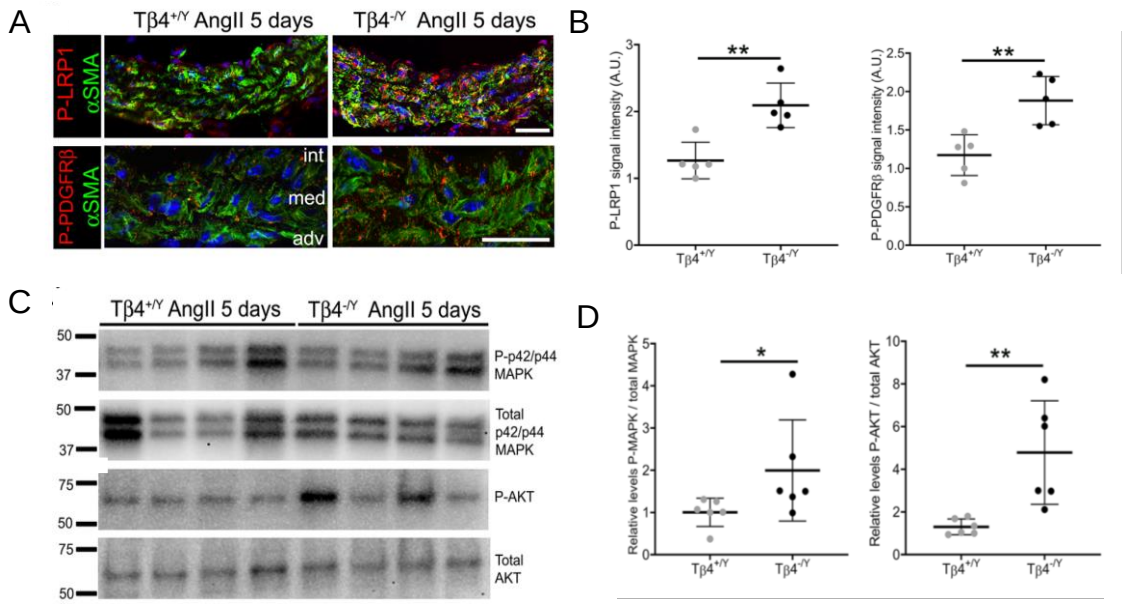



***Figure 5.2: Dysregulated LRP1-PFGRβ signalling in aneurysmal Tβ4 null aortas.*** *(**A**) Phospho-LRP1 (Tyr 4507) and Phospho-PDGFRβ (Tyr1021) levels in VSMCs after 5 days' AngII infusion, detected by immunofluorescence and quantified in (**B**) with n=5. Western blotting of downstream effectors, phosphorylated p42/p44 MAPK and AKT (**C**), expressed relative to total p42/p44 MAPK and AKT, n=6 respectively (**D**). Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using a Mann Witney non-parametric test. * P≤ 0.05; ** P ≤ 0.01. int: intima; med: media; adv; adventitia. Scale bars: **A**: 50μm. Data is published in[170], © J.Clin.Invest.*

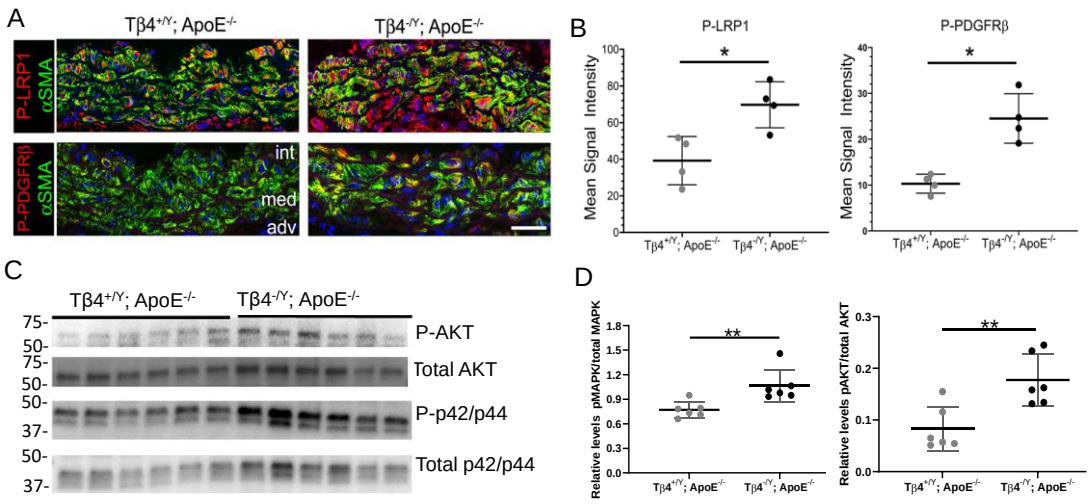



***Figure 5.3: Dysregulated LRP1- PFGRβ signalling in atherosclerotic Tβ4 null aortas.*** *(**A**) Phospho-LRP1 (Tyr 4507) and Phospho-PDGFRβ (Tyr1021) levels in aortic VSMCs of ApoE$^{-/-}$; Tβ4$^{+/Y}$ and ApoE$^{-/-}$; Tβ4$^{-/Y}$ mice fed on WD for 3 weeks, (**B**) quantification from aortic sections from 4 animals and (**C**) western blotting of downstream effectors, phosphorylated p42/p44 MAPK and AKT, expressed relative to total p42/p44 MAPK and AKT from n=6, respectively (**D**). Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using a Mann Witney non-parametric test. * P≤ 0.05; ** P ≤ 0.01. int: intima; med: media; adv; adventitia. Scale bars: **A**: 50μm.*

***Acknowledgement of Contribution:*** *Mean signal intensity quantification from immunostained sections were made by Dr. Nicola Smart.*

### 5.3.2 Tβ4 regulates LRP1 mediated endocytosis of matricellular proteins

To further confirm the notion that LRP1-mediated endocytosis is affected with loss of Tβ4, endocytosis of other LRP1 ligands and matricellular proteins, such as CTGF, HTRA1[379] and PAI-1[391], were assessed. By immunofluorescence, an increased accumulation of CTGF, HTRA1 and PAI-1 was observed at day 10 in the medial VSMCs

of aneurysmal Tβ4$^{-/Y}$, compared with Tβ4$^{+/Y}$ aorta (**Figure 5.4 A-B**). Furthermore, increased punctate staining extracellularly, particularly of CTGF (**Figure 5.4 A, boxed region**), suggested a possible defect in LRP1 ligand internalisation. Similarly, increased accumulation of HTRA1 was observed in Tβ4$^{-/Y}$; ApoE$^{-/-}$ compared to Tβ4$^{+/Y}$; ApoE$^{-/-}$ mice fed on WD for 9 weeks (**Figure 5.4 C**). Unfortunately, CTGF staining was unsuccessful on atherosclerotic aortic sections from 7-, 9- and 12 weeks WD fed mice, while it is difficult to pinpoint the exact reason, a more likely explanation could be that the expression changes with VSMC phenotype/death in chronic disease. Increased levels of CTGF and HTRA1 were observed in VSMC-specific Lrp1 knockout mice[379] consistent with the possibility of a common regulatory mechanism involving LRP1 and Tβ4.

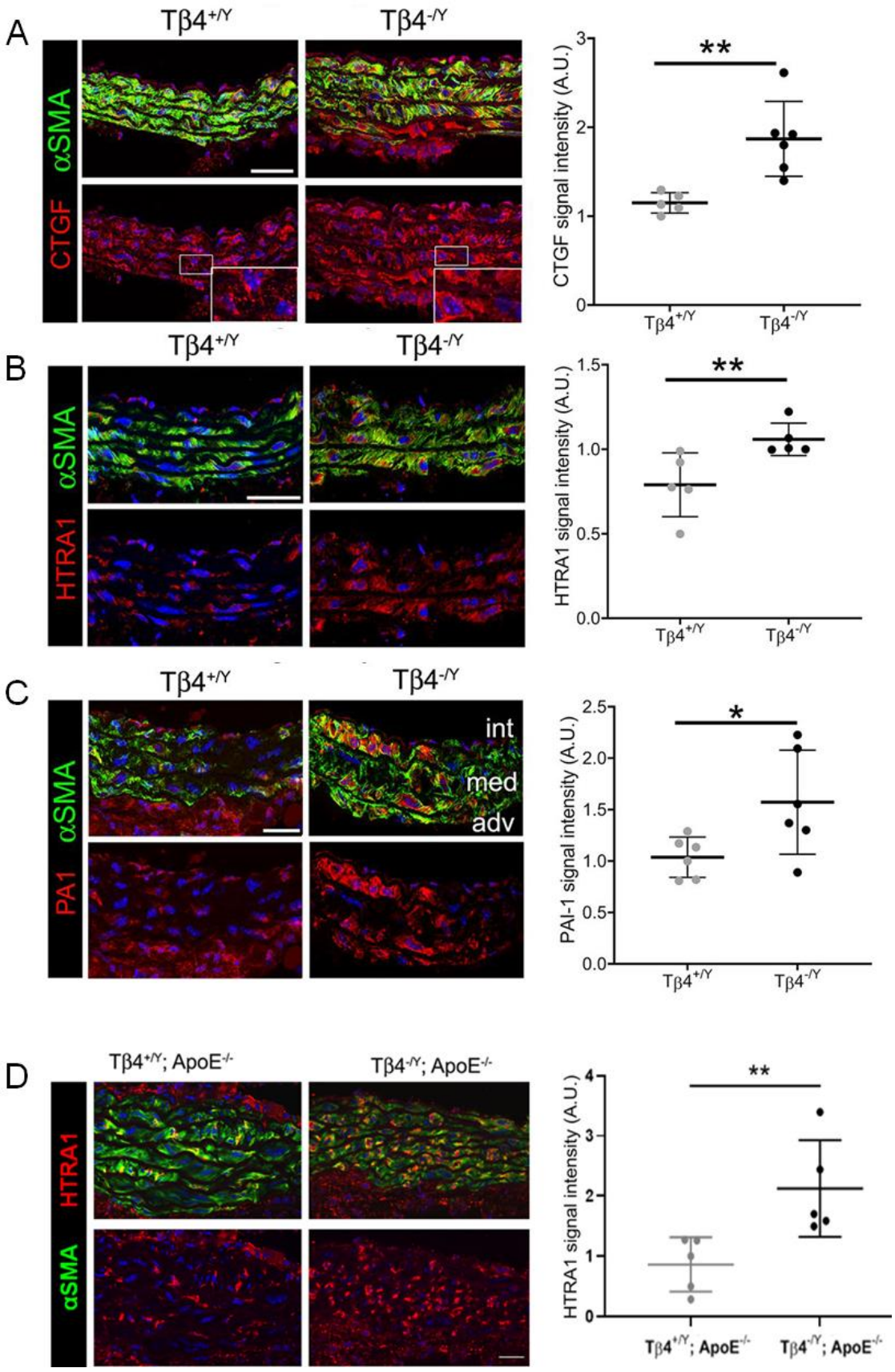

A
Tβ4+/Y
Tβ4-/Y
αSMA
CTGF
CTGF signal intensity (A.U.)
**
B
HTRA1
HTRA1 signal intensity (A.U.)
C
PA1
int
med
adv
PAI-1 signal intensity (A.U.)
*
D
Tβ4+/Y; ApoE-/-
Tβ4-/Y; ApoE-/-

***Figure 5.4: Loss of Tβ4 affects endocytosis of LRP1 ligands.*** *Matricellular proteins CTGF and HTRA1 are LRP1 ligands regulated by endocytosis. Aneurysmal aortas from Tβ4$^{-/Y}$ show accumulation of CTGF (**A**), HTRA1(**B**) and PAI-1 (**C**) in medial VSMCs after 5 days' AngII infusion, (**D**) increased accumulation of HTRA1 was also observed in Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice fed on WD for 9 weeks, indicating dysregulated endocytosis of LRP1 ligands in the absence of Tβ4. Data are presented as mean ± SD, with each data point representing an individual animal with aortic sections from 5 to 6 animals assessed in **A**, **B**, **C** and **D**. Significance was calculated using a Mann Witney non-parametric test. * P≤ 0.05; ** P ≤ 0.01. Scale bars: **A-B**: 50μm, **C**: 100μm. Immunostaining of aortic section for CTGF, HTRA1 and PAI-1 in AAA (panels **A-C**) published in[170], © J.Clin.Invest.*

### 5.3.3 Tβ4 modulates intracellular trafficking of LRP1-PDGFRβ complexes

To evaluate the mechanism through which loss of Tβ4 results in dysregulated LRP1-PDGFRβ signalling, *in vitro* studies were performed on mouse aortic smooth muscle cells (MOVAS-1) by Dr Susann Bruche in the Smart group. As shown in **Appendix Figure 2 A,** MOVAS-1 cells treated with 20ng/ml PDGF-BB show activation of the signalling cascade and, interestingly, the activation was both enhanced in magnitude and more sustained in duration in Tβ4 knockdown (Tβ4 siRNA) cells. To explore this, cell surface levels of LRP1 and PDGFRβ were assessed using surface biotinylation assays over the same 60-minute time course of PDGF-BB treatment. Surface levels of both LRP1 and PDGFRβ peaked 5 minutes after PDGF-BB treatment both in control and knockdown cells, following which the levels declined up to 30 minutes, suggesting rapid internalisation of the receptors. However, whereas levels of LRP1 continue to decline further and remained significantly below baseline through to 60 minutes in the control cells, receptor levels in Tβ4 siRNA-treated cells recovered, almost to baseline, by 45 minutes (**Appendix Figure 2 B**). Similar profiles were observed for PDGFRβ except that the recovery of the receptor in Tβ4 siRNA-treated cells after 45mins was much steeper (**Appendix Figure 2 C**). Results from the surface biotinylation assays suggested that

Tβ4 is not required for translocation of receptors to the membrane or the initial steps of receptor-mediated endocytosis, but that, in the absence of Tβ4, LRP1-PDGFRβ complexes preferentially recycled to the cell surface, rather than being targeted for lysosomal degradation, which may explain enhanced and sustained pathway activation in absence of Tβ4.

These results were encouraging and demanded a more rigorous study to determine how Tβ4 regulated LRP1-PDGFRβ signalling. Thus, the subcellular distribution of LRP1-PDGFRβ complexes was tracked as proximity ligation assay (PLA) signals through successive endocytic compartments within primary aortic VSMCs by Dr. Nicola Smart. Serum-starved primary murine aortic VSMCs from $T\beta4^{+/Y}$ and $T\beta4^{-/Y}$ mice were treated with PDGF-BB over a 60-minute time course. The proportion of LRP1-PDGFRβ co-localisation to early endosomes (EEA1), late endosomes (Rab7), recycling endosomes (Transferrin receptor; TfR), and lysosomes (LAMP-1) were quantified using the ImageJ plug-in JACoP v2.0[257]. In both $T\beta4^{+/Y}$ control cells and $T\beta4^{-/Y}$ cells, LRP1-PDGFRβ internalisation increased over the first 10- and 30-minutes treatment, evidenced by increased distribution of LRP1-PDGFRβ was assessed within EEA1+ early endosomes and Rab7+ late endosomes. However, the level of the receptor complex was much higher in $T\beta4^{-/Y}$ cells. At 60 mins the levels of the receptor complex declined to below baseline levels in $T\beta4^{+/Y}$ control cells, in contrast to $T\beta4^{-/Y}$ cells where LRP1-PDGFRβ remained elevated in both early and late endosomes over the 60-minute time course, as shown in **Figure 5.5 A-C**, suggesting that receptor complexes were retained for longer in active signalling compartments in cells lacking Tβ4.

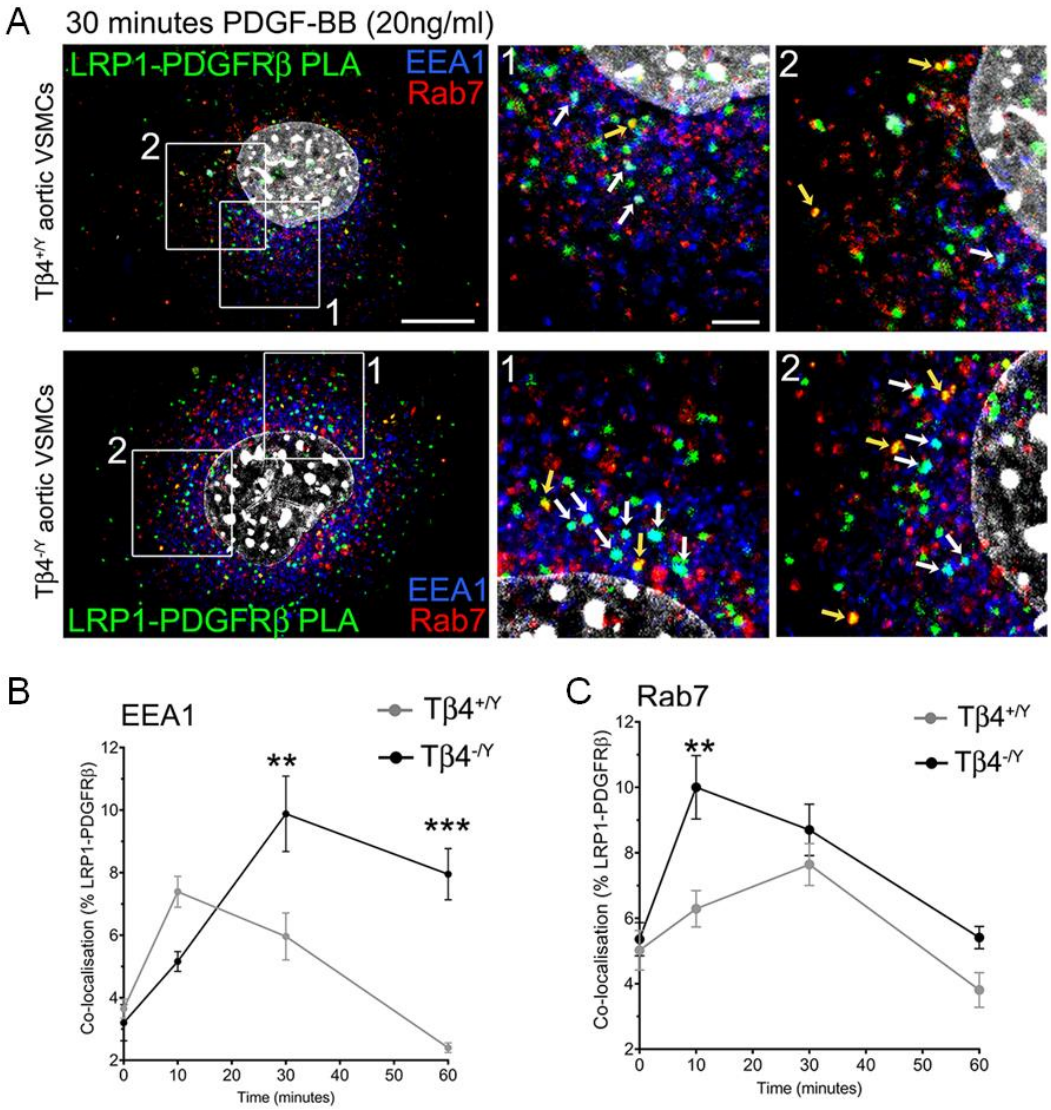



***Figure 5.5: Dysregulated LRP1-PDGFRβ receptor trafficking in cells lacking Tβ4.***

*LRP1-PDGFRβ complexes, indicated by proximity ligation assay (PLA), trafficking through endocytic compartments of primary aortic VSMCs over a 60-minute time course (**A-C**), Shown at 30 minutes, co-localising with early endosomes (EEA1; white arrows, **A**) and late endosomes (Rab7; yellow arrows, **A**). Quantification of co-localisation in **B**, **C**. Data are presented as mean ± SEM; n=3 experiments. Significance was calculated using one-way ANOVA with Tukey's post-hoc tests. ** P ≤0.01; *** P ≤0.001. Scale bars: **A:** 5μm (applies to all whole cell views in **A**); boxed areas 1 and 2 shown magnified to Left, with scale 2μm (applies to all magnified views). Data is published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution:***

*Intracellular trafficking studies were performed by Dr. Nicola Smart*

Next, the distribution of LRP1-PDGFRβ complexes in lysosomes and recycling endosomes of Tβ4$^{+/Y}$ control cells and Tβ4$^{-/Y}$ cells were compared. Most of the LRP1-PDGFRβ complexes were targeted for degradation and their levels in LAMP-1+ lysosomes peaked after 30 minutes' treatment in Tβ4$^{+/Y}$ control cells; only a small degree of the receptor complex was recycled. However, interestingly, LRP1-PDGFRβ was significantly elevated at all time points in TfR+ recycling endosomes in Tβ4$^{-/Y}$, compared with Tβ4$^{+/Y}$ control cells (**Figure 5.6 A-C**). The unexpected elevation of LRP1-PDGFRβ complex levels in lysosomes at 0 mins in Tβ4$^{-/Y}$ cells (**Figure 5.6 C**) could be an adaptive response to suppress PDGF-BB hypersensitivity during serum starvation.

These findings imply that Tβ4 is required to attenuate PDGFRβ signalling, following PDGF-BB stimulation, by targeting LRP1-PDGFRβ complexes to lysosomes for degradation. Dysregulation of receptor trafficking resulting in failure to down-modulate signalling may explain the advanced dedifferentiation (synthetic phenotype) and proliferation observed at baseline and more prominently during disease.

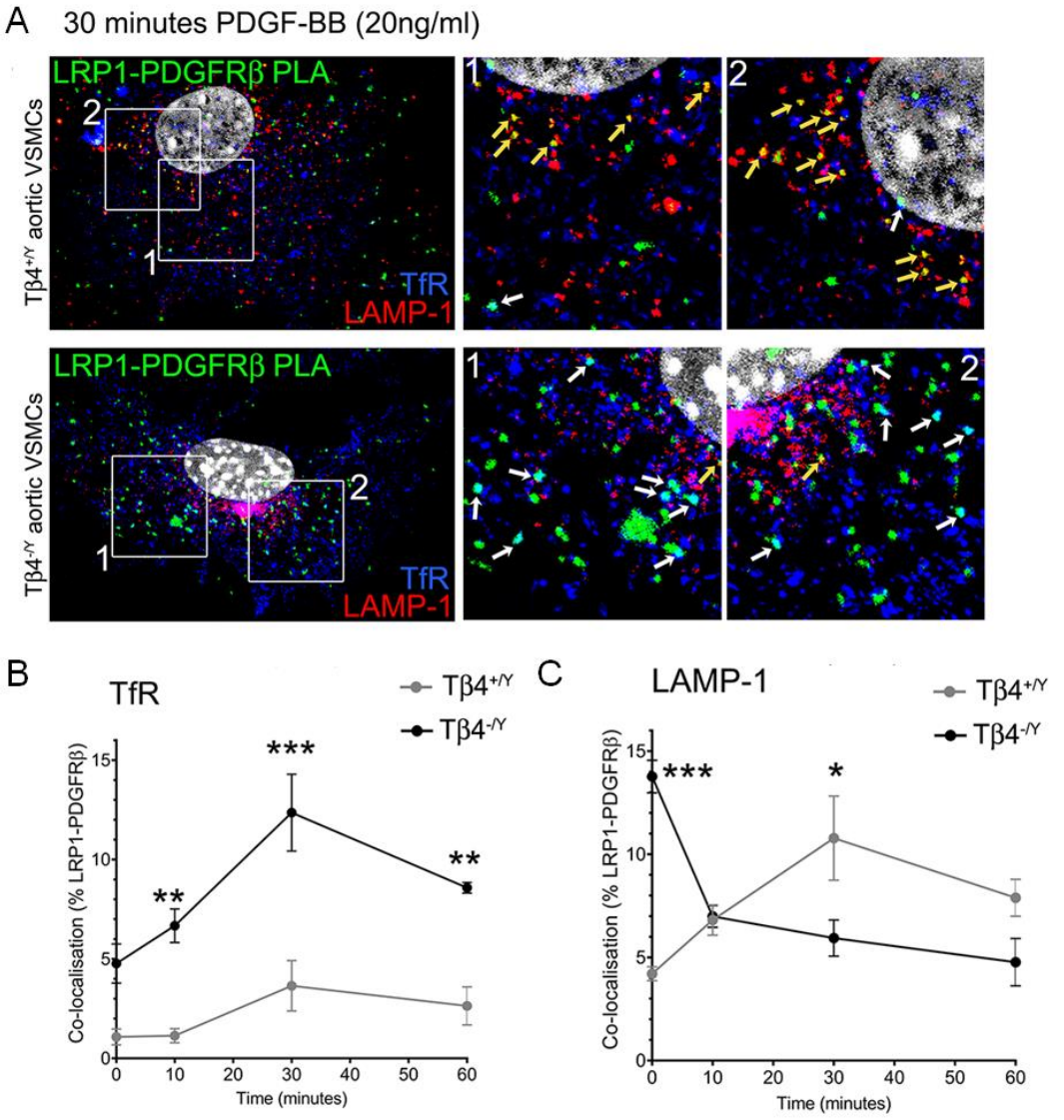



***Figure 5.6: Loss of Tβ4 leads to dysregulated LRP1-PDGFRβ receptor trafficking.***

*LRP1-PDGFRβ complexes, indicated by proximity ligation assay (PLA) in primary aortic VSMCs, trafficking through endocytic compartments over a 60-minute time course (**A-C**). Shown at 30 minutes, co-localising with recycling endosomes (TfR; white arrows, **A**) and lysosomes (LAMP-1; yellow arrows, **A**). Quantification of co-localisation in **B, C**. Data are presented as mean ± SEM; n=3 experiments. Significance was calculated using one-way ANOVA with Tukey's post-hoc tests. * P ≤ 0.05; ** P ≤0.01; *** P ≤0.001. Scale bars: **A:** 5μm (applies to all whole cell views in **A**); boxed areas 1 and 2 shown magnified to left, with scale 2μm (applies to all magnified views). Data is published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution**: Intracellular trafficking studies were performed by Dr. Nicola Smart*

### 5.3.4 Tβ4 regulates LRP1-PDGFRβ signalling in vascular disease

To directly test if dysregulated growth factor signaling is responsible for accelerated disease progression in $T\beta4^{-/Y}$ mice, we sought to block activation of PDGFRβ in a disease model to determine whether the disease phenotype could be ameliorated. Pharmacological inhibition of PDGFRβ signaling via administration of Imatinib was previously shown to reduce atherosclerotic lesion formation and AAA progression, correlating with attenuated VSMC phenotypic modulation [164, 397]. Imatinib is relatively unstable at body temperature which precludes administration via osmotic mini pump. Oral gavage [398] was therefore the preferred route for the Imatinib administration in this study. However, daily oral gavaging over a long period of time leads to chronic esophageal irritation and increases the likelihood of inadvertent tracheal administration, reflux, esophageal perforation, pneumonia and death[399-403]. As the hypercholesteremia model is a chronic model, which would necessitate daily gavaging for a long time, the AngII induced AAA model was selected for this study.

As Tβ4 is naturally expressed in all mice $T\beta4^{+/Y}$ are essentially C57BL6 mice hence a combination of $T\beta4^{+/Y}$, C57BL6 (brought in from Jax) were used as control and $T\beta4^{-/Y}$ mice were administered a previously tested dose of Imatinib (10mg/kg/day) or sterile water daily via oral gavage from 2 days prior to AngII infusion via osmotic mini-pump implantation (1mg/kg/day AngII) until harvest, as described in[166]. The experimental setup is summarized in **Figure 5.7**. Upon harvest, activation of the LRP1 and PDGFRβ receptors and the downstream effector molecules, AKT and p42/p44 were evaluated by immunofluorescence staining and western blotting of aortic samples, respectively.

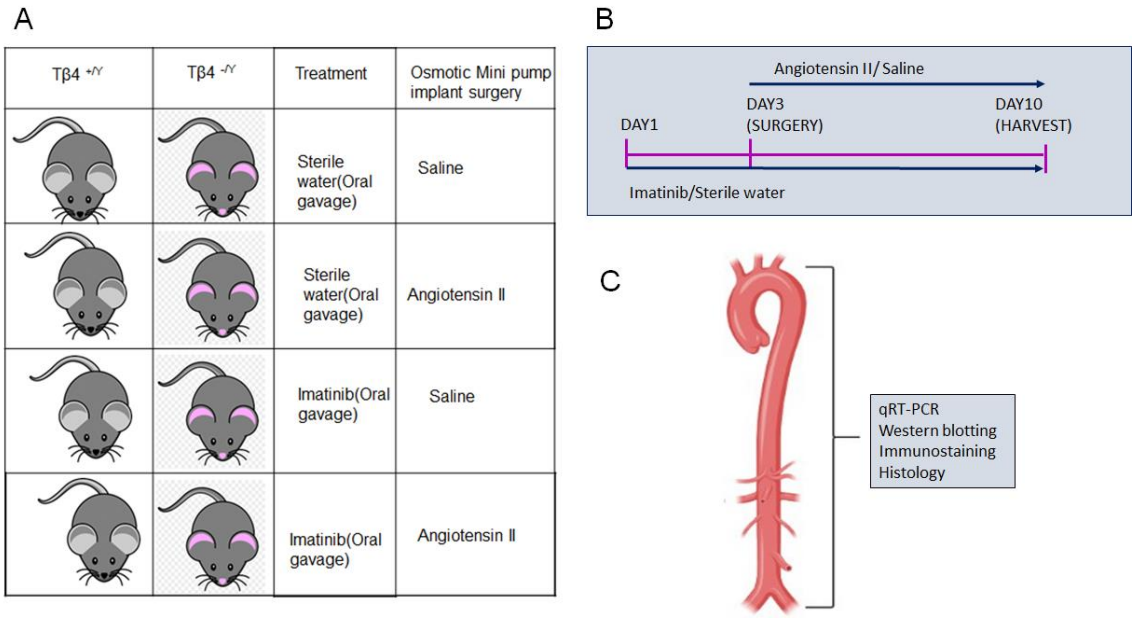



***Figure 5.7: Experimental design to evaluate if blocking LRP1-PDGFRβ can protect against aortic aneurysm in mice lacking Tβ4.*** *Mice were randomly grouped based on treatment (**A**), a daily dose of 10mg/kg/day Imatinib or saline was administered via oral gavage from two days prior to osmotic mini pump implantation to deliver AngII 1mg/kg/day or saline up to 10 days (**B**). Upon harvest, the aortas were subjected to histological and pathological assessments by qRT-PCR, western blotting, and immunostaining (**C**).*

As expected, Imatinib administration attenuated activation of both LRP1 and PDGFRβ in AngII treated Tβ4$^{+/Y}$ and Tβ4$^{-/Y}$ mice compared to vehicle treated mice. It must be noted that Tβ4$^{+/Y}$ refers a mix of the two control strains. Moreover, imatinib treatment restored activation of PDGFRβ and LRP1 in aneurysmal Tβ4$^{-/Y}$ mice to similar levels as in AngII treated control mice (**Figure 5.8, A, B**). Next, the levels of phospho-AKT and phospho-p42/44 in aortic arch lysates from aneurysmal mice treated with vehicle and saline were assessed using western blotting. Attenuating activation of PDGFRβ resulted in

decreased activation of AKT and p42/p44 in Tβ4$^{-/Y}$ mice, to a level which was also comparable to that of Tβ4$^{+/Y}$ mice.

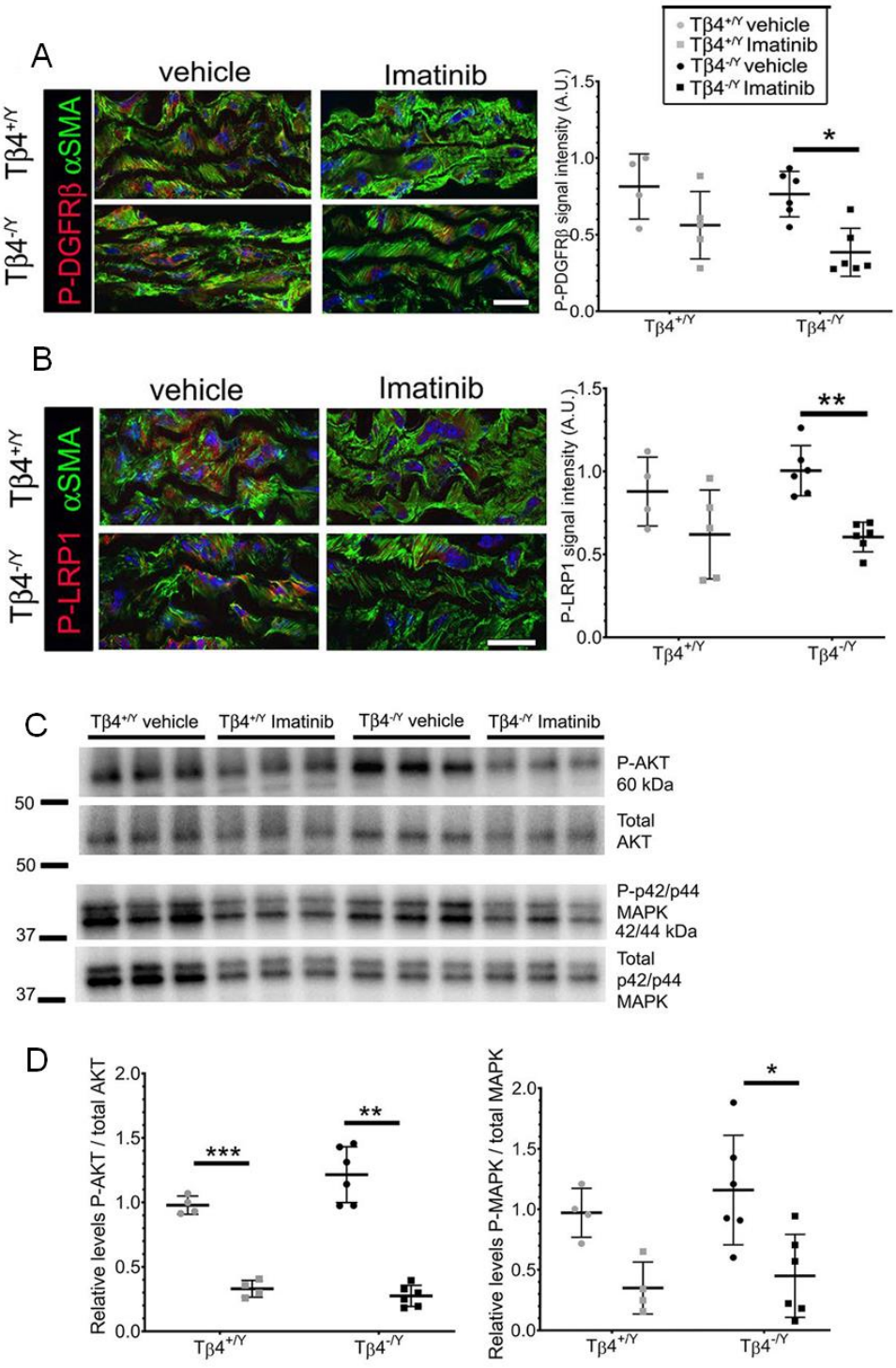


***Figure 5.8: Blocking PDGFRβ activation attenuated downstream signalling in aneurysmal Tβ4$^{-/Y}$.*** *Imatinib attenuated PDGFRβ pathway activity in aortas of AngII-infused Tβ4$^{+/Y}$(Tβ4$^{+/Y}$ refers a mix of the two control strains) and Tβ4$^{-/Y}$ mice, confirmed by immunostaining for phospho-PDGFRβ (Tyr1021) (**A**), phospho-LRP1 (Tyr 4507) **(B**). **C**: western blotting of downstream effectors, phosphorylated AKT and p42/p44 MAPK, expressed relative to total AKT and p42/p44 MAPK, respectively, and quantified by densitometry in (**D**). Data are presented as mean ± SD, with each data point representing an individual animal. n=4 Tβ4$^{+/Y}$ mice where Tβ4$^{+/Y}$ refers a mix of the two control strains and n=6 Tβ4$^{-/Y}$ mice subjected to AngII treatment were treated with vehicle and Imatinib. Significance was calculated using two-way ANOVA with Tukey's multiple comparison tests. * P ≤ 0.05; ** P ≤0.01; *** P ≤0.001. Scale bars: **A**, **B**: 50μm. Data is published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution:*** *Mean signal intensity quantification from immunostained sections were made by Dr Nicola Smart.*

### 5.3.5 Attenuating PDGFRβ signalling ameliorates the aneurysmal phenotype of Tβ4 null mice

To determine if restoration of signalling in Tβ4$^{-/Y}$ aortas was sufficient to preserve vascular integrity, histological analysis was performed on aortic sections from the abdominal region (**Figure 5.9 A**). Imatinib administration reduced aortic diameter by 1.3-fold in aneurysmal Tβ4$^{+/Y}$ and 1.7-fold in aneurysmal Tβ4$^{-/Y}$ (**Figure 5.9 B**), suggesting that Imatinib prevented aneurysm-associated aortic dilatation both in Tβ4$^{+/Y}$ and Tβ4$^{-/Y}$. Noticeably higher elastin degradation was evident in vehicle-treated aneurysmal Tβ4$^{-/Y}$ mice compared to aneurysmal Tβ4$^{+/Y}$; however, Imatinib administration restored elastin integrity and reduced degradation by 1.6-fold in aneurysmal Tβ4$^{-/Y}$, as shown in **Figure 5.9 C**.

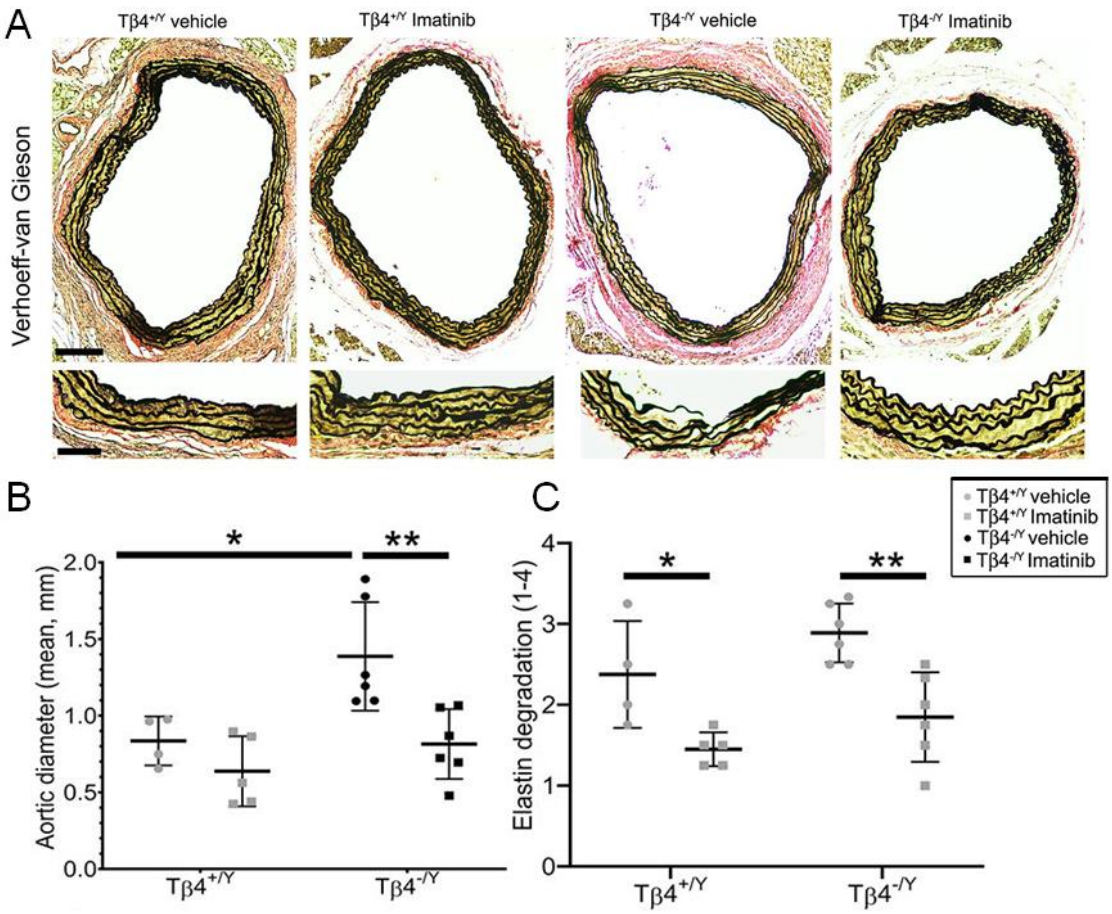



***Figure 5.9: Attenuating PDGFRβ signalling ameliorates the aneurysmal phenotype of Tβ4 null mice.*** *Verhoeff-van Gieson staining (**A**), to quantify aortic diameter (**B**) and assess elastin integrity (degradation score)(**C**) shows normalisation of aortic diameter and preserved elastin integrity in aneurysmal Tβ4 null aortas upon imatinib administration. Data are presented as mean ± SD, with each data point representing an individual animal. n=4 Tβ4$^{+/Y}$ mice where Tβ4$^{+/Y}$ refers a mix of the two control strains and n=6 Tβ4$^{-/Y}$ mice subjected to AngII treatment were treated with vehicle and Imatinib. Significance was calculated using two-way ANOVA with Tukey's multiple comparison tests. * P ≤ 0.05; ** P ≤0.01. Scale bars: **A**: 500μm; inset 100μm. Data published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution**: Histological studies were performed by Dr Nicola Smart.*

Next, VSMC phenotype was evaluated by immunofluorescence staining, western blotting, and qRT-PCR. VSMC contractile phenotype was better preserved in Imatinib administered aneurysmal Tβ4$^{-/Y}$ mice. Immunostaining data and western blotting on aortic biopsies reveal striking retention of canonical contractile markers, such as αSMA (**Figure 5.10 A-B**) and Calponin (**Figure 5.10 C-D**), relative to synthetic marker Caldesmon (**Figure 5.10 A-C and D**) in Imatinib administered aneurysmal Tβ4$^{-/Y}$ mice compared to Tβ4$^{+/Y}$ mice. Furthermore, an increase in the ratio of Sm22α/*Tropomyosin* (**Figure 5.10 B**) in aneurysmal Tβ4$^{-/Y}$ mice administered with Imatinib further confirms preservation of contractile VSMC phenotype at the transcript level, in contrast to the decreased ratio observed in vehicle-treated aneurysmal mice infused, which is indicative of phenotypic switching to a more synthetic state[34].

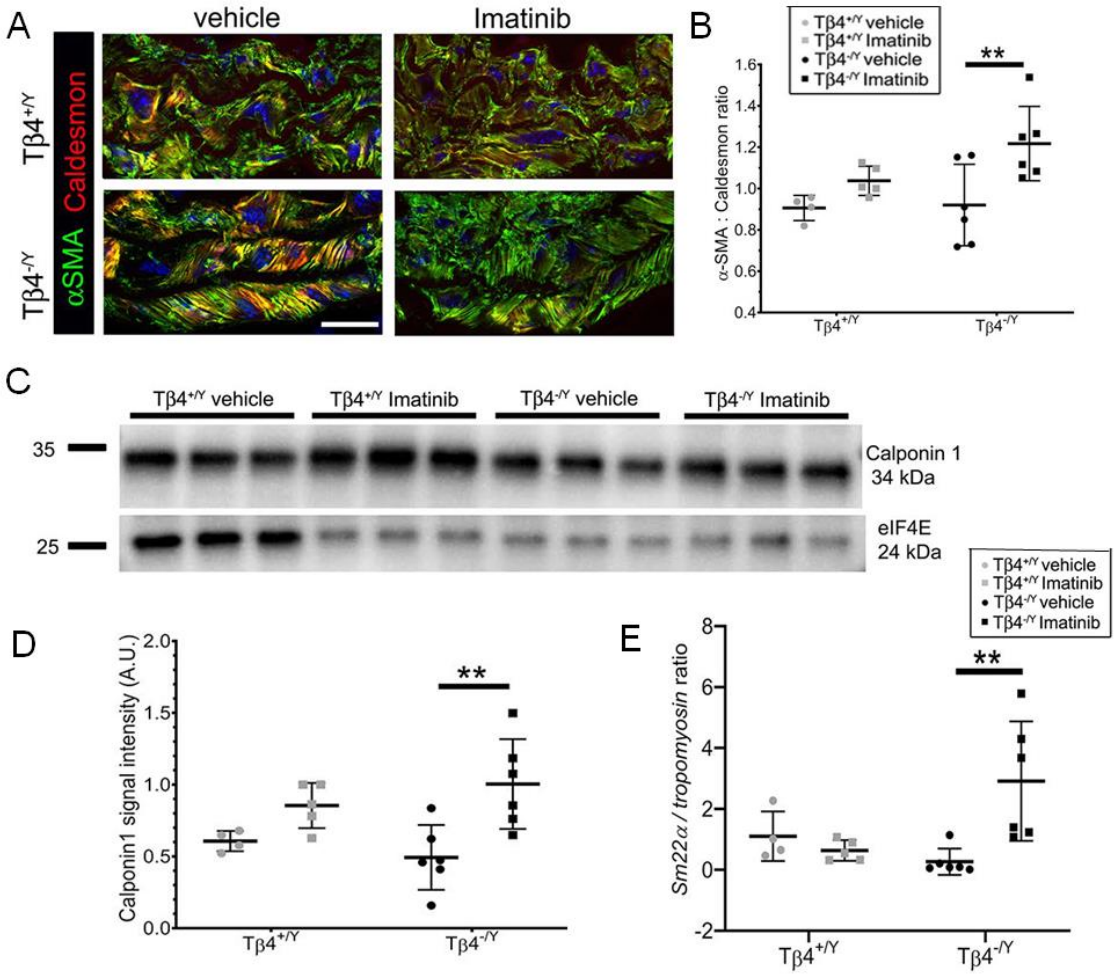



***Figure 5.10: Imatinib treatment attenuated PDGFRβ signalling and preserves the contractile VSMC phenotype of Tβ4 null AAA mice.*** *(**A**) Immunofluorescence staining to evaluate VSMC phenotype modulation in aneurysmal mice treated with Imatinib and vehicle, (**B**) Quantification. Preserved VSMC contractile phenotype in Imatinib treated mice was confirmed by western blotting (**C**), quantification of which shows comparable levels of Calponin1 (contractile marker) in aneurysmal Tβ4$^{+/Y}$ and Tβ4$^{-/Y}$ aortic lysates (**D**). (**E**) qRT-PCR for Sm22α (contractile) and Tropomyosin (synthetic) show increased contractile/synthetic ratio in Imatinib treated Tβ4$^{-/Y}$ aortic lysates. All samples were harvested after 8 days' AngII infusion. Data are presented as mean ± SD, with each data point representing an individual animal. n=4 Tβ4$^{+/Y}$ mice where Tβ4$^{+/Y}$ refers a mix of the two control strains and n=6 Tβ4$^{-/Y}$ mice subjected to AngII treatment were treated with vehicle and Imatinib. Significance was calculated using two-way ANOVA with Tukey's multiple comparison tests.* P ≤ 0.05; ** P ≤0.01. Scale bars: **A**: 50μm. Data published in [170], © J.Clin.Invest.*

## 5.4 Discussion

Identifying the main molecular mechanisms that underpin the pathogenesis of atherosclerosis and AAA is crucial to provide a better insight into the identification of novel therapeutic targets to protect against disease. VSMC autonomous Tβ4 was identified as an important player in protection against AAA[170] and atherosclerosis, as shown in Chapters III and IV. Using *in vivo* and *in vitro* studies, Tβ4 was shown to play an important role in regulating LRP1 mediated PDGFRβ signalling. Vascular defects in Tβ4 null mice characterised by increased contractile-synthetic VSMC phenotypic switching are underpinned by dysregulated growth factor signalling. Alongside data presented in this chapter, published data from the group[170] confirm dysregulated intracellular trafficking of LRP1 and its co-receptor PDGFRβ in cells lacking Tβ4.

Previous studies reveal that LRP1 regulates internalisation and endocytosis of PDGFRβ, however, the mechanistic insight into intracellular trafficking remained elusive[164, 168]. Immunostaining and western blotting data presented in this chapter confirm that loss of Tβ4 expedites activation of LRP1 and PDGFRβ. Hyperactivation of the receptor translates into dysregulated downstream signalling and escalates susceptibility to AAA and atherosclerosis. Moxon *et al* and Chan *et al* proposed an inverse association between the levels of infra-renal aortic LRP1 expression and AAA presence in human patients[153, 404]. A plausible explanation for this discrepancy is that the aortic biopsy specimens collected in both studies were from patients undergoing open surgery with large AAA at an advanced stage. Samples with fewer viable VSMCs would contain less LRP1, whereas the samples showing an increase in activated/ phosphorylated LRP1 in VSMCs shown in this chapter were obtained from early disease stages (5-days of AngII infusion and 3- weeks WD feeding). Besides, studies conducted by Boucher *et al* in smLRP1$^{-/-}$ mice demonstrate that excessive activation of PDGFRβ leads to medial layer

defects, and increase susceptibility to AAA and atherosclerosis[150], suggesting that Tβ4 null mice phenocopy LRP1$^{-/-}$ mice both in health and disease.

Furthermore, blocking the activation of PDGFRβ by administration of Imatinib, in aneurysmal Tβ4$^{-/Y}$ mice demonstrated a protective effect, suggesting that dysregulated PDGFRβ signalling is responsible for the VSMC phenotypic modulation and elastin degradation that leads to medial degeneration. Similar results have also been observed in LDLR$^{-/-}$; smLRP1$^{-/-}$ hypercholesteremia mice, where blocking PDGFRβ activation preserved vessel wall integrity in atherosclerosis[164] and AAA[166]. Although Tβ4$^{+/Y}$ mice have been maintained on a C57BL6 background for more than 20 generations, the C57BL6 mice used in the imatinib experiment showed more susceptibility than Tβ4$^{+/Y}$ mice, and some even more than Tβ4$^{-/Y}$ mice, in the absence of Imatinib. Although we used the same sub-strain of C57BL6/J in this study, a number of studies in past decade have reported multiple genetic differences between different genetic strains of C57BL which may affect results. In addition to sub-strain differences, the inhouse littermate Tβ4$^{+/Y}$ have been subjected to a different environment over several generation and the possibility of genetic drift with natural mutations in an isolated breeding population cannot be ignored[405]. Therefore, in future, littermate controls should be used for such experiments.

Assembly of actin filaments is essential for remodelling of structures at the cell surface to allow inward movement of vesicles. However, the initial activation and internalisation of the LRP1 and PDGFRβ are unaffected by the loss of Tβ4. The endocytosis data reveal that Tβ4 influences the differential sorting of LRP1-PDGFRβ complexes either for lysosomal destruction or receptor recycling to potentiate PDGF-BB signalling. It is well accepted that under physiological conditions most of the ligand-receptor complex that undergoes endocytosis is degraded upon fusion of the late endosomes with

lysosomes[406]. Tβ4 has previously been shown to mediate actin polymerisation required for the fusion of late endosome with lysosome[407] and might explain the reduced lysosomal targeting of the receptor complex in primary aortic VSMC that lacked Tβ4, however, this does not explain the increased distribution of the receptor complex to recycling endosomes. Recycling of signalling receptors has previously been proposed to occur via 'Actin-Stabilized Sequence-dependent Recycling Tubule (ASSERT)' scaffold formation, which requires actin polymerization[408].

Work done by others in the Smart group demonstrated dramatic upregulation of Filamin A (FLNa) levels in Tβ4 KD VSMCs[170] within 10-30 minutes of PDGF-BB treatment. FLNa has previously been shown to crosslink F-actin to mediate recycling of a broad range of membrane receptors, including the chemokine receptor CCR2b, β2-adrenergic receptor, and calcitonin receptor, by controlling receptor entry into endosomal actin microdomains that recruit cargo for the rapid recycling pathway[409, 410]. This may be a more likely explanation for augmented receptor recycling in absence of Tβ4.

In addition to regulating endocytosis and intracellular trafficking of PDGFRβ, the data further advocate a potential role for Tβ4 in endocytosis of other LRP1 ligands, such as HTRA1 and CTGF, which are involved in vascular protection. Loss of Tβ4 leads to accumulation of these proteins in the ECM, which may contribute towards the increased elastolysis in knockout mice in AAA and atherosclerosis. Several studies reveal that LRP1 mediates endocytic clearance of a multitude of extracellular ligands[379, 411]. Earlier studies on aortas from *smLRP1*$^{-/-}$ mice suggest defective endocytosis may lead to increased accumulation of HTRA1 and CTGF resulting in medial degeneration[379]. These findings further support the conclusion that, in absence of Tβ4, LRP1-mediated endocytosis is dysregulated.

Despite the association of LRP1 with several CVDs including AAA and atherosclerosis, the lack of mechanistic insight has been a major limiting factor in the understanding of the disease. While *TMSB4X* is the most abundant transcript in the healthy aorta[345] and has been shown to play an important role in VSMC differentiation during vascular development and in maintain differentiated VSMCs at baseline, yet the role of Tβ4 in vascular protection and regulation of LRP1-mediated growth factor signalling had not been recognised. Results in this chapter are relevant from a clinical perspective as they delineate a mechanism by which endogenous Tβ4 controls LRP1-mediated VSMC responses to protect against vascular disease.

## 5.5 Limitations

It is important to note that intracellular trafficking of the receptor is a dynamic, non-synchronised event and despite serum starvation and PDGF-BB treatment of aortic VSMCs at the same time, some receptor molecules will be internalising, while others recycling. Therefore, it is not possible to estimate the time it takes for each cycle in various endosomes within the cell. Thus, the results are a comparison of the relative level in any endocytic compartment at any given time.

Secondly, in addition to endocytosis, ubiquitination of signalling receptors also increases the efficacy of internalization and sorting to the lysosomes[412] and LRP1 is shown to modulate PDGF signalling by controlling the ubiquitination of the PDGFRβ[381]. The study did not investigate if, and how, ubiquitination of the PDGFRβ is affected in absence of Tβ4 as this may additionally affect receptor activity.

VSMCs of both human and murine AAA express both PDGFR-α and -β[413, 414]. It is also important to note that imatinib used to block activation of PDGFRβ in aneurysmal mice, also regulates several other RTKs including PDGFR-α and bcr–abl fusion protein, c-kit, and Flt3[415]. Therefore, studies using more specific inhibitors are warranted in the future. Alternatively assessing the changes in the activation of PDGFRα and comparing it with the PDGFRβ data may be useful.

Compelling evidence from NMR studies and intracellular trafficking data define a crucial role of Tβ4 in regulating degradation versus recycling of LRP1. However, the study is limited in the sense that it does not define the specific subcellular compartments in which Tβ4 interacts with LRP1 and which specific aspects of receptor endocytosis are controlled by the complex.

## 5.6 Conclusions

In conclusion, the data presented in this chapter provide insight into the molecular mechanism that contributes towards the VSMC phenotypic switching in AAA and atherosclerosis. Receptor recycling plays a critical role in controlling the rate of cellular re-sensitization to signals[416, 417]. Endogenous Tβ4 emerges as an important molecule that mediates vasculoprotective effects, at least in part, via control of LRP1-PDGFRβ exposure on the cell surface, to influence the sensitivity of VSMCs to PDGF-BB and potentially other ligands that are regulated by LRP1.

## Acknowledgement of contributions

*In vitro* signalling, studies were performed by Dr Susann Bruche.

The intracellular trafficking studies and elastin integrity studies were conducted by Dr Nicola Smart.

Dr Andia Redpath performed osmotic mini-pump implantation surgeries and assisted with harvesting mice in the Imatinib cohort.

# Chapter VI

# Exploring the therapeutic potential of exogenous Tβ4

## 6.1 Introduction

Maintaining aortic wall integrity to prevent aneurysmal dilatation and rupture is crucial to overcome potentially life-threatening consequences. Endothelial dysfunction initiates a cascade of events that induce dedifferentiation of VSMCs and degeneration of medial elastin, leading to compromised stability and susceptibility to aneurysm. Prevalence of AAA is 5% in the elderly and increased risk of mortality from aneurysmal rupture demands effective and efficient disease management. However, the asymptomatic nature of the disease poses a major challenge in disease diagnosis and treatment.

In recent years, considerable efforts have focused on identifying potential therapeutic targets for developing novel treatments using animal models and studies in human explant cultures to reverse, regress, or retard the progression of AAA. Some of these are listed in (**Figure 6.1**)[418, 419]. Intriguingly, most of the pharmacological therapies proposed so far have focused on reducing inflammation, oxidative stress, or inhibiting proteases that destroy the ECM. However, despite some preclinical success, these therapeutics have generally failed clinical validation.

Lack of efficient pharmacological therapeutics means surgical intervention remains the only proven management strategy currently. Despite some benefits, surgical interventions performed at an advanced stage are associated with several risks including postoperative mortality due to infection and organ failure, thus demanding an urgent need to develop effective pharmacological treatments to protect against AAA. Findings

from many laboratories, along with the data previously presented in this thesis, strongly emphasise the importance of VSMCs both in the early and late stages of the disease, suggesting the potential to develop novel therapeutics that protect medial VSMCs.

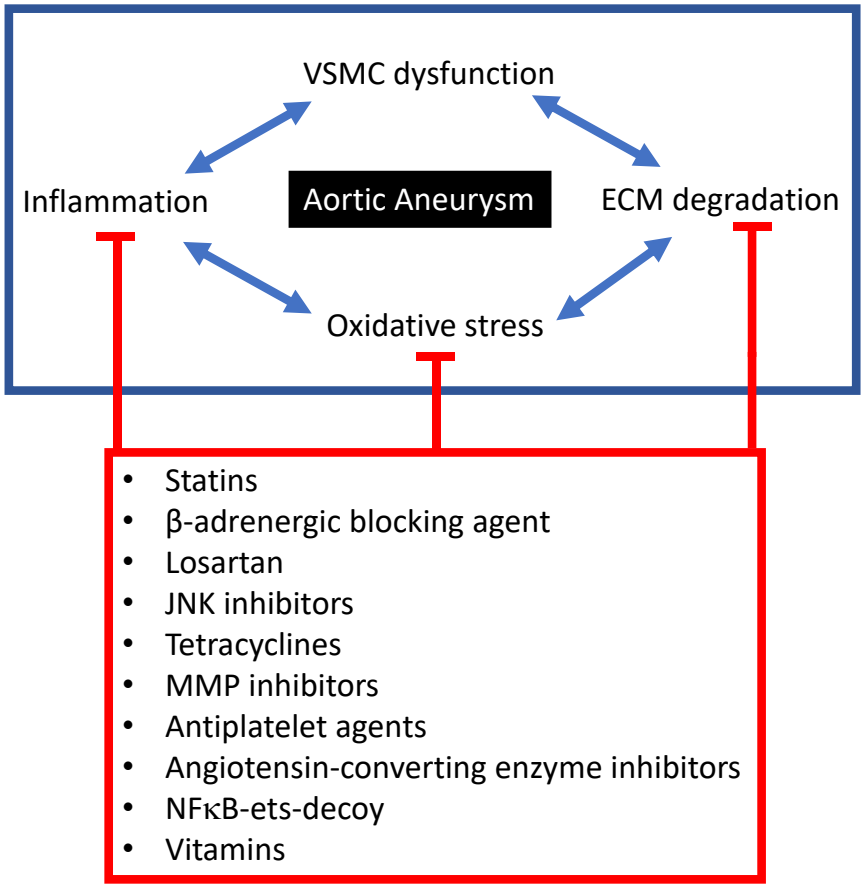



***Figure 6.1: Pharmacological therapies that have been tested to treat AAA.*** *Oxidative stress, Inflammation, VSMC dysfunction, and ECM degradation are the major pathological changes associated with aneurysm development and are shown in the blue box. Listed in the red box are therapeutic compounds that have so far shown preclinical beneficial effects but either did not directly treat the condition (statins, β-adrenergic blocking agents) or failed clinical validation.*

### 6.1.1 Is Tβ4 a potential therapeutic agent for the treatment of AAA?

Tβ4 has recently emerged as a key player in vascular protection[170, 206, 243]. Evidence provided in Chapters III & IV of this thesis demonstrates the vascular protective role of endogenous Tβ4 against atherosclerosis and AAA. We identified an interaction between Tβ4 and the GWAS-implicated vasculoprotective receptor LRP1, which is associated with signal transduction and receptor recycling[170]. LRP1 is involved in vascular remodelling, inflammation, differentiation and cell migration[150], roles shared with Tβ4. Chapter V defined an endogenous role for Tβ4 in controlling endocytic trafficking of the LRP1-PDGFRβ complex to prevent growth factor controlled VSMC phenotypic modulation, which underlies aortic disease progression.

Whilst Tβ4 has several endogenous functions, there has been increasing interest to understand the role of exogenous Tβ4 as a potential therapeutic agent. Tβ4 is found in almost every mammalian cell at concentrations of 0.3–0.4 mM [175, 420], yet exogenous administration of even small concentrations of the peptide is shown to have potent benefits in a range of diseases e.g. myocardial infarction [191, 421]. There is a paucity of information with regards to the tissue pharmacokinetics of Tβ4 and the optimal therapeutic levels in blood or tissue[422], however, Tβ4 is shown to be well tolerated over a wide range of doses both in humans trials and animal studies[217, 423, 424]. Human clinical trials studied the efficacy of Tβ4 over a dose range of 42-1260 mg [425], in the treatment of venous stasis ulcers[234] and dermal wound healing, and reported a biphasic response in patients and rapid wound healing at the mid-dose level of Tβ4[234]. In animal studies, a dose range of 6-12mg/kg is shown to be well tolerated without signs of adverse reactions[238, 422, 426].

Recently, it was identified that the N-terminal ac-SDKP tetrapeptide is responsible for some of the anti-inflammatory and anti-fibrotic properties of Tβ4, additionally, the N-terminal (ac-$^{1}$SDKPDMAEIEKFDKS$^{15}$) sequence was shown to regulate the anti-apoptotic functions[191, 194, 214, 427]. Additionally, Tβ4 has been shown to regulate endocytosis even when administered exogenously[208, 209]. Pharmacological application of Tβ4, either via intraperitoneal or intracardiac routes in experimentally induced MI dramatically reduced apoptosis of cardiomyocytes in the infarct zone[191]. Tβ4 administration was shown to protect human corneal epithelial cells against oxidative damage by upregulating anti-oxidative enzymes[428].

Tβ4 is a small intracellular peptide (4.9kDa) found in the cytosol and the nucleus[178]. Due to the small size and lack of a signal sequence, earlier studies suggested that it readily diffused in and out of the cell. Moreover, it is postulated that after secretion or exogenous administration, once Tβ4 enters the circulation, it is rapidly oxidized to produce Tβ4 sulfoxide; while oxidation of the peptide attenuates its intracellular actin sequestering activity, it enhances its extracellular signalling properties[182], albeit these mechanisms are yet to be elaborated. More recently, Freeman *et al* (2011) identified $F_1$-$F_0$ ATP synthase as an extracellular receptor to which Tβ4 could bind, to induce ATP synthesis near the plasma membrane, and demonstrated that silencing of the ATP-responsive purinergic receptor P2X4 blocked Tβ4-induced HUVEC migration in culture[207]. Tβ4 treatment was shown to prevent cell death and promote cell survival and migration post coronary ligation in adult mice via ILK-mediated AKT activation[191]. Due to the ability of Tβ4 to promote and preserve VSMC differentiation[170, 206], and its role in cell survival[191, 421, 429], wound healing[430] and in beneficially modulating inflammation[217, 220], Tβ4 is a promising therapeutic candidate to protect against AAA.

## 6.2 Aim

Using the well-established AngII-induced AAA model, the studies described in this chapter aimed to examine the therapeutic potential of exogenously administered Tβ4 to protect against AAA formation and progression.

## 6.3 Results

### 6.3.1 Exogenous Tβ4 attenuates aneurysm formation in experimental mice

In order to determine the effect of exogenous Tβ4 in vascular protection in AAA, age-matched adult male C57B6/J mice were simultaneously infused with AngII (1mg/kg/day) and either Tβ4 (12mg/kg/day) or saline, using two subcutaneous osmotic mini pumps implanted for 14 days. Whole-mount imaging of the aortas at the 14-day time point revealed a high incidence of aneurysm, both in the aortic arch and descending aorta of AngII+Saline treated mice while a reduced incidence of aneurysm was observed at both sites in mice treated with Tβ4, as shown in **Figure 6.2**, suggesting a vascular protective role of exogenous Tβ4 against aortic aneurysm.

Additionally, a mouse from each of the AngII+Saline and AngII+Tβ4 cohorts suffered fatal rupture. While the mouse from the saline cohort was found dead at 6 days of AngII infusion, the mouse from the Tβ4 cohort survived 13 days post- surgery, this warranted a detailed analysis of the changes in the aortic media in terms of elastin integrity, VSMC morphology, marker expression and inflammation in the presence of exogenous Tβ4 in the AngII-induced aneurysm model.

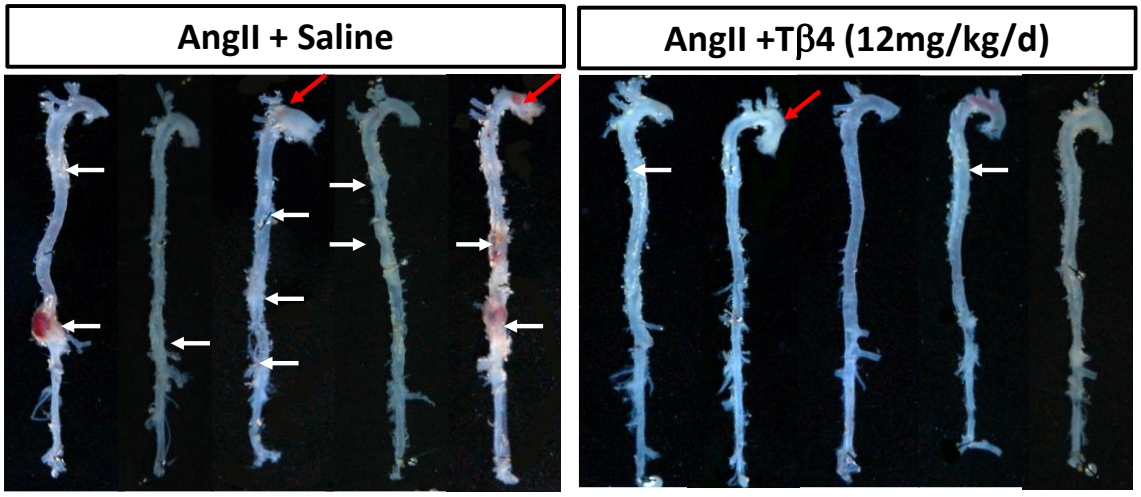



***Figure 6.2: Exogenous Tβ4 reduced AngII-induced aneurysm formation in both the ascending and descending aorta.*** *Whole mount images of aortas from AngII+Saline and AngII+Tβ4 treated mice 14 days post-surgery. Red arrowheads indicate ascending and white arrowheads indicate descending aortic aneurysms. Representative images of n=6.*

***Acknowledgement of Contribution****: Dissections and whole mount imaging were performed by Dr Nicola Smart.*

### 6.3.2 Tβ4 treatment preserved medial layer integrity in AngII induced AAA

#### 6.3.2.1 Reduced elastin fragmentation in Tβ4-treated mice

Verhoeff van Gieson staining on paraffin-embedded aortic sections indicated a striking reduction in the number of elastin breaks in the aortas of AngII+Tβ4 treated mice, compared to those from AngII+Saline treated mice, as shown in **Figure 6.3 A.** Due to the known antifibrotic properties of Tβ4, expression of CTGF- a known fibrosis marker was assessed, levels of which were previously shown to be reduced in models of pulmonary hypertension and right ventricular hypertrophy upon Tβ4 administration[223]. Quantification of the immunostained sections demonstrate a reduction in CTGF expression, in Tβ4 treated mice compared to saline-treated mice (**Figure 6.3 B**) and supports the anti-fibrotic property of Tβ4.

#### 6.3.2.2 Tβ4 treatment reduced AngII-induced aortic dilatation

An increase in aortic diameter is a critical indicator of disease progression, hence aortic diameter measurements were made on histological sections. As expected, AngII significantly increased aortic diameter, compared with its vehicle (P=0.0009) an effect that was significantly attenuated by exogenous Tβ4 treatment (P=0.0052), as shown in **Figure 6.3 C.** The aortic diameter of AngII+Tβ4 treated mice was increased compared with non-AngII treated mice, albeit not significantly (**Figure 6.3 C**), suggesting that Tβ4 did not entirely negate the effect of AngII, despite being administered simultaneously, however, Tβ4 infusion reduced the severity of the disease.

#### 6.3.2.3 Tβ4 treatment reduced AngII induced aortic rupture

In addition to fatal rupture, contained, non-fatal ruptures were observed in both cohorts. Contained ruptures were recognised by hematoma sealed within the aorta[431]. **Figure 6.3 D** is an example of a mouse aorta treated with AngII+Saline showing contained rupture with blood leak around the aorta. Interestingly, despite the rupture, the mouse survived until harvest on day 14. 80% of aneurysmal mice treated with saline suffered from non-fatal, contained ruptures, compared to only 10% of mice in the Tβ4 treated cohort (**Figure 6.3 E**).

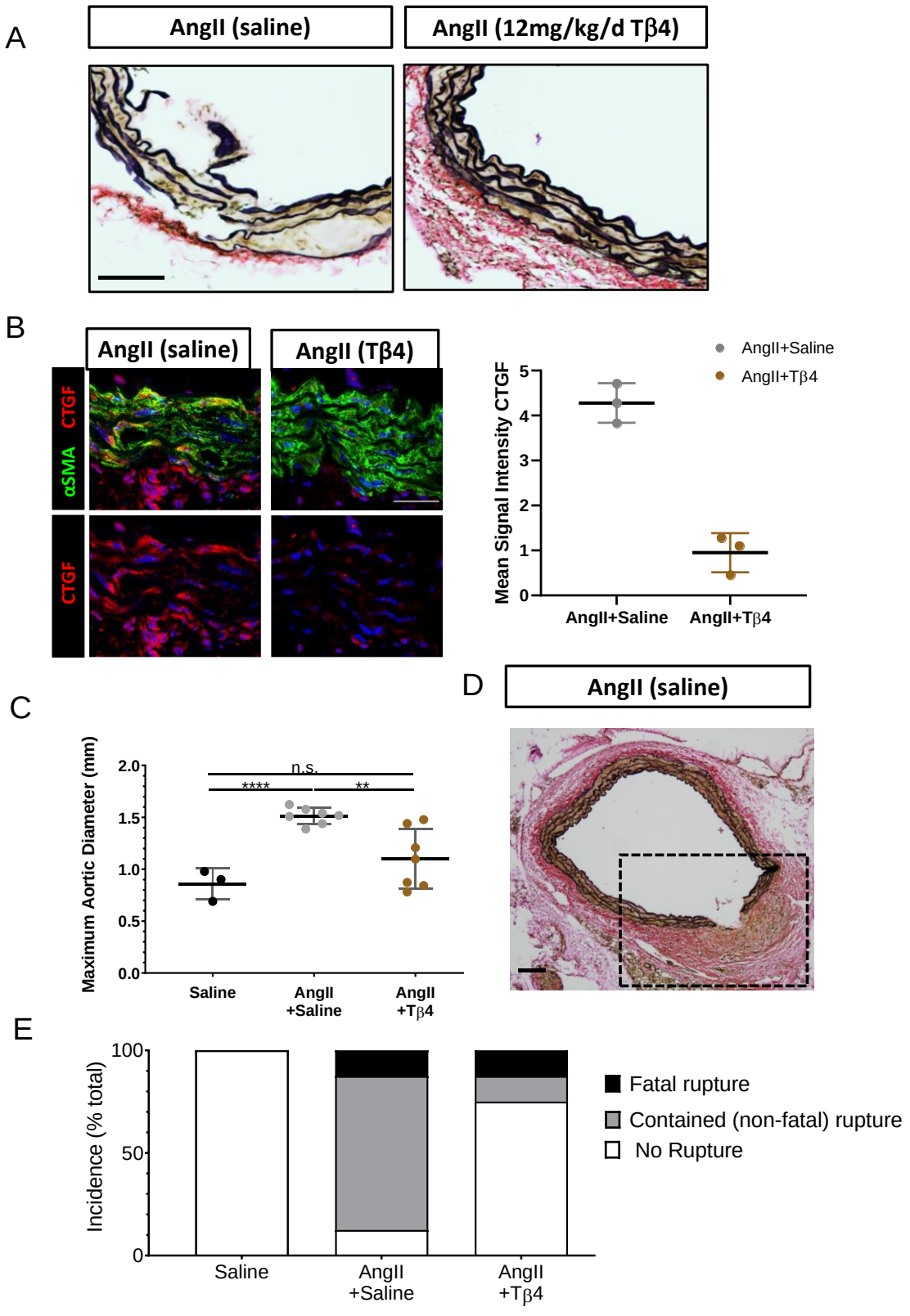



***Figure 6.3: Tβ4 administration preserved elastin integrity and reduced fibrosis and aortic dilatation in AngII model.*** *Aortic sections from AngII+Saline and AngII+Tβ4 mice*

*14 days post infusion was assessed using **A**) Verhoeff-van Gieson staining to evaluate elastin integrity; **B**) immunostaining with αSMA and CTGF and quantification from n=3. **C**) Comparison of maximum aortic diameter between Saline (n=3), AngII+Saline and AngII+Tβ4 treated mice (n=7). (**D**) Aortic section from an AngII+Saline treated mouse harvested at 14 days showing a contained rupture; (**E**) Incidence plot comparing intact aortas versus fatal and non-fatal ruptures in Saline, AngII+Saline, and AngII+Tβ4 treated mice. Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using Mann Witney non-parametric test in **B** and one-way ANOVA in **C**. n.s= not significant *** P ≤ 0.001. Images in **A** and **B** are representative images of n = 6.Scale bars: **B & D**=100μM.*

***Acknowledgment of Contribution**: Histology and elastin integrity in panels A and D performed by Dr Nicola Smart.*

### 6.3.3 Exogenous Tβ4 preserved VSMC phenotype in the AngII model of AAA

To determine the effect of exogenous Tβ4 on VSMC phenotype, gene expression analysis was performed using qRT-PCR and the ratio of contractile (*Sm22α and Myh11)* to synthetic *(Caldesmon, Tropomyosin, and Vimentin)* gene expression was determined. Despite the biological variability between samples, a significant increase in contractile-synthetic gene ratio was observed in aneurysmal mice treated with Tβ4 compared to AngII+saline treated mice (**Figure 6.4 A-B**). These results imply that Tβ4 treatment preserved contractile phenotype and retarded the switch to the synthetic state in AngII-treated mice.

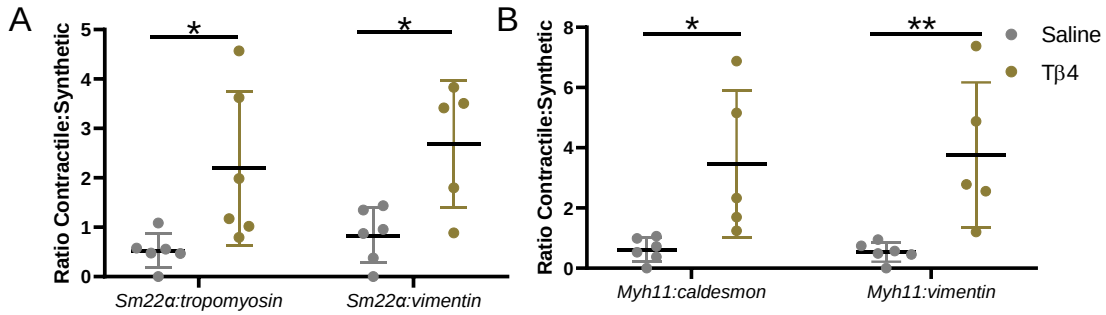


***Figure 6.4: Gene expression analysis indicates preserved contractile phenotype in aneurysmal mice treated with Tβ4****. qRT-PCR on aortic biopsies indicate a significant increase in* ***A****) Sm22α: tropomyosin, Sm22α: vimentin; (****B****) Myh11: caldesmon, Myh11: vimentin; expression in AngII+Tβ4, compared to AngII+Saline aortic biopsies. Data are presented as mean ± SD, with each data point representing an individual animal where n=5. Significance was calculated using two-way ANOVA with Šídák's multiple comparisons test for* ***A*** *and* ***B****. *p ≤ 0.05, ** P ≤ 0.01.*

Results at the transcriptional level encouraged the evaluation of VSMC phenotype by immunostaining, hence cryosections were immunostained with contractile (αSMA) and synthetic (Caldesmon) markers. Sections from AngII+Saline mice showed multiple regions within the aortas devoid of cells, which could be due to increased VSMC death, compared to AngII+Tβ4, as a result, quantification of marker expression was problematic. Despite the difficulty this may present, taking a ratio of the channels for the respective markers suggested increased intensity and expression of Caldesmon, relative to αSMA, in aortic VSMCs of mice treated with AngII+Saline compared to those in AngII+Tβ4 aortas, as shown in **Figure 6.5 A**.

Expression of Caldesmon was also observed in peri-adventitial cells. **Figure 6.5 B** shows high-power image of aortic sections from a mouse treated with AngII+Saline with substantial upregulation of Caldesmon in peri-adventitial cells. Intriguingly, some of these cells co-expressed CTGF and HTRA1 (**Figure 6.5 C-D**). While αSMA expression was mostly confined to the medial VSMCs, weaker expression was also observed in some cells in the peri-adventitial layer. Based on these findings, it can cautiously be speculated that some of the cells in the peri adventitial layer might be modulated smooth muscle cells. However, to ascertain this, lineage-tracing studies are recommended in the future.

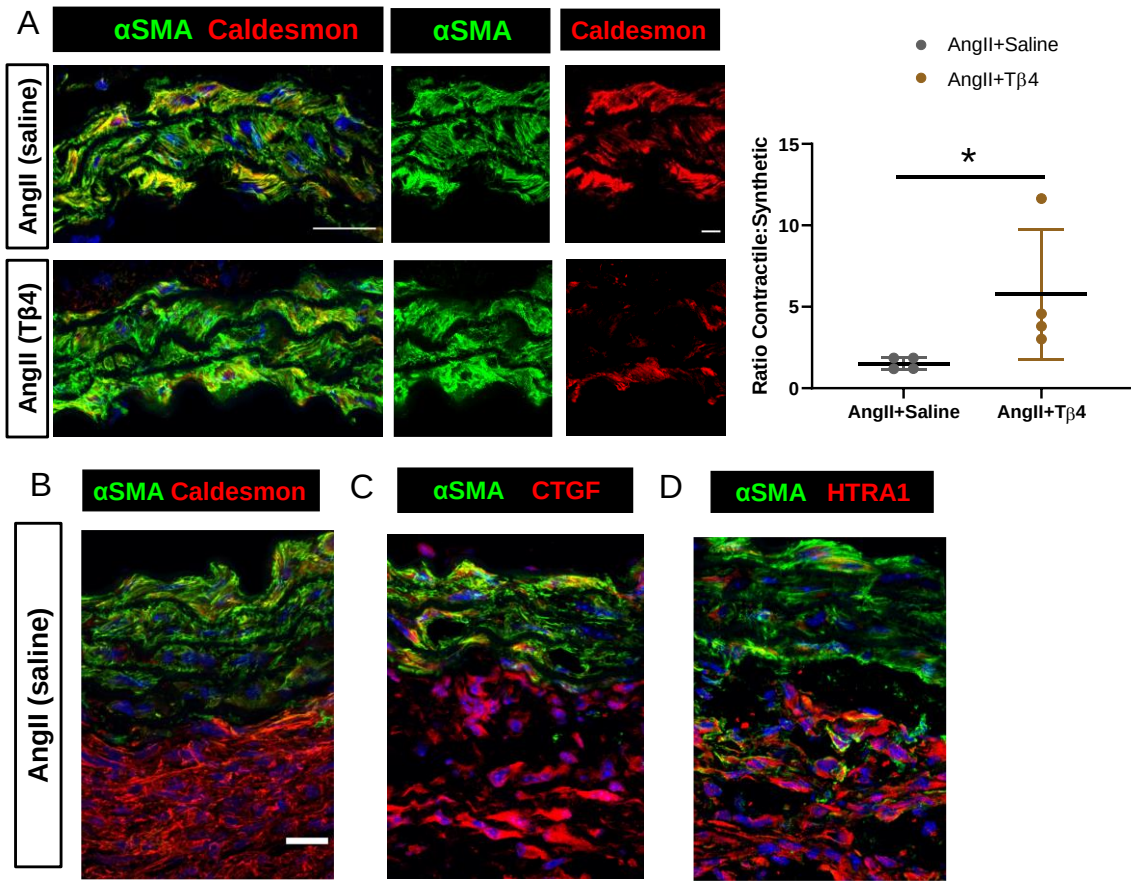



***Figure 6.5: Exogenous Tβ4 treatment preserved contractile phenotype in aneurysmal mice***. *Immunostained aortic sections from AngII+Saline and AngII+Tβ4 treated mice (**A**) at high power(63x) showing αSMA+Caldesmon expression and quantification showing expression of Caldesmon relative to αSMA. A region of immunostained aortic section from a mouse treated with AngII+Saline with increased peri-adventitial expansion is zoomed to show increased αSMA+Caldesmon expression (**B**), αSMA+CTGF expression (**C**), and αSMA+HTRA1 expression (**D**). Images in **A** are representative images of n = 6. Data are presented as mean ± SD, with each data point representing an individual animal. Significance was calculated using Mann Witney non-parametric test. *p ≤ 0.05. Scale bars: **A**: Merged panel (left): 100μM, red and green panels (right): 10μM, **B-D**: 100μM.*

### 6.3.4 Exogenous Tβ4 attenuates inflammation in the advanced stages of aneurysmal disease.

To evaluate if exogenous Tβ4 affects inflammation, aortic sections from mice treated with AngII+Saline and AngII+Tβ4 for 14 days were immunostained with CD68 and αSMA. Based on the expression of CD68, it was apparent that compared to aortic sections from AngII+Saline treated mice, a reduced density of CD68+ macrophages were observed in the aortas of AngII+Tβ4 treated mice, suggesting that administration of Tβ4 in AngII treatment reduces inflammation. Additionally, most aortas from AngII+Saline treated mice showed massive expansion of the peri-adventitial layer predominantly made up of CD68+ macrophages. Expansion of the peri-adventitial layer was also observed in aortas from AngII+Tβ4 mice, however this ranged from minimal to moderate. Although 6 aortas from each group were used for the study, 2 from AngII+Saline cohort were not included in quantification as the aorta were either folded or a small portion of the aorta was missing which may lead to erroneous quantification. This finding supports the anti-inflammatory property of Tβ4[219, 220, 432], however, it is not clear if reduced inflammation is due to a reduction in macrophage proliferation, survival, migration, or promoting macrophage dispersal and will require further study.

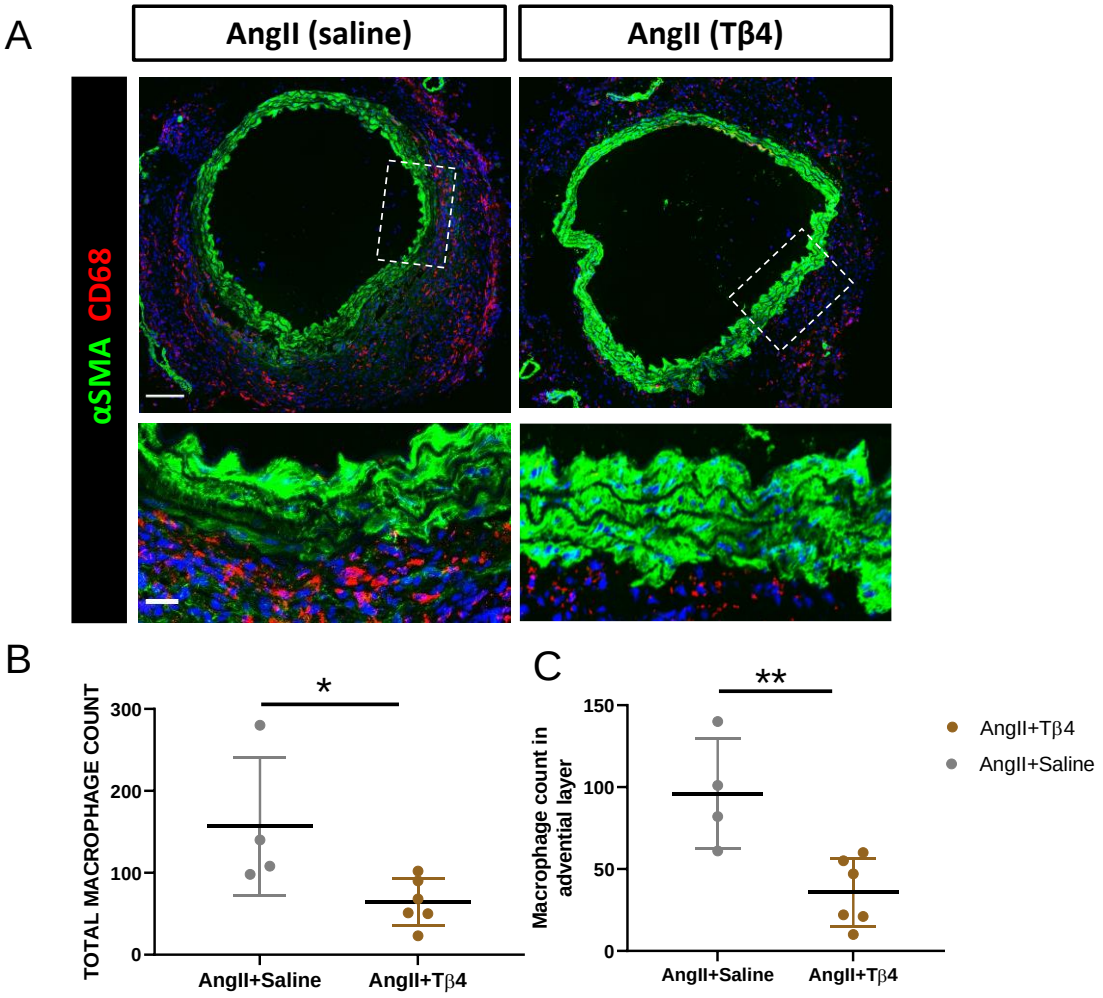



***Figure 6.6: Reduced inflammatory cell infiltration in aneurysmal mice treated with Tβ4 for 14 days.*** *The anti-inflammatory role of Tβ4 was demonstrated based on CD68 staining (macrophages).* ***A****) Aortic sections from AngII+Saline and AngII+Tβ4 treated mice were stained with CD68 and αSMA,* ***B****) quantification of number of macrophages in the medial and adventitial layer and* ***C****) number of macrophages in the adventitial layer. Representative images of n = 6 in* ***A****. Data are presented as mean ± SD, with each data point representing an individual animal where n=4 for AngII+Saline and n=6 for Ang+Tβ4. Significance was calculated using Mann Witney non-parametric test. *p ≤ 0.05; **p≤0.01. Scale bars: Top panel: 500μM, bottom panel:100μM.*

### 6.3.5 Inflammatory responses remain unaltered with Tβ4 treatment at an early stage of aneurysm development

Modulated smooth muscle cells exacerbate inflammation, therefore to determine whether the inflammatory response that initiates aneurysm formation is affected by Tβ4 or if the differences only manifest at later stages, inflammation was evaluated after 5 days of AngII infusion. Firstly, peripheral blood levels of cytokines that are known to drive inflammation in AAA, such as CCL2, IFNγ, IL-6 and TNFα, were measured using automated multiplexed ELISA. No significant difference in the levels of circulating proinflammatory cytokines was observed between the two treatment groups (**Figure 6.7 A-D**).

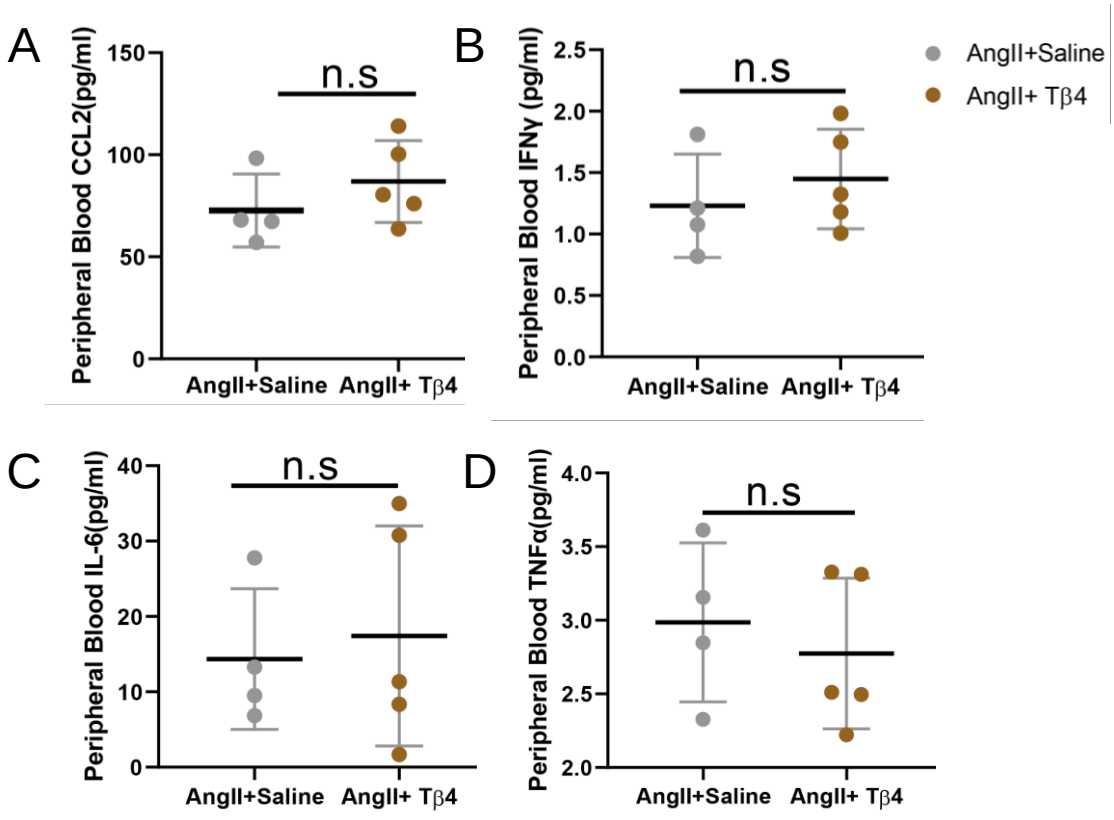



***Figure 6.7: Proinflammatory cytokines in the peripheral blood are unaltered in aneurysmal mice treated with Tβ4 in the early stage of the disease (5 days' AngII).*** *Peripheral blood from AngII+Saline and AngII+Tβ4 treated mice were used to measure levels of proinflammatory cytokines (**A**) CCL2, (**B**) IFNγ, (**C**) IL-6 and (**D**) TNFα using*

*automated multiplexed ELISA. Data are presented as mean ± SD, n=4 with each data point representing an individual animal. Significance was calculated using Mann-Witney non-parametric tests for **A-D**. n.s.=not significant.*

To determine the number of inflammatory macrophages recruited to the descending aorta 5 days post surgery, the aortic region immediately below the arch to just above the iliac bifurcation was excised from AngII+Saline and AngII+Tβ4 treated mice and digested for FACS. Interestingly, there was no significant difference in the number of $CCR2^+$ macrophages recruited between the two treatment groups. In contrast to the immunostaining results observed at 14 days, the FACS data indicated that exogenous Tβ4 did not affect early AngII-induced inflammation, ruling out the possibility that Tβ4 prevented aneurysm induction by directly negating the pro-inflammatory effect of AngII.

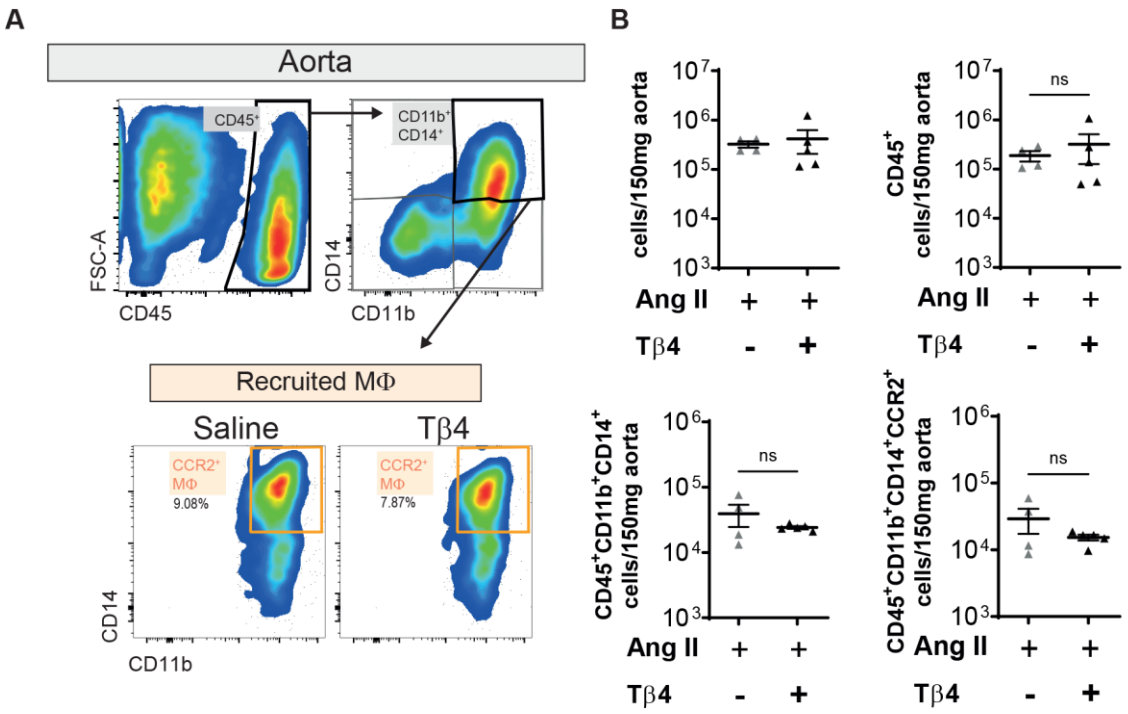



***Figure 6.8: Inflammatory responses are not significantly altered with Tβ4 treatment at an early stage of aneurysm development.*** *Flow cytometry was used to*

*quantify macrophages (MΦ) recruited to aortas of aneurysmal mice treated with saline and or Tβ4 at 5 days post-surgery (gating strategy shown in (**A**), with the percentage of CCR2$^+$ macrophages out of total live cells shown. (**B**) Quantification of total CD45$^+$ leukocytes, CD11b$^+$CD14$^+$ monocytes, and CCR2$^+$ recruited macrophages. n=4 AngII+Saline and n=5 AngII+Tβ4. Significance n.s.= not significant was calculated using unpaired t-test with Welch's correction.*

***Acknowledgment of Contribution**: The flow cytometry assay was performed under the supervision and guidance of Dr Andia Redpath, and analysed by Dr Redpath.*

### 6.3.6 Exogenous Tβ4 exerts a biphasic effect on VSMC migration

To begin to investigate the mechanisms of protection by Tβ4, consideration was given to the PDGF signalling pathway, as endogenous Tβ4 was previously shown to regulate intracellular trafficking and signalling via PDGFRβ in VSMCs of healthy and AAA aorta. PDGF signalling has been implicated in VSMC migration during vascular development and in response to injury[433-435]. Tβ4 is also known to promote the migration of several different cell types[209]. To determine if migration of VSMCs via the PDGF pathway is regulated by exogenous Tβ4, a simple scratch wound assay was conducted in cultured mouse aortic smooth muscle cells (MOVAS-1 cell line)[436]. VSMCs in culture proliferate and migrate in response to growth factors present in serum; therefore, to investigate migration, it is essential to block proliferation, which may otherwise contribute to a decrease in wound size. Addition of Hydroxyurea (HU) to cultured cells has been shown to arrest cell proliferation without affecting cell migration [437]. To determine the optimal dose of HU that arrests VSMC proliferation without causing cell toxicity, a dose-response (25, 30, 40μM HU) study was performed on MOVAS-1 cultured in media containing 0.1% and 1% foetal bovine serum (FBS), the reduction in serum considered necessary to reduce baseline stimulation, in order to determine migration in response to exogenous PDGF-BB. Very high cell death after 24 hrs was observed at all HU concentrations in

0.1% serum. Compared to cells in 0.1%, cells in medium containing 1% serum showed better survival, higher cell survival to cell proliferation ratio was observed at 25µM HU (data not shown) and hence, subsequent migration assays were performed using 25µM HU in 1% serum.

To investigate the effect of Tβ4 on VSMC migration under non-proliferative conditions, MOVAS-1 cells were seeded in 12 well plates in 10% FBS medium and, upon confluency, the medium in the well was replaced with 1% FBS medium. After overnight serum reduction, a scratch wound was inflicted on the MOVAS-1 monolayer, as described in Section 2.7.3, and the wells were treated with 20ng/ml PDGF-BB and Tβ4 (0, 0.5, 5 & 10 µM). Wells were imaged at 0, 6, and 24 hours to assess cell migration. By 24 hrs, wounds treated with 0.5 and 5µM Tβ4 were completely sealed, therefore a 6 hrs time point was used to determine the % of wound closure.

4 independent repeats of the migration assay were performed on MOVAS-1 cells from consecutive low passages as they become increasingly synthetic. MOVAS-1 migration was enhanced by 0.5 and 5 µM Tβ4, whereas this effect was abolished with higher concentrations, as shown with 10µM Tβ4, suggesting a biphasic effect of Tβ4 on the migratory response of MOVAS-1 cells.

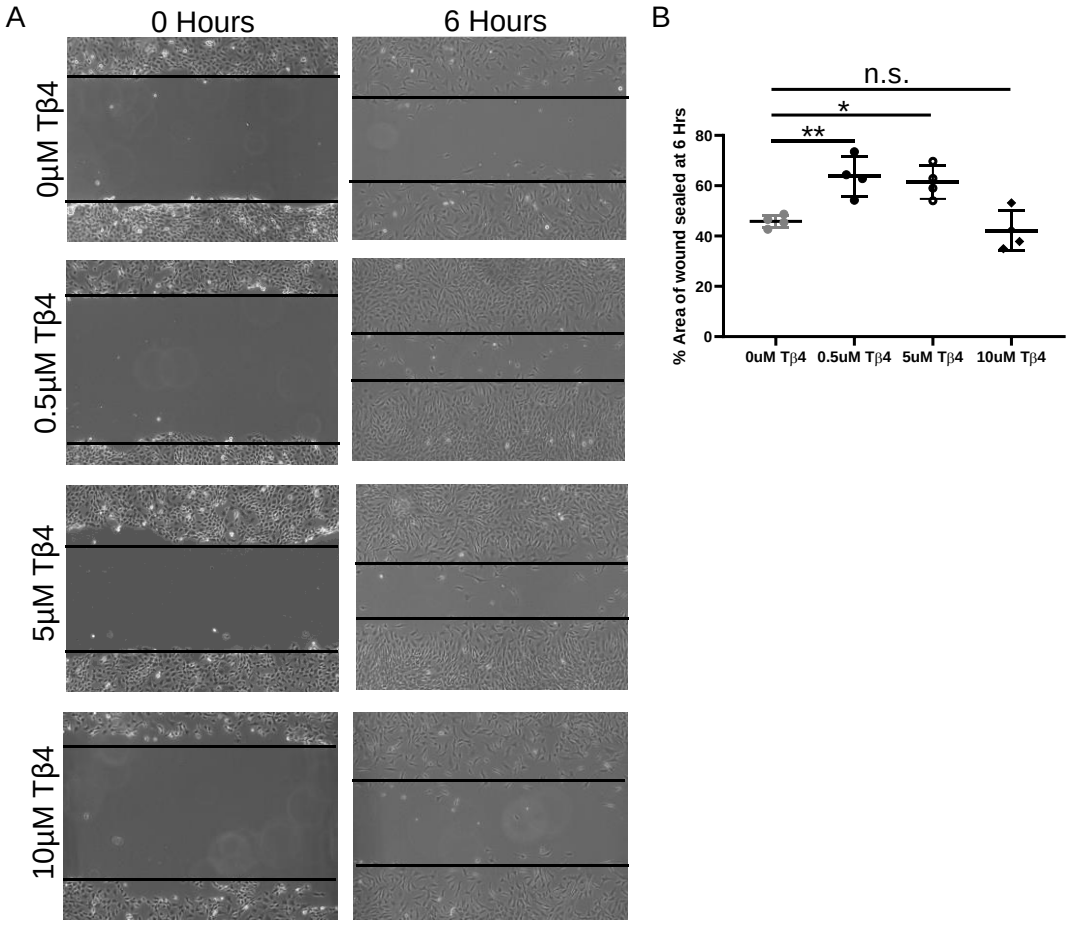



***Figure 6.9: Effect of Tβ4 on PDGF-BB stimulated MOVAS-1 cell migration.*** *PDGF-BB stimulated MOVAS-1 show a biphasic response to Tβ4 treatment. (**A**) Image of the scratch injury on a confluent cell monolayer treated with different doses of Tβ4 at 0 and 6 hrs after injury. (**B**) Effect of different doses of Tβ4 on cell migration was quantified as a percentage of the scratch area after 6 hrs of Tβ4 treatment, relative to 0 hrs. Significance n.s. = not significant, *p ≤ 0.05, ** P ≤ 0.01 was calculated using one-way ANOVA, Dunnett's multiple comparisons test. n=4.*

In an attempt to relate the biphasic effect of Tβ4 on MOVAS-1 migration with altered sensitivity of the cells to exogenous PDGF-BB, signalling pathway activity was investigated in primary aortic VSMCs and MOVAS-1 cells, however, the hypothesised

effect that exogenous Tβ4 may downregulate activation of the PDGFRβ signaling pathway was not observed (data not shown).

The effect of exogenous Tβ4 on PDGFRβ signalling was further examined *in vivo* in aortic sections from mice treated with AngII+Tβ4 and AngII+Saline, with assessment of receptor activation by immunostaining. Although the intensity of phospho-PDGFRβ signal appeared to be reduced in AngII+Tβ4 treated mice, compared to AngII+Saline treated, as shown in **Figure 6.8 A**, quantification shown in **Figure 6.8 B** revealed no significant difference. The analysis is clearly underpowered, however, the p=0.0571 by Mann-Witney test suggests a trend towards a reduction in the level of phospho-PDGFRβ, which may be substantiated with increased sample size.

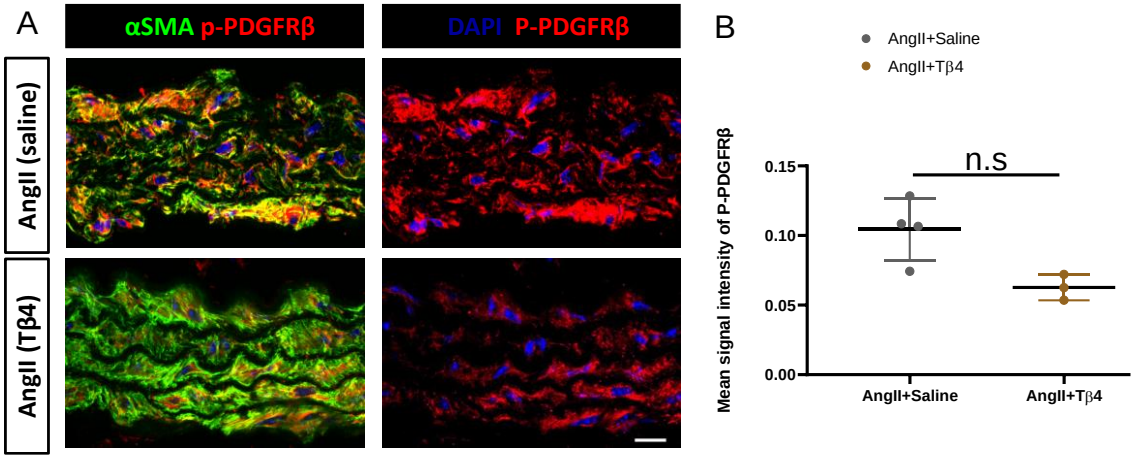



***Figure 6.10: No significant difference in the activation of PDGFRβ in VSMCs of aneurysmal mice treated with Tβ4 treatment**. Aortic sections from AngII+Saline and AngII+Tβ4 treated mice immunostained with αSMA and P-PDGFRβ (Tyr-1021), showing attenuated activation of PDGFRβ in mice treated with Tβ4 (**A**), and quantification in (**B**) Right hand panels showing αSMA and P-PDGFRβ staining and left-hand panels showing DAPI and P-PDGFRβ staining. Scale bars: 100μM for all four panels. Data are presented as mean ± SD, AngII+Saline (n=4) and AngII+Tβ4 (n=3) with each data point*

*representing an individual animal. Significance was calculated using Mann-Witney non-parametric tests. Significance n.s.= not significant.*

Taken as a whole, these studies demonstrate that exogenous Tβ4 has a profound effect on preserving VSMC differentiation, elastin integrity, reducing fibrosis, and resolving inflammation in the advanced stages of AAA, to prevent disease and thus, this is the first description of Tβ4 as a promising candidate in protection against vascular disease.

## 6.4 Discussion

Results in this chapter identify Tβ4 as a potential therapeutic agent to protect against AAA. Administration of Tβ4 at the dose rate of 12mg/kg/day for a period of 14 days, simultaneously with AngII, augmented VSMC protection and attenuated the development of aortic aneurysms. Reduced incidence of aneurysm, along with fewer contained ruptures, were reported in mice treated with Tβ4. While contained aneurysms were non-fatal during the course of this study, in patients, they contribute to 4% of all ruptured aortic aneurysms[438]. In addition to the contained rupture, one fatal rupture was reported in each of the Tβ4 and Saline treated mice treated with AngII, however, death occurred much earlier at 6 days in vehicle treated mouse, compared to 13 days in the mouse treated with Tβ4.

Tβ4 was administered at the rate of 12mg/kg/day, as it has previously been shown to be effective and well tolerated in mice [238], using osmotic minipumps to minimise experimental variability and ensure continuous administration of the peptide[243]. Aneurysms were previously noted (Chapter IV) in some $T\beta4^{-/Y}$ mice between 5-10 days,

therefore, to assess the potential to rescue a moderate to severe phenotype in C57B6/J mice, a 14-day time point was selected for this study. Exogenously administered Tβ4 in AngII-treated mice reduced the extent of medial elastin degradation. Elastin is an important structural protein that is relatively resistant to elastolysis and synthesised by VSMCs during development. Injury-induced release of proteases from activated VSMCs and macrophages promotes elastin degradation. A previous study reported a lower elastin/collagen ratio in AAA[439]. While collagen content was not measured directly, the data reveal a trend to reduction in expression of CTGF in VSMCs. These results are in keeping with the previous study that describes the anti-fibrotic property of Tβ4[223].

Several researchers have demonstrated a role for exogenous Tβ4 in reducing fibrosis in models of eye, skin, lung, liver, and renal disease It is suggested that fibrosis is an adaptive response in the initial stages of vascular disease to support the medial layer, however, as disease progresses, it increases vascular stiffness. In fact, vascular stiffness in large vessels leads to hemodynamic damage to peripheral tissues[440]. Taken together, these findings suggest that exogenously administered Tβ4 prevents lowering of the elastin: collagen ratio to maintain structural integrity.

In addition to maintaining structural proteins, exogenously administered Tβ4 in aneurysmal mice preserved smooth muscle contractile phenotype to provide vascular stability. An increased ratio of contractile to synthetic markers both at the gene and protein level in aorta from AngII+Tβ4 treated mice indicates that exogenous Tβ4 provides vascular protection by preserving differentiated VSMC phenotype over synthetic phenotype. Results presented previously in Chapter III & Chapter IV demonstrate that loss of endogenous Tβ4 increases susceptibility to atherosclerosis and AAA, with the accelerated transition of VSMCs to a synthetic state and enhanced elastin degradation

being notable characteristics of the more severe disease phenotypes in mice lacking Tβ4.

Tβ4 has previously been shown to reduce inflammation, and to inhibit the influx and adhesion of inflammatory cells in several disease models[217, 219, 220, 317, 432]. Moreover, in cardiac wound healing, Tβ4-SO acts as the immune cell dispersal signal to prevent chronic inflammation[220]. In keeping with these studies, reduced inflammation was observed in aortas from AngII+Tβ4 mice at an advanced stage of the disease. However, these studies do not inform if exogenous Tβ4 affects macrophage migration, survival, proliferation, or egress, which requires further investigation. Moreover, studies performed at the earlier stage indicated that inflammation was unchanged between mice treated with Tβ4 or saline. These data confirm that exogenously administered Tβ4 did not negate the effect of AngII, despite the concurrent administration.

Results in Chapter V demonstrate that endogenous Tβ4 regulates PDGFRβ signalling via LRP1 mediated lysosomal targeting, whether exogenous Tβ4 regulates PDGFRβ signalling via LRP1 may be an important question for future study. In an attempt to tease out the underlying mechanisms through which Tβ4 protects differentiated VSMC phenotype, the functional effects of Tβ4 were investigated *in vitro* using a scratch wound assay. Tβ4 acts in a biphasic manner, to promote the migration of PDGF-BB stimulated MOVAS-1 cells at lower concentrations and accelerating wound closure while reducing the rate of migration at higher concentrations. This was essential because 1) PDGF signalling regulates VSMC phenotype, 2) endogenous Tβ4 regulates PDGFRβ signalling to protect against AAA and atherosclerosis. A previous study using human HSC treated with 50ng/ml PDGF-BB, 10ng/ml Tβ4 suggested that Tβ4 inhibits the reappearance of the receptor once it is degraded by downregulating the expression of PDGFRβ [441]. However, Tβ4 treatment showed no significant effect on PDGFRβ levels or activation of

the signalling pathway in aortic VSMCs *in vitro.* Whilst the activation of PDGFRβ in AAA did not show a significant difference, a trend towards reduced activation of PDGFRβ was observed in AngII+Tβ4 treated mice. It must also be noted that while administration of exogenous Tβ4 in the AngII model was chronic, in the *in vitro* assay the cells were treated only for a short period of time (60 minutes).

Although direct changes in the intracellular levels of Tβ4 post-administration were not measured, either *in vivo* or *in vitro,* the data demonstrate that Tβ4 plays a significant role in maintaining VSMC contractile phenotype and vascular integrity *in vivo*. Given the high abundance of Tβ4 in the cytosol and nucleus, it is difficult to envisage that the modest increases that might have been achieved by the doses administered in our study that could significantly alter the intracellular Tβ4 pool, thus an alternative mechanism appears likely. A study by Freeman and colleagues demonstrated that exogenous Tβ4 increases cell surface ATP levels by binding to $F_1F_0$-ATP synthase, to promote HUVEC migration through activation of the purinergic receptor P2X4[207]. The presence of purinergic receptor P2X has been confirmed on VSMCs[442] and an exciting area of further study would be to employ purinergic receptor antagonists to determine whether any of the effects of exogenous Tβ4 on VSMCs are dependent on purinergic receptor activation.

## 6.5 Limitations

Whilst this chapter highlights the therapeutic potential of exogenous Tβ4 to protect against AAA by preserving VSMC contractile phenotype and elastin integrity, it fails to delineate the molecular mechanism by which exogenous Tβ4 confers vascular protection. As shown in Chapters III, IV & V, endogenous Tβ4 protects the vasculature via interaction with LRP1 to control PDGFRβ trafficking. Thus, an important question to

evaluate would be whether exogenous Tβ4 mediates vascular protection via this or another pathway.

Apoptosis is a classical hallmark of AAA both in humans and mice, and anti-apoptotic properties of Tβ4 are well recognised[443]. Thus, future studies must evaluate if exogenous Tβ4 reduces VSMC apoptosis in addition to reducing contractile-synthetic VSMC phenotypic switching to maintain medial stability. Considerable differences exist in the pathophysiology of vascular diseases in humans and experimental animal models, therefore, whether Tβ4 will have a similar effect in humans cannot be assumed. To overcome this limitation, future experimental designs should incorporate the use of *ex vivo* cultures of human AAA tissue, in addition to further animal studies.

## 6.6 Conclusion

In summary, this study postulates that exogenous Tβ4 partially protects the vasculature against AAA. Data from this chapter, together with its role in VSMC differentiation[170, 206], cell survival[191, 429], wound healing[217], ECM remodelling[430] and resolving inflammation[182], suggest that Tβ4 could be a promising therapy for the treatment of AAA. However, the precise pharmacotherapeutic mechanisms by which exogenous Tβ4 promotes vascular stability warrant further study.

## Acknowledgement of contributions

The AngII osmotic mini pump implantation surgeries and Flow cytometry experiments were conducted together with and analysed by Dr Andia Redpath.

Whole-mount and elastin integrity studies presented in this chapter were conducted by Dr Nicola smart.

# Chapter VII

# General Discussion, Conclusions and Future Work

## 7.1 Discussion

Chronic diseases, such as atherosclerosis and AAA, lead to loss of vascular stability with life-threatening consequences. In addition to maintaining vascular stability and tone, VSMCs of the healthy medial layer contribute to the production of ECM proteins and proteases[26, 444-447], therefore maintaining the structure and function of fully differentiated VSMCs is important in vascular homeostasis. Owing to the well-defined role of Tβ4 in VSMC differentiation during development[206] and maintaining VSMC differentiation postnatally at baseline[170], this thesis focused on the endogenous and exogenous roles of Tβ4 to protect against vascular disease. This chapter summarises the main findings of the thesis, the limitations associated with the methodology used for the study, important avenues that can be further explored in future and finishes with the primary conclusions.

### 7.1.1 The main objectives of this thesis

The findings of the thesis are discussed in the context of the aims listed in Chapter I:

- To determine if endogenous Tβ4 protects against atherosclerosis and aortic aneurysm.
- To delineate the mechanisms involved in phenotypic switching of VSMCs, ECM modulation, and inflammation.
- To explore the therapeutic potential of exogenous Tβ4 in vascular protection.

#### 7.1.1.1 Endogenous Tβ4 protects against vascular disease

Tβ4 is a small peptide, abundantly expressed (up to 500μM) in almost all cell types and known to regulate cellular morphology, proliferation, migration, and cell survival[191, 209, 218, 420]. Endogenous Tβ4 is important to maintain vascular homeostasis in the healthy vessel, and loss of endogenous Tβ4 either globally or specifically in VSMCs in mice leads to increased VSMC phenotypic switching, aberrant elastin deposition, aortic dilation, and increased vascular compliance resulting in compromised vascular stability[170]. Beyond a protective role in vessel homeostasis[170], data presented in this thesis demonstrate that endogenous Tβ4 protects against atherosclerosis and AAA. These conclusions were based on the use of well-established experimental disease models, namely:

- Atherosclerosis – hypercholesterolemia by modified diet on the ApoE$^{-/-}$ background.
- Aortic aneurysm – continuous AngII infusion via osmotic mini pump implantation.

Many previous studies in the field have characterised disease phenotype at a single time point and hence lack information on the changes that occur at different stages of disease progression, therefore this study was designed to examine disease phenotype at both early and late stages, to separate events at discrete stages in disease development. However, it must be noted that, in our hands, disease progression even in our control animals, was greatly accelerated both in atherosclerosis and AAA, relative to timescales reported in the literature. Recently, studies from number of laboratories have reported several factors that are institutional specific including housing, diet, gut microbial population that may influence disease progression differently in mice from different facilities[361, 448], for example- Karbach *et al* indicated that colonization of germ-free mice with regular gut microbiota promotes AngII induced arterial hypertension and

vascular dysfunction[449]. Another study demonstrated that the gut microbiota promoted arterial thrombosis in high fat-fed Ldlr$^{-/-}$ mice[450]. Whilst the animals used for this project were maintained in a pathogen-free facility, environmental differences or stress may have contributed to accelerated disease progression.

Accelerated disease progression in mice lacking Tβ4 was characterized by significantly compromised elastin integrity, augmented contractile-synthetic VSMC phenotypic switching, increased VSMC proliferation and apoptosis, compared to the respective control mice. Owing to the crucial role of contractile VSMCs in vascular stability, contractility, regulation of vascular tone and ECM homeostasis, maintaining VSMCs in their differentiated state is crucial to protect against diseases that can be fatal. The VSMC phenotypic changes observed in mice lacking Tβ4 recapitulate previous studies where VSMC phenotypic switching is induced with vascular injury, including atherosclerosis, hypertension, post-angioplasty restenosis, as well as aneurysm[27-29, 51].

VSMCs undergo multiple processes including phenotypic switching, proliferation, necrosis, apoptosis, often simultaneously, at different stages and in different regions of the aorta, therefore, the role of VSMC phenotypic switching in atherosclerosis is still not completely resolved[451]. The analysis of previously published scRNA-seq data of lineage traced VSMCs from the ascending aorta and aortic arch of ApoE$^{-/-}$ mice at baseline and after high-fat diet feeding[21] was performed to evaluate transcriptomic changes, especially with regard to the expression of *Tmsb4x.* Atherosclerotic aortas were characterised by an increase in the population of mSMCs and these expressed significantly reduced *Tmsb4x* in comparison to the cells that comprised the contractile VSMC clusters.

To more specifically define the role of endogenous Tβ4 in medial stability in atherosclerosis, assessments were made on aortas from Tβ4$^{+/Y}$; ApoE$^{-/-}$ (control) and Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice fed on WD. Compared to the control, Tβ4$^{-/Y}$; ApoE$^{-/-}$ mice developed larger atherosclerotic plaques, characterised by large necrotic cores devoid of VSMCs, accelerated shift of VSMCs to a synthetic phenotype with plaque caps predominantly composed of synthetic VSMCs, which is predicted to make them vulnerable to rupture. Considerable debate exists over the role of VSMCs in plaque; while an increase in the synthetic VSMCs population is considered beneficial in advanced plaques, as these cells promote ECM deposition in the fibrous cap, which may prevent rupture, studies have also shown that loss of contractile VSMCs increases ECM degradation. It is therefore important to understand VSMC heterogeneity using scRNA-seq in healthy and atherosclerotic vessels[21]. Such studies may improve our understanding of the function of an individual cell in the context of its microenvironment and enable the identification of the regulators of contractile-synthetic switching, which may allow for modulation of VSMC phenotype at various stages of disease to promote repair.

Similarly, evidence provided in Chapter IV confirms that adult Tβ4$^{-/Y}$ and VSMC-specific Tβ4 KD (Myh11$^{CreERT2}$; Hprt$^{Tβ4shRNA}$) mice, infused with AngII, show increased susceptibility to AAA, with a higher incidence of dissection, rupture, and mortality. Inducible Myh11$^{CreERT2}$ was preferred due to the specificity of *Myh11* to mature VSMCs[366], in contrast to constitutive sm22α$^{Cre}$ that was used in earlier studies[164], with the serious limitation that sm22α, is transiently expressed in a broad range of non-VSCM cell types during development, moreover, despite being active in embryonic VSMC progenitors, young mice were reported to show no aortic defects[2]. Whilst AngII infusion in mice is associated with aortic dilatation, accelerated elastin degradation, VSMC apoptosis and VSMC phenotypic switching, these processes were both augmented and accelerated in AngII-infused Tβ4$^{-/Y}$ and VSMC specific KD mice. In fact,

the results demonstrate that VSMC phenotypic switching occurs as early as 5 days of AngII infusion in Tβ4 null mice. These results confirm an important cell-autonomous requirement for Tβ4, similar to that observed at baseline, to protect the vasculature[170]. A recent study has demonstrated the importance of autophagy in eliminating 'synthetic' cells to preserve vessel integrity and reduce the occurrence and severity of aortic dissection[366].

Given the key role of macrophages in plaque progression and AAA, inflammation was assessed. Intriguingly, the levels of proinflammatory cytokines and inflammatory macrophages remained unaltered between Tβ4 null and the respective controls in the early stages of atherosclerosis (3 weeks WD) and AAA (after 5 days of AngII infusion) between the genotypes, suggesting that inflammation is not the primary cause of the exacerbated disease phenotype. Although no significant difference was observed in the number of $CD45^{+}CD11b^{+}CD14^{+}CCR2$ macrophages, an increasing trend in $CD45^{+}$ cells was observed in atherosclerosis, moreover, a low SSC scatter suggests that the cells are not granular and could likely be lymphoid cells. It is well established that disease progression in atherosclerosis and AAA involves a dynamic interplay between a number of different immune cell types, including tissue-resident macrophages, neutrophils, dendritic cells, T helper cells, Treg cells, cytotoxic T cells, and B cells[452], demanding a comprehensive evaluation of different immune cells in relation to disease. Recently, mass cytometry called Cytometry by time of flight (CyTOF), which utilizes rare metal isotopes instead of fluorophores for antibody labelling has emerged as a powerful single-cell proteomic analysis technique[453], for myeloid cell discrimination and comprehensive mapping of the immune cell in atherosclerotic aortas[454].

It has been shown that deletion of the *Lrp1* gene results in early embryonic lethality (E13.5) in mice due to extensive hemorrhaging[159, 455] and, of note, these

developmental defects are similar to those observed in Tβ4 null embryos[206]. Equally, in adult disease, the vascular defects including increased VSMC phenotypic switching, elastin degradation and aortic dilation observed in Tβ4 null mice in atherosclerosis and AAA were comparable to Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$ mice used in this study and previously reported VSMC-specific loss of LRP1 mice in disease [2, 164, 308]. Taken together, these studies suggest that Tβ4 null mice phenocopy LRP1 null mice during development and postnatally in health and disease. Another interesting observation was that the Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$ mice used in this study recapitulated the previously published SM22Cre$^{+}$; LRP$^{fl/fl}$ mice[164], despite the loss of LRP1 in Myh11$^{CreERT2}$; Lrp1$^{fl/fl}$ mice being induced postnatally. The data in Chapters III and IV confirms that LRP1, like Tβ4, is not only required during development, but is required throughout life to maintain vascular homeostasis and protect against disease.

AngII infusion has been widely used to induce aortic aneurysms in a number of studies including this research project, and has reported that approximately 10% of the mice die between 4-10 days of AngII infusion due to ruptured AAAs[133]. However, Davis *et al* reported that chronic AngII infusion (1000ng/kg/day for 28 days) in smLRP1$^{-/-}$ mice exacerbated superior mesenteric arterial dilatation, hematomas and ascending aortic aneurysm and had no effect on AAA formation. An important shortcoming of this study was that although the authors report the death of several mice during the AngII infusion, no post-mortem details were provided. It is therefore possible that the mice that died during the AngII infusion suffered from AAA, aortic dissection or rupture.

#### 7.1.1.2 Tβ4 regulates LRP1-mediated PDGF signalling to protect against vascular disease

Identifying the regulators of contractile-synthetic switching is crucial to enable the fine-tuning of VSMC phenotypes that are beneficial for repair. It is well established that PDGF signalling is a potent modulator of VSMC phenotypic switching during development and disease[26]. LRP1, a GWAS candidate associated with several CVDs, including atherosclerosis and AAA[147, 149, 456], is an endocytic regulator of the PDGFRβ signalling pathway[156, 247]. Hyperactivation of PDGFRβ and augmented phosphorylation of downstream signalling molecules, such as Erk1/2 are reported in the aortic walls of the conditional LRP1-defective animals[164]. This thesis provides compelling evidence of dysregulated LRP1-PDGFRβ signalling in aortic VSMCs of mice lacking Tβ4 in both disease models. Dysregulated LRP1-PDGFRβ signalling was attributed to defective intracellular trafficking of the receptor in the absence of Tβ4 as shown in **Figure 7.1**.

Under physiological conditions, following signalling, most of the receptor is degraded, either within the lysosome or proteasome, to dampen continuous signalling [384, 385] and the balance between endocytic uptake and recycling is tightly regulated[386]. However, an increased recycling of the LRP1-PDGFRβ complex was observed in cells lacking Tβ4, resulting in hyperactive signalling, which may be responsible for augmented VSMC phenotypic modulation. These results are in keeping with studies demonstrating that enhanced development of atherosclerosis in smLRP1$^{-/-}$ mice fed on WD is also accompanied by significantly enhanced PDGF signalling in VSMCs[164, 308]. In addition to endocytosis, ubiquitination is shown to promote internalization and intracellular sorting of receptors[457]. *In vitro* studies (human fibroblast cells) have shown that deletion of ubiquitin ligases c-Cbl and Cbl-b affect PDGF-BB induced PDGFRβ internalisation and activation, without affecting PDGFRβ degradation [458].

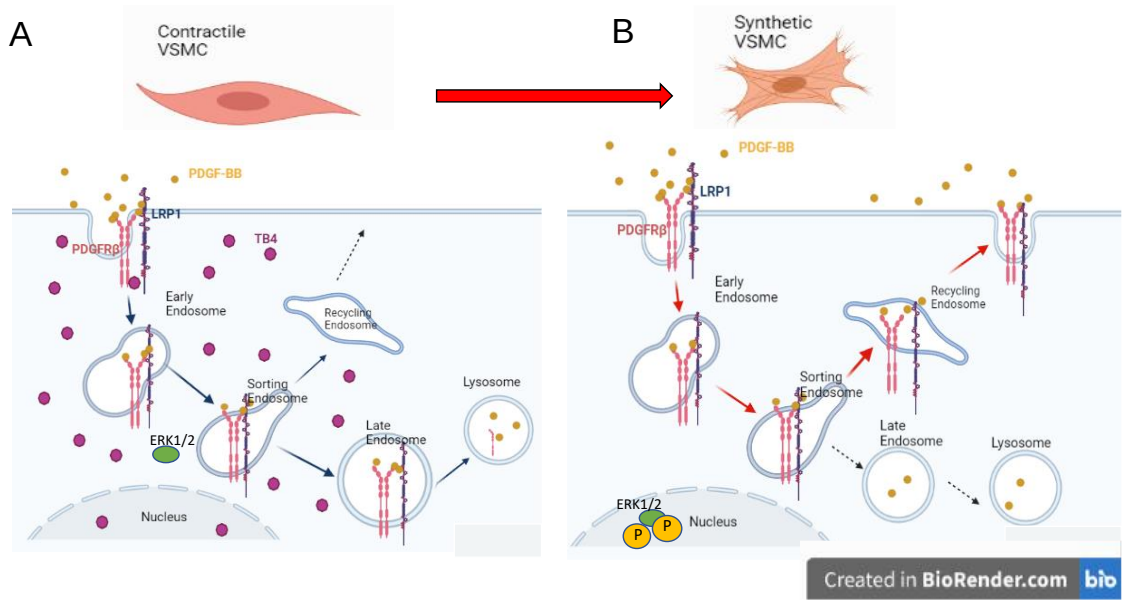



***Figure 7.1: Endogenous Tβ4 regulates intracellular trafficking of LRP1-PDGFRβ receptor complex to maintain VSMC phenotype.*** ***A****) Intracellular activation and degradation of the LRP1-PDGFRβ complex is tightly regulated in the presence of endogenous Tβ4, thus maintaining VSMC contractile state.* ***B****) In VSMCs lacking Tβ4, the LRP1-PDGFRβ complex is increasingly recycled rather than being targeted for degradation, resulting in augmented signalling which accelerates VSMC phenotypic modulation to a synthetic state, affecting vascular stability and susceptibility to disease. Image created using BioRender.com.*

Dysregulated LRP1 endocytosis in Tβ4 mice increased accumulation of LRP1 ligands, specifically matricellular proteins, such as CTGF and HTRA1, associated with elastin degradation, fibrosis, and defective elastinogenesis in atherosclerosis and AAA. Muratoglu *et al* [379] demonstrated exacerbated ECM degradation in smLRP1$^{-/-}$ mice due to dysregulated endocytosis and increased accumulation of LRP1 ligands CTGF and HTRA1. Earlier studies in mammalian A431 cells (epithelial cells) demonstrated a biphasic effect of exogenous Tβ4, where treatment with low concentrations (<0.5μM) of Tβ4 increased receptor-mediated endocytosis, while high concentrations (10μM) led to

complete inhibition of endocytosis[204]. This is unsurprising as Tβ4 is a major actin-binding protein that regulates its polymerization and depolymerization.

Interestingly, a striking 43-fold up-regulation of FLNa, implicated in controlling the trafficking and fate of diverse receptors[409, 410], was reported in Tβ4-knockdown MOVAS-1[170] which may explain increased partitioning of the LRP1-PDGFRβ complex to recycling endosome in the absence of Tβ4. Interestingly, it was reported that expression of FLNa is marginally (0.76 fold) reduced in VSMCs of smLRP1$^{-/-}$ mice[161]. The discrepancy between these findings may perhaps be explained by differential roles relating, directly or indirectly, to FLNa expression which, like Tβ4, has roles in actin filament formation. Alternatively, there may be contrasting requirements to compensate for loss of Tβ4, compared with loss of LRP1, or the difference may simply reflect differences in acute versus chronic adaptation to loss of Tβ4 after 1-2 days' siRNA treatment, versus constitutive knockout of LRP1 from embryonic stages. It is also postulated that FLNa mediates the signaling that regulates vasoconstriction [459]. FLNa has previously been shown to regulate VSMC proliferation and migration[460] and downregulation of the protein is reported in patients with aortic dissections[461]. Based on these findings, FLNa may emerge as an important link that may define the molecular mechanism by which Tβ4 regulates endocytosis of LRP1-PDGFRβ in VSMCs.

Additionally, a direct interaction was identified between Tβ4 and LRP1-ICD[170], which may explain the remarkable similarity between Tβ4 and LRP1 mutants both in their developmental and disease phenotypes and, most importantly, the selective role of Tβ4 in the regulation of LRP1-PDGFRβ. In addition to vascular diseases explored in this thesis, LRP1 is also clinically relevant in the pathogenesis of Alzheimer's disease[462]. It is, therefore, important to understand more precisely how Tβ4 regulates LRP1 internalisation and activation and whether this mechanism is disrupted in other diseases.

#### 7.1.1.3 Therapeutic potential of exogenous Tβ4 for vascular protection

The data presented in Chapter VI of this thesis indicate that Tβ4 may have potential to protect against AngII induced AAA. Controlled release of Tβ4 using osmotic mini-pumps in C57B6/J mice over a period of 14 days significantly reduced aortic rupture and mortality, and preserved the structural integrity of the medial layer by maintaining contractile phenotype, reducing medial elastin fragmentation and limiting the accumulation of CTGF[463], that modulates cell-matrix interactions and HTRA1, an elastolytic serine protease[390] in VSMCs. This anti-fibrotic property of exogenous Tβ4 is in keeping with previous studies[220, 464]; notably exogenous Tβ4 treatment in pulmonary hypertension and right ventricular hypertrophy, induced by AngII, demonstrated decreased expression of Col 3a and CTGF resulting in decreased fibrosis in the right ventricle and lung tissue[223].

Importantly, systemically administered Tβ4 was well tolerated at the dose rate of 12mg/kg/day for period of 14 days. Although we did not actively investigate the side effects of exogenous Tβ4, these would seem unlikely as it is a highly abundant, naturally occurring peptide. Administration of Tβ4 via topical application and the intravenous route was shown to be safe and well-tolerated in human clinical trial studies[425]. To increase the potency and reduce systemic side effects, the delivery of therapeutics via a local route is recommend [465]. Recently, site-specific drug delivery using nanoparticles has been shown as an attractive strategy to inhibit aneurysm progression[466]. Polyethylene glycol-based polymers are biodegradable and offer a promising solution to deliver small compounds and peptides[467].

Whilst the percentage mortality between aneurysmal mice treated with Tβ4 and saline was similar, death occurred much later in the mouse treated with Tβ4 and the histological data from surviving mice suggested that Tβ4 treatment may reduce the rate of AAA progression. Importantly, assessment of aneurysmal aortas revealed significant preservation of contractile VSMC phenotype in mice treated with Tβ4. While it was difficult to quantify the expression of contractile and synthetic markers from immunostained sections, as large areas of aortic media in mice treated with saline were devoid of VSMCs, probably due to apoptosis, the proportional expression of αSMA relative to Caldesmon appeared higher in mice treated with Tβ4. It must be noted that the Caldesmon antibody used for immunostaining does not differentiate between the h- (high molecular weight) and l- (low molecular weight) isoforms of Caldesmon associated with contractile and synthetic phenotype, hence evaluation using other synthetic markers or western blotting that allows separation of high and low molecular weight Caldesmon isoforms is recommended.

Results in Chapter V of this thesis confirm that loss of Tβ4 leads to dysregulated LRP1 mediated PDGFRβ signaling and increases susceptibility to AAA and atherosclerosis. In rat liver, Reyes-Gordillo and colleagues reported that exogenous Tβ4 treatment prevented up-regulation of α1(and 2) collagen, α-SMA, PDGFRβ, and fibronectin[441]. Examination of aortic sections from aneurysmal mice suggested that, in comparison to saline-treated mice, Tβ4 treated mice show lower levels of phospho-PDGFRβ, however, further experiments are required with increased sample size to confirm or exclude if exogenous Tβ4 regulate activation of PDGFRβ and the molecular mechanism involved. Based on the literature, two distinct mechanisms through which exogenous Tβ4 regulates PDGFRβ signaling can be speculated:

- Given that exogenously administered Tβ4 can regulate endocytosis[204], it is possible that exogenous Tβ4 may internalize through cell surface pores or channels or may diffuse through the cell membrane due to its small size[468]. Once inside the cell, Tβ4 may increase lysosomal targeting of the receptor rather than recycling as shown in **Figure 7.2**. However, this seems unlikely as Tβ4 is present in high concentrations (up to 500μM) in most cells, is especially high in the aorta, and the amount of Tβ4 administered exogenously is infinitesimal in comparison.
- A second, arguably more likely, possibility is that Tβ4 may activate a cell surface receptor which may, in turn, regulate PDGF signaling. For example, exogenous Tβ4 was shown to increase extracellular ATP levels upon binding to $F_1$-$F_0$ ATP synthase, which has been shown in turn to activate signaling via the purinergic receptor[207]. In further support of this potential mechanism, a previous study demonstrated that paracrine or exogenous Tβ4, once it enters the circulation, rapidly becomes oxidized to a sulfoxide form; Tβ4-SO has significantly reduced actin binding capacity but much more potent extracellular signalling properties than the native form[182], albeit the precise signalling pathways remain to be determined.

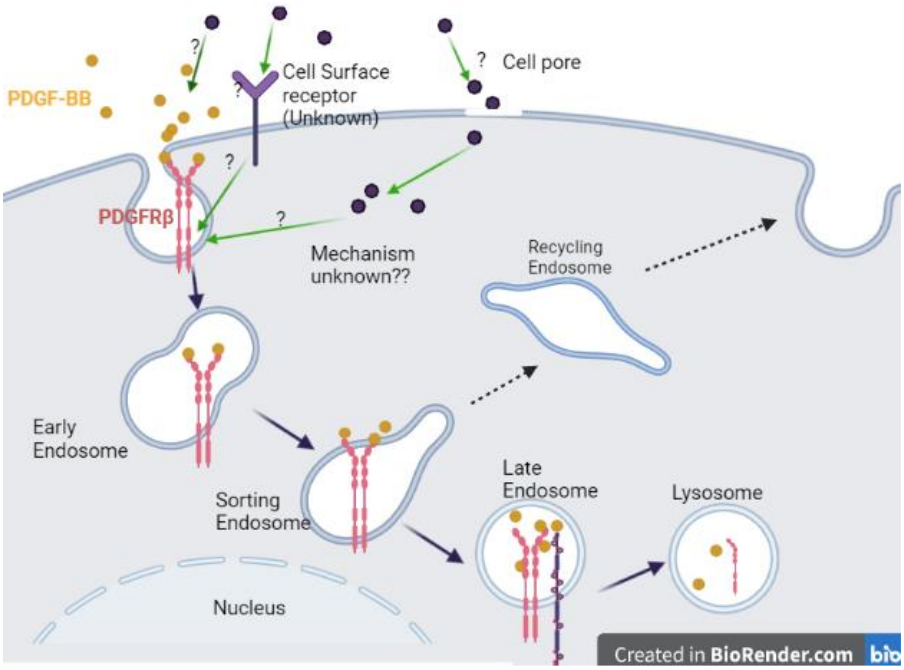



***Figure 7.2: Exogenous Tβ4 regulates PDGFRβ signaling to protect against AAA.*** *Exogenous Tβ4 emerges from this study as a potential therapeutic to protect against AAA. The mechanism by which exogenous Tβ4 regulates PDGF signaling remains unknown, however, owing to the small size of the peptide, it could be internalized through a cell surface pore or channel to act intracellularly to regulate intracellular signaling. A second possibility is that exogenous Tβ4 signals through an unknown cell-surface receptor. Image created using BioRender.com.*

*In vitro*, PDGF-BB stimulated MOVAS cells treated over a range of Tβ4 doses confirmed a biphasic effect on MOVAS migration, with increased migration at lower doses of Tβ4 (0.5 and 5 μM) and reduced migration at 10μM Tβ4. Exogenous Tβ4 was previously found in wound closure assay to show a biphasic effect on the migration of ECs and keratinocytes[209]. It must be noted that differentiated VSMCs show very low migration, however VSMCs in culture are subjected to various stimuli and may change or lose some of their contractile properties, hence future experiments are recommended in early passage primary VSMCs.

Due to the known role of macrophages in AAA progression and the anti-inflammatory properties of Tβ4 reported previously[219], the extent of inflammatory macrophage recruitment at an early stage of aneurysm formation was determined. *In vivo*, there were no differences in the number of recruited macrophages between Tβ4 and saline-treated aneurysmal mice. However, after 14 days' AngII infusion, qualitative analysis suggests a substantial reduction in peri-adventitial macrophage content by Tβ4 treatment. Together, these data suggest that exogenous Tβ4 did not negate the inflammatory property of AngII and allowed disease initiation, however, at advanced stages, Tβ4 either prevented secondary inflammatory cell influx, which may be exacerbated by VSMC modulation, or it promoted macrophage egress, as shown in other disease contexts, to limit disease progression.

## 7.2 Limitations of this study

There are several limitations associated with this study, some of which are listed below and must be taken into consideration while designing future experiments. This research study extensively uses experimental animal models to identify the key regulators and underlying mechanisms involved in the maintenance of vascular stability. Due to the inherent differences between mouse and human pathology, translating some of these findings from mouse studies to humans may be problematic. As an example, AAA is a chronic condition in humans and develops over decades, however, the disease pathogenesis is acute, initiating within days in experimental mouse models. Even if this was considered normal based on the average life span of mice (1.5-2 years), the disease was initiated in mature adult mice (8-12 weeks) a human equivalent of 20-30 years of age[469, 470] instead of aged mice, when the disease manifests in humans (AAA

prominent after 65 years in humans). Despite the limitations associated with the use of mouse models, they remain a useful tool to demonstrate proof of concept and determine the efficacy of potential drugs in preclinical studies.

Another limitation that will require attention in the future is VSMC heterogeneity within the aorta. It has been well recognised that vascular heterogeneity has direct implications on VSMC function during injury responses[471]. To delineate VSMC heterogeneity, profiling of individual cells based on several hundred transcripts using scRNA-seq, in conjunction with VSMC lineage tracing that allows unambiguous tracking of VSMCs, even if they downregulate or completely lose markers that originally defined them as VSMCs [56, 57], will be an important approach. Combining scRNA-seq with lineage tracing will allow the integration of information both on cell lineage and cell state in disease[472]. The study extensively uses immunostaining to evaluate spatial and temporal distribution of contractile and synthetic VSMC proteins within diseased mouse aortic sections, although the technique is semi-quantitative. Thus, immunostaining data are backed up with western blotting and qRT-PCR. A major shortcoming of using selected VSMC markers is that they are seldom unique, rather expressed by multiple cell types within vascular lesions[56, 366]. scRNA-seq offers the greatest opportunity to identify cell type-specific markers for more definitive characterisation in future studies.

Elastin and collagen are important structural proteins in the media, synthesized by VSMCs. A decrease in the elastin to collagen ratio has previously been reported in AAA[473]. Data presented in this thesis confirm significantly higher elastin fragmentation and reduced elastin integrity in aortas lacking Tβ4 in both disease models. While elastin integrity was measured from diseased aortas using Verhoeff Van-Gieson stain, collagen content was not measured directly, for example using Masson's trichrome or Picrosirius Red stains, which have been extensively used to detect tissue collagen[474]. The

expression of CTGF, a known fibrosis marker, was measured on aortic sections in both disease models and increases in CTGF indirectly suggested that fibrosis might be exacerbated in the aorta of Tβ4 null mice. While injury-induced elastin fragmentation destabilises vessels, in contrast, collagen synthesis is upregulated to provide mechanical support to the vessel wall; however, excessive collagen synthesis leads to excessive fibrosis and vessel wall stiffening. It has been suggested that loss of vascular elasticity accompanied with increased stiffness leads to increased systolic pressure, cardiac load and risk of cardiovascular events[475].

It is important to recognise that the VSMC is not an independent entity, rather a constant cross-talk exists between the VSMC and the surrounding ECM, and VSMCs interact with cells in other layers of the aorta to promote vascular homeostasis. For example, shear stress is primarily sensed by the ECs which, in turn, influence VSMC phenotype[476]. In addition to cell-autonomous regulators, several paracrine signals may also affect VSMC structure and function in health and disease. Despite the limitations, this thesis provides compelling evidence of the crucial roles played by both endogenous and exogenous Tβ4 in vascular protection.

## 7.3 Conclusions

VSMCs of the medial layer are inherently plastic. Mature VSMCs in healthy vessels are involved in maintaining vascular tone and contractility, however, in response to injury-evoked stimuli, they undergo phenotypic modulation to a less differentiated state. An increase in VSMC phenotypic modulation in the early stage of disease promotes vascular remodelling; over time, this aggravates inflammation, which may eventually impair vascular stability. In the present study, the importance of endogenous and exogenous Tβ4 in maintaining medial layer stability to protect against vascular diseases, using well-

established mouse models, is described. Loss of endogenous Tβ4 in mice increases VSMC phenotypic switching, proliferation, apoptosis and elastin degradation, resulting in susceptibility to atherosclerosis and AAA. Moreover, vascular defects observed in Tβ4-null mice were comparable to $Myh11^{CreERT2}$; $Lrp1^{fl/fl}$ mice in both disease models, in keeping with their functioning in a common molecular pathway that we defined. Vascular defects observed in mice lacking Tβ4 were underpinned by hyperactivation of LRP1-PDGFRβ signalling, likely due to dysregulated intracellular trafficking of the LRP1-PDGFRβ complex, as characterised *in vitro*. Accordingly, inhibiting PDGFRβ activation in aneurysmal mice lacking Tβ4 preserved medial VSMC phenotype and elastin integrity, confirming that dysregulated growth factor signalling was responsible for accelerated disease progression due to loss of Tβ4.

To establish the therapeutic potential of Tβ4, preclinical studies in experimental AAA mice were performed. Pharmacologically administered Tβ4 reduced aneurysm development by protecting elastin integrity and preserving VSMC contractile phenotype, underpinned by a reduction in PDGFRβ activation, suggesting that the vascular protection functions of exogenous Tβ4, at least in part, are mediated via PDGF signalling. Preliminary investigation into the molecular mechanisms suggests that Tβ4 has a biphasic effect on VSMC migration in response to PDGF-BB, however, further *in vivo* and *in vitro* studies are warranted to elaborate the precise mechanisms by which exogenous Tβ4 confers vascular protection. These studies will provide useful insights into how to design potent Tβ4-based therapeutic strategies to promote vascular stability in health and disease.

## 7.4 Future work

Data presented in this thesis delineate the mechanism by which Tβ4 regulates LRP1-mediated growth factor signalling to protect against vascular disease. Combining proximity ligation assay and immunostaining, dysregulated intracellular trafficking of LRP1-PDGFRβ was identified as an underlying mechanism for increasing the sensitivity of VSMCs to the destabilising effects of PDGF-BB. However, the question of when, and in which endocytic compartment, Tβ4 binds to LRP1 needs attention.

LRP1 is a common integrator of both the PDGF-BB and TGFβ signalling pathways and, as such, it may play other, distinct functional roles in vascular protection. Interestingly, Tβ4 has been shown to regulate the TGFβ signalling pathway during development[206], in addition to PDGF signalling postnatally[170], to maintain vascular health. Thus, future studies can be directed towards evaluating whether Tβ4 additionally regulates TGFβ signalling to regulate vascular protection, given the importance of this pathway in promoting VSMC differentiation.

The loss of endogenous Tβ4 was shown to affect LRP1-PDGFRβ signalling (Chapter V) in AAA and atherosclerosis and exogenous Tβ4 reduced pathway hyperactivation in aneurysmal C57B6/J mice (Chapter VI). Given that increased susceptibility to atherosclerosis and AAA and accelerated disease progression in sm LRP1$^{-/-}$ mice was shown to occur via hyperactivation of PDGFRβ signalling, with similar VSMC and elastin defects to those seen in Tβ4 null mice, it is of considerable interest to determine whether exogenous Tβ4 can rescue LRP1 mutant mice from AAA and atherosclerosis. A negative result, relative to protection in control mice, would implicate LRP1 in mediating most of the benefits of exogenous Tβ4 treatment while demonstrating rescue would suggest one or more LRP1-independent effects of Tβ4.

Based on the data presented in Chapter VI, Tβ4 emerges as a promising treatment for AAA. Owing to the difference in the etiology between AAA and atherosclerosis, it will be worthwhile to determine if Tβ4 can similarly be used to treat atherosclerosis, or whether these diseases need to be targeted differently. Tβ4 and LRP1 are both abundantly expressed in a number of cell types, including ECs and macrophages, therefore it would be of interest to further investigate whether Tβ4 functionally regulates LRP1 also in these cell types to influence vascular disease outcome.

Another area of interest is the investigation of endothelial effects on the medial layer. Tβ4 and LRP1 are expressed both in ECs and VSMCs[170, 206, 308, 477]. Moreover, endothelial secreted Tβ4 is essential for VSMC differentiation during development[206], thus suggesting the importance of evaluating the role of paracrine Tβ4 on VSMC phenotype in steady-state and disease. A preliminary study from our laboratory suggests that, while aortas of postnatally induced endothelial-specific Tβ4 knockdown were minimally affected at baseline, these mice appeared to show a severe aneurysmal phenotype after AngII administration. If substantiated in a larger study, these findings would suggest that, although endothelial Tβ4 is dispensable in vascular homeostasis, it may play an important protective role in disease, similar to that in development.

Tβ4 treatment is shown to modulate the MMP/TIMP balance and decrease corneal inflammation to promote corneal wound repair[430]. During vascular disease, it is well established that upregulation of proteolytic enzymes enhances ECM degradation, which promotes VSMC migration, proliferation and immune cell infiltration. Thus, it will be interesting to investigate if exogenous Tβ4 promotes vascular protection by regulating the MMP/TIMP balance.

## 7.5 Final Remarks

In summary, studies in this thesis provide relevant preclinical proof-of-concept data using experimental mouse models. Despite an incomplete understanding of the molecular mechanisms by which exogenous Tβ4 protects AAA, the data highlight the potential therapeutic properties and efficacy of exogenous Tβ4. Taken together, this thesis identifies Tβ4 as a key player in maintaining vascular stability and protection against vascular disease by regulating LRP1-mediated endocytosis of PDGFRβ.

# Appendix

## Publications

- Thymosin β4 protects against aortic aneurysm via endocytic regulation of growth factor signaling.

  Published: *J.Clin.Invest*. May 2021, 17;131(10):e127884,

  doi: 10.1172/JCI127884

- Thymosin β4 mediates vascular protection via regulation of Low Density Lipoprotein Related Protein 1 (LRP1) in atherosclerosis.

  Sonali Munshaw, Andia Redpath, Benjamin Pike & Nicola Smart.

  Manuscript in revision.

- Paracrine and exogenous Thymosin β4 potently protect against aortic aneurysm

  Sonali Munshaw, Andia Redpath, Georgina Sanderson, Jyoti Patel, Keith Channon & Nicola Smart.

  Manuscript in preparation.

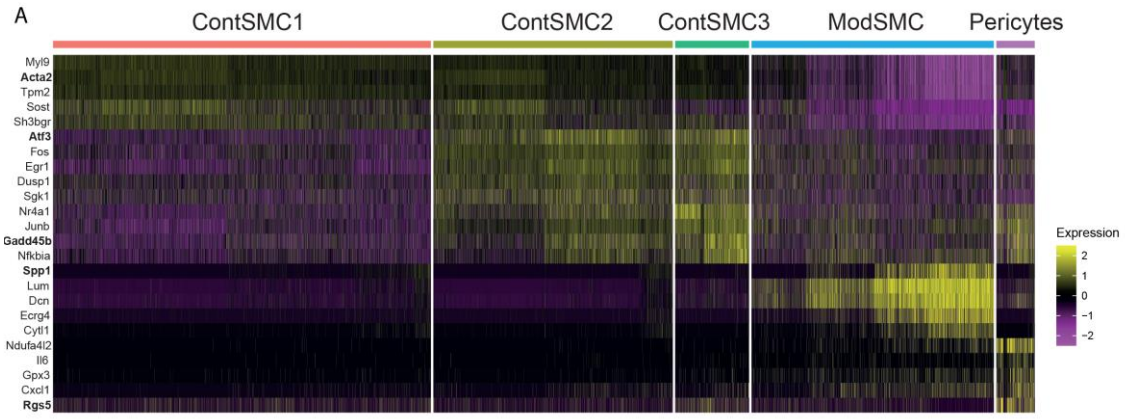
- Influence of extracellular matrix on vascular smooth muscle cell phenotype and function in atherosclerotic plaque development.

  Vignesh Murugesan, Sonali Munshaw & Nicola Smart.

  Review article: manuscript in preparation.

## Appendix Figure 1 :

### Heatmap illustrating the top 10 marker genes and their expression levels to characterise VSMC clusters




*Computational analysis of aortic scRNA-seq data derived from ApoE$^{-/-}$ mice with SMC lineage tracing characterised 5 different subsets of VSMCs based on the expression of specific marker genes. Heatmap illustrating the top 10 marker genes and their expression levels to characterise each cluster.*

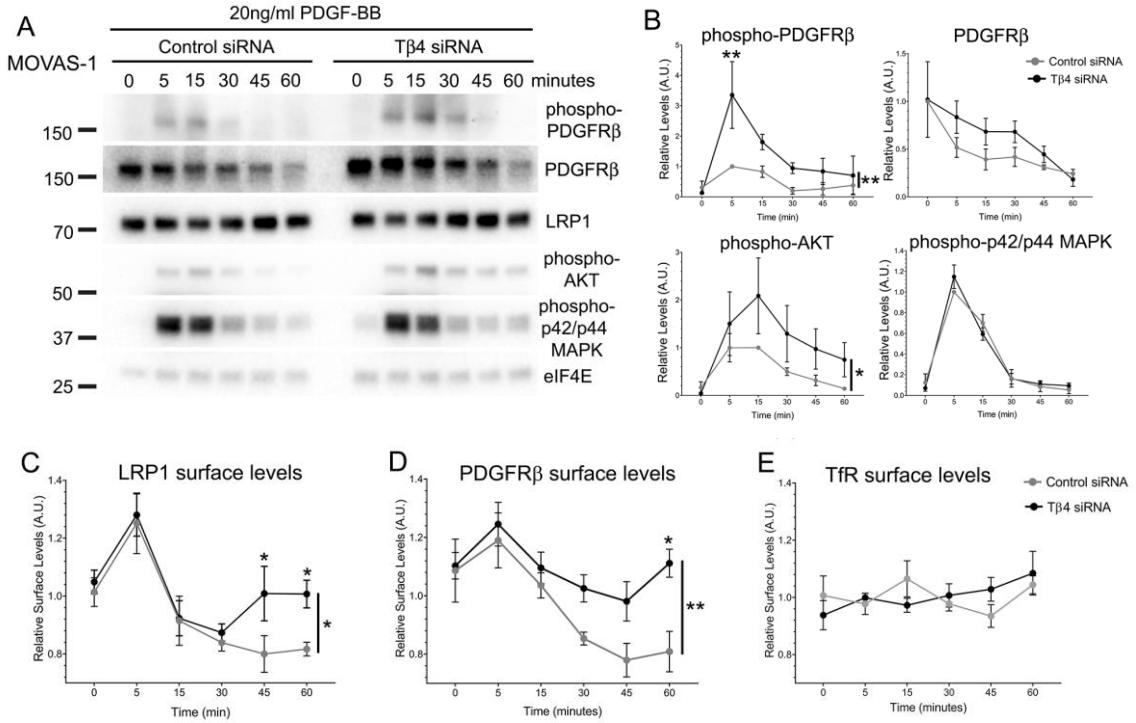
***Acknowledgement of Contribution:***

*Previously published data sets were used for analysis[21].*

*Computational analysis of scRNA-seq data was performed by Dr. Andia Redpath and Benjamin Pike (unpublished).*

## Appendix Figure 2 :

**VSMCs lacking Tβ4 show enhanced sensitivity to PDGF-BB resulting in increased LRP1-PDGFRβ cell surface exposure.**




*MOVAS-1 cells treatment with 20ng/ml PDGF-BB over a 60-minute time course show activation of PDGFRβ pathway demonstrated by western blotting in (**A**), quantified in **B**. Cell surface levels of LRP1 (**C**), PDGFRβ (**D**) and TfR (**E**) were measured by surface biotinylation assays. Data are presented as mean ± SEM; n=3 experiments (**A-B**); n=4 experiments (**C-E**). Significance was calculated using one-way ANOVA with Tukey's post-hoc tests. * P ≤ 0.05; ** P ≤0.01. Data published in[170], © J.Clin.Invest.*

***Acknowledgement of Contribution:*** *In vitro assays described in Appendix figure 2 were performed by Dr Susann Bruche.*

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