The Role of Vitamin D in the Development and Progression of Experimental Abdominal Aortic Aneurysm

Thesis Submitted by

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DECLARATION OF SOURCES

I, the undersigned declare that this research investigation has not been previously submitted anywhere for another degree or diploma at any university or institution of tertiary education in or out of Australia. Information derived from the published or unpublished works of others has been acknowledged in the text and a list of reference is given.

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted in accordance with guidelines for research ethics outlined in by the James Cook University Ethics Committee and adhered to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). All animal studies and experimental protocols were performed in accordance with institutional and ethical guidelines approved by the James Cook University Animal Ethics committee (Approval numbers: AEC#: 1970 & AEC#: A2354).

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ABSTRACT

Vitamin D deficiency has been associated with human abdominal aortic aneurysm (AAA) in epidemiologic studies; however, its role in AAA pathogenesis and progression is unclear. Recently, it was reported that within Apolipoprotein E (*ApoE*$^{-/-}$) mice in which sclerostin (SOST) was over expressed were protected from AAA induction by angiotensin II (AngII) infusion. Vitamin D has been reported to upregulate the expression of *SOST* in osteoclasts; however, its effect on the aorta and vascular smooth muscle cells (VSMCs) is unknown. The main hypothesis of this thesis was that vitamin D limited AAA development, growth and rupture through upregulation of SOST, particularly within the suprarenal aortic (SRA) wall. Effects of vitamin D on AAA development, growth and rupture were investigated *in vivo* using the AngII infusion *ApoE*$^{-/-}$ mouse model. The effect of vitamin D on VSMCs *in vitro* was also investigated. This thesis had four aims(1) to examine whether the active metabolite of vitamin D, 1,25(OH)2D3, induced SOST expression in VSMCs *in vitro*; (2) to investigate whether dietary-induced vitamin D deficiency reduced circulating levels of SOST and SRA SOST expression in *ApoE*$^{-/-}$ mice; (3) to investigate if dietary-induced deficiency of vitamin D promoted AAA development and rupture; and (4) to examine if raising circulating 25(OH)D levels through cholecalciferol (CCF) supplementation limited progression of established AAA.

Incubation of VSMCs with increasing concentrations of 1,25α-dihydroxyvitamin D3 (1α,25(OH)$_2$D3; 0, 0.1, 1, 10, 100 nmol/L) over 48 hours dose-dependently increased gene expression for *SOST* (P<0.001), vitamin D receptor (*VDR*; P<0.001), and 25-hydroxyvitamin D-24-hydroxylase (*CYP24A1;* P<0.001). In contrast, expression of 25-hydroxyvitamin D3 1-alpha-hydroxylase (*CYP27B1*; P<0.001), osteopontin (*OPN*; P<0.001), matrix metalloproteinases-2 (*Mmp-2*; P=0.004), and β-catenin (*CTNNB1*; P<0.001) were dose-dependently downregulated. Vitamin D deficiency in mice (no measurable circulating 25-hydroxyvitamin D) was associated with decreased circulating and aortic levels of SOST. In a separate study, AngII infusion in vitamin D deficient *ApoE*$^{-/-}$ mice resulted in the development of larger AAAs as assessed by ultrasound (P=0.034) and *ex vivo* morphometry [median (inter-quartile range) maximum suprarenal aortic diameter 2.34(2.08-3.1) mm vs 1.83(1.27-2.52) mm; P=0.041] that ruptured more commonly (48% vs 19%; P=0.028) than controls. Vitamin D deficiency was also associated with increased aortic expression of osteopontin (*Opn*), and *Mmp-2* and *Mmp-9* than controls. In the final study, CCF

administration to mice with ***pre-established*** AAA limited growth of AAA as assessed by ultrasound (P<0.001); and *ex vivo* morphometry [median (inter-quartile range) maximum suprarenal aortic diameter 3.03 (2.56-5.27) mm vs 2.54 (1.82-2.69) mm; P=0.035] and reduced rupture rate (8% vs 46%; P=0.031) which was associated with upregulation of circulating and aortic SOST. Additionally, CCF supplementation was shown to reduce SRA mRNA expression for a number of markers for that are involved in AAA progression, including *Mmp-2* (P=0.035), *Mmp-9* (P=0.020) and *Opn* (P=0.035).

In conclusion, the investigation suggested that vitamin D deficiency promotes development of large AAA with more propensity to rupture in an experimental mouse model. Furthermore, it was shown that CCF administration could limit both growth and rupture of AAA in mice with ***pre-established*** AAA. These effects of vitamin D appear to be mediated via changes in genes involved in extracellular matrix remodelling, particularly SOST. Findings from this thesis are supportive of future clinical trials aiming at evaluating therapeutic benefits of vitamin D supplementation in patients presenting with small AAA.

PUBLICATIONS

Published

Nsengiyumva V, Fernando ME, Moxon JV, Krishna SM, Pinchbeck J, Omer SM, Morris DR, Jones RE, Moran CS, Seto SW and Golledge J (2015). The association of circulating 25-hydroxyvitamin D concentration with peripheral arterial disease: A meta-analysis of observational studies. *Atherosclerosis,* 243(2), 645-651.

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Nsengiyumva V, Krishna SM, Moxon JV, Moran SM, Golledge J. A potential role of vitamin D in the pathogenesis of aortic aneurysms: A systematic review and meta-analysis. (In preparation).

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LIST OF ABBREVIATIONS AND ACRONYMS

1α,25(OH)$_2$D:	1α,25-dihydroxyvitamin D
25(OH)D:	25-hydroxvitamin D
2D UPLC-MS/MS	2-dimensional ultra-performance liquid chromatography separation coupled tandem mass spectrometry
AAA:	Abdominal aortic aneurysm
ABS:	Australian bureau of statistics
ACTA2:	Smooth muscle alpha (α)-2 actin
AMPK:	AMP-activated protein kinase
AngII:	Angiotensin-II
AP-1:	Activator protein-1
ATR1:	Angiotensin receptor-1
BM-MSCs:	Bone marrow-derived mesenchymal stem cells
BMI	body mass index
BSA	Bovine Serum Albumin
CALD1:	Caldesmon 1
CCF:	Cholecalciferol
CCL-1:	Chemokine (C-C motif) ligand
CNN1:	Calponin-1
CVD:	Cardiovascular disease
CYP24A1:	Cytochrome P450 family 24 subfamily A member 1
DAPI	4′,6-diamidino-2-phenylindole
Dkk-1	Dickkopf Wnt signalling pathway inhibitor 1
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
ECM:	Extracellular matrix
EGFR:	Epidermal growth factor receptor
Epi-C3-25(OH)D:	Epi-C3-25-hydroxyvitamin D
FGF23:	Fibroblast growth factor 23
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GSK-3	Glycogen synthase kinase 3
hrSOST	human recombinant SOST
HBSS	Hank's Balanced Salt Solution
HDL:	High-density lipoprotein
HIF1α:	Hypoxia-inducible factor 1-alpha
IHC:	Immunohistochemistry
IL-1:	Interleukin-1
IU	International Unit
IQR:	Interquartile range
JCU/AEC	James Cook University/Animal Ethics Committee
LV:	Left ventricle
MAPK	mitogen-activated protein kinase
MMP:	Matrix metalloproteinase

MYH11:	Myosin, Heavy Chain 11, Smooth Muscle
NFκB:	Nuclear factor-κB
NHF:	National Heart Foundation
NOTCH:	Notch homolog, translocation-associated
OCN	Osteocalcin
OCT:	Optimum cutting temperature
OPG	Osteoprotegerin
OPN	Osteopontin
PAD:	Peripheral artery disease
p-GSK-3:	Phosphorylated-glycogen synthase kinase 3
PPARα/γ:	Peroxisome proliferator–activated receptor α/γ
PTH:	Parathyroid hormone
RXR:	Retinoid X receptor
SBP	Systolic blood pressure
SMD:	Standard mean difference
SMTN:	Smoothelin
SNPs	single nucleotide polymorphisms
SOST	Sclerostin
SRA	Suprarenal aorta
TAA:	Thoracic aortic aneurysm
TAD:	Thoracic aortic dissection
TAGLN:	Transgelin
TGF-β:	Transforming growth factor-β
TNF-α:	Tumour necrosis factor-α
UK:	UK small aneurysm trial
UKSAT:	United Kingdom
USA:	United States of America
UVB:	ultraviolet B
VDBP:	Vitamin D binding protein
VDD	Vitamin D deficient
VDR	Vitamin D receptor
VDS	Vitamin D sufficient
VSMCs	Vascular smooth muscle cells
µL	Microliter
%	Percentage
<	Less than
≥	Greater than or equal to
≤	Less than or equal to
>	Greater than
º	Degree
µg	Microgram
µM	Micro molar

CHAPTER 1:

REVIEW OF THE LITERATURE

1.1. ABDOMINAL AORTIC ANEURYSM

1.1.1. The aorta in health and disease

Physiologically, the aorta channels oxygenated blood to the rest of the systemic arterial tree from the heart. Anatomically, the aorta extends from the valve adjoining the left ventricle (LV) where it facilitates post contraction outflow of blood and supply of the coronary circulation. The aorta then ascends slightly (≈ 5.0 cm long), before arching and extending through the thorax (thoracic aorta) and the abdomen (abdominal aorta). The thoracic aorta is posteriorly located in the mediastinal cavity, running longitudinally to the spinal cord, and to the diaphragm hiatus. The abdominal aorta extends from diaphragm hiatus, superior to renal arteries, (suprarenal), then infra-renally, before reaching the point where it bifurcates into the common iliac arteries (**Figure 1.1**). In the human adults, the normal aortic diameter is about 3.5 cm at the aortic root, and diminishes gradually to approximately 2.0 cm in the abdominal region. From early embryogenesis, the abdominal aorta is physiologically conditioned to effectively deliver blood directly to vital organs via its branching arteries, including the celiac, mesenteric (superior and inferior), renal (right and left) gonadal (right and left), common iliac (right and left) and spinal cord arteries.

Although it has been previously described as a conduit, the aorta is not merely a tube. It exhibits a highly organised physiology that facilitates an ever-continuous and dynamic function (Belz, 1995). The aorta plays a central role as an elastic buffer following every ventricular stroke (systole-diastole) through its recoil and distention (the Windkessel function) (Belz, 1995). Characteristically, the aortic wall consists of three concentrically arranged layers. The intima (innermost), media (middle) and adventitia (external) (**Table 1.1**). Each of three layers presents with unique histological features that are known to contribute to the homeostasis of the aorta (Stenmark et al., 2013). The aortic intimal lining is a selectively permeable and plays a critical role in the regulation of vascular tone, haemostasis and cellular leukocyte extravasation. The intima consists of endothelial cells, sub-endothelial basement and the fenestrated membrane. Endothelial function is important in the regulation of the blood flow due to their ability to provide an antithrombotic lining surface that is conducive to the smooth flow of blood and blood constituents (**Table 1.1**). This uniform blood flow is regulated via secretion and uptake of vasoactive factors that dilate and constrict neighbouring vascular smooth muscle cells (VSMCs) in a paracrine manner.

While earlier research had directed more interests to the function of the endothelium, recent progresses in vascular biology now recognizes an important role of all three layers in the regulation arterial function (Baltgaile, 2012). In addition, originally only thought to be a supportive tissue and extension of perivascular fats, adventitia is presently known to play distinct roles response to vascular hypoxia, injury and pulmonary hypertension (Stenmark et al., 2013; F. Xu, J. Ji, L. Li, R. Chen, & W. Hu, 2007; F. Xu, J. Ji, L. Li, R. Chen, & W. C. Hu, 2007). The adventitia has been shown to respond to both physiological and pathophysiological conditions including vascular remodelling, tissue repair and extracellular matrix (ECM) deposition. The aortic wall consists of elastic fibres (≈40%); however, elastin content diminishes progressively from towards distal aorta (Bader, 1967). Elasticity in the human aorta predicts the aortic stiffness; and this has been shown to correlate with age and physiological pressure (Lindesay, Ragonnet, Chimenti, Villeneuve, & Vayssettes-Courchay, 2016).

Table 1.1: The aortic wall structure

Aortic layer	**Major component**	**Main physiological function**
Intima	Basal lamina	Structural support and permeability control
	Endothelial cells	Secretion vasoactive factors and vascular tone
	Sub-endothelial tissue	Endothelial support & connective tissue
Media	Concentric VSMCs	Distention and recoiling and synthesis of ECM
	Elastic lamellae	Structural support of the aorta
	Elastin	Viscoelasticity; recoil and distention properties
	Collagen	Tensile strength, resilience to over-distention
Adventitia	ECM	Integral support, aortic wall structural resilience

Abbreviation: VSMCs: vascular smooth muscle cells; ECM: extracellular matrix

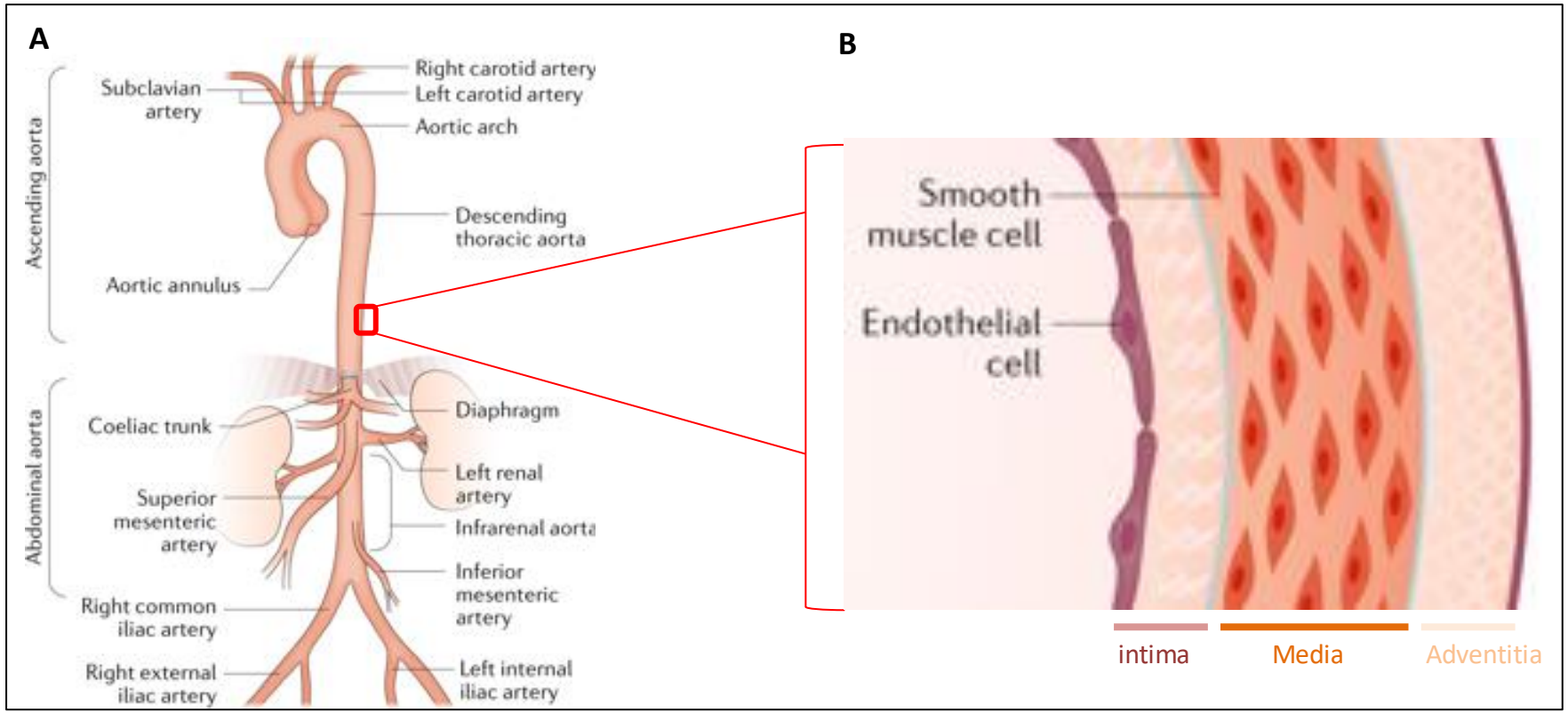


Figure 1.1: Schematic diagram of aortic anatomy

(A) The descending thoracic aorta spans from distal to the origin of the left subclavian artery to the diaphragm. Abdominal aortic aneurysms commonly develop in the infrarenal region. The normal aortic wall structure. **(B)** Normal aortic wall comprises of intima (innermost layer), media (the middle layer) and adventitia (the outer layer). Aortic diameter can be measured based on inner wall-to-inner wall which is the inside space of the aorta, or can be measured from outer layer-to-outer layer which indicates the maximal aortic diameter when measured at systole. Adapted from Sakalihasan et al (2018)

1.1.2. Abdominal aortic aneurysm, prevalence and risk factors

Abdominal aortic aneurysms (AAA) is a multifactorial cardiovascular pathology characterised by gradual weakening in the aortic wall (Johnston et al., 1991; Joseph V Moxon et al., 2010; Natzi Sakalihasan, Limet, & Defawe, 2005). In human, AAA is commonly defined as localised expansion of the infrarenal aorta ≥3.0 cm. Contemporary reports suggest that AAA affects between 1-2% of men and about 1% of women in the general population (Golledge et al., 2019). Described as an age-related disease, AAA is currently recognised as one of the causes of premature deaths in people aged >65 years old (Sampson et al., 2014). AAA rupture is a life-threatening emergency with up 90% risk of death (Budd, Finch, & Carter, 1989; Johansson & Swedenborg, 1986; Moll et al., 2011; Natzi Sakalihasan et al., 2005). The natural course of AAA is progressive expansion, which increases aortic rupture risk (Baxter et al., 2016; Longo et al., 2002; Wassef et al., 2001). Ruptured AAA is a life-threatening medical emergency that has been associated with almost 95% mortality (Budd et al., 1989; Johansson & Swedenborg, 1986; Moll et al., 2011; Natzi Sakalihasan et al., 2005), although recent advances in AAA patient care now suggest >50% mortality (Chaikof et al., 2018). The prevalence of AAA varies among epidemiological studies depending on the target population (Moukarzel, Mao, & Eraso, 2013; Tang et al., 2016). Actual mortality data resulting from AAA is sometimes hampered by possible undocumented causes of deaths (Golledge, Muller, Daugherty, & Norman, 2006; Moukarzel et al., 2013).

Despite the significant public and healthcare burden posed by the disease, there still exists no effective pharmacological treatment for AAA (Golledge & Norman, 2011; Golledge & Powell, 2007; Kokje, Hamming, & Lindeman, 2015; Lindeman & Matsumura, 2019). Repair surgery remains the only medical option presently afforded to AAA patients (Golledge & Norman, 2011; Golledge & Powell, 2007; Kokje et al., 2015; Lindeman & Matsumura, 2019). Guidelines recommend that small AAA (3.0-5.0 cm) be conservatively managed with serial ultrasound surveillance until they reach the elective diameter for surgery (i.e. 5.5 cm for men and 5.0 for women) (Golledge & Norman, 2011; Golledge & Powell, 2007; Kokje et al., 2015; Lindeman & Matsumura, 2019). Although rupture risk in small aneurysms is minimal, the majority of AAAs continue to expand thus increasing the risk of rupture (Golledge, Norman, Murphy, & Dalman, 2017). AAA repair surgery is also associated with a significant mortality rate, and cost-effectiveness of the procedure remains debatable (Dua, Kuy, Lee, Upchurch Jr, & Desai, 2014). For instance, with approximately 25000 AAA repair procedures (endovascular and open surgery) performed in the USA yearly, the median in-hospital cost has been estimated

to be $58,305 USD (Suckow et al., 2018). The figure markedly goes up for procedures performed on ruptured AAA (84,744 USD) (Dua et al., 2014). The figures from Centre for Disease Control and prevention (USA) indicated that, in 2009, AAA was the primary killer in 10,597 deaths and an accomplice in another 17,215 deaths (Go et al., 2013; Kochanek, Xu, Murphy, Minino, & Kung, 2011; Suckow et al., 2018). Although women are underrepresented in epidemiological studies compared to men, it has been suggested that the prognosis of small AAA is worse in women; and that women have a higher post-operative mortality compared with men (Hultgren, Vishnevskaya, & Wahlgren, 2013; Ulug, Sweeting, von Allmen, Thompson, & Powell, 2017).

The majority of AAAs are diagnosed when they are small to medium in size (i.e. 3.0-5.0 cm) (J. Golledge, Norman, Murphy, & Dalman, 2016). Most AAAs continue to expand to >5.5 cm; a diameter at which surgical repair may be required to prevent aortic rupture because the risk of rupture increases significantly from this diameter (Powell et al., 2017). Although the commonly reported average expansion rate has varied between 0.25 to 0.50 cm per year, larger AAAs tend expand much faster than smaller aneurysms (J. Golledge, Norman, et al., 2016). Recent reports have shown that about 25000 AAA repair procedures (endovascular and open surgery) are performed in the USA, annually (Dua et al., 2014).

Ultrasound is a useful tool in both AAA research and clinical diagnosis. Ultrasound assessment is a non-invasive accurate and specific modality for AAA diagnosis. It however remains an unmet need to find biomarkers that can be specifically used for AAA diagnosis. Until now, no single biomarker that has been found to be highly sensitive and specific enough for prediction of AAA rupture (Filis et al., 2014; Golledge, Tsao, Dalman, & Norman, 2008; Hellenthal, Buurman, Wodzig, & Schurink, 2009; Moxon et al., 2014; Moxon et al., 2010; Stather et al., 2014). Moreover, AAA is understood to be a multifactorial and a multistage disease, suggesting that factors in early stages of the disease could be different from those involved in the disease progression (Nordon, Hinchliffe, Holt, Loftus, & Thompson, 2009). Well-documented risk factors for AAA include advanced age (≥ 60), cigarette smoking, male gender and genetic predisposition (Björck & Wanhainen, 2013; Nordon et al., 2009). Other AAA risk factors, also including uncontrolled hypertension, dyslipidaemia and presence of cardiovascular events comorbidity (Björck & Wanhainen, 2013; Brewster et al., 2003; Nordon et al., 2009). AAA risk factors can also be classified as modifiable and non-modifiable. Lifestyle modification such smoking cessation, dietary and exercise regimen are among modifiable risk factors while genetic predisposition, age and race are non-modifiable risk factors (Björck & Wanhainen,

2013; Brewster et al., 2003; Nordon et al., 2009). Given that individuals who are smokers are seven times more likely to develop AAA compared with than non-smokers, cigarette smoking remains biggest modifiable risk factor (Aggarwal, Qamar, Sharma, & Sharma, 2011; Aune, Schlesinger, Norat, & Riboli, 2018). In addition, AAA has been reported to expand faster in smoking patients compared with non-smokers (Aggarwal et al., 2011; Aune et al., 2018). Therefore, smoking cessation is the most important lifestyle modification that patients are advised to make as this has been shown to regress the risks (Aune et al., 2018). Dietary regimen such as increase intake of fruits and vegetables has also been reported to lower the risk of AAA development (Nordkvist, Sonestedt, & Acosta, 2018; Stackelberg, Bjorck, Larsson, Orsini, & Wolk, 2013).

1.1.3. AAA pathophysiology

Current understanding of the mechanisms implicated in AAA pathogenesis remains somewhat incomplete. However, it is likely that aortic wall inflammation and degradation of the adventitial layer influence both development and progression of AAA (Golledge et al., 2019; Nordon et al., 2009; Wassef et al., 2001). Depletion of vascular smooth muscle cells (VSMCs) and destruction of the supportive extracellular matrix (ECM) are the primary characteristics of AAA diseased tissue (Nordon et al., 2009). Infiltration of inflammatory and immune cells has also been consistently reported in the pathogenesis of AAA (Krishna et al., 2017; Krishna et al., 2015; Nordon et al., 2009). Chronic transmural inflammation contributes to AAA development through infiltration of mononuclear phagocytes and lymphocytes in the media and adventitial layer (Krishna et al., 2017). The infusion of AngII in *ApoE*$^{-/-}$ mice, one of the most commonly used rodent models, induces inflammation, atherosclerosis and ECM proteolysis (Daugherty & Cassis, 2004; Davis, Rateri, & Daugherty, 2014; Krishna et al., 2017; Seto, Krishna, Moran, Liu, & Golledge, 2014). Within this mouse model, collagen and elastin are degraded due chronic production of inflammatory mediators, including leukocyte adhesion molecules and chemokines is understood to drive the progression of AAA growth (Krishna et al., 2017; Krishna et al., 2015). The infiltration of lymphocytes expressing IL-4, IL-5, IL-8 and IL-10 and tumour necrosis factor (TNF-alpha), which regulate the local immune response, have also been reported as important contributors in AAA formation (Daugherty & Cassis, 2004; Davis et al., 2014; Juvonen et al., 1997; Krishna et al., 2017; Seto et al., 2014). Involvement of mast cells has also been highlighted in AAA formation due to their release of several proteases, growth factors, cytokines, and chemokines through degranulation (Lindholt & Shi, 2006). Furthermore, mast cells are likely implicated in AAA development by inducing the activation

of T-lymphocytes and macrophages which in turn release various pro-inflammatory cytokines (Lindholt & Shi, 2006). It was suggested that the peptide fragments resulted from the degradation of the ECM might serve as chemotactic agents for the infiltrating macrophages (Lindholt & Shi, 2006). Secretion of chemokines such as interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1) continually trigger leukocyte recruitment in the microenvironment of the aortic wall, resulting in chronic inflammation (Juvonen et al., 1997; Krishna et al., 2017; Lindholt & Shi, 2006).

Experimental studies on AAA formation and progression have relied heavily on rodent models (*in vivo*) and cell experiments (*in vivo*) (Golledge & Norman, 2010; Nordon et al., 2009). Descriptive assessments of diseased AAA tissue and circulating biomarkers have also informed the current understanding of AAA pathogenesis. Among other hallmark features, presence of inflammatory cell infiltrates within the aortic wall, depletion of medial VSMCs and degradation of ECM in the adventitia have been consistently reported in AAA tissue (Boddy et al., 2008; Maegdefessel, Dalman, & Tsao, 2014). A number of circulating biomarkers have also been associated with AAA presence (Golledge, Walker, Norman, & Golledge, 2009; Golledge et al., 2008). Among these are matrix degrading proteins, thrombogenic markers and inflammatory markers (Golledge et al., 2008; Hellenthal et al., 2009; Urbonavicius et al., 2008). Interestingly, there has been strong evidence on association between AAA and atherosclerosis, although a causal relationship between the two conditions has not yet been established (Golledge & Norman, 2010). Similarly, within a mouse model of AAA, a diseased supra-renal aortic (SRA) wall is characterised by presence of immune and inflammatory cells as well as proteolytic degradation of ECM (Krishna et al., 2017). Other features include presence of atherosclerotic plaques and intraluminal thrombus (ILT) (Nordon et al., 2009).

1.1.4. Medical management of AAA

The lack of pharmacological therapy for AAA patients unfortunately means that small AAAs are managed through serial surveillance with ultrasound. Theoretically, all AAAs continue to expand and may ultimately require prophylactic measures to slow or limit their progression to rupture (Chaikof et al., 2018; Tang et al., 2016). In male AAA patients, surgery is indicated in patients with AAA $\geq$ 5.5 cm while in female surgery is recommended when the diameter is equal or exceeds 5.0 cm (Chaikof et al., 2018; Golledge, Norman, et al., 2016). Additionally, prophylactic AAA repair may be recommended when there is rapid diameter expansion >1.0 cm/year or when the patient complains of symptoms such as abdominal and/or back pain and

distal embolization (Chaikof et al., 2018; Robinson, Mees, Verhagen, & Chuen, 2013; Natzi Sakalihasan et al., 2005). In Western countries, vascular surgery centres are reported to electively perform about 75-85% of AAA repairs for intact aneurysms (Beck et al., 2016; Karthikesalingam et al., 2016). AAA surgery is categorised into two main options: open AAA repair surgery or endovascular AAA repair (EVAR) surgery (Chaikof et al., 2018). Irrespective of whether a less invasive EVAR or an invasive open surgery is carried out, AAA repair surgery always comes with a number of significant risks ranging from short-term and long-term risks. Short-term risks may include perioperative complications such as death and renal failure while long-term risks include graft failure requiring repeat surgical procedures or death resulting from AAA rupture (Chaikof et al., 2018; Golledge, Norman, et al., 2017; Natzi Sakalihasan et al., 2005). Open surgery is an invasive procedure usually performed with laparotomy and consists of the interposition of an arterial prosthesis to contain the aneurysmal sac (Chaikof et al., 2018). Open AAA repair procedure is important for patients in whom EVAR is contraindicated, patients with persistent endoleak, graft infection or mycotic AAA (Chaikof et al., 2018). Reports suggest that young and healthy patients, with an AAA 5.0 - 5.4 cm, may benefit from early repair, especially young women (IMPROVE Trial Investigators: Ulug P et al, 2017). However, older patients with significant comorbidities or other frailty risk factors that are considered risky for surgery may be delayed even for AAA diameter greater than 5.4 cm (IMPROVE Trial Investigators: Ulug P et al, 2017; Powell et al., 2017). In contrast to asymptomatic AAA, ruptured AAA are regarded as life-threatening emergencies. Ruptured AAA requires immediate and prompt surgical repair, and both EVAR and open surgery are currently offered to patients with ruptured AAA (Chaikof et al., 2018). Contemporary reports suggest that short and long-term benefits may be better in AAA patients undergoing EVAR surgery compared with open surgery (IMPROVE Trial Investigators: Ulug P et al, 2017; Powell et al., 2017).

1.1.5. Pharmacological management for AAA

A common theme in findings from clinical trials and pharmacological intervention for AAA remains that there is an unmet need in AAA research (Golledge et al., 2019; Golledge, Norman, et al., 2017; Lindeman & Matsumura, 2019; Sakalihasan et al., 2018; Yoshimura et al., 2018). In essence, continuous expansion of AAA and consequent aortic rupture may be inevitable in many cases unless AAA repair surgery is offered to patients at the right time. Pharmacological therapy to prevent AAA progression could have significant impacts on the management of patients. Indeed, AAA pathophysiology is thought be categorised into 3 stages which are

initiation, progression and rupture (Bi et al., 2014; Nordon et al., 2009). The initiation stage has been suggested to be characterised by immune response, inflammation, atherosclerosis and matrix dysfunction, suggesting that these pathways are attractive targets to inhibit AAA formation. The progression stage is understood to exhibit exacerbated chronic inflammation, matrix degradation and VSMCs depletion (Bi et al., 2014; Krishna et al., 2015; Nordon et al., 2009). Biomechanical disturbance is also understood to play an adverse role during AAA progression, particularly at the point where the aortic wall integrity can longer withstand internal hemodynamic stress (Aronow et al., 2011; Bi et al., 2014). Indeed, the majority of previous and recent studies have focused on effects of pharmacological interventions that can specifically target to lower blood pressure, eliminate *Chlamydia pneumoniae* infection, correct dyslipidaemia and prevent plaque formation, or limit inflammation and matrix remodelling (**Table 1.2 & Table 1.3)** (Golledge et al., 2019; Lindeman & Matsumura, 2019).

Examples of targeted inflammatory pathways are summarised herein; and have included inhibition of NFκB (Saito et al., 2013), inhibition of the Rho kinase signalling (Wang et al., 2005) and modulation of immune and inflammatory cells (Eliason et al., 2005) **(Table 1.2 & Table 1.3)**. Notably, inhibition of antigen-induced allergic reactions, a mast cell target, pemirolast, was investigated in a randomized clinical trial (Sillesen et al., 2015). Another interesting pharmacological target for AAA formation has been inhibition of proteases such as MMPs (Ennis et al., 2012), serine protease (Deng et al., 2003) and cysteine protease (Sun et al., 2012) **(Table 1.2 & Table 1.3)**. In particular, inhibition of MMPs such as MMP-2 and MMP-9 are an interesting target since these proteases are consistently demonstrated within the aortic tissue of AAA patients and in blood markers (Golledge et al., 2009; Meijer et al., 2013; Mosorin et al., 2001). However, translation of pre-clinical findings into clinical trials has continued to yield disappointing outcomes. A comprehensive systematic review and meta-analysis by Golledge *et al* (2019) that examined the effect of different antibiotic regimens on AAA progression in randomised placebo-controlled trials showed no significant effects on AAA growth rate (mm/year). Further analysis on effects of different antihypertensive drugs, including propanol, perindopril and amlodipine on reduction of AAA rupture or delaying AAA surgical repair (defined as clinical events) in randomised placebo-controlled trials showed marginal benefits (Golledge et al., 2019).

Most pre-clinical investigations in AAA research have been investigated in mouse models (Davis et al., 2014; N. Sakalihasan et al., 2018). Given, the differences between humans and

animal models, it is also important that effectiveness of pharmacological interventions be studied using human cells and possibly in human AAA explant culture (Davis et al., 2014; Manning, Cassi, Huang, Szilvassy, & Daugherty, 2002). Regulation of pro-inflammatory mediators, the renin-angiotensin system (RAS) are thought to be critical targets in order to halt the development and progression of pre-existing AAA have also been investigated (Golledge et al., 2019; Sakalihasan et al., 2018; Natzi Sakalihasan et al., 2005). In addition, abrogation of ECM degradation through inhibition of MMPs is also believed to be an effective approach in preventing AAA development and progression (Golledge et al., 2019; Golledge, Norman, et al., 2017; Sakalihasan et al., 2018). Previously, treatment with HMG-CoA reductase inhibitors (statins) or angiotensin-converting enzyme inhibitors was also effective in reducing inflammatory responses in human AAA tissue culture (Yoshimura et al., 2018). In addition, AAA cell therapy that has been performed using local infusion of bone marrow-derived mesenchymal stem cells (BM-MSCs), endothelial cells or VSMCs, as well as systemic injection of BM-MSCs, was shown to be effective in suppressing the progression of pre-existing AAA in animal models (Allaire et al., 2004; Schneider et al., 2013; Yamawaki-Ogata et al., 2014).

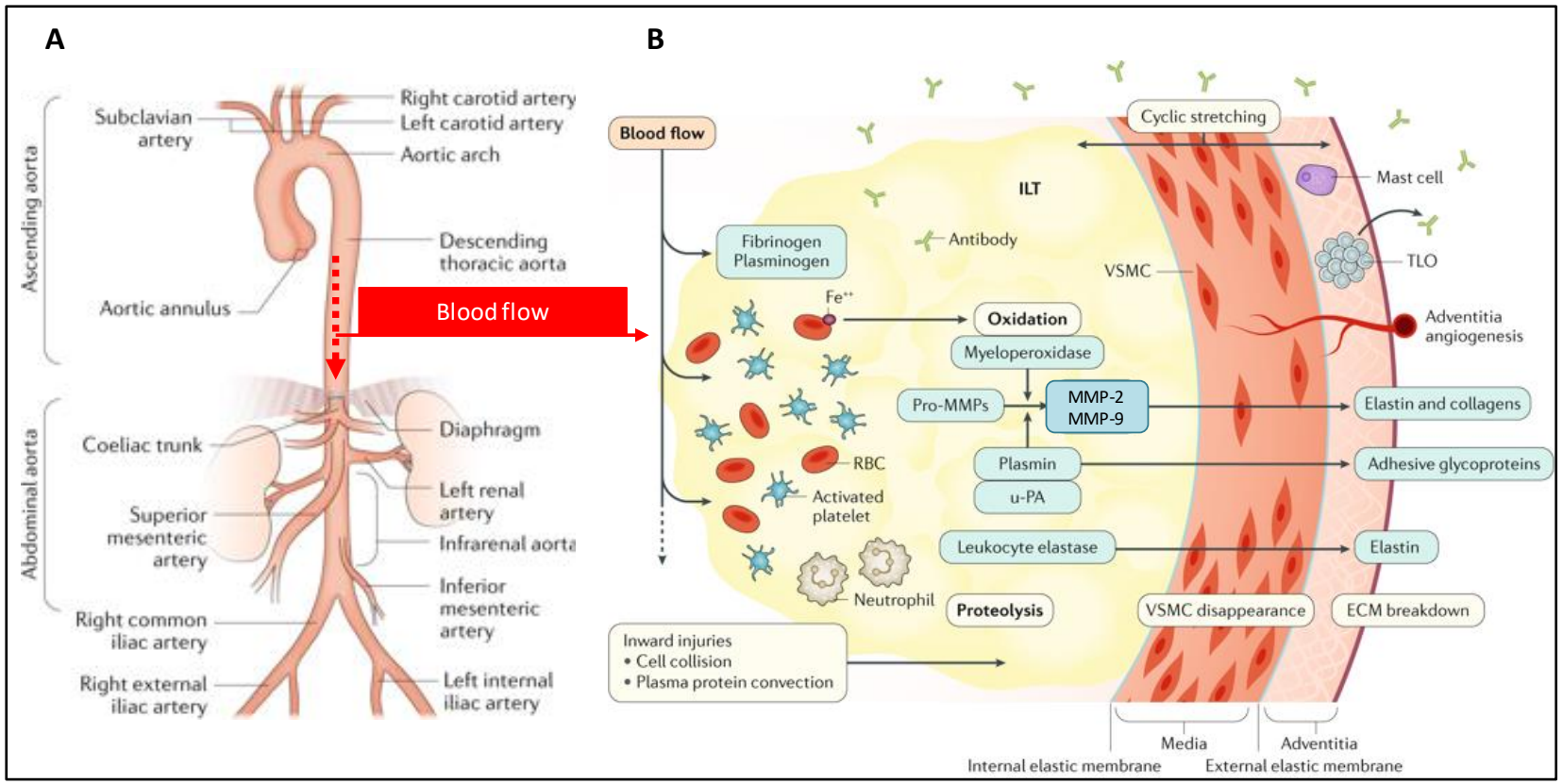


Figure 1.2: Hypothetical model of AAA pathogenesis.

Development and progression of AAA is believed to be driven by inflammation and matrix remodelling. These processes result in breakdown of extracellular matrices (ECM), apoptosis of vascular smooth muscle cells (VSMCs) and activation of immune response. AAA tissue samples exhibit increased local production of collagenases, elastase, and MMPs which degrade collagen and elastin ECM proteins. Serine proteases, neutrophil elastase, as well as cysteine proteases are also elevated in AAA tissue. These proteinases are produced by macrophages, ECs, VSMCs and fibroblasts within the arterial wall. Adapted from Sakalihasan *et al* (2018)

High blood pressure is thought to play an important role in the development and progression of AAA in a sense that hypertension affects the aortic wall ability to withstand the haemodynamic stress in AAA patients (Kobeissi, Hibino, Pan, & Aune, 2019). Current clinical guidelines suggest that CVD related events and risk factors should be promptly managed in patients with AAA (Golledge & Norman, 2011; Golledge & Powell, 2007; Lindeman & Matsumura, 2019). Propranolol, a β-blocker, was one of the first drugs to be proposed for inhibition of AAA development in animal models (Lindeman & Matsumura, 2019). Seemingly, the choice to test propranolol in subsequent clinical trials is because pre-clinical studies in animals had used this beta-blocker, suggesting that other beta-blocker agents would perhaps work in similar manner (Brophy, Tilson, & Tilson, 1989; Ricci et al., 1996; Simpson, 1972; Simpson & Boucek, 1983; Simpson, Boucek, & Noble, 1976; Slaiby, Ricci, Gadowski, Hendley, & Pilcher, 1994). However, in a randomized trial, AAA patients did not tolerate propranolol well, and the drug did not significantly affect the growth rate of AAA (Investigators., 2002). Randomized clinical trials of antibiotics, including doxycycline and azithromycin, were conducted previously, but did not clearly demonstrate the effect of reducing AAA growth rate (Golledge et al., 2019; Lindeman & Matsumura, 2019; Meijer et al., 2013). Disappointingly, another randomised trial that investigated effects of doxycycline, an MMP inhibitor, in a larger number of patients for a longer period (18 months) still reported that doxycycline treatment did not reduce AAA growth or delay the need for patients to require AAA surgical repair (Meijer et al., 2013). Recently, pemirolast, a mast cell inhibitor, and perindopril, an ACE inhibitor, have been investigated in patients but neither drug reduced AAA growth rate (Sillesen et al., 2015). Several small observational studies demonstrated an association between statin administration and decreased AAA growth, although the beneficial effect of statins has not been confirmed in larger clinical trials (Kokje et al., 2015; Lysgaard Poulsen, Stubbe, & Lindholt, 2016; Yoshimura et al., 2018). There is therefore no scientific evidence currently to support the efficacy of pharmacological treatment to reduce AAA growth in humans (Golledge et al., 2019; Lindeman & Matsumura, 2019; Wang et al., 2017).

Table 1.2: Examples of targeted pathways and mechanisms in previous pharmacotherapy studies		
Targeted cluster	Reported mechanisms	References*
Antibiotics	Elimination of *Chlamydia pneumoniae*‡	Karlsson, *et al* (2009)
Anti-inflammatory	Inhibition of NFκB AP-1	Saito, *et al* (2013) Yoshimura, *et al* (2005)
	Inhibition Rho kinase	Wang, *et al* (2005)
	Inhibition of IL1 TNFα CCL-1	Johnston, *et al* (2013) Xiong, *et al* (2009) Wang, *et al* (2014)
	Depletion of B cell or γδT-cells	Schaheen, *et al* (2016) Zhang, *et al* (2018)
	Neutrophil inhibition	Eliason, *et al* (2005) Baxter & Pipinos (2006)
	Mast cell inhibition‡	Sillesen, *et al* (2015)
	Complement inhibition	Zhou, *et al* (2013)
	Oxilipin inhibition	Ahluwalia, *et al* (2007)
	Immune suppression	Marinkovic, *et al* (2013)
Protease inhibition	MMP inhibition‡	Allaire, *et al* (1998) Ennis, *et al* (2012)
	Cysteine protease inhibition	Sun, *et al* (2012)
	Serine protease inhibition	Deng, *et al* (2003)
Oxidative stress	Antioxidant enzymes	Maiellaro-Rafferty, *et al* (2011)
	Secondary antioxidants	Gavrila, *et al* (2005)
Blood pressure lowering	β-Blockers‡	Lindholt, *et al* (1999) Propranolol Aneurysm Trial (2002)
	Ca antagonists	Miao, *et al* (2015)
	ACE inhibitors‡	Xiong, *et al* (2014) Liao, *et al* (2001)
	ATR1 antagonists	Iida, *et al* (2012)
	iNOS inhibition	Lizarbe, *et al* (2009)

Lipid metabolism	Statins	Shiraya, *et al* (2009) Kalyanasundaram, *et al* (2006)
	HDL	Delbosc, *et al* (2016)
	PPARα/γ activation ‡	Golledge, *et al* (2010) Krishna, *et al* (2012)
	RXR	Escudero, *et al* (2015) Martorell, *et al* (2016)
Cell therapy	Mesenchymal stem cells	Blose, *et al* (2014)
	Fibroblasts	Giraud, *et al* (2017)
Matrix/morphogens	Interference with TGF-β signalling	Gaoand, *et al* (2014)
	Interference with NOTCH signalling	Cheng, *et al* (2014)
	Interference with Wnt signalling	Krishna, *et al* (2017)
	Thrombospondin inhibition	Krishna, *et al* (2015)
	EGFR inhibition	Obama, *et al* (2015)
Metabolism	Inhibition of HIF1α	Yang, *et al* (2016)
	Activation of AMPK	Yang, *et al* (2017)
Nutraceuticals	Polyphenols	Wang, *et al* (2017)
	Phytoestrogens	Lu, *et al* (2014)
	Omega-3 fatty acids	Meital, *et al* (2019)
	Resveratrol	Moran, *et al* (2017)
Sex hormones	Castration	Zhangand, *et al* (2015)
	Oestrogens	Martin-McNultyand, *et al* (2003)

Abbreviations: ACE: angiotensin-converting enzyme; AMPK, 5' AMP-activated protein kinase; AP-1, activator protein-1; ATR1, angiotensin II receptor type 1; CCL-1, chemokine (C-C motif) ligand; EGFR, epidermal growth factor receptor; HDL, high-density lipoprotein; HIF1α, hypoxia-inducible factor 1-alpha; IL-1, interleukin-1; iNOS, inducible NO synthase; MMP, matrix metalloproteinase; NFκB, nuclear factor-κB; NOTCH, notch homolog, translocation-associated; PPARα/γ, peroxisome proliferator–activated receptor α/γ; RXR, retinoid X receptor; TGF-β, transforming growth factor-β; and TNF-α, tumour necrosis factor-α. * Not an exhaustive list of references; ‡ Indicates targeted mechanisms

which have progressed to randomised clinical trials. Adapted from Lindeman & Matsumura (2019)

1.2. SOST AND WNT/B-CATENIN SIGNALLING IN AAA

AAA is a multifactorial and multistage disease; and given the complex pathophysiological mechanisms, potential therapies should plausibly possess pleiotropic effects. One interesting pathways that is emerging as important in cardiovascular and vascular wall function is Wnt/β-catenin signalling (Foulquier et al., 2018). Wnt/β-catenin signalling negative regulators play an important role in bone diseases and other pathophysiological conditions (Marinou, Christodoulides, Antoniades, & Koutsilieris, 2012; Mason & Williams, 2010; Nusse & Clevers, 2017). Mechanistically, in the absence of competitive ligands, Wnt molecules interact with the frizzled receptors, mainly LRP5/6, which leads to downstream effects on cytosolic subunits such as phosphorylation of the GSK-3α/β and upregulation of axin (Jho et al., 2002). Phosphorylated GSK-3α/β results in dissociation and accumulation of the β-catenin complexes in the cytoplasm. The β-catenin complex then translocates to the nucleus and mediates gene transcription through TCF/LEF transcriptional factors. In the presence of competitive Wnt inhibitors, however, non-phosphorylation of the GSK-3α/β subunits results in proteasomal degradation of β-catenin thus limiting further translocation into the nucleus. Activation of Wnt/β-catenin signalling releases the β-catenin from the binding protein subunits such as axin and increases the cytoplasmic accumulation of β-catenin subunits, eventually promoting nuclear translocation of these subunits. Within the nucleus, β-catenin interacts with transcriptional factors, initiating the expression of several target genes (Nusse & Clevers, 2017). Conversely, in the absence of Wnt binding, perhaps due to competitive inhibitors such as *Sost* or Dkk-1, β-catenin is in a steady state, and free β-catenin is eventually degraded by 26S proteasome (Nusse & Clevers, 2017). Wnt/β-catenin signalling controls the expression of OPG, OPN and matrix metalloproteinase (MMP) -2, -7 and -9 which have all been implicated in AAA pathogenesis (Maruotti, Corrado, Neve, & Cantatore, 2013; Wu, Crampton, & Hughes, 2007; Zhang, Gaspard, & Chung, 2001).

Table 1.3: Examples of pharmacological agents tested in pre-clinical AAA studies and have progressed to randomised clinical trials			
Agent	**Reported-findings from pre-clinical studies***	**Clinical trial**	**Trials outcomes on AAA diameter**
Propranolol	**Findings:** Propranolol preserved structural morphology aortic elastic fibres which was associated with reduction of aortic rupture in turkeys whereby AAA was induced by β-aminopropionitrile (BAPN) (Charles F. Simpson et al., 1976). In normotensive and genetically hypertensive rats in which AAA was induced by perfusion of the isolated infrarenal aorta with elastase; propranolol slowed AAA size postoperatively	Propranolol versus placebo trial (Lindholt et al., 1999)	AAA annual growth rate was similar between the placebo and the propanol group.

	which was assessed at days 7 and 14 (Ricci et al., 1996; Slaiby et al., 1994). **Mechanisms:** Increased aortic tensile strength, decrease in blood pressure, lower heart rate and reduced myocardial contractility (Simpson, 1972; Simpson & Boucek, 1983; Simpson et al., 1976). Reduced systolic tail blood pressure monitored with a plethysmograph (Ricci et al., 1996; Slaiby et al., 1994).	Propranolol for Small Aneurysms (Investigators., 2002).	Patients in the propranolol group had significantly poorer quality of life scores in the physical functioning, which questioned the safety and tolerability of the drug (Investigators., 2002; Lindholt et al., 1999).
Azithromycin & Roxithromycin	**Findings:** Treatment of *Chlamydia pneumoniae* infected rabbits with azithromycin at early stage prevented atherosclerosis which was thought to be a cause for AAA development (Fong et al., 1999; Kol, Bourcier, Lichtman, & Libby, 1999). **Mechanism:** Antibiotic (Macrolide) were associated with presence of *Chlamydia*	Azithromycin Trial (Karlsson et al., 2009)	No effect with Azithromycin on AAA expansion and no correlation was found between serological markers for *Chlamydia pneumoniae* and AAA expansion which suggested that no clinical relevance for *Chlamydia pneumoniae* testing in AAA surveillance.

	pneumonia which results in atherosclerosis plaques formation within the aorta, which was thought to be a cause for AAA development. Both Azithromycin and Roxithromycin are antibiotics that limited growths of *Chlamydia pneumoniae.*	Roxithromycin Trial 1 (Vammen, Lindholt, Ostergaard, Fasting, & Henneberg, 2001)	During the first year the mean annual expansion rate of AAAs was reduced by 44% in the Roxithromycin group. During the second year the difference was only 5%. Multiple linear regression analysis showed that Roxithromycin treatment and initial AAA size were significantly related to AAA expansion when adjusted for smoking; diastolic blood pressure and immunoglobulin A.
		Roxithromycin Trial 2 (Hogh et al., 2009)	Intermittent, long-term Roxithromycin-treatment reduced mean annual growth rate by 36% compared with placebo after adjustment for potential confounders. Long-term Roxithromycin-treated patients had a 29% lower risk of being referred for surgical evaluation, increasing to 57% after adjusting for potential confounders.

Doxycycline	**Findings:** Doxycycline inhibited MMP activity and elastin degradation of the aortic wall and attenuated AAAs development in individual animals (Pyo et al., 2000). AAA-suppressing effects of doxycycline were reported to be dose-dependent and were different from its antibiotic activities, and they coincide with the structural preservation of medial elastin fibres (Curci et al., 2000; Curci, Petrinec, Liao, Golub, & Thompson, 1998). **Mechanism:** Antibiotic was shown to limit *Chlamydia pneumoniae* and limit MMP degradation of the aortic wall.	Doxycycline Trial I (Mosorin et al., 2001)	AAA expansion rate in the AAA diameter was lower in doxycycline treated group compared with placebo group during the 6- to 12-month follow-up period.
		Doxycycline Trial II (Baxter et al., 2016)	The outcome is yet to be reported.
		PHAST Trial (Meijer et al., 2013)	Treatment with doxycycline surprisingly increased AAA growth rate compared with placebo group during the 18-month follow-up period
Pemirolast	**Findings:** reported that human AAA lesions had high numbers of chymase-immunoreactive mast cells and serum chymase level were found to be correlated with AAA growth rate AAA in in wild-type mice and mice deficient in mast cell protease-4 (Sun et al., 2009).	AORTA trial (Sillesen et al., 2015)	There was no difference in AAA growth between patients who were treated with pemirolast and placebo. Pemirolast intervention did not affect the need for AAA surgery repair.

	Inhibition of chymase by a specific chymase inhibitor, NK3201, was shown to suppress MMP-9 in a dog AAA model (Furubayashi et al., 2007), inhibited AAA development in a hamster experimental model (Tsunemi et al., 2004); and inhibited elastase-induced AAA in mice (Sun et al., 2007). **Mechanism:** Mast cells develop from bone marrow-derived progenitor cells and are present as two different types (one type, which expresses tryptase, and another type, which expresses chymase, carboxypeptidase and cathepsin G). Mast cells are known to limit inflammation and chronic MMP proteolysis in the aortic wall. Mast cells are already known to reduce antigen-induced allergic reactions in patients.		
Fenofibrate	**Findings:** Administration of Fenofibrate or pioglitazone in AngII-infused *ApoE*$^{-/-}$ mice resulted in reduced suprarenal aortic diameter and reduced OPN cytokines and macrophage	FAME-2 Trial (Pinchbeck et al., 2018)	Fenofibrate did not reduce AAA growth rate in comparison with placebo controls

	infiltration compared to vehicle control mice (J. Golledge et al., 2010). Also, Fenofibrate administration in low-density lipoprotein receptor-deficient resulted in reduced suprarenal aortic diameter, reduced aortic arch atherosclerosis reduced infiltration of macrophages, T lymphocytes compared with controls receiving vehicle (Krishna et al., 2012). **Mechanism:** Fenofibrate is PPARα ligand that limits osteopontin and macrophages infiltration in the aortic wall.		
Perindopril OR Amlodipine	**Findings:** Perindopril was reported to inhibit aortic degeneration and AAA formation in a rat model of AAA (F. Xiong et al., 2014). **Mechanism:** ACE-Inhibitors that are effective for reducing blood pressure. However, effects of Perindopril appear to be independent of its influence on blood pressure but rather its ability to inhibit inflammatory cell influx and protection	AARDVARK (Kiru, Bicknell, Falaschetti, Powell, & Poulter, 2016)	Neither perindopril nor amlodipine significantly reduced the AAA growth rate over 24 months of follow-up.

	against intimal thickening and the preservation of aortic medial elastin.		
Ticagrelor	**Findings:** Treatment with AZD6140, a P2Y(12) receptor antagonist, inhibited platelet activation and attenuated of experimental AAA in rats in which AAA was induced by implanting a segment of sodium dodecyl sulphate-decellularised guinea pig aorta in rat aortas (Dai, Louedec, Philippe, Michel, & Houard, 2009). **Mechanism:** As a platelet inhibitor, Ticagrelor inhibits platelet aggregation and prevents ILT formation.	TicAAA Trial (Wanhainen et al., 2019)	Ticagrelor did not reduce growth of small AAAs
AAA: abdominal aortic aneurysm. NK3201: 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[[3,4-dioxo-1-phenyl-7-(2-pyridyloxy)]-2-heptyl]acetamide; AngII: Angiotensin II; *ApoE*$^{-/-}$: Apolipoprotein E-null; PPARα: peroxisome proliferator–activated receptor α/γ; AT1: Angiotensin II receptor 1; MMP: matrix metalloproteinases; ILT: intraluminal thrombus * References to preclinical studies is not an exhaustive list.			

The Wnt signalling pathway regulates cell-to-cell interactions, including cell polarity, differentiation, migration and apoptosis as well as overall tissue morphogenesis (MacDonald, Tamai, & He, 2009). SOST is a protein product of the *SOST* gene, a potent bone morphogenic protein antagonist previously known to be exclusively expressed by osteocytes. SOST has been shown to modulate osteoblast proliferation, differentiation, apoptosis and matrix formation and mineralization of mature osteoblasts (Keller & Kneissel, 2005; Sutherland, Geoghegan, Yu, Turcott, et al., 2004; van Bezooijen, ten Dijke, Papapoulos, & Löwik, 2005; Winkler et al., 2003). As a competitive inhibitor for Wnt signalling, SOST binds to the low-density lipoprotein receptor-related proteins 5 and 6, thereby acting as a Wnt antagonist (Brandenburg et al., 2015; Krishnan, Bryant, & MacDougald, 2006; Xiaofeng Li et al., 2005; Vaclav Veverka et al., 2009). In the presence of the Wnt ligand and its interaction with the Frizzled receptors and low-density lipoprotein receptor-related protein 5/6 co-receptors, the β-catenin complex is stabilised and able to translocate to the cell nucleus where it modulates gene transcription. (MacDonald et al., 2009). In the absence of the Wnt ligand, proteasomal degradation of the β-catenin complex occurs prior to reaching the nucleus and before modulating gene transcription (MacDonald et al., 2009).

Data published from our laboratory previously showed that an experimental mouse model of AAA exhibited 3-fold downregulation in aortic expression of the SOST gene compared to mice that did not develop AAA (Rush et al., 2009). Subsequently, results from human AAA and experimental mouse models corroborated these findings showing that SOST was downregulated in human AAA tissue as well as in mice that developed larger AAA (**Figure 1.3A—B**) (Krishna et al., 2017). Additionally, it was demonstrated that *SOST* injection and transgenic *SOST*-overexpression in mice inhibited AAA formation (**Figure 1.3C**) (Krishna et al., 2017). Nonetheless, although these data support the role of *SOST* deficiency in AAA development, the exact underlying mechanisms that lead to downregulation of *SOST* protein during AAA pathogenesis remain unknown (Lenk et al., 2007; Rush et al., 2009).

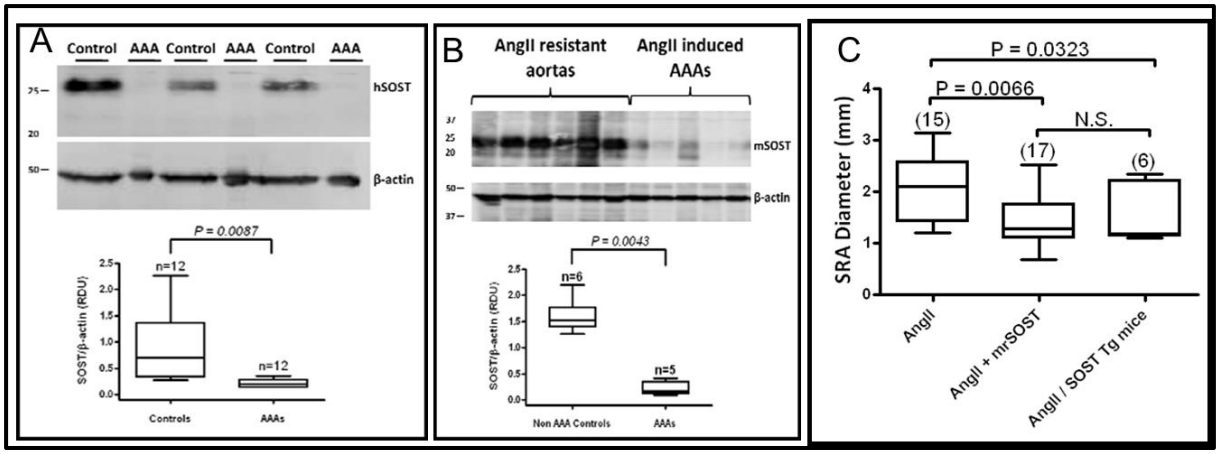


Figure 1.3: Human and mice expression of SOST

Western blot showing downregulation of *SOST* in human (**A**) and AngII-infused mice (**B**). SRA diameter was decreased in response to mr*SOST* injection and in *SOST* tg mice (C). **Abbreviations:** AAA: abdominal aortic aneurysm; SRA suprarenal aortic, tg: transgenic; AngII: angiotensin II; mrSOST: human recombinant sclerostin. Adapted from (Krishna et al., 2017).

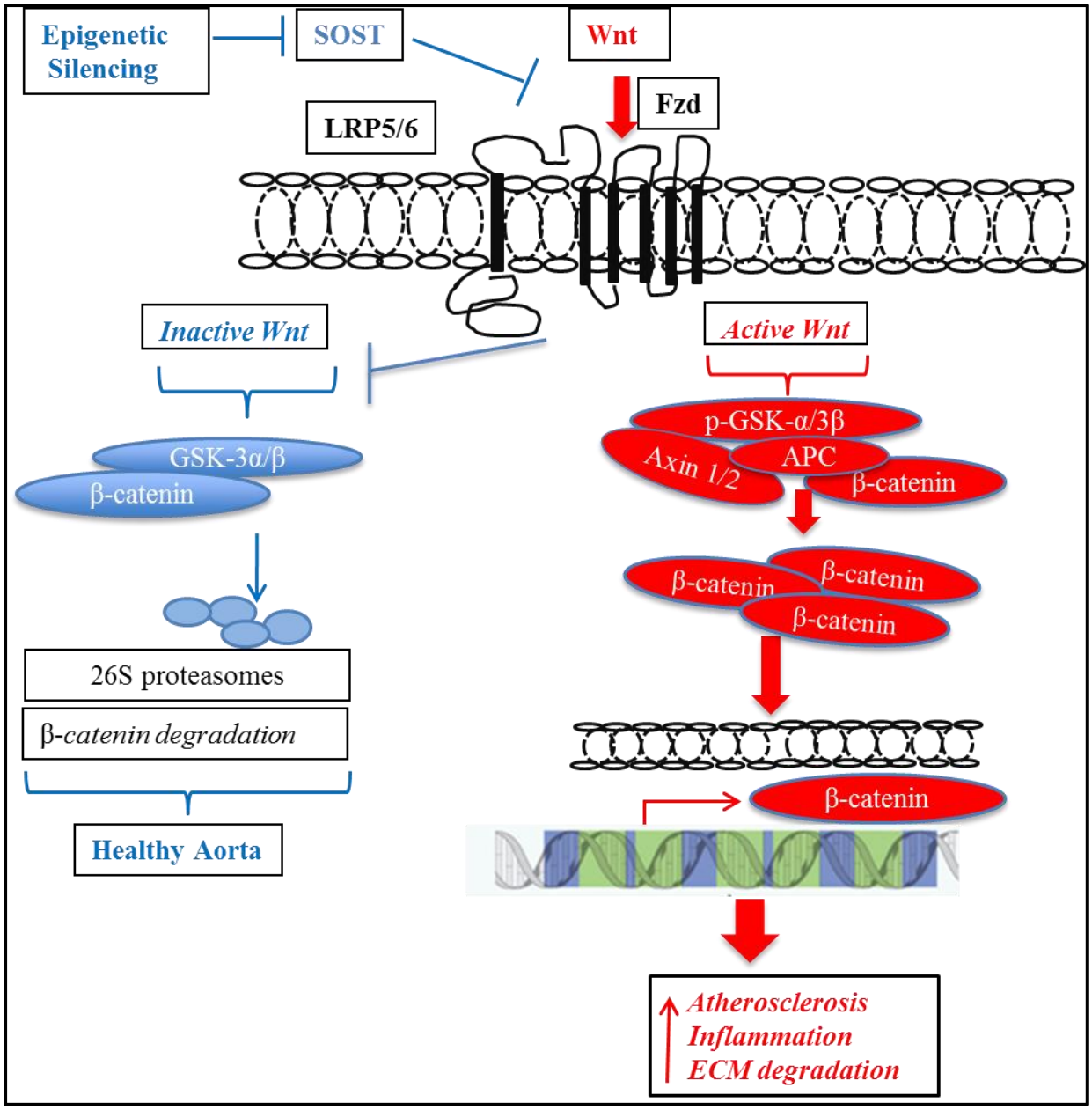


Figure 1.4. : SOST downregulation is implicated in AAA development

When active, SOST binds to Wnt co-receptor LRP5/6 to inhibit Wnt1 class signalling. Wnt interacts at the cell surface with frizzled receptor (Fzd) and low-density lipoprotein receptor-related 5/6 (LRP5/6). In the cytoplasm, β-catenin binds to the multiprotein complex containing kinases such as Gsk3-α/β and Casein kinase-1, which phosphorylates specific amino acids on the β-catenin. In the absence of Wnt binding, β-catenin is in a steady state and free β-catenin is eventually degraded by 26S proteasome. In contrast, activation of Wnt binding releases the β-catenin from the binding proteins, inhibits its phosphorylation, resulting in accumulation of β-catenin cytoplasmic levels and eventually promotes its translocation into the nucleus. After translocation, β-catenin interacts with transcriptional factors initiating the expression of several

target genes. It is possible that the SOST promoter undergoes inactivation due to DNA methylation; resulting in the activation of Wnt signalling which then promotes atherosclerosis, inflammation and degradation of ECM, thus promoting development of AAA. (Red arrows show activated pathway and blue shows inhibition). **Abbreviations**: p-Gsk3-α/β: phosphorylated glycogen synthase kinase 3-α/β, SOST, sclerostin; TF, transcription factor. Adapted from Krishna, *et al* (2017).

1.3. VITAMIN D

The term "vitamin D" is a misnomer usually used to describe CCF (vitamin D3) and ergocalciferol (vitamin D2) as well as their derived metabolites. The active metabolite of vitamin D is 1α,25(OH)D which has to undergo a number of hydroxylation steps before activation. It was not until 1930s when a German team led by Windaus clarified the chemical structure of vitamin D (Hirsch, 2011). Vitamin D is a group of fat-soluble seco-steroids (steroids with broken ring) which take the responsibility of regulating calcium and phosphorus balance in the body (Holick, 2006). The structure of vitamin D is almost similar to that of cholesterol, except vitamin D has double bonds between C-7 and C-8, and C-10 and C-19, and an open B ring structure (Hollis, 2005). There are two main forms of vitamin D in the human body, CCF (vitamin D3) and ergocalciferol (vitamin D2) and both have four intact rings in their inactive form. When the strength of sunlight UVB reaches the wavelength ranging between 290-315 nm, the bond between C-9 and C-10 of the B ring breaks down (Hollis, 2005). Consequently, a double bond is formed between C-10 and C-19, making the pre-vitamin D3. Thermogenic isomerization plays the major role in turning pre-vitamin D3 to CCF. In a similar manner, ergosterol from plant sterols or yeast can be irradiated to form supplemental ergocalciferol.

CCF is produced endogenously and is transported to the liver using D-binding protein (DBP), while supplemented D2 or D3 within the dietary fats are primarily absorbed in the duodenum. Both CCF and ergocalciferol are then transported to the liver through lymph channels. Within the liver, CP27A1, one of the monooxygenase enzymes of the cytochrome P450 family, adds a hydroxyl group to C-25 forming 25-hydroxyvitamin D3 or D2 [25(OH)D] (Christakos, Dhawan, Verstuyf, Verlinden, & Carmeliet, 2016). In turn, 25(OH)D is further metabolized to the "active" form of vitamin D by hydroxylation of C-1 in the kidney. This role is accomplished by another member of cytochrome P450 family, CYP27B1, to produce 1-alpha-25-

dihydroxyvitamin D3 or D2 (1α,25(OH)2D), widely known as calcitriol (Christakos et al., 2016). Although, the conversion of pre-vitamin D to 25(OH)D is not well regulated, the conversion to the active 1α,25(OH)2 form is tightly regulated by high parathyroid hormone (PTH) and low levels of calcium and phosphorus (Christakos et al., 2016). When circulating levels of calcium decrease and/ or the serum phosphate level increases, it triggers the release of calcium from the bones, enhances active reabsorption of calcium distal tubules and increases the absorption of calcium in the intestine (Bikle & Bouillon, 2018).

Table 1.4: Vitamin D and derivatives—nomenclature

Name	Source	Function	Different names
7-DHC	cholesterol precursor from skin pigmentation	biologically inactive	previtamin-D_3
vitamin D_2	created from irradiation of plants and fungi	biologically inactive	ergocholecalciferol
vitamin D_3	made in the skin when exposed to light; sourced from fish and meat	biologically inactive	cholecalciferol; activated 7-dehydrocholesterol
25(OH)D	A product of hydroxylation of vitamin D (mainly) in the liver	Main circulating form of vitamin D (considered best indicator of vitamin D status)	25-hydroxyvitamin D; calcidiol; calcifediol
1α,25$(OH)_2$D	A product of 25(OH)D hydroxylation in renal tubules and other target organs by enzyme 1-alpha-hydroxylase	primary biologically active metabolite; activates the vitamin D nuclear receptor	1α,25-dihydroxycholecalciferol; calcitriol

3-epi-25(OH)D	Epimerisation of circulating 25(OH)D	Low-activity form; significant levels present in infants	3-epi-25-hydroxyvitamin D
Notes: Both vitamin D2 and vitamin D 3 must undergo the same pathway for downstream metabolism with derivatives having comparable biological actions. The distinction between their derivatives is connoted 2 or 3 at the end (e.g. 25(OH)D3 or 25(OH)D2. Abbreviations: 1α, 25-dihydroxyvitamin D; 7-DHC: 7-dehydrocholesterol			

It is thought that almost a sixth of people worldwide do not have adequate levels of circulating 25(OH)D in their blood (Gordon, DePeter, Feldman, Grace, & Emans, 2004; Holick, 2007). Contemporary reports suggest that there is a worldwide deficiency of vitamin D in most populations, especially infants, pregnant and lactating women, people living away from the equator, persons who are not exposed to the sun, and populations with dark skin pigmentation (Jamil et al., 2018; Kuhnlein, 2018; Macdonald et al., 2011). A cross-sectional study including 727 females living in Sweden found that serum levels of 25(OH)D3 are positively associated with age, oral contraceptive use, and seasonal variations in blood collection (summer / autumn) (Macdonald et al., 2011). Circulating levels of 25(OH)D3 were also positively associated with a high dietary intake of vitamin D and serum concentrations of creatinine, phosphate and calcium. The positive association between vitamin D and contraceptives can be explained by the fact that oestrogen increases the levels of 25(OH)D-binding proteins (Grzechocinska, Warzecha, Szymusik, Sierdzinski, & Wielgos, 2018; Mayor, 2016). However, there is currently strong evidence showing that vitamin D deficiency is predominantly prevalent among elderly people (Boucher, 2012; MacLaughlin & Holick, 1985). Possible explanations for this association have been related to poor cutaneous photosynthesis, decreased intestinal absorption, impaired renal function and restricted sun exposure (Boucher, 2012; MacLaughlin & Holick, 1985; Tsiaras & Weinstock, 2011).

Activated vitamin D exerts its cellular functions by modulating the transcription of target genes after binding to the nuclear Vitamin D Receptor (VDR) (Rachez & Freedman, 2000). Interaction of VDR with its ligand 1α,25(OH)2D3 induces formation of two independent protein interaction sur-faces on the VDR, one that facilitates association with the retinoid X receptor (RXR) necessary for DNA binding, and one that is required for recruitment of co-

regulators necessary for gene modulation (Christakos et al., 2016; Rachez & Freedman, 2000). Following interaction with 1α,25(OH)2D3-VDR dimerizes with RXR and translocates to the nucleus where it binds to vitamin D response elements (VDRE) in vitamin D responsive genes (Christakos et al., 2016). Depending on the target gene, either co-activators or co-repressors are attracted to the VDR/RXR complexes to induce or repress gene transcription (Christakos et al., 2016). VDR was found to be expressed in almost all body cells, including immune, myocardial or vascular cells, points to involvement of vitamin D-mediated effects in several other systems not only the musculoskeletal system (Bikle & Bouillon, 2018; Christakos et al., 2016; Giustina et al., 2019). These actions involve the regulation of calcium homeostasis, cell growth and differentiation and modulation of adaptive and innate immunity (Christakos et al., 2016; Di Rosa, Malaguarnera, Nicoletti, & Malaguarnera, 2011). VDR expression and activity are important for all stages of a T cells life, ranging from development to differentiation and elicitation of effector functions (Cantorna, Snyder, Lin, & Yang, 2015; Di Rosa et al., 2011). The genes encoding for 1α,25(OH)2D3-catabolizing cytochrome P450 enzyme (*CYP24A1*) and the human cathelicidin antimicrobial protein (CAMP) are examples of 1α,25(OH)2D3-regulated target genes (Heulens et al., 2016). Previous evidence suggests that differential expression of *CYP24A1* and CAMP may affect vitamin D status (Heulens et al., 2016) and susceptibility to infectious diseases (Cantorna et al., 2015; Di Rosa et al., 2011).

1.3.1. Vitamin D and cardiovascular disease

Physiologically, vitamin D is sourced from cutaneous radiation by ultraviolet B (UVB) and dietary intake, followed by hepatic hydroxylation to 25(OH)D (main circulating metabolite) and renal hydroxylation to 1α, 25-dihydroxyvitamin D [1α,25$(OH)_2$D] (hormonally bioactive metabolite) (Dusso, Brown, & Slatopolsky, 2005; Holick & Chen, 2008). 25(OH)D circulates bound to DBP and accounts for endogenous and exogenous sources. Assay of 25(OH)D metabolite is recommended for assessment of vitamin D status (Holick et al., 2011). In addition to transportation of vitamin D metabolites in the circulation, DBP also regulates the uptake of 25(OH)D and 1α,25$(OH)_2$D by target cells (Dusso et al., 2005). The classic role of vitamin D is to regulate calcium and bone homeostasis. However, an increasing body of evidence shows that vitamin D exerts numerous biological benefits in extra-skeletal tissue and cells (Holick & Chen, 2008). Interestingly, pathological links between mineral and bone disorders, renal diseases and cardiovascular diseases (CVD) has been indicated by previous evidence (Brandenburg et al., 2015; Lampropoulos, Papaioannou, & D'Cruz, 2012). Recent studies have shown that deficiency of vitamin D is an important risk factor for CVD, including ischemic

heart disease, stroke, hypertension and atherosclerosis (Kim, Sabour, Sagar, Adams, & Whellan, 2008). Observational studies have shown that low 25(OH)D is associated with peripheral arterial diseases (PAD) (Fahrleitner-Pammer et al., 2005; Fahrleitner et al., 2002; McDermott et al., 2014; Michal L. Melamed et al., 2008; Zagura et al., 2011), a common comorbidity with AAA (Olin & Sealove, 2010). Finally, a meta-analysis performed on these cross-sectional studies demonstrated that lower levels of 25(OH)D were associated with PAD presence, particularly in patients with advanced PAD symptoms (Nsengiyumva et al., 2015).

The rennin-angiotensin-system (RAS) plays an essential role in the pathogenesis of cardiovascular diseases and has been consistently implicated in AAA formation (Carrara et al., 2014). Paricalcitol, a bioactive analog of 1α,25(OH)2D3 was shown to prevent cardiac hypertrophy in rats infused with a moderate dose of AngII (800 ng/kg/min) over a 14-day period (Chen & Gardner, 2013). In addition, administration of paricalcitol (intraperitoneal injection of 300 ng/kg every48 hours) resulted in partial reversal of AngII effects (Chen & Gardner, 2013). Moreover, Kong, *et al* (2010) previously showed that a combination of vitamin D analog with losartan generates a degree of therapeutic synergism in the attenuation of left ventricular hypertrophy.

Previously, 1α,25$(OH)_2$D has been shown to inhibit the RAS lowering the blood pressure and improve endothelial function through regulation of endothelial cell-dependent vasodilation (Al Mheid & Quyyumi, 2017; Carrara et al., 2014; Schroder-Heurich et al., 2019). The relationship between vitamin D and hypertension was also reported by the NHANES III study which demonstrated that the average systolic blood pressure (SBP) was nearly 3 mm Hg lower in subjects with the highest quartile of 25(OH)D compared with those in the lowest quartile (Scragg, Sowers, & Bell, 2007). A meta-analysis of 3 cohorts showed that lower circulating 25(OH)D were associated with an 80% higher risk of the incidence of hypertension (Pittas et al., 2010). However, a separate analysis of 10 clinical trials suggested reported that vitamin D supplementation reduced SBP by only 2 mm Hg and had no effect on diastolic blood pressure (Pittas et al., 2010). Nonetheless, a meta-analysis of 10 randomized controlled trials that examined the effects of vitamin D supplementation (with or without calcium) on blood pressure and incident hypertension found a very modest and non-significant improvement in SBP and diastolic blood pressure with supplementation. A higher dose (1000 IU/day) of vitamin D, compared with lower doses, had no effect on SBP but a small effect on diastolic blood pressure (Pittas et al., 2010). Upregulated expression of *CYP24A1* gene, which encodes an enzyme that

catalyses 25(OH)D and 1α,25(OH)2D3 into 24-hydroxylated products has been associated with higher SBP and DBP (Kunutsor, Burgess, Munroe, & Khan, 2014).

It has been previously suggested that obesity is risk factors for AAA development, albeit inconsistently (Cronin, Walker, & Golledge, 2013; Stackelberg, Bjorck, Sadr-Azodi, et al., 2013). Wortsman and colleagues (2000) reported that obesity is associated with decreased bioavailability of vitamin D characterised by lower 25(OH) in obese patients. Two main explanations were hypothesized for this relationship, the first one was that continuous vitamin D deposition in adipose tissue leads to lower circulating 25(OH)D levels in the blood while the other theory posited that vitamin D deficiency may be a contributing factor leading to obesity (Foss, 2009; Wortsman et al., 2000). However, a bi-directional Mendelian Randomization study lately showed a one directional causal relationship supporting that obesity leads to lower vitamin D levels (Vimaleswaran et al., 2013). In this study, 21 cohorts were included, comprising a total number of 42,024 adult patients and 12 well-known single nucleotide polymorphisms (SNPs) related to body mass index (BMI) and four typical vitamin D-related SNPs were analysed. It was demonstrated that each unit increase of BMI was associated with a 1.15% decrease of 25(OH)D after adjustments for other confounders. Conversely, genetically determined 25(OH)D levels were not significantly related to BMI, suggesting that obesity may be causal for lower 25(OH)D and not vice-versa (Vimaleswaran et al., 2013).

Several recent studies have reported high rates of CV diseases in patients with lower circulating levels of 25(OH)D, suggesting that deficiency of vitamin D plays a role in cardiac pathology. In 1,739 Framingham Offspring Study participants who were free of CV disease at baseline, the rate of major CV disease events was 53% to 80% higher among those with low vitamin D levels, with the increased risk magnified among those with hypertension (Wang et al., 2008). It was however suggested that a slight increase in CV risks was also associated with higher 25(OH)D levels (Wang et al., 2008). In an analysis of 13,331 adults from the NHANES III study followed up for a median of 8.7 years, mortality was inversely associated with vitamin D levels, with the lowest quartile of 25(OH)D (<17.8 ng/ml) having a 26% increased mortality compared with the highest quartile (Melamed, Michos, Post, & Astor, 2008). A prospective study of 3,258 consecutive patients scheduled for coronary angiography, low 25(OH)D and 1α,25$(OH)_2$D levels were independently associated with all-cause and CV mortality (Dobnig et al., 2008). Moreover, a 10-year follow-up of 18,225 men in the Health Professionals Follow-up Study reported that low levels of 25(OH)D were associated with higher risk of myocardial

infarction, even after controlling for other coronary heart diseases risk factors (Giovannucci, Liu, Hollis, & Rimm, 2008). It should however be noted that not all studies reported on a significant association between low 25(OH)D levels and increased risk of CVD (Welsh et al., 2012). Furthermore, the efficacy of vitamin D supplementation on CVD risks remains topically debatable. A meta-analysis that summarized randomized trials of vitamin D supplementation (until 2010) reported no significant effect of vitamin D on death, stroke, myocardial infarctions, lipid fractions, blood pressure, or blood glucose values. It is also important to mention that they analysed randomized trials with enrolled participants without severe vitamin D deficiency (Elamin et al., 2011). Comprehensively, an umbrella review of systematic reviews and meta-analyses performed on observational studies and randomised trials on vitamin D and multiple health outcomes reported found that available evidence is less convincing on effects of vitamin D and multiple health outcomes (Theodoratou, Tzoulaki, Zgaga, & Ioannidis, 2014).

1.3.2. Vitamin D and Inflammation

Administration of 1α,25$(OH)_2$D to subjects with vitamin D deficiency has been shown to down-regulate inflammatory biomarkers such as C-reactive protein (Christakos et al., 2016). VDR have a broad tissue distribution that includes VSMCs, macrophage, and lymphocytes (Christakos et al., 2016; Zhang et al., 2012). Moreover, there is evidence that VSMCs and endothelial cells possess the enzyme 25(OH)D-1 α-hydroxylase, which is responsible for the conversion of 25(OH)D to 1α,25(OH)2D (Norman & Powell, 2014). Vitamin D induces prostacyclin in VSMCs, which prevents thrombus formation, cell adhesion, and VSMC proliferation (Norman & Powell, 2014). Furthermore, vitamin D regulates the expression of a number of other proteins relevant to the arterial wall, including vascular endothelial growth factor (VEGF), MMP-9, myosin, elastin, type I collagen, and γ-carboxyglutamic acid, a protein that protects against arterial calcification (Norman & Powell, 2014). Vitamin D suppresses pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) *in vitro* and *in vivo* (Gardner, Chen, Glenn, & Ni, 2011). Low circulating vitamin D has been reported to be implicated in aortic inflammation localised within the adventitial layer in patients (Oma et al., 2017).

1.3.3. A role of vitamin D in AAA formation

The realisation that VDR are expressed within the heart and arterial cells has suggested that vitamin D plays an important role within the cardiovascular system (Christakos et al., 2016). While circulating levels of 25(OH)D are the best indicator of individual vitamin D status

(Dusso et al., 2005; Holick et al., 2011; Kleerekoper et al., 2011). Active vitamin D, 1α,25(OH)D, is the active metabolite that mediates both genomic and non-genomic functions (Christakos et al., 2016). Vitamin D supplementation and consequent achievement of optimal circulating levels has been reported to confer extra-skeletal benefits that include cardio-protective effects (Lee, O'Keefe, Bell, Hensrud, & Holick, 2008). Moreover, findings from cross-sectional studies have previously suggested that low levels of 25(OH)D are associated with vascular-related diseases, including aneurysmal subarachnoid haemorrhage (Alvarado Reyes, Perez, & Rodriguez-Vega, 2017; Guan et al., 2016), coronary arterial diseases (CAD) (Gondim, Caribe, Vasconcelos, Segundo, & Bandeira, 2016; Kunadian, Ford, Bawamia, Qiu, & Manson, 2014) and peripheral arterial diseases (PAD) (Nsengiyumva et al., 2015).

Vitamin D can be implicated in AAA formation through several mechanisms. AAA is an age-related disease and given that cutaneous synthesis of sunlight-derived vitamin D diminishes over time due to aging, it is possible that limited bio-availability of 25(OH)D in the elderly people may cause AAA formation (Fahrleitner et al., 2002; Mosekilde, 2005). This notion is well supported by previous reports showing that older people are at risk of severe vitamin D deficiency characterised low circulating 25(OH)D levels (Fahrleitner et al., 2002; Grober, Spitz, Reichrath, Kisters, & Holick, 2013; Melamed et al., 2008). Numerous clinical trials have showed that optimal levels of 25(OH)D may indeed prevent bone fracture in institutionalised patients. Indeed, beneficial effects of vitamin D in bone health remains the highest available level of evidence. Emerging findings however suggest that dysregulation of a number of bone proteins, including osteoprotegerin (OPG), osteopontin (OPN), osteocalcin (OCN) and sclerostin (SOST) is an independent risk factor for development of aortic aneurysms (Lerchbaum, Schwetz, Pilz, Boehm, & März, 2014; Li et al., 2016; Moran, Jose, Erik Biros, & Golledge, 2014; Vianello et al., 2017). Findings carried out in rodent models and human AAA tissue have already shown that bone proteins, including OPG, OPN and SOST are implicated in the pathogenesis of aortic aneurysms (Lerchbaum et al., 2014; Li et al., 2016; Moran et al., 2014). However, mechanistic understanding of how vitamin D is implicated in AAA formation remain somewhat elusive.

Previously, observational studies have reported an inverse relationship between circulating levels of 25(OH)D and AAA presence (**Table 1.6 & Figure 1.6**). Wong, *et al* (2013) reported that lower circulating levels of 25(OH)D were associated with AAA diameter in a dose-dependent manner. An inverse association remained statistically significant even after adjustment for several traditional AAA risk factors and other potential confounders. Further

analyses demonstrated that AAA patients in the lowest quartile of 25(OH)D were at an increased risk of 5 times likely to develop larger AAAs (≥ 4.0 cm) compared healthy participants (Wong et al., 2013). Similarly, Demir *et al* (2012) showed that patient with thoracic aortic aneurysm (TAA) had lower levels of 25(OH)D compared with the control group. The study (2012) included 87 patients with thoracic aortic dissection (TAD) who were aged 40—70 years old and found that vitamin D deficiency was as an independent factor associated TAD presence. Another study by Van De Luijtgaarden *et al* (2012) examined the association of vitamin D deficiency with aneurysmal diseases patient and PAD patients; and concluded that low 25(OH)D levels were strongly associated with both arterial conditions, independent of traditional cardiovascular risk factors. While these findings showed no significant difference in circulating levels of 25(OH)D between PAD and AAA patients, a moderate decrease in 25(OH)D levels was noted within AAA patients.

Preliminary evidence to suggest that vitamin D may be implicated in AAA development probably comes from proteomic analyses performed on human AAA patients' tissue showing that VBP was altered in AAA patients (Gamberi et al., 2011; Spadaccio et al., 2016; Urbonavicius et al., 2010). Proteomic analyses in AAA tissue and intraluminal thrombus and plasma were performed and expression of VDBP was characterised (Gamberi et al., 2011; Spadaccio et al., 2016; Urbonavicius et al., 2010). Urbonavicius, *et al.* (2010) first showed that VBP expression was positively correlated with AAA presence in aortic tissue. However, Gamberi *et al* (2011) found a negative correlation between plasma VBP expression and AAA presence. In later study, Spadaccio *et al* (2016) reported that expression of plasma VBP increased by 1.92-folds in AAA patients compared with controls. It was proposed that the discrepancy between the two studies would be attributed to differences in the origin of tissue used. VBP mainly acts as a plasma carrier protein for 25(OH)D and other vitamin D metabolites, although its role in vascular remodelling has also been suggested (Gamberi et al., 2011).

Almost all cells that are implicated AAA development and progression, including VSMCs, endothelial cells and macrophages and immune cells have been shown to have the ability to synthesise 1α,25(OH)D_2 from locally circulating 25(OH)D (Christakos et al., 2016; Dusso et al., 2005). Valcheva *et al* (2014) reported that lack of VDR signalling in VSMCs increased local production of AngII, which then contributed to vascular ageing, oxidative stress and premature senescence. A number of studies that investigated molecular link between vitamin D and AAA are presented in **Table 1.5**. In a recent study, Martorell *et al* (2016) showed that

incubation of active vitamin D3, calcitriol, with human umbilical vein endothelial cells inhibited AngII-induced leukocyte-endothelial cell interactions and decreased production of endothelial pro-inflammation. Furthermore, it was shown that abrogation of VDR-RXR interaction limited these effects, suggesting the importance of vitamin D metabolism pathway. Moreover a review by Vanherwegen *et al* (2017) reported on possible preferential immunomodulation mediated by 1α,25(OH)D_2 on macrophages, dendritic cells, T cells and B cells, all of which contribute to AAA formation.

Table 1.5: Mechanistic assessments performed on tissues collected from aortic aneurysm patients and animal models	
References	**Main findings**
Nieuwland et al (2016)	Daily injection of 1 μg of paricalcitol to patients who were scheduled for open AAA was associated with reduced inflammation compared with controls.
Spadaccio et al (2016)	Western blot analysis of 10 AAA patients' tissue versus 10 control (no-AAA) tissue showed that expression of vitamin D-binding protein (VDBP) increased 1.7-fold in AAA patients.
Gamberi et al (2011)	Western blot analysis AAA tissue showed a negative correlation between the vitamin D-binding proteins (VDBP) with AAA presence.
Urbonavicius et al (2010)	A proteomic approach on AAA tissue using 2D-PAGE and LC-MS/MS showed positive correlation of DBP (p=0.042) with AAA presence.
Lutsey et al (2018)	A total of 12,770 participants for AAA incidence and 449 AAA cases occurred over a median follow-up duration of 19.7 years. Markers for vitamin D metabolism, including 25(OH)D, FGF23, phosphorus and PTH were not associated with risk of AAA incidence.
Martorell et al (2016)	The study showed that within the AngII infused *ApoE*$^{-/-}$ mice, oral administration of calcitriol resulted in reduced AAA dissection.
Abbreviations: AA: aortic aneurysm; AAA: abdominal aortic aneurysm; FGF23: Fibroblast growth factor 23; VDBP: vitamin D-binding protein; 2D-PAGE: Two-dimensional poly-acrylamide gel electrophoresis; LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry; AngII: angiotensin II; PTH: parathyroid hormone; 25(OH)D: 25-hydroxyviatmin D.	

Table 1.6: Cross-sectional studies examining 25(OH)D levels in aortic aneurysm patients and controls					
References	***AA patients***		***No-AA controls***		***Summary of the findings***
	Sample	25(OH)D (nM)	Sample	25(OH)D	
Wong et al (2013)	311	65.0±22.8	3922	68.6±23.3	Comparing AAA patients with AAA-free healthy controls, circulating levels of 25(OH)D levels were significantly lower among AAA patients compared with controls. Furthermore, the data revealed a graded inverse relationship between circulating 25(OH)D and AAA size.
Demir et al (2012) *	87	29.7±18.8	93	39.9±12.4	Comparing the patients with TAD and healthy controls as diagnosed or excluded by transthoracic echocardiography, TAD patients had significantly lower 25(OH)D levels.
van de Luijtgaarden (2012) †	236	57±31.0	254	59.2±27.0	Comparing patients with AAA or TAD and controls who were PAD patients, it was demonstrated that low 25(OH)D levels were independently associated with both arterial diseases with the levels being slightly lower among AA patients compared with PAD patients.
Results are presented as mean ± SD. *This study investigated groups of patients who hath thoracic aneurysm. †This studies included patients presenting with AA and patients who had PAD were chosen as controls. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; PAD: peripheral arterial diseases; Abbreviations: 25(OH)D: 25-hydroxyvitamin D; AA: Aortic aneurysm; TAD: thoracic aortic aneurysm, PAD: peripheral arterial diseases					

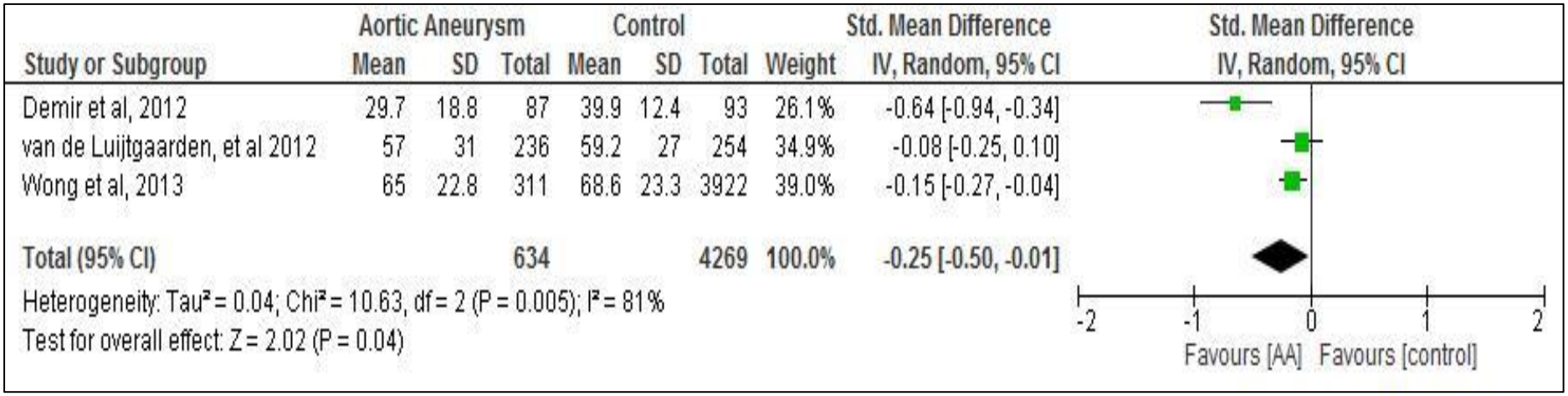


Figure 1.5: Forest plot showing the overall and subgroup concentrations of 25(OH)D (nmol/L) among aortic aneurysm patients and controls participants

Forest plot of standard mean difference (SMD) and 95% confidence interval of 25(OH)D concentrations in patients with aortic aneurysm compared with control participants. Standard mean difference (SMDs) and 95% confidence interval were computed in a random effects model using Review Manager (Version 5.3). Abbreviations: 25(OH)D: 25-hydroxyvitamin D, AA: aortic aneurysm.

Nieuwland, *et al* (2016) recently reported that daily administration of 1 μg of paricalcitol to patients on daily basis attenuated inflammation; characterised by aortic tissue immunohistochemistry (IHC) and gene expression. A relatively long-term follow-up study recently assessed the link between AAA development and vitamin D metabolism (Lutsey et al., 2018). The study used a cohort of 12, 770 black and white individuals and examined the serum collected from ARIC to assess whether low serum 25(OH)D levels, elevated calcium, FGF23, phosphorus, and PTH were associated with AAA. Findings from this study concluded that current evidence is insufficient to establish whether vitamin D plays a role in AAA development. Genomic and non-genomic effects of VDR and subsequent downstream signalling owing to vitamin D supplementation is an important factor. The paucity of evidence on VDR activation in AAA formation and AAA tissue is in in fact a limiting factor in our understanding of mechanistic insights of vitamin D and AAA formation. Interestingly, so far, only one study has investigated the effects of vitamin D in aortic aneurysm development within a mouse model (Martorell et al., 2016). Findings from this study showed that within the AngII-infused *ApoE*$^{-/-}$ mice, oral administration of 1α,25(OH)D resulted in reduced aortic dissection. These effects were reported to be unrelated to SBP or plasma cholesterol concentrations (Martorell et al., 2016). Furthermore, examination of mice SRA tissue showed significant decrease in macrophage infiltration and neovascularisation which was accompanied by downregulated expression of *Mmp-2* and *Mmp-9* (Martorell et al., 2016). Currently, the mechanism by which vitamin D deficiency promotes AAA remains unknown. Moreover, there is lack of clinical and pre-clinical evidence exploring therapeutic potentials of vitamin D supplementation that can be better translated to AAA management in human.

Cellular experiments using 1α,25$(OH)_2$D3 have shown that vitamin D can be metabolised by these cells resulting in VDR activation and other downstream pathways (Christakos et al., 2016; Norman & Powell, 2014). Within VSMCs, deletion of the VDR was shown to induce premature senescence, possibly through upregulation of the cyclin-dependent kinase inhibitor (Valcheva et al., 2014). Although the cells primarily implicated in AAA formation are VSMCs, effects of vitamin D on other cell lines provide invaluable information on the mechanisms implicated. *In vitro* effects of 1α,25$(OH)_2$D3 have been investigated in a number of cell lines that are known to contribute to aortic wall homeostasis, notably, endothelial cells and inflammatory cells and immune cells (Querfeld, 2013; Schroder-Heurich et al., 2019; Vanherwegen et al., 2017; Yong Zhang et al., 2012). Norman *et al* (1995) previously suggested that excess vitamin D may have a detrimental role in arterial wall and a possible cause for AAA

development. Hypothetically, it was argued that exposure to excessive vitamin D during maternal development or in early infancy could potentially result in an individual being predisposed to AAA formation in later life. A recent study that prospectively followed up 12,770 participants for incident of AAA and 449 AAA incidents occurred over a median follow-up of 19.7 years. Recently, Lutsey et al (2018) reported that a number of vitamin D metabolism markers, including 25(OH)D, FGF23, phosphorus, and PTH were not associated with risk of AAA incidence. Well-designed experiments using AAA animal models are a driving force for basic research and a foundation for further translational research. Given that vitamin D is a well-known nutraceutical with limited side effects profile, it is important that new biological effects are examined using animal models which are suited for AAA management. Of note, while a meta-analysis of epidemiologic studies clearly shows that lower circulating 25(OH)D levels are associated with AAA presence (**Figure 1.5**), the levels reported in these studies cannot necessarily be interpreted as deficient (Holick et al., 2011). Currently, the mechanism by which vitamin D deficiency may promote AAA remains unknown. There is therefore lack of clinical and pre-clinical evidence to demonstrate therapeutic potential of vitamin D supplementations in AAA management. Findings from cross-sectional studies are largely prone to limitation since they do not provide conclusions on cause-and-effect.

In conclusion, a relatively low circulating concentration of 25(OH)D has been associated with human aortic aneurysms in a number of cross-sectional studies (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). Contrary to this evidence, however; it has been reported that the prevalence of AAA is highest in Australasia where levels of ultraviolet exposure (and thus likely vitamin D) are very high (Li, Zhao, Zhang, Duan, & Xin, 2013; Sampson et al., 2014). Excess vitamin D has also been reported to have potential pathological effects such as inhibiting production of elastin (an important aortic matrix protein) by VSMCs (Hinek, Botney, Mecham, & Parks, 1991; Norman et al., 1995; Norman, Moss, Sian, Gosling, & Powell, 2002). Therefore, further experimental research is currently needed to resolve the importance of vitamin D in AAA pathogenesis and progression.

1.4. HYPOTHESES AND AIMS

The present thesis hypothesized that vitamin D protects against AAA pathogenesis through upregulation of SOST (**Figure 1.6**). Therefore, effects of vitamin D on AAA development, growth and rupture were investigated *in vivo* using the *ApoE*$^{-/-}$ mouse model in which AAA was induced by AngII infusion. The AngII infused *ApoE*$^{-/-}$ mouse model is well accepted for its pathological characteristics that it shares with human AAA (Daugherty & Cassis, 2004; Patelis et al., 2017; Senemaud et al., 2017; Trollope, Moxon, Moran, & Golledge, 2011). This thesis also investigated effects of vitamin D on human aortic VSMCs *in vitro*.

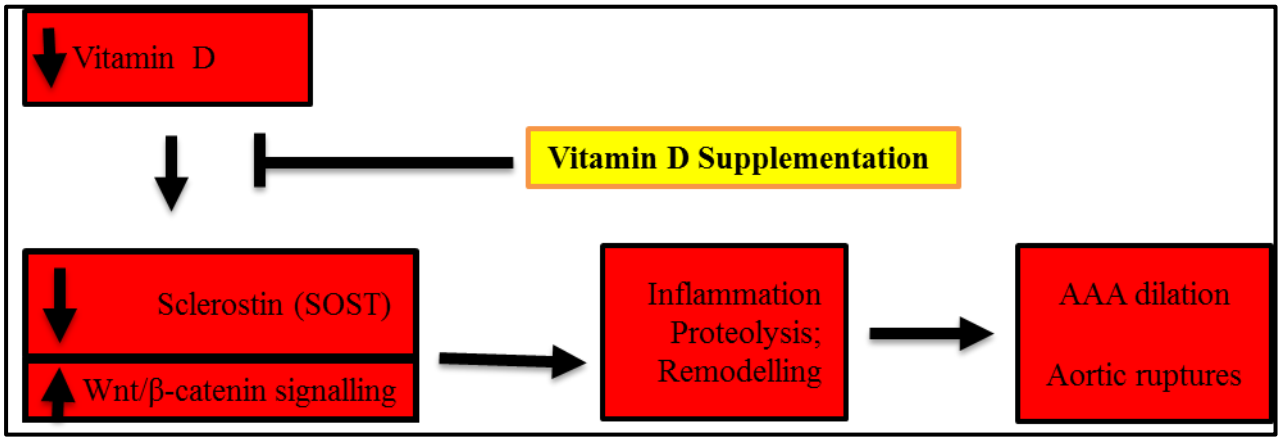


Figure 1.6: Hypothetical Mechanism on the role of vitamin D in AAA development

Vitamin D deficiency promotes downregulation of SOST, which results in activation of Wnt/β-catenin activation. Cytoplasmic accumulation of β-catenin and translocation of β-catenin complex in the nuclear promotes genes that are implicated in pro-inflammation, extracellular matrix degradation and aortic wall remodelling, resulting in focal expansion of the aortic wall and aortic. Plausibly, replenishment or up-titration of vitamin D may protects against AAA development and rupture or reverse these effects through upregulation of SOST and downregulation of β-catenin.

Specifically, the following hypotheses were proposed:

1. 1α,25(OH)$_2$D3 promotes SOST expression in human aortic VSMCs
2. Dietary restriction of vitamin D reduces SOST in *ApoE*$^{-/-}$ mice.
3. Dietary restriction of vitamin D promotes AAA development and rupture in the *ApoE*$^{-/-}$ AngII)-infused mouse model.
4. CCF supplementation attenuates AAA growth and rupture in AngII-infused *ApoE*$^{-/-}$ mice.

These aims were developed:

1. To examine whether 1α,25(OH)$_2$D3 induced *SOST* expression in human VSMCs.
2. To examine if dietary deficiency of vitamin D reduced SOST in the *ApoE*$^{-/-}$ mice.
3. To investigate if dietary deficiency of vitamin D promoted experimental AAA growth and rupture in a mouse model.
4. To examine whether up-titration of 25(OH)D levels through CCF supplementation slowed expansion and rupture of pre-established AAA in a mouse model of AAA.

CHAPTER 2:

GENERAL METHODS

2.1. Introduction

Validity, feasibility and reproducibility of methodologies in basic science are of paramount importance (Baker, Lidster, Sottomayor, & Amor, 2014; Kilkenny, Browne, Cuthill, Emerson, & Altman, 2011). Findings from preclinical studies, particularly animal studies are increasingly becoming scrutinised due to poor translatability into subsequent clinical investigations (Baker et al., 2014; Kilkenny et al., 2011). Major critiques suggest that some of findings generated from preclinical studies remain irreproducible by peer researchers and therefore may not be translated to clinical trial (Baker et al., 2014). ARRIVE guidelines underscore a number of checkpoints that are required to be considered by publishing journals (Baker et al., 2014). This chapter therefore aims to provide details on the general materials and methods used across all animal experiments (i.e. Chapters 3, 4 & 5).

2.2. Animal studies

The mice used in animal studies (Chapters 3, 4 & 5) were all male, *ApoE*$^{-/-}$ mice (C57BL/6J background). All mice were sourced from the Animal Resource Centre (Manning Vale, Western Australia) at 10-12 weeks old. Gene expression was confirmed in these mice through routine genotyping. All animal procedures described herein were conducted in personal containment levels 2 laboratory in the established animal house at the James Cook University. Mice husbandry and agistment were carried out in the PC2 laboratory in individually ventilated, temperature/humidity-controlled cages (Aero IVC Green Line; Tecniplast). Mice were housed in temperature controlled rooms under a 12-hour light-dark cycle (relative humidity: 55-60%; temperature: 22±1ºC) and given portable water *ad libitum*. The animal weight was monitored on a weekly basis. All animal studies described in this thesis were approved by the James Cook University/Animal Ethics Committee (JCU/AEC) and adhered to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA.

2.3. Angiotensin-II infusion in Apolipoprotein E-null mice

AngII infusion results in the development of aneurysm in the suprarenal region of the aorta in the majority of *ApoE*$^{-/-}$ mice (Patelis et al., 2017; Senemaud et al., 2017; Trollope et al., 2011). Mice were surgically implanted with osmotic minipumps (Model 1004, Alzet, Durect Corporation) that have been programmed to deliver 1μg/kg/min of human AngII (Sigma-Aldrich, NSW, Australia) over 28 days (Equation 5.1). The AngII vials contained 50 mg (in powder form) of synthetic peptide human AngII ($C_{50}H_{71}N_{13}O_{12}$) were first reconstituted with 2 ml of ultrafiltered, autoclaved milli-Q H_2O to make up a stock solution of 25mg/ml. The

solution was then diluted to get the actual concentration per mouse. Each osmotic mini-pump was filled up to ≥90% which was ensured by measuring the before and after filling pump weight. Each mouse was weighed on the day and the weight was used to determine the required amount of AngII required for that particular mouse (Equation 5.1). Prior to surgery, the mouse was sedated under 2.4% of isoflurane inhalation and the adequacy of anaesthesia was monitored by continuous monitoring of physiological parameters and by performing a reflex response test. The hair was removed around the mouse's dorsal area using a hair clipper or depilatory cream and a small lateral incision was made between the scapulae. The osmotic mini-pump was subcutaneously inserted and positioned in the ventral region in a small pocket created by separating the skin from the connective tissue using a haemostat. The incision was then closed with polyamide monofilament non-absorbable sutures (Dafilon®; B. Braun Surgical, Spain). Topical antiseptic (Betadine, Purdue Products LP, USA) was applied to prevent infection to the wound. Mice were then allowed to recover under a heated lamp and monitored twice daily until terminal dissection. Mice were checked daily for general health, including eating habits (weight loss), docility (locomotion), behaviour (stress, depression aggressiveness), and appearance (wound infection and limping). In general, the mice remained lethargic and fairly docile on the day of surgery but were in a normal state within 2-3 days; with no wound infection or other complications.

2.4. Tissue collection

2.4.1. Aortic rupture post angiotensin-II infusion

Aortic rupture and dissection are common in the first 14 days of AngII infusion in this animal model, accounting for nearly 26% of mortality (Cao, Amand, Ford, Piomelli, & Funk, 2010). Therefore, necroscopic analysis was performed within 12 hours on all mice that died post-AngII infusion. Necroscopic dissection to expose the full aorta from the heart and to the point of bifurcation was carefully carried out under a dissection microscope. In particular, signs of aortic rupture and intramural thrombus presence were noted and photographed. In these mice, aortic rupture was commonly characterised by presence of blood clot around the juxtarenal region (retroperitoneal regions) or around the aortic arch. Mice which died from aortic rupture were not included in subsequent experiments (i.e. tissues were not stored for further analysis).

2.4.2. Collection of blood

Both the source and method of blood collection can have been shown to affect blood parameters, and consequently affect the quality of findings (Parasuraman, Raveendran, & Kesavan, 2010). In this study, serial blood collection was achieved via tail vein bleeding; and at terminal procedure, the blood was collected via cardiac puncture. Both methods typically yield a mixture of venous and arterial blood (Parasuraman et al., 2010). During the experiment, mice were comfortably secured in the restrainer and a heating lamp was placed above the tail region at the temperature of 24—27^{0}C. Topical anaesthesia (Emla® 5% cream, Australia) containing lignocaine (25mg/g) and prilocaine (25mg/g) was applied and left to absorb through the skin on the mouse tail. A sterile surgical scalpel was then used to nick the vein and the blood was collected into lithium heparin coated tubes (BD Bioscience, USA). In accordance with to the AEC recommendation, up to 200 µl of blood volume could be collected from each mouse.

2.5. Assessment of AAA growth

2.5.1. Ultrasound assessment of AAA

ApoE$^{-/-}$ mice usually develop AAA in the suprarenal aortic region (P. E. Norman & Powell, 2010). In order to assess in vivo growth of AAA, the SRA diameter was monitored first at baseline (day 0, before AngII) and then monitored serially every after 14 days throughout the study in all AngII-infused mice (cf. Chapter 4 and 5). The mice were briefly sedated with 3.5% isoflurane inhalation and the hair was removed in the abdominal area using depilatory cream. The ultrasound was perform in B-mode using a MyLab™ 70 VETXV platform (Esaote, Italy) which was connected to an LA435 linear transducer (Esaote, Italy). Warm transmission ultrasound gel was applied on the exposed skin surface. The ultrasound transducer was then positioned perpendicular to the animal ventral region and to acquire the SRA region in a trans-axial plane. Anatomical landmarks, including spinal cord and renal arteries were used to facilitate proper orientation. The application on the machine was set-up to an automatic focal zone and frequency of 'tendon equine' 'mouse' in a sagittal mode. Maximum SRA diameter was measured at peak systole using an in-built calliper feature from outer wall to outer wall.

Given the importance of SRA diameter in this study, inter-assessor repeatability of ultrasound assessment was carried out by two assessors, whereby the second observer was blinded to

experimental groups. The repeatability was ensured early on during the habituation of animals (i.e. prior to commencing the actual data collection).

2.5.2. Morphometric assessment and classification of AAA

AAA severity was also assessed in harvested aortas *ex vivo* (morphometry) by measuring the maximum diameter of the aortic arch, thoracic aorta (TA), SRA and infra-renal aorta (IRA). During dissection, phosphate buffered saline (PBS) was carefully perfused through the aortas for complete removal of blood clots. The aorta was then excised by ensuring complete removal of perivascular fats, and then the whole aorta was placed on a graduated template and digitally photographed (Coolpix 4500, Nikon). Analysis was performed on the images using a computer-aided software (Adobe® Photoshop® CS5 Extended version 12, Adobe Systems Incorporated). In order to establish an acceptable inter-observer reproducibility, a second observer who was blinded to samples labelling repeated measurements. Morphometric analyses were performed on aortas of mice that were alive until terminal dissection (i.e. ruptured aortas were excluded). It has been previously demonstrated that both ultrasound and morphometric measurements can be performed with excellent inter-observer reproducibility (Krishna et al., 2015).

In addition, a scale-based assessment of gross appearance was performed on excised and digitally photographed aortas according to the protocol devised by Daugherty et al (2001) (Daugherty, Manning, & Cassis, 2001). Briefly, during the assessment, aneurysms were scored and classified as type I if they manifested a dilated lumen in the supra-renal region of the aorta with no thrombus. Aneurysms were scored as type II if they manifested a remodelled tissue in the SRA region, accompanied by presence of thrombus. Type III aneurysms were characterised by pronounced bulbous form of the type II and contained thrombus. Aneurysms were classified as type IV if multiple, overlapping aneurysms were present within the SRA whereas type 0 meant absence of aneurysm or dissection within the aorta. This classification of aneurysms was complemented by histological staining of the aortic section by haematoxylin and eosin (H&E) as described below (Section 2.8.1). Assessments were performed by two assessors whereby the secondary assessor (Dr Smriti Krishna) was blinded to animal groups. The final score was achieved with a final 100% concordance. Ruptured aortas (confirmed by necroscopy) were excluded in the analysis.

2.6 Non-invasive tail-cuff plethysmography

SBP, diastolic blood pressure (DBP) and heart rate were measured in mice at baseline, prior to starting AngII infusion and 14 and 28 after AngII infusion started. Measurements were performed using a computerized non-invasive tail-cuff system (CODA Monitor, Kent Scientific). Mice were habituated to the device by handling them on a daily basis for one week. Mice were allowed to settle for 2 hours at room temperature prior to assessment. An average of five consistent readings that were consecutively recorded were taken. Excellent reproducibility for this technique has been previously reported (Krishna et al., 2016; Krishna et al., 2015; Seto et al., 2014).

2.7. Plasma analyses

Collection of blood was performed into heparin coated tubes (BD Microtainer). The plasma was recovered by centrifugation at 3000 x g for 10 minutes at 4^0C and then stored at -80^0C until use. Cell-free plasma was obtained by centrifugation at 2000 x g at 4^0C for 10 minutes followed by a further 10 minutes centrifugation at 15000 x g at 4^0C if required. The plasma samples were snap-frozen in liquid nitrogen and stored -80^0C for later use.

2.7.1. Measurement of plasma 25(OH)D3, 25(OH)D2 and epi-C3-25(OH)D

There is ongoing debate surrounding vitamin D assays (Carter, 2011; Holick et al., 2011; Holick, 2009; Kleerekoper et al., 2011). According to a prominent panel of experts within the vitamin D research field (Kleerekoper et al., 2011), 25(OH)D is the metabolite that most accurately depicts an individual's vitamin D status and assays of this metabolite by mass spectrometry (MS) is a gold standard. Measurement of 25(OH)D was therefore performed as previously described (Clarke, Tuckey, Gorman, Holt, & Hart, 2013). Plasma 25(OH)D was thawed at room temperature and diluted (1:2) in RPMI Media 1640 (i.e. 75µl of serum + 75µl 1640 RPMI Media) to make up 150µl and shipped on dry ice for assaying in a vitamin D assay accredited laboratory (Centre for Metabolomics, WA, Australia). The assay for 25(OH)D was performed using a two-dimensional ultra-performance liquid chromatography separation coupled tandem mass spectrometry (2D UPLC-MS/MS) (Clarke et al., 2013). The lower detectable limit for 25(OH)D was 2.0 nM. The 2D UPLC-MS/MS technique has been previously demonstrated to accurately quantitate 25(OH)D3, 25(OH)D2 and epi-25(OH)D3 metabolites (Albarhani et al., 2015; Clarke et al., 2013).

2.7.2. Measurement of plasma SOST

Plasma SOST was assayed using a Quantikine® ELISA kit (R&D Systems; cat#:MSST00). Briefly, samples and standards were prepared according to the manufacturer's instructions. Samples (50 µl) were tested in duplicates along with assay diluent and standards to their respective wells followed by incubation for three hours at room temperature. The plate was washed five times with 400µl of the wash buffer and 100µl of mouse SOST conjugate was added to each well and incubated for 1 hour at room temperature. The plate was washed five times, 100 µl of the substrate solution was added, followed by a 30 minutes incubation at room temperature. The reaction was stopped by addition of the stop solution (100 µl). The plate optical density was read at 540 λ with wavelength correction option (Omega, BMG Labtech).

2.8. Assessment of atherosclerosis lesion

The Sudan IV stain was prepared by weighing 5.0 g of Sudan IV and then mixed in 500.0 ml of acetone and 500.0 ml of 70% ethanol. The mixture was dissolved on a stir plate for 30 minutes. 80% ethanol was used as a decolorizing solution. Aortic arches were individually collected and stored in optimum cutting temperature (OCT, ProSciTech) and stored at -80^{0}C until dissection. For staining, aortic arches were brought at room temperature, serially washed with PBS (3x) to remove the residual OCT and fixed in 70% ethanol overnight. Excess perivascular fatty tissue was carefully trimmed, including removal of brachiocephalic arteries. The arches were then cut open longitudinally and stained for 15 minutes with Sudan IV solution [0.1% Sudan IV dissolved in 1:1 acetone and 70% ethanol (v/v)]. This was followed by with differentiation with 80% ethanol for 5 minutes and a wash with water for 5 minutes, a decolorizing solution. Stained arteries were then rinsed with running H_2O for several minutes until the background was cleared. All stained aortic arches were immediately digitally photographed and the atherosclerotic plaque areas were analysed using a computer-aided software (Adobe®Photoshop® CS5 Extended version 12, Adobe Systems Incorporated) as previously reported (Krishna et al., 2016; Krishna et al., 2012; Krishna et al., 2015). *En face* Sudan IV staining was quantitated as percentage of stained area over the whole tissue area. An acceptable reproducibility of this technique has been previously reported (Krishna et al., 2015; Krishna et al., 2012).

Aortic arches were individually collected and stored in optimum cutting temperature (OCT, ProSciTech) and snap-frozen in liquid N_2 and stored at -80^{0}C during dissection. *En face* Sudan

IV staining was performed on these aortic arch samples to identify intimal atherosclerotic plaque as previously reported (Krishna et al., 2012).Aortic arches were thawed at room temperature, washed PBS to remove residual OCT and fixed in 70% ethanol overnight. The arches were then cut opened longitudinally and stained for 15 minutes with Sudan IV solution [0.1% Sudan IV dissolved in 1:1 acetone and 70% ethanol (v/v)]. This was followed by tissue decolourisation with 80% ethanol for 5 minutes and wash by H_2O for several minutes until artefact stain disappeared. All stained aortic arches were immediately digitally photographed and the atherosclerotic plaque areas were analysed using a computer-aided software (Adobe® Photoshop® CS5 Extended version 12, Adobe Systems Incorporated).

2.9. Histological and histopathological assessments

In these experiments, aortic segments were collected and processed for assessments. Histological assessments are commonly used to characterise the gross appearance of the SRA tissue within the AngII infused *ApoE*$^{-/-}$ mouse model (Cao et al., 2010; Krishna et al., 2017; Krishna et al., 2015). Additionally, histopathological assessments usually aim to characterise the pathological effects within the SRA tissue in this mouse model (Cao et al., 2010; Krishna et al., 2017; Krishna et al., 2015). During terminal dissection, the SRA segments were isolated and stored in OCT compound (ProSciTech) for later histological analysis. SRA segments from all mice from each group were processed for sectioning. SRA segments were first washed of OCT by serial rinsing in the PBS solution then fixed in 70% EtOH overnight. The sections were then orientated in cassettes with OCT as frozen sections at -20^{0}C.

2.9.1. Morphology of suprarenal aortic by Haematoxylin and Eosin

Serial cryostat sections (5µm) were cut from each SRA segment and air dried on silane coated slides for 30 min. For nuclei staining sections were covered with haematoxylin for 4 minutes and then rinsed with running milliQ-H_2O. Slides were dipped into 0.25% hydrochloric acid in absolute EtOH (v/v), 10x, then rinsed in milliQ-H_2O for 2 min. Slides were then differentially stained by dipping them 10x into a 0.09% ammonia chloride solution in milliQ-H_2O (v/v) followed with rinsing under running milliQ-H_2O and then immersed into 95% EtOH for 10 sec. Sections were then counterstained with eosin for 15 sec and dehydrated by immersion into 95% EtOH (5 minutes) and then into absolute EtOH (5 minutes). Final clearance was achieved by immersions of slides into xylene for 5 min (2x) followed by mounting with in Entellan

mounting medium (ProSciTECH, Australia). Stained sections were then left to be air dried in fume cupboard for 24 hr prior to visualisation under a microscope (Nikon, Japan).

2.9.2. Assessment of suprarenal aortic wall elastin

SRA sections were stained with Elastin Van Giessen (EVG) staining (Polysciences, Inc, Australia) to measure the elastin content. Differentiation of all connective tissue components cannot be readily distinguished by gross morphology staining using H&E. Elastin Verhoeff's Van Gieson (EVG) stain is an iron-haematoxylin stain that specifically binds to elastic fibres. The stain was freshly made using 5% alcoholic haematoxylin, 10% aqueous ferric chloride and iodine solution. The tissue was first rehydrated and OCT removed using PBS for 3 minutes and then stained in the EVG solution for one hour. Sections were then rinsed 3 times with tap water and differentiated using 2% ferric chloride for 2-5 minutes. Since elastin has a strong affinity for the iron-haematoxylin complex in the stain, it is expected that elastin fibres holds the dye longer than other tissue components. Elastin therefore remains stained even after other tissue elements are decolourised. The differentiation was stopped by washing the section through several changes of tap water. Stained sections were photographed using a Nikon Eclipse 50i microscope fitted with a CCD Camera (DSFi1) and digital images captured to a PC supported with image analysis software (NIS Elements, version F2.30). Qualitative evaluation of elastin fibre integrity was performed on digital images by semi-quantitative grading by a blinded observer as follows; 1- no elastin degradation or mild degradation; 2- moderate; 3- moderate to severe; and 4- severe elastin degradation following previously published protocols and showed good reproducibility (Krishna et al., 2015; Wang, 2014).

2.9.3. Assessment of suprarenal aortic collagen

Collagen is an important structural protein that controls the distension in the aortic wall by maintaining its tensile strength. It is the key element of the ECM in the aortic wall and its depletion can significantly reduce the aortic wall structural integrity by up to 50 times. Type I and III collagen are the most abundant proteins within the aortic wall. The picrosirius red staining is an effective method commonly used to characterise collagen I (old) and III (newly forming) within the tissue. This technique also aids to semi-quantify the amount of collagen in a given area of aortic wall tissue. Collagen type I and III are distinguished from each other under polarized light microscopy.

For the animal studies, SRA segments were collected and placed in OCT, then stored at -80ºC. In this experiment, SRA frozen sections were serially sectioned to 5-6 µm using a microtome. Slide sections were air dried and dehydrated using 75% EtOH, then stored at -20^{0}C until use. Slides were then retried and brought to room temperature for 30 minutes then immersed in PBS for 10 minutes. Sections were fixed on slides using 10% formalin for 5 minutes and rinsed in water. Sections were then stained with Weigert's Haematoxylin for 5 minutes, then rinsed in Milli-Q H_2O (3X), then placed in phosphomolybdic acid for 2 minutes. Slides were then immersed in picrosirius red F3BA solution for 90 minutes followed by 5 rinses in milli-Q H_2O. Slides were then immersed into 0.1 N hydrochloride acid for 2 minutes, then rinsed in milli-Q H_2O. Slides were then serially rehydrated in absolute, 90% and 70% EthOH for 2 minutes each, then covered using mounting media. Sections were then visualised under polarised light microscope and digitally photographed using. Stained tissue showed that collagen fibres stained red, type I collagen stained yellow while type III collagen stained green. It was previously reported that all these histological measurements could be repeated with good reproducibility (Krishna et al., 2015).

2.10. Suprarenal aortic tissue protein assay by ELISA

2.10.1. Preparation of suprarenal aortic tissue samples

The SRA tissue was thawed on ice, cleared of OCT, rinsed in PBS, transferred to a clean microfuge tube and snap frozen in liquid nitrogen. The tissue was then minced manually with a pestle with addition of 500µl of ice-cold radioimmunoprecipitation assay (RIPA; i.e. 1 x RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl; 2 mM EDTA, 1% TritonX-100, 0.1% SDS, and 0.1% sodium deoxycholate) buffer (Cell Signalling Technology) which also contained protease inhibitors (Roche) and phosphatase inhibitors (PhoSTOP). The tissue was then homogenised with the pestle attached to a handheld drill and the homogenised samples were centrifuged for 5 minutes at 80000xg at 4°C. The supernatant was collected and protein concentration was quantitated by the BioRad protein assay (BioRad, USA).

2.10.2. Estimation of protein concentration by BioRad protein assay

A standard was first constructed using IgG standards (range 0-8µg) which were prepared by dilution IgG (1mg/ml) with milli-Q water. From each sample, 2µl was drawn and diluted to

1:1000 in milliQ water and used for the assay to determine the protein concentration. Each well for sample (160µl) or standard (160µl) was mixed well with 40µl Bradford reagent in a flat bottom 96-well plate followed by 30 minutes of incubation away from light. The optical density (OD) of the wells were read at 595nm in the Omega plate reader. Excel data from the plate reader were transferred to GraphPad prism software to interpolate unknown concentrations of the diluted samples using the standard curve. The interpolated values were multiplied by the dilution factor (x1000) for the protein concentrations of the extracted proteins for each sample.

2.10.3. Mouse suprarenal aortic SOST protein assay

The measurement SOST protein was performed using the Quantikine® ELISA kit as described in manufacturer's instruction (R&D Systems; cat#:MSST00). A 7 points standards was serially prepared with an 8th blank and loaded. Samples and standards were loaded in duplicates followed by incubation for three hours at room temperature. The plate was then washed four times with 400µl of the wash buffer and 100µl of mouse SOST conjugate was added to each well and incubated for 1 hour at room temperature. The plate was then washed five times before addition of the substrate solution. This was followed by a 30 minutes incubation at room temperature and the reaction was stopped by addition of the stop solution. The plate optical density was read at 540 nm and 450 nm (Omega, BMG Labtech). Plate's optical wavelength correction was achieved by subtracting the readings at 540 from 450nm).

2.10.4. Mouse suprarenal aortic phospho-GSK-3α/β (S21/S9) protein assay

Mouse phospho-GSK-3α/β (S21/S9) levels were determined using DuoSet® IC ELISA in accordance with manufacturer's instructions (R&D systems®, Cat#: DYC2630-2). Briefly, the mouse phospho-GSK-3α/β (S21/S9) capture antibody was reconstituted and diluted to a working concentration of 4.0 µg/ml in PBS and then coated into a 96-well micro-plate (100 µl) per well. The plate was sealed and incubated overnight at room temperature. On the following day, the plated contents were aspirated and the plate was washed 4x with was buffer (0.05%Tween-20 in PBS). 300 µl of blocking buffer (1% BSA, 0.05%NAN_3 in PBS) was added to the plate and incubated for 2 hours at room temperature (RT). During the incubation time, a 7 standards points was serially prepared with a two-fold dilution starting from a working concentration of 10000µg/ml while the 8th standard was a blank diluent. All samples were centrifuged at 3000g for 10 minutes before addition. The plate was then washed as above and 100 µl of sample or standards were added. The plate was sealed and incubated for 2 hours. The

plate was washed again and mouse GSK-3β and GSK-α detection antibodies were added. The plate was sealed again and incubated for another 2 hours. The plate was washed and incubated with 100µl/ well of diluted streptavidin-HRP for 20 minutes (in a dark place). The plated was again washed followed by addition of 100µl (1:1) of the substrate solution [Colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine)] and incubated for 20 minutes. The reaction was stopped after two minutes by addition of 50µl of the stop solution ($2NH_2SO_4$) to each well. The plate was read at 540 nm and at 450 nm (Omega, BMG Labtech). The wavelength correction was achieved by subtracting the optical density readings at 540 from those at 450nm.

2.11. Extraction and purification of mRNA

In all related experiments, extraction and of RNA was based on five principles tissue homogenisation, phase separation, RNA precipitation, RNA purification. First, the SRA tissue was homogenised using mechanical blender. RNA extraction was performed using an established phenol-chloroform extraction techniques (Toni et al., 2018). TRIzol reagent, a mono-phasic solution of phenol, guanidine isothiocyanate (Qiagen) was used for tissue lysis. Tissues segments were removed from the RNAlater and placed into Trizol (250 µL) for 30 minutes while kept on ice. The segment was mechanically homogenised using sterile rods prior to addition of another 250µL of Trizol. The samples were then left on ice for an extra 20 min for proper lysis. Phase separation was achieved by addition of 400 µL of chloroform then vortexed. This mixture was then incubated for 5 minutes at RT. The tubes were then centrifuged for 10 minutes at 12000 x g at 4^0C. The upper aqueous phase chloroform (Fisher) was carefully pipetted into a new tube. RNA precipitation was achieved by addition of 200µL of molecular grade isopropyl alcohol (2-propanol) (Fisher) followed by quick vortex. The samples were then left at 4^0C for overnight to allow for RNA precipitation. The samples were then vortexed and centrifuged for 10 minutes at 12,000xg at 4ºC to recover the precipitated pellet and the supernatant were carefully discarded. The pellets were resuspended in 500 µL of 75% ethanol in RNase free water (Qiagen). RNA resuspension and purification was achieved by repeated pipetting followed by quick vortex (in most of the tubes, precipitated RNA strands could be seen floating).

This was then transferred into column tubes (Qiagen). The column tubes were centrifuged at 8000x g for 30 secs and flow-through were discarded. Pellets were then washed with the RWI

buffer (700µL) and centrifuged at 8000xg for 15 minutes. Further purification was achieved through DNA digestion by addition of DNase I (Qiagen) to the column. This was then incubated for 15 min at RT (DNase was kept on ice). RNA was then washed in with 500 µL of RPE buffer and spun at 8000xg for 15 secs followed by another with the same buffer but spun for 2 minutes. The spin column were then transferred to new collection tubes (RNase free collection tubes, Qiagen). Excess ethanol left to evaporate at RT for 5 minutes. RNA elution was then achieved by adding 25 µL of warm RNase free water (55^0C) and spun at 8000xg for 30 secs. This was repeated twice, thus achieving a total collection of 50 µL per sample. RNA tubes were then kept on ice at all times. RNA quality and concentration was determined for each sample by placing 1µL of original stock on a Nano-spectrophotometer (Nanodrop), blanked against the RNA free water. The ratio (A260/A280) range of 1.8-2.0 was assumed to be of good RNA quality (Toni et al., 2018). Extracted RNA was stored at -20^0C into 20µL aliquots.

2.12. Analysis of gene expression by quantitative real time polymerase chain reaction

Quantitative real time polymerase chain reaction (qRT-PCR) was used to assess relative gene expression. Total mRNA was extracted from cells using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions as detailed above. Quantification of RNA per sample was performed using a nano-spectrophotometer machine (Nanodrop2000). The forward and reverse primers were designed using (Premier 6 software, Premier Biosoft) and purchased from Sigma (Australia). A table comprising an exhaustive list of primers and target genes is provided in each study. Relative gene expression was calculated by using an auto-generated and corrected concentration-Ct-standard curve and normalized to relative expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* as a housekeeping gene. *GAPDH* was chosen as housekeeping gene since its expression was similar across the samples analysed. All reactions were independently repeated in duplicate.

2.13. Statistical analysis

D'Agostino and Pearson test was used to test the normality of the data. Results were expressed as median and interquartile range (IQR) for non-normally distributed data; and as mean ± SEM for normally distributed data. For non-normally distributed data, comparisons were made using Mann-Whitney U-test or Kruskal-Wallis test followed by Dunn's multiple comparisons test, where appropriate. Normally distributed data were compared using ANOVA followed by Bonferroni's multiple comparisons test. Mortality rate was tested using a survival Kaplan

Meier curve. Differences were considered to be statistically significant at $P < 0.05$. Elastin grading was analysed using Fischer exact test. Repeated measures were analysed using repeated measures ANOVA. All analyses were performed using GraphPad Prism 8.4.2 software (GraphPad Software, Inc., USA).

CHAPTER 3:

IN VITRO EFFECTS OF VITAMIN D ON SOST IN HUMAN AORTIC SMOOTH MUSCLE CELLS AND *IN VIVO* EFFECTS OF VITAMIN D IN APOLIPOPROTEIN E-NULL MICE.

3.1. INTRODUCTION

Vitamin D is an umbrella term non-specifically used to describe cholecalciferol (CCF) and ergocalciferol as well as their downstream metabolites (Demer, Hsu, & Tintut, 2018; Holick, 2007). Sunlight-derived (exogenous) and nutritional (endogenous) vitamin D must first undergo a step of hydroxylation to 25(OH)D which occurs in the liver (Demer et al., 2018; Holick, 2002; Holick, 2007). During the activation stage, circulating 25(OH)D is hydroxylated to produce the bioactive metabolite of vitamin D known as 1α,25$(OH)_2$D (Demer et al., 2018; Holick, 2002; Holick et al., 2011; Holick, 2007). While it was previously thought that renal tubules was the only site for 25(OH)D hydroxylation to the bioactive 1α,25$(OH)_2$D, current evidence indicates that a number of other extrarenal tissue targets have the ability to locally mediate this process (Christakos et al., 2016; Holick, 2007). Although the bioactive form of vitamin D is 1α,25$(OH)_2$D, levels of circulating 25(OH)D is the best indicator for individual's vitamin D status (Holick et al., 2011). Therefore, measurement of circulating 1α,25$(OH)_2$D is not clinically recommended except in extenuating circumstances where renal insufficiency or genetic defects associated with vitamin D metabolism are present (Holick et al., 2011).

A well-recognised role of vitamin D is to regulate calcium and phosphorus metabolism (Holick, 2005; van Driel & van Leeuwen, 2017). Therefore, studies on the role of 1α,25$(OH)_2$D have traditionally focused on bone and mineral homeostasis (Acibucu et al., 2016; Christakos et al., 2016; Holick, 2005; van Driel & van Leeuwen, 2017). Nonetheless, emerging findings are now suggesting that deficiency of vitamin D is implicated in the pathophysiology of several diseases, including cancers, infectious diseases and cardiovascular diseases (Christakos et al., 2016; Holick, 2002; Holick & Chen, 2008). The realization that many cells of the cardiovascular system, including immune cells, endothelial cells and VSMCs possess the enzymatic machinery to convert circulating 25(OH)D to active 1α,25$(OH)_2$D have provided new insights into how vitamin D may be implicated in cardiac and vascular functions (Christakos et al., 2016; Demer et al., 2018; Holick, 2002; Holick et al., 2011; Holick, 2007; Norman & Powell, 2014). 1α,25$(OH)_2$D is the most potent metabolite of vitamin D and a ligand for VDR (Carlberg & Molnar, 2012; Christakos et al., 2016). Therefore, almost all vitamin D actions are mediated through the binding of 1α,25$(OH)_2$D to its cognate VDR, a transcriptional receptor located within the cell nucleus (Christakos et al., 2016). VDR is a nuclear receptor that regulates transcription of target genes through recruitment of ligand-dependent co-regulators (Rachez & Freedman, 2000).

SOST is Wnt/β-catenin negative regulator with a seminal role in bone mineralisation (Moester, Papapoulos, Lowik, & van Bezooijen, 2010; van Bezooijen et al., 2005). Previously, there was no known role in the function of arterial wall; however, a recent study from our laboratory showed for the first time that downregulation of SOST is associated with AAA presence in human AAA tissue (Krishna et al., 2017). For the first time, the report provided new insights into the role of SOST in the pathogenesis of AAA. For instance, it was demonstrated that SOST plays a protective role in maintaining the aortic wall homeostasis via several mechanisms such as anti-atherosclerotic, anti-inflammatory, and anti-ECM degrading functions. Reduced SOST was reported to activate Wnt/ β-catenin signalling, which regulates downstream genes that are important in maintaining the aortic wall integrity (Krishna et al., 2017). Furthermore, it was demonstrated that both transgenic SOST overexpression and administration of exogenous human recombinant SOST were associated with reduced inflammation and matrix remodelling (Krishna et al., 2017). Two studies recently reported *in vitro* effects of 1α,25$(OH)_2$D3 on *SOST* expression in human osteoclast cells (Wijenayaka et al., 2016; Wijenayaka et al., 2015). In both studies, 1α,25(OH)2D was shown to increase *SOST* gene expression in a dose-dependent manner (Wijenayaka et al., 2016; Wijenayaka et al., 2015). Administration of ergocalciferol has previously been reported to increase circulating SOST concentrations in patients with osteoporosis (Sankaralingam, Roplekar, Turner, Dalton, & Hampson, 2014). Moreover, a positive correlation between circulating 25(OH)D and SOST levels has been previously reported in human (Acibucu et al., 2016; Ardawi, Al-Kadi, Rouzi, & Qari, 2011; Pirgon, Sandal, Cetin, & Dundar, 2016). However, it remains currently unclear whether 1α,25(OH)2D upregulates SOST expression in VSMCs. It is also unknown whether dietary deficiency of vitamin D plays a direct role in reducing SOST and thus compromising the aortic wall integrity. Given that the key characteristics of AAA development include arterial wall remodelling due to focal inflammation and marked depletion of medial VSMCs (Aggarwal et al., 2011), it is plausible that Wnt/β-catenin signalling plays an important role in AAA development. Additionally, focal loss of contractile phenotype markers within VSMCs is also understood to be among the main causes for aortic wall disintegration and continuous remodelling (Peng et al., 2018).

In this chapter, it was hypothesized that 1α,25$(OH)_2$D3 upregulates *SOST* expression in human aortic VSMCs and that dietary restriction of vitamin D reduces circulating SOST and aortic wall SOST expression in *ApoE*$^{-/-}$ mice. Therefore, this study aimed to examine if

1α,25(OH)$_2$D3 upregulated *SOST* expression in human aortic VSMCs and investigate whether dietary restriction of vitamin D reduced SOST in *ApoE*$^{-/-}$ mice.

***The hypotheses for this study were that*:**

1. 1α,25(OH)$_2$D3 upregulates *SOST* expression in human aortic VSMCs.
2. Dietary restriction of vitamin D reduced circulating SOST and aortic wall SOST expression in *ApoE*$^{-/-}$ mice.

***Specifically, the aims of this study were*:**

1. To examine if 1α,25(OH)$_2$D3 induced *SOST* expression in human aortic VSMCs.
2. To assess whether dietary restriction of vitamin D reduced circulating SOST and aortic wall SOST expression in *ApoE*$^{-/-}$ mice.

3.2. MATERIALS AND METHODS

3.2.1. CELL CULTURE STUDY

Commercially available human aortic VSMCs (were purchased (CC-2571, Lonza). Cells were originally isolated from healthy young donors without a history of AAA or athero-occlusive diseases. All cell culture procedures were performed in an isolated PC2 room, in a laminar flow hood, under sterile conditions. Experiments were performed on cells from passage 4 to passage 9. Cells were initially seeded in T75 flask and maintained in Dulbecco`s Modified Eagle Media (DMEM) which was supplemented with 10% foetal calf serum. Cells were then incubated in a humid-controlled atmosphere at 37°C, 5% CO_2.

3.2.2 Cell counting, passaging and storage

3.2.2.1. Cell counting

A cell count was performed to determine the seeding density before they were passaged or stored. A cell count technique was first performed using a haemocytometer whereby the dye exclusion test also known as trypan blue exclusion test was used to determine the number of viable cells. Trypan blue cell exclusion test is based on the principle that healthy cells possess

an intact membrane; and therefore are not permeable by heavy dyes (e.g. trypan blue, eosin or propium) whereas this ability is compromised in dying cells (Strober, 2015). In this experiment, viable cells manifested a clear cytoplasm with a circular or spindle-like shape while non-viable cells had a blue-stained cytoplasm.

In preparation for trypan blue cell exclusion test, cell suspensions were centrifuged for 5minutes at 100g x g and supernatants were discarded. Cell pellets were resuspended in 1 ml PBS and then mixed with 0.4% trypan blue (1:1 v/v). The mixture was then incubated at RT for ~2-3 minutes to avoid cell deaths that may be due to longer incubation times (Strober, 2015). A drop of the mixture (cell suspension in PBS and 0.4 % trypan blue dye) was applied to the haemocytometer and the number of stained (dead) and unstained (viable) cells were counted. The haemocytometer cell count technique suggests that the average number of cells per ml (viable or non-viable) per ml is obtained by multiplying the total number of cells by the dilution factor of the dye then converted to (10000 $x10^4$). In addition, a cell count was also performed electronically using Scepter™ 2.0 Cell Counter (Sigma) with a sensor size operating range for 40-µm; 50,000–1,500,000 cells/mL; and for 60-µm 10,000–500,000 cells/ml.

3.2.2.2. Cell Passaging

Cells were passaged when they reached 80% to 90% confluency. VSMCs are adherent cells, therefore detachment of cells was required prior to passaging. The cell culture medium was discarded; and cells were washed (X 3) using a saline solution, Hanks' Balanced Salt Solution (HBSS; Gibco) that keeps the osmotic pressure and pH in cells. Cell detachment was performed by addition of Trypsin-EDTA (0.25% trypsin, 1 nM EDTA-4NA, Gibco) to cover the monolayer surface of cells. The flask was then placed in an incubator at 37°C in an atmosphere of 5% CO_2 for ~3 minutes. The cell detachment process, also known as trypsinisation, was confirmed by visualisation of floating cells using light microscope. In order to neutralise the trypsinisation enzymatic process, DMEM culture medium containing 10% FCS was added and cells were resuspended by gently pipetting up and down while avoiding foaming. Suspension of cells was then divided equally into the number of required flasks for platting (e.g. in a T75 flask, a total volume of 25 ml was achieved).

3.2.2.3. Cells storage

For cell storage, the suspension was transferred into a sterile centrifuge tube and spun at 200g for 10 minutes. Cells were stored when they were actively proliferating at early passages (i.e. passages 4 to 6). All procedures in preparation for cells storage were performed in a laminar floor hood under sterile conditions. Culture medium was washed off by HBSS buffer and the monolayer of cells was trypsinised (as above) to obtain single cell suspension. The suspension was then transferred into a sterile centrifuge tube and spun at 200g for 10 minutes. The supernatant were carefully discarded and cell pellets were resuspended into required volumes of DMEM medium. Resuspension DMEM medium was reconstituted with 20% FCS and 10% dimethyl sulfoxide (DMSO) which was to be aliquoted as 1 ml/cryovial. The cryovials which are made from cryogenic elements were designed to store cells to low temperature (-196^0C) without brittle breakage. Cell counting was performed to determine a dilution factor required in order for each cryovial to receive a minimum of $1x10^6$ viable cells per ml. Cryovials were placed into freezing chambers (Mr Frosty®) containing isopropanol; and placed in a freezer at -80^0C. After 12 hours, the cryovials were transferred into liquid nitrogen ($\geq 120^0$C) for long-term storage.

3.2.3. *In vitro* incubation of VSMCs with 1α,25(OH)$_2$D3

At 80% to 90% confluency, cells in T75 flasks were washed, trypsinised and resuspended as above. Cells were then plated into 6 separate 6-well cultures plates at $1x10^5$ cells/ml. Plating of cells was performed using the growth media and incubated at 37°C with 5% CO_2 atmosphere. Confluency at 70—80% was confirmed after 72 hours of cell platting. Cells were then synchronised to the G_0 phase by draining out the initial media and supplementation of FCS poor media for 12 hours. The FCS-poor medium was then drained out and cells were incubated with 1α,25(OH)$_2$D3 (Sigma).

Briefly, a 10 µg, vial of 1α,25(OH)$_2$D3 (≥99% HPLC grade, molecular weight 416.64, Sigma) was dissolved with 1 ml of DMSO to make a stock concentration of 10µg/ml or (24 µM). The required doses (molarity) of 1α,25(OH)$_2$D3 for this experiment were 0nM, 0.1M, 1nM, 10nM and 100nM. The preparation of 1α,25(OH)$_2$D3 is depicted below (**Figure 3.1)**. The final volume for media culture was 2ml per well.

3.2.4. RNA extraction and assessment of gene expression

At the end of 48 hours incubation, supernatants were drained out by pipetting out and mRNA was extracted using TRIzol reagents (Qiagen) as previously described in Chapter (Section 2.11). Details on genes that were assessed in human aortic VSMCs are presented **Table 3.1**. Primers were designed against human *SOST* (5'-CTAACTTGCTGTGTAACC -3' and 5'-TATGCCAATAGTCTCCTC-3'), *TNFRSF11B* (5'-AATGTGGAATAGATGTTACC-3' and 5'-TCTACCAAGACACTAAGC-3'), *OPN* (5'-AATGATGAGAGCAATGAG-3' and 5'-GTCTACAACCAGCATATC-3') using the Premier 6 Software (Premier Biosoft). Other primers were purchased pre-designed (Qiagen).

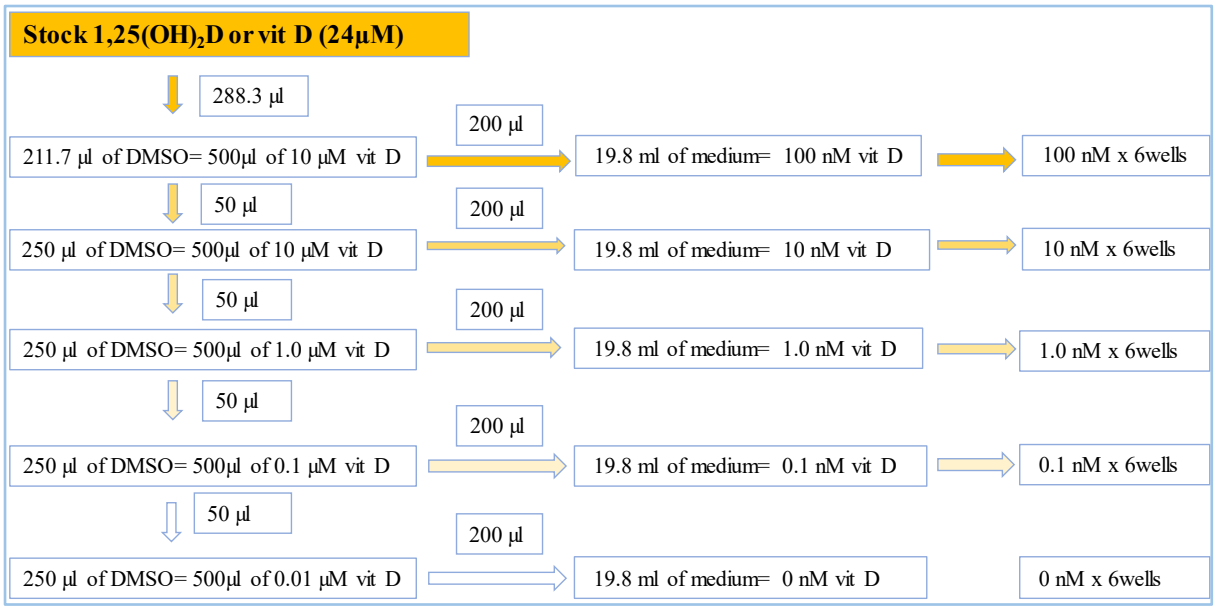


Figure 3.1: Preparation of 1α,25(OH)2D3 doses and study design

The molecular weight of 1α,25$(OH)_2$D3 is 416.64 g/mol. Therefore, 1α,25$(OH)_2$D3 was dissolved in DMSO then diluted to the required concentrations by addition of the DMEM media. Increasing concentrations of 1α,25$(OH)_2$D3 were then added to 60% to 70% confluent human aortic VSMCs in six well plates (i.e. n= 6/dose of 1α,25$(OH)_2$D3). Abbreviations: Vit D: vitamin D; 1α,25$(OH)_2$D3: 1α-dihydroxyvitamin D3; DMSO: dimethyl sulfoxide; DMEM: Dulbecco's modified eagle medium; VSMCs: vascular smooth muscle cells.

3.2.5. Characterisation of VSMCs *in vitro*

In order to characterise contractile phenotype markers within human aortic VSMCs in response to 1α,25$(OH)_2$D3 incubation, immunofluorescence staining was performed for α-actin smooth muscle (α-SMA). Analyses for gene expression were performed on VSMCs contractile

phenotype markers. For immunostaining, cells were grown on sterile coverslips for 24 hours in 37^0C/5% CO_2 incubator. Cells were rinsed with HBSS and fixed using 70% methanol (in HBSS) for 10 minutes. For staining the cells were rinsed 3X with HBSS and incubated for 20 minutes at RT with 10% normal goat serum (Vector Laboratory, CA; Cat# S-1000) PBS to block non-specific binding of IgG. Cells were then rinsed twice with HBSS and incubated with 1µg/ml of α-SMA in HBSS -BSA solution for 1 hour at RT. Following 3 times washes with HBSS, cells were incubated with 1:250 (or 4µ/ml) biotin-conjugated secondary antibody for 1 hour. Cells were then washed three times with HBSS before the coverslips on which cells were attached was removed from the plate wells. The coverslips were mounted inverted (i.e. the cells layer facing the glass slide surface) using 10µl Vectashield mounting media (Vector Laboratory, CA; Cat# S-1000) or DAPI medium. Excess media was removed using a fibre-free paper. The edges were sealed using regular transparent nail polish and slides were allowed to air dry for 5 minutes for long-term preservation of slides. The cytoskeleton filaments were visible in all differentiated healthy VSMCs (**Figure 3.2)**.

Table 3.1: List of Human primers for quantitative real time polymerase chain reaction

Gene Primers	**Encoded protein**	**Source**	**Catalog #**
H_SOST	Sclerostin	Sigma	Designed in house
H_TNFRSF11B	Osteoprotegerin	Sigma	Designed in house
H_OPN	Osteopontin	Sigma	Designed in house
H_MMP2	Matrix metalloprotenase-2	Qiagen	PPH00151B
H_VDR	Vitamin D receptor	Qiagen	PPH02123F
H_CYP27B1	25-Hydroxyvitamin D3 1-alpha-hydroxylase	Qiagen	PPH01242A
H_CYP24A1	1α,25-dihydroxyvitamin D3 24-hydroxylase	Qiagen	PPH01279A
H_DKK-1	Dickkopf Wnt signalling inhibitor 1	Qiagen	PPH01752C

H_OCN	Osteocalcin or bone gamma-carboxyglutamic acid-containing protein (BGLAP).	Qiagen	PPH01898A
H_CTNNB1	β-catenin	Qiagen	PPH00643F
H_AGTR1	Angiotensin-II receptor 1	Qiagen	PPH02362F
H_MCP-1	Monocyte chemoattractant protein-1	Qiagen	PPH00192F
H_MYH11	myosin heavy chain 11	Qiagen	PPH02469A
H_CNN1	Calponin-1	Qiagen	PPH02065A
H_SMTN	Smoothelin	Qiagen	PPH12727A
H_CALD1	Caldesmon 1	Qiagen	PPH21139A
H_TAGLN	Transgelin	Qiagen	PPH19531F
H_ACTA2	α-smooth muscle actin-2	Qiagen	PPH01300B
H_GAPDH	Glyceraldehyde 3-phosphate dehydrogenase		
Shown are the primers used to assess gene expression in human aortic smooth muscle cells.			

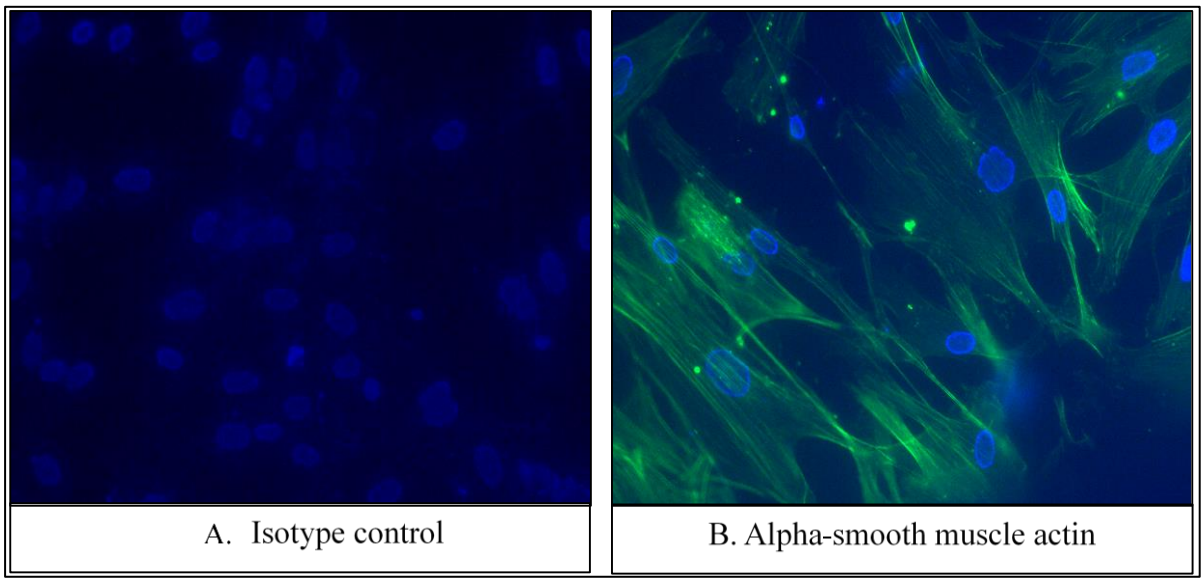


Figure 3.2: Analysis of VSMCs actin filaments.

Microscopic photographs of cytoskeletons in human aortic VSMCs (passage 9). **A.** An isotype of cell nuclei are counterstained blue. **B.** Green filaments showing immuno-fluorescent polymerized filaments of α-smooth muscle actin. Microscopic cell count showed that more than 95% of cells were positively stained by α-SMA.

3.2.6. MOUSE STUDIES

ApoE$^{-/-}$ mice on C57BL/6J background were used in this study. Mice were sourced from Animal Resource Centre (ARC, Western Australia) and housed in cages that were individually ventilated and temperature/humidity-controlled (Aero IVC Green Line; Tecniplast). Mice were maintained under a 12-hour light-dark cycle (relative humidity: 55-60%; temperature: 22±1ºC) and given portable water *ad libitum*. The animal's welfare, including weight and general health were monitored in accordance with internal regulations. All procedures in this study were approved by the James Cook University Animal Ethics Committee, Australia; and adhered to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA). Animals experiments described in this chapter were approved under (AEC#: 1970; Appendix 1A).

3.2.7. Design of mouse studies

At 13 weeks of age, mice were randomly split into two groups (n=8 per group) using Microsoft excel random number generator function (Microsoft EXCEL; 2016). One group was allocated to a vitamin D sufficient (VDS) diet while the other group was allocated to a vitamin D deficient diet (VDD) diet. The VDS group was allocated to a vitamin D3 enriched diet (2,200 IU/ kg of feed) while the VDD group was maintained on a CCF-depleted diet (**Table 3.2**). Blood was collected prior to starting the diets; and every month thereafter. Mice were maintained on their respective diets for 5 months and then sacrificed at the terminal endpoint. At the endpoint (5 months), mice were euthanized by CO_2 asphyxiation, followed by dissection and collection of samples

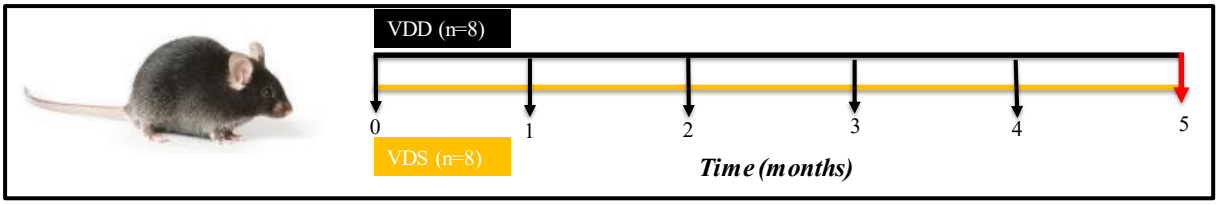


Figure 3.3: Experimental design

Thirteen weeks old mice (n=8/groups) were assigned to the vitamin D deficient diet (VDD; 0 IU/kg cholecalciferol) or vitamin D sufficient (VDS; 2200 IU/kg of cholecalciferol) for five months. Time points (1-5 months) indicates the interval at which blood collection were performed.

3.2.8. Animals and Diet

Dietary formulations were organised through Speciality Feeds (WA, Australia). Dietary formulations were balanced to ensure that nutritional intake in both diets, including minerals, fats, proteins and vitamins were similar. CCF levels were 2200 IU/kg of feed for the VDS and was non-traceable in the VDD diet (**Table 3.2**). Mice were initially maintained on the normal mice chow that contained CCF levels of 3000 IU/kg of feed. When mice were 13 weeks old, they were randomly split into two groups. One group was allocated to the CCF-enriched diet (2200IU/kg of feed) while the other group was maintained on a control cholecalciferol-depleted diet for five months.

Table 3.2: Nutritional parameters in the vitamin D deficient and vitamin D sufficient dietary formulations		
Nutritional Parameters	**SF05-034 (VDS)**	**SF05-033 (VDD)**
Protein	19.40%	19.40%
Total Fat	7.00%	7.00%
Crude fibre	4.70%	4.70%
Adequate dietary fibre	4.70%	4.70%
Digestible Energy	15.00 MJ / Kg	15.80 MJ / Kg
Total energy from lipids	19.00%	19.00%
Total energy from protein	23.00%	23.00%
Calcium*	1.00%	2.00%
Phosphorus*	0.70%	1.20%
Vitamin D3 (Cholecalciferol)	2200 IU/kg	Not traceable
*Calcium and phosphorus minerals were balanced between the diets. The vitamin D sufficient (VDS) diet is formulated as the control diet for the vitamin D deficient (VDD) diet according the manufacturer. IU: international units.		

3.2.9. Plasma analyses

Blood collection was performed by tail bleeding during the experiments and at the dissection end-point by cardiac apex puncture. The blood was collected into heparin-coated tubes (BD Microtainer). Plasma was isolated by centrifugation at 6400x g for 10 minutes at 4^0C and stored at -80^0C until analysed. Assays for 25(OH)D, epi-25(OH)D3, and 25(OH)D2 were performed as previously detailed in Chapter 2 (Section 2.7.1). Briefly, plasma was diluted in RPMI Media 1640 (ThermoFisher Scientific) (1:3 v/v) and assayed in a vitamin D assay accredited laboratory at the Centre for Metabolomics (WA, Australia). The assay was performed using a two-dimensional (2D) ultra-performance liquid chromatography (UPLC) separation coupled

to tandem mass spectrometry (MS/MS). The detectable levels for serum 25(OH)D were >5.0 nmol/L. 25(OH)D assay methodology has been previously demonstrated to accurately detect and quantitate 25(OH)D3, epi-25(OH)D3, and 25(OH)D2 (Albarhani et al., 2015; Clarke et al., 2013).

Plasma SOST assay was performed as previously detailed in Chapter 2 (Section 2.7.2). Briefly, the assay was performed according to the manufacturer's instruction. Serum SOST was measured using a Quantikine® enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems; Cat#: MSST00) according to the manufacturer's instructions. Serum samples required a 2-fold dilution using the calibrator diluent (RD6-12) provided with the kit. All samples and standards and were performed in duplicates. Mean intra-assay and inter-assay coefficient of variation (CV) were 5.1% and 5.8%, respectively. This described in details Chapter 2 (Section 2.7.2).

3.2.10. Suprarenal aortic protein assay by ELISA

SRA segments were cleared of OCT and homogenized in the presence of the radio-immunoprecipitation assay buffer (1 x RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl; 2 mM EDTA, 1% TritonX-100, 0.1% SDS, and 0.1% sodium deoxycholate) supplemented with protease inhibitors and phosphatase inhibitor (Roche). Mouse SRA tissue SOST protein were assayed using a Quantikine® ELISA (R&D systems) as described above. Phospho-GSK-3α/β (S21/S9) protein levels within the SRA were determined using DuoSet® IC ELISA (R&D systems) following the manufacture's instruction. Briefly, a 96 well ELISA plate was coated with immobilized capture antibodies specific to mouse phospho-GSK-3α/β (S21/S9) and incubated overnight. After washing, a biotinylated detection antibody specific only to mouse phospho-GSK-3α/β (S21/S9) was used to detect phosphorylated protein levels using a standard streptavidin-horse radish peroxidase (HRP). All samples and standards were assayed in duplicates with a mean intra-assay coefficient of variation (CV) of 4.7% and 4.9% for SOST and phospho-GSK-3α/β (S21/S9) assays, respectively. This is described in details in Chapter 2 (Section 2.10.4)**.**

3.2.11. RNA extraction and assessment of gene expression

The SRA tissue that was previously stored in RNAlater was homogenised and mRNA was extracted using TRIzol reagents (Qiagen) as previously described in Chapter 2 (Section 2.11).

Details on genes that were assessed in mice SRA tissue are presented **Table 3.3**. Primers for mouse *Sost* (5'-CTTAAAGGGAAGGGAGTG-3' and 5'-TTACATTTGGGTGGAAGG-3'), mouse *Tnfrsf11b* (5'-AAGATGGCTTCTATTACC3' and 5'-GCTGAAGATAGTCTGTAG-3') and mouse *Opn* (5'-ACTCTTCCAAGCAATTCC3' and 5'-GTCTCCATCGTCATCATC-3') were designed using the Premier 6 Software (Premier Biosoft) and purchased from Sigma (**Table 3.3**). Other primers were purchased pre-designed (Qiagen).

Table 3.3: Mouse Primers			
Primers	**Encoded protein**	**Source**	**Catalog #**
mm-Sost	Sclerostin	Sigma	Designed in house
mm_Tnfrsf1	Osteoprotegerin	Sigma	Designed in house
mm_Opn	Osteopontin	Sigma	Designed in house
mm_Vdr	Vitamin D receptor	Qiagen	PPM05132C
mm_Cyp27b1	25-Hydroxyvitamin D3 1-alpha-hydroxylase	Qiagen	PPM03992A
mm_Dkk-1	Dickkopf WNT signalling pathway inhibitor 1	Qiagen	PPM05476F
mm_Ocn	Bone gamma-carboxyglutamic acid-containing protein (BGLAP) or osteocalcin	Qiagen	PPM04465F
mm_Ctnnb1	β-catenin	Qiagen	PPM03384A
mm_ Agtr1	Angiotensin-II receptor type 1	Qiagen	PPM05162A
mm_ Mcp-1	Monocyte chemoattractant protein 1	Qiagen	PPM03151G
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	Qiagen	QT01658692
Primers used for real-time quantitative polymerase chain reaction mice experiments. Shown are the primers used to assess gene expression within the suprarenal aortas of mice.			

3.2.12. Statistical analysis

The D'Agostino and Pearson test were used to test normality of the data. Non-normally distributed data were expressed as median and interquartile range (IQR) and normally distributed data were expressed as mean ± SEM. For non-normally distributed data, comparisons were made using Mann-Whitney U-test or Kruskal-Wallis test followed by

Dunn's multiple comparisons test. Normally distributed data were compared using ANOVA followed by Bonferroni's multiple comparisons test. Animal experiments described in this chapter were primarily conducted as a pilot study to investigate whether dietary restriction of cholecalciferol (CCF) affected circulating levels of 25(OH)D and circulating SOST and SRA SOST in *ApoE*$^{-/-}$ null mice. Therefore, sample size calculation was not determined for this study. Changes in 25(OH)D levels over time were analysed by repeated measures ANOVA. All data were analysed using GraphPad Prism 8.4.2 software (GraphPad Software, Inc., USA). Differences were considered statistically significant at $P < 0.05$.

3.4. RESULTS

3.4.1. 1α,25(OH)$_2$D3 upregulated SOST and attenuated Wnt/β-catenin signalling in human aortic vascular smooth muscle cells.

Incubation of 1α,25(OH)$_2$D3 with human aortic VSMCs for 48 hours resulted in dose-dependent upregulation of *SOST* (P<0.001, **Figure 3.4A**). In contrast, *CTNNB1* was dose-dependently downregulated in human aortic VSMCs following incubation of with 1α,25(OH)$_2$D3 (P=0.007, **Figure 3.4B**).

3.4.2. 1α,25(OH)$_2$D3 modulated expression of genes implicated in vitamin D metabolism in human aortic vascular smooth muscle cells.

Incubation of human VSMSCs with 1α,25(OH)2D3 significantly upregulated expression of *VDR* in a dose-dependent manner (**P<0.001, Figure 3.5A**) in VSMCs. Expression of *OCN* was also significantly upregulated in dose-dependent manner (**P=0.112, Figure 3.5B**). Similarly, *CYP24A1* was significantly upregulated (**P<0.001, Figure 3.5C**). In contrast, expression of *CYP27B1* which encodes an enzyme that catalyses the conversation of 25(OH)D to 1α,25(OH)$_2$D3, was significantly downregulated **(P<0.001, Figure 3.5D**).

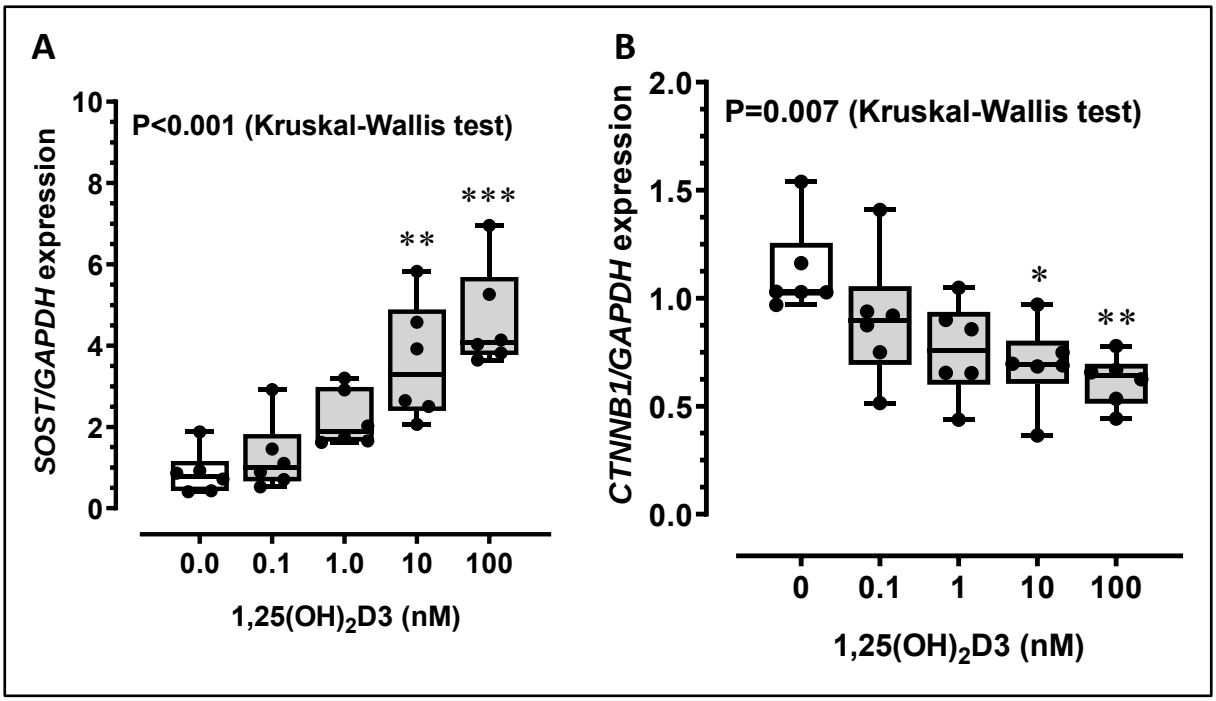


Figure 3.4: Expression of SOST and CTNNB1 in VSMCs following incubation with 1α,25(OH)$_2$D3.

The graph shows gene expression of (A) *SOST* and (B) *CTNNB1* expression (relative to *GAPDH*) in response to increasing concentration of 1α,25(OH)$_2$D3. Data expressed as median and interquartile range with maximum and minimum data points (whiskers) and compared with Kruskal-Wallis test (A, P<0.001; B, P=0.007). Gene expression at individual 1α,25(OH)$_2$D3 concentrations were compared to 0.0 nM (negative control) using Dunn's multiple comparison test; *P<0.05 and **P<0.01.

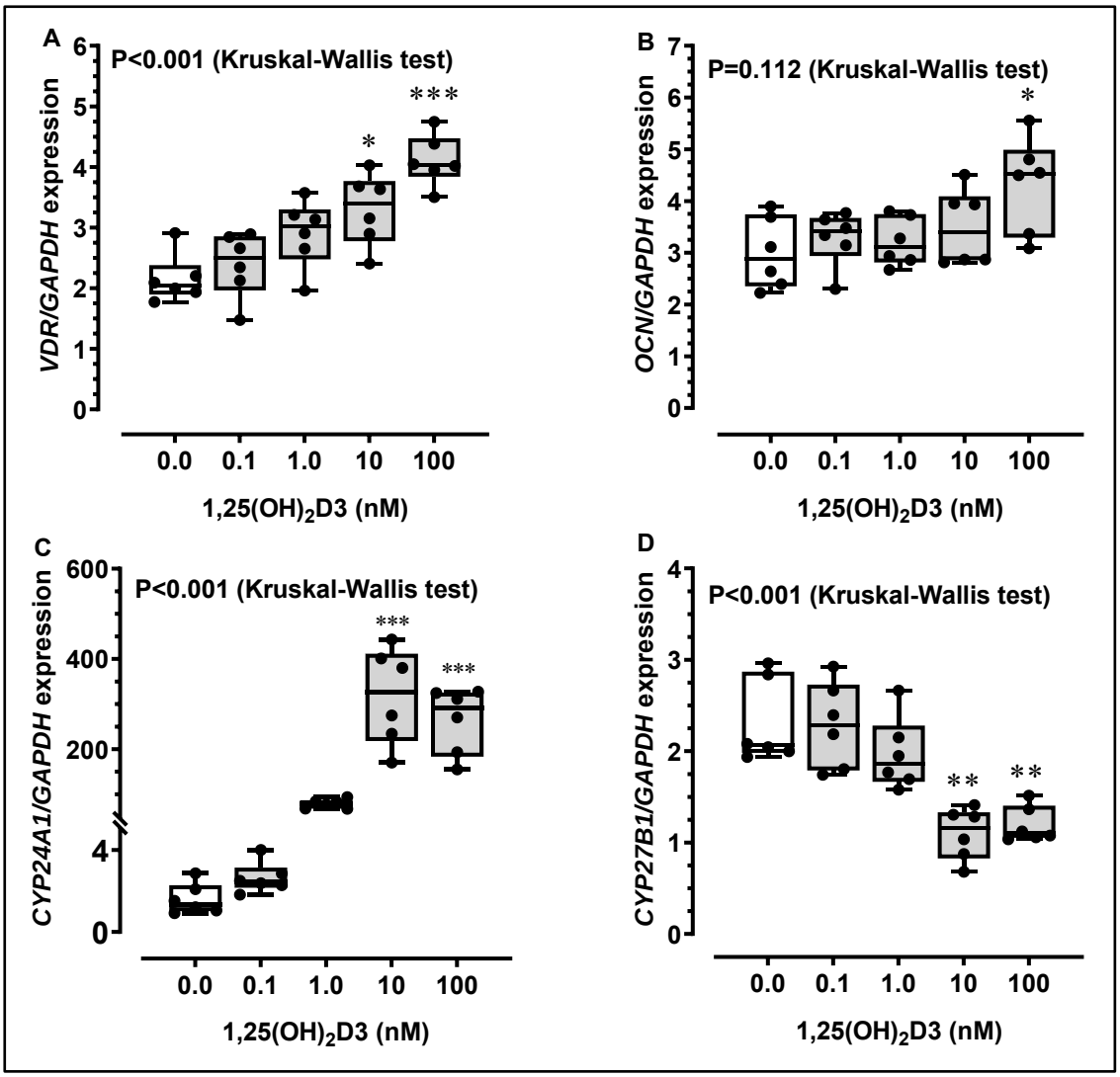


Figure 3.5: Effects of 1α,25(OH)$_2$D3 on vitamin D metabolism in VSMCs.

The graph shows gene expression of (A) *VDR* and (B) *OCN*, (C) *CYP24A1* and *CYP27B1* expression relative to *GAPDH*. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared with Kruskal-Wallis test. 0.1 nM to 100 nM concentrations were compared to 0.0 nM (negative control) using Dunn's multiple comparison test. Statistical significance is shown as *P<0.05 and **P<0.01.

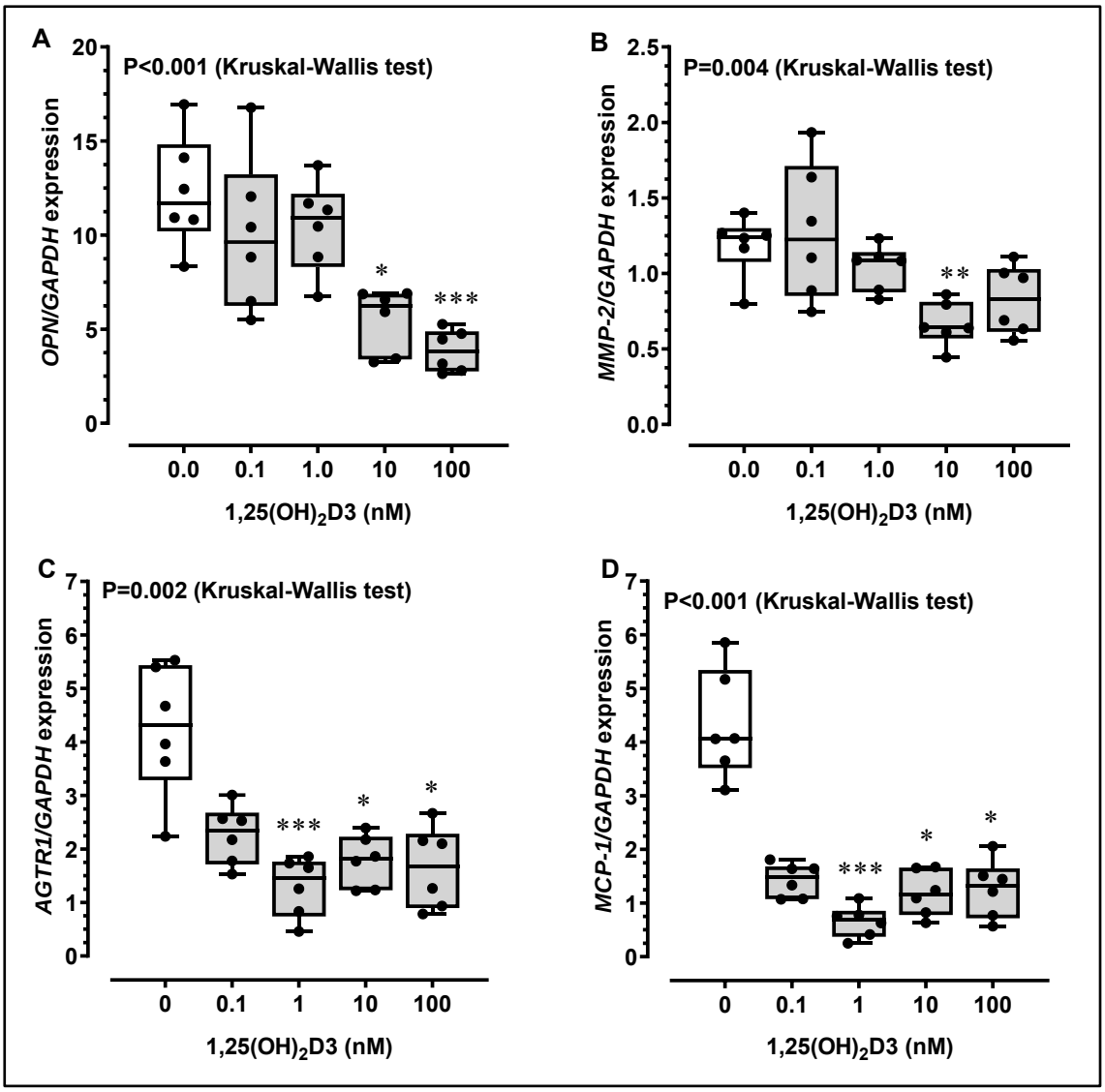


Figure 3.6: Expression of genes implicated inflammatory and matrix degradation following incubation of 1α,25(OH)$_2$D3 with VSMCs.

Following incubation of 1α,25(OH)2D3 with human aortic VSMCs for 48 hours expression of (A) *OPN*, (B) *MMP-2*, (C) *AGTR1* and (D) *MCP-1* was dose-dependently downregulated. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared among concentrations with Kruskal-Wallis test. 0.1 nM to 100 nM concentrations were compared to 0.0 nM (negative control) using Dunn's multiple comparison test. Statistical significance is shown as *P<0.05 and **P<0.01.

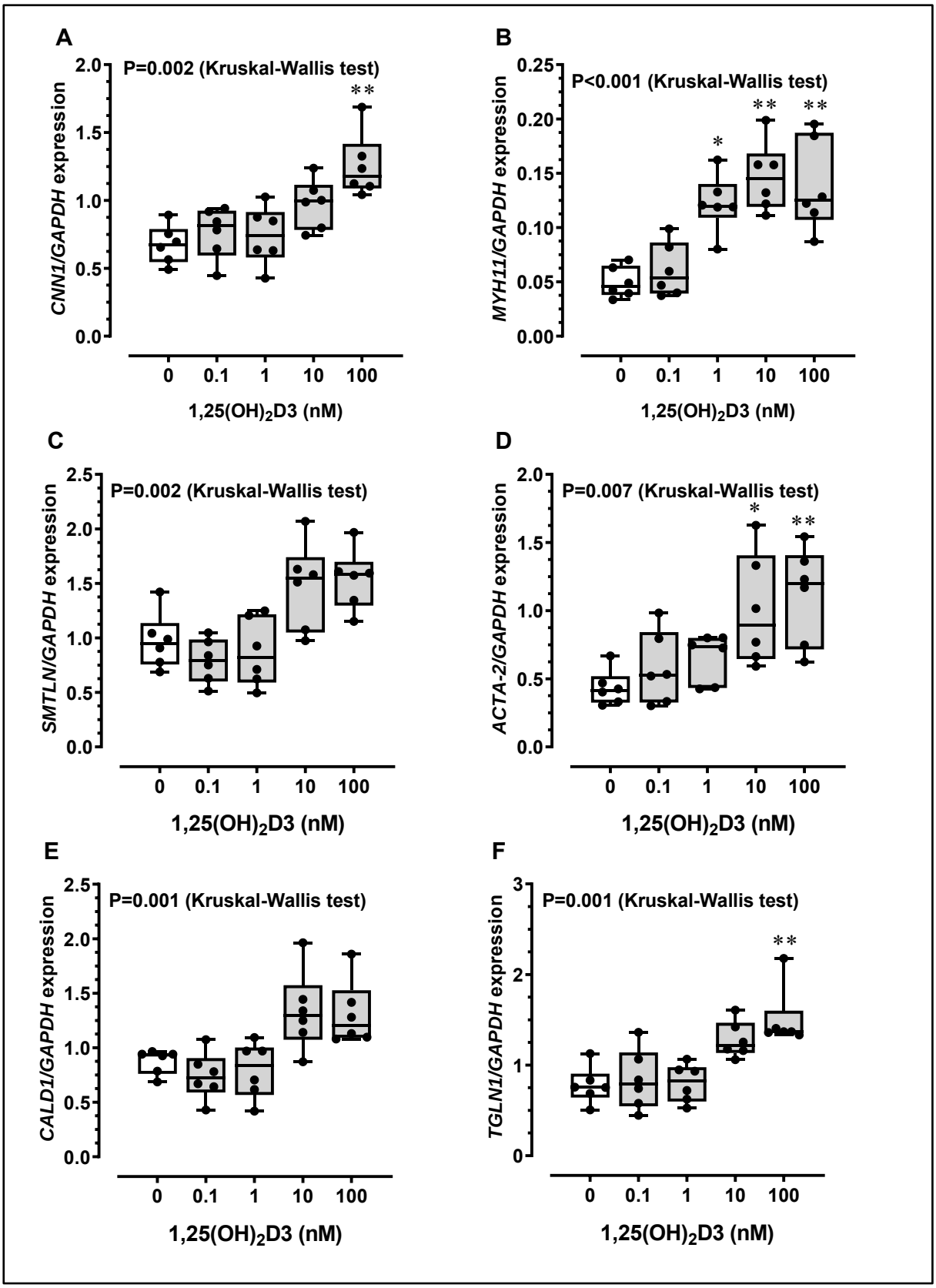

A
P=0.002 (Kruskal-Wallis test)
CNN1/GAPDH expression
2.0
1.5
1.0
0.5
0.0
**
0
0.1
1
10
100
1,25(OH)2D3 (nM)
B
P<0.001 (Kruskal-Wallis test)
MYH11/GAPDH expression
0.25
0.20
0.15
0.10
0.05
0.00
*
**
**
0
0.1
1
10
100
1,25(OH)2D3 (nM)
C
P=0.002 (Kruskal-Wallis test)
SMTLN/GAPDH expression
2.5
2.0
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,25(OH)2D3 (nM)
D
P=0.007 (Kruskal-Wallis test)
ACTA-2/GAPDH expression
2.0
1.5
1.0
0.5
0.0
*
**
0
0.1
1
10
100
1,25(OH)2D3 (nM)
E
P=0.001 (Kruskal-Wallis test)
CALD1/GAPDH expression
2.5
2.0
1.5
1.0
0.5
0.0
0
0.1
1
10
100
1,25(OH)2D3 (nM)
F
P=0.001 (Kruskal-Wallis test)
TGLN1/GAPDH expression
3
2
1
0
**
0
0.1
1
10
100
1,25(OH)2D3 (nM)

Figure 3.7: Expression of VSMCs contractile phenotype markers after incubation with 1α,25(OH)2D3.

Following incubation of 1α,25(OH)2D3 with human aortic VSMCs for 48 hours, expression of (A) *CNN1*, (B) *MYH11*, (C) *SMTLN*, (D) *ACTA-2*, (E) *CALD1* and (F) *TGLN1* was dose-dependently upregulated. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared among concentrations with Kruskal-Wallis test. 0.1 nM to 100 nM concentrations were compared to 0.0 nM (negative control) using Dunn's multiple comparison test. Statistical significance is shown as *P<0.05 and **P<0.01.

3.4.3. 1α,25(OH)$_2$D3 promoted expression of contractile vascular smooth muscle cells phenotype

Molecular regulation of contractile phenotype markers in VSMCs is important in atherosclerosis and aortic aneurysms (Ailawadi et al., 2009; Airhart et al., 2014; Rzucidlo, Martin, & Powell, 2007). Effects of 1α,25(OH)$_2$D3 on human aortic VSMCs contractile markers were investigated *in vitro*. Treatment with 1α,25(OH)$_2$D3 resulted in a dose-dependent upregulation of *CNN1* (P=0.002); *MYH11* (P<0.001), *SMTN* (P=0.002), *CALD1* (P=0.001), *TAGLN* (P=0.001) and *ACTA2* (P=0.007) (**Figure 3.7A-F)**.

3.4.4. 1α,25(OH)$_2$D3 downregulated pro-inflammatory and matrix degrading genes

Incubation of VSMCs with 1α,25(OH)$_2$D3 resulted in dose-dependent downregulation of constitutive genes that are known to be implicated in inflammation and ECM degradation. Expression of *OPN* (P<0.001), *MMP-2* (P=0.004) *AGTR1* (P=0.002), *MCP-1* (P<0.001) (**Figure 3.6**) and MAPK13 (P<0.001) (**Figure 3.8**) were all downregulated in a dose-dependent manner following incubation of 1α,25(OH)$_2$D3 with VSMCs for 48 hours.

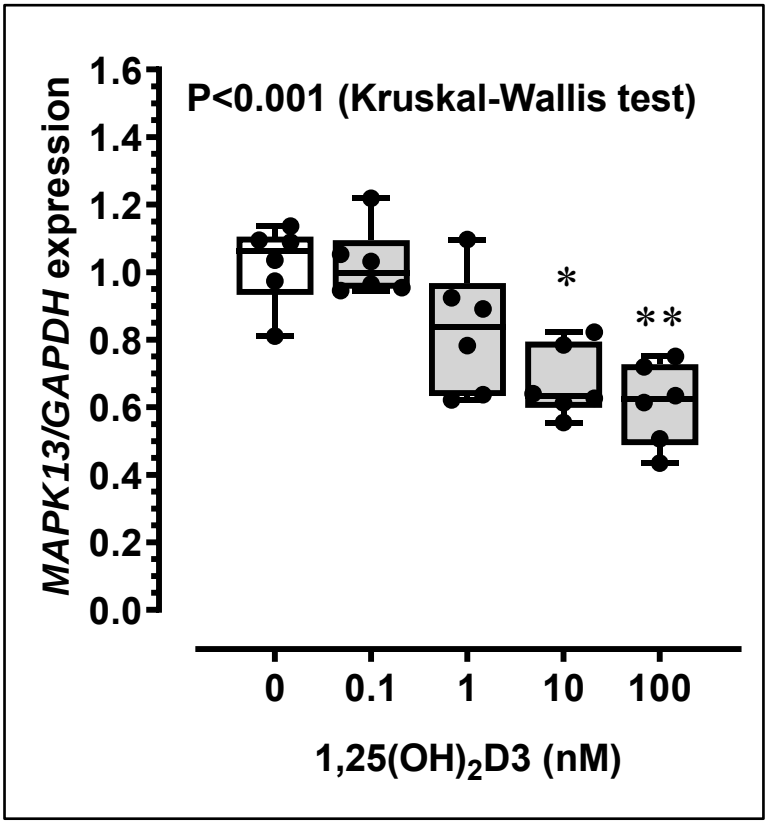


Figure 3.8: Expression of MAPK13 in VSMCs following incubation with 1α,25(OH)$_2$D3.
Incubation of 1α,25(OH)$_2$D3 with human aortic VSMCs for 48 hours resulted in in dose-dependently downregulation of MAPK13. Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared among concentrations with Kruskal-Wallis test. 0.1 nM to 100 nM concentrations were compared to 0.0 nM (negative control) using Dunn's multiple comparison test. Statistical significance is shown as *P<0.05 and **P<0.01.

4.4.5. Dietary restriction of vitamin D reduced plasma 25(OH)D, circulating SOST and suprarenal aortic SOST expression, resulting in activation of Wnt/β-catenin signalling in apolipoprotein E-null mice

In this experiment, effects of dietary restriction of vitamin D on circulating plasma 25(OH)D, plasma SOST, SRA SOST protein, SRA p-GSK-3α/β protein and SRA *Sost* gene expression were assessed. At baseline, plasma levels of 25(OH)D were similar between the VDS and the VDD groups. After one month, circulating levels of 25(OH)D were no longer detectable in all mice that were receiving the VDD diet while no significant changes were observed in the VDS

group. Overall, 25(OH)D levels were significantly lower in mice that were maintained on the VDD diet compared with those on VDS mice (**P<0.001; Figure 3.9A**). At baseline, plasma SOST levels were similar between both groups. However, after five months of dietary supplementation, the mice receiving the VDD diet had significantly reduced SOST levels compared with baseline (**P=0.016**) while the levels did not significantly change in mice receiving the VDS diet. At the five months endpoint, the mice that received the VDD diet had significantly lower SOST levels compared the group of mice that received the VDS (**P= 0.028; Figure 3.9B**). SOST protein levels measured from the homogenised SRA tissue were significantly lower in mice that received the VDD diet compared to mice maintained on the VDS diet (**P=0.015; Figure 3.9C**). Conversely, protein expression of phosho-GSK-3α/β were significantly higher in mice receiving the VDD diet compared with those fed the VDS diet (**P=0.001; Figure 3.9C**).

SRA gene expression was examined using rt-qPCR and normalised to the housekeeping gene *Gapdh* (**Table 3.4**). SRA *Sost* gene expression was significantly downregulated in mice that were fed the VDD diet compared with mice that received the VDS diet (**P<0.001**). Conversely, expression of *Ctnnb1* was significantly upregulated in mice receiving the VDD diet compared to mice maintained on the VDS diet (**P=0.007**). Expression of *Dkk-1* was similar in both groups of mice (**Table 3.4**).

4.4.6. Dietary restriction of vitamin D modulated suprarenal aortic expression of genes that are implicated in vitamin D metabolism and upregulated pro-inflammatory genes within the apolipoprotein E-null mice

SRA gene expression of *Cyp27b1* (**P=0.001**) and *Ocn* (**P=0.028**) were significantly upregulated in the mice fed the VDD diet compared to mice fed the VDS diet and, respectively. However, expression of *Vdr* was not significantly different between both groups of mice. Expression of pro-inflammatory genes, including *Opn* (**P<0.001**), *Agtr1* (**P=0.038**) and *Mcp-1* (**P<0.001**) was significantly upregulated in mice that received the VDD diet compared the group of mice that was fed the VDS diet. However, expression of *Opg* was not significantly different between the groups (**Table 3.4**).

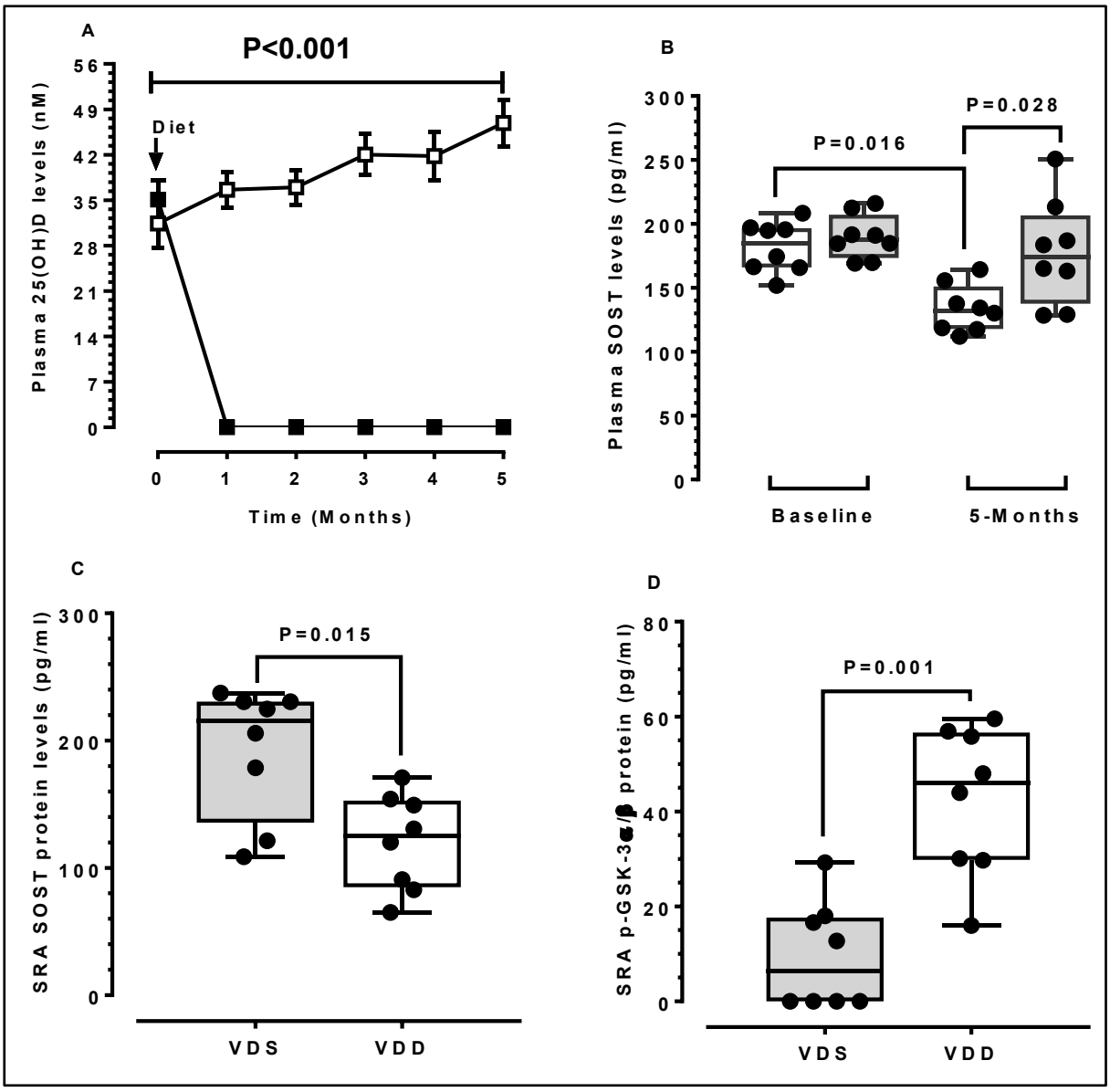


Figure 3.9: Effects of dietary restriction of vitamin D on plasma SOST and the Wnt/ß-catenin signalling in the aortic wall of ApoE$^{-/-}$ mice.

The graph shows (**A**) monthly plasma levels of circulating 25(OH)D, (**B**) plasma levels of SOST at baseline and endpoint, (**C**) SRA protein expression and (**D**) SRA p-GSK-3α/β protein expression. **A.** Compared with mice that received the VDS diet, the VDD diet resulted in depletion of circulating levels of 25(OH)D (below detectable levels; repeated measures ANOVA were performed to compare differences). Plasma SOST levels were compared between baseline and 5-months endpoint using by Wilcoxon paired test. SRA tissue SOST protein expression and phosphorylated GSK-3α/β (p-GSK-3α/β) complex were compared between groups with Mann Whitney U test. Statistical significance is shown as actual *P*-values. **Abbreviations**: 25(OH)D: 25-hydroxyvitamin D; SRA: suprarenal aorta; p-GSK-3α/β: phosphorylated glycogen synthase kinase 3α/β.

Table 4: Effect of dietary vitamin D restriction on gene expression in the suprarenal aorta of apolipoprotein E-null mice

Gene	VDS (n=8)	VDD (n=8)	Expression	P
Sost	1.32 [1.06-2.02]	0.14 [0.07-0.30]	↓	<0.001
Opg	0.07 [0.07-0.08]	0.08 [0.06-0.08]	↔	0.863
Opn	1.25 [0.76-1.81]	4.67 [2.97-8.26]	↑	<0.001
Vdr	0.42 [0.26-0.89]	0.53 [0.18-0.91]	↔	0.959
Cyp27b1	0.35 [0.24-0.44]	0.55 [0.47-0.77]	↑	0.001
Dkk-1	0.47 [0.32-0.66]	0.24 [0.17-0.40]	↔	0.065
Ocn	0.71 [0.44-107]	1.10 [0.91-1.31]	↑	0.028
Ctnnb1	0.69 [0.33-0.99]	1.11 [1.02-1.26]	↑	0.007
Agtr1	0.59[0.37-0.80]	0.83[0.70-0.97]	↑	0.038
Mcp-1	0.16[0.13-0.26]	0.39[0.30-0.47]	↑	0.001

Shown are relative gene expression as median (inter-quartile range) relative to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression. Inter-group comparison was analysed using Mann-Whitney U test (n=8/group). **Abbreviations:** *Sost*: gene encoding sclerostin; *Opg*: osteoprotegerin; *Opn*: osteopontin (also known as secreted phosphoprotein 1, *Spp1*); *Vdr*: vitamin D receptor; *Cyp27b1*: gene encoding for 25-hydroxvitamin D3 hydrolase; *Dkk*: *Wnt* inhibitor Dickkopf-1; *Ocn*: osteocalcin (also known as bone-Gla matrix); *Ctnnb1:* gene encoding for β-catenin; *Agtr1*: angiotensin-II receptor 1; *Mcp-1*: monocyte chemoattractant protein-1.

3.4. DISCUSSION

The main findings from this study were that 1α,25(OH)$_2$D3 induced *SOST* expression in human aortic VSMCs; and that dietary restriction of vitamin D reduced SOST in *ApoE*$^{-/-}$ mice. Incubation of 1α,25(OH)$_2$D3 with human aortic VSMCs for 48 hours resulted in upregulation of *SOST* and downregulation of *CTNNB1* in a dose-dependent manner. This resulted in dose-dependent downregulation of genes that are implicated in inflammation and upregulation of contractile phenotype marker genes. Conversely, dietary restriction of vitamin D resulted in increased circulating SOST and SRA wall SOST expression in *ApoE*$^{-/-}$ mice. Expression of pro-inflammatory and matrix degrading genes were downregulated within the SRA wall of these mice. These findings suggest that vitamin D modulates both *in vitro* expression of SOST within VSMCs and *in vivo* SOST in *ApoE*$^{-/-}$ mice. Downstream effects of SOST were demonstrated by activation of *Wnt*/β-catenin signalling. Effects of reduced SOST on the aortic wall inflammation and matrix degrading markers are supported by a previous study, which showed that transgenic downregulation of SOST promoted aortic aneurysm in experimental mice (Krishna et al., 2017).

Findings from this study suggested that VDD diet resulted in downregulation of SOST and activation of the Wnt/β-catenin signalling within the SRA wall of *ApoE*$^{-/-}$ mice. *In vitro* data showed that 1α,25(OH)$_2$D3 upregulated contractile phenotype makers in VSMCs, which is an important manifestation AAA (Peng et al., 2018). Activated Wnt/β-catenin signalling promotes mammalian aging and aging-related phenotype changes. In a recent study, findings from our group reported that SOST is epigenetically silenced in patients who are susceptible to AAA development as well as in mice (Krishna et al., 2017). Of importance, the role of vitamin D in epigenetic silencing is currently of interest as emerging evidence suggests that has vitamin D has the ability to mediate DNA demethylation in cells (Fetahu, Höbaus, & Kállay, 2014). It has also been previously reported that during arterial aging, VSMCs switch from a proliferative response to non-proliferative response as a result of Wnt/β-catenin activation (Carlberg, 2019; X. Gao, Zhang, Schottker, & Brenner, 2018; Marchand et al., 2011). This suggests that lower circulating 25(OHD)D which is a common finding in elderly populations (Fahrleitner et al., 2002; Grober et al., 2013; Holick, 2007) may be implicated. Additionally, it has been reported that VSMCs in the vessels of elderly subjects may not proliferate efficiently in response to β-catenin activation (Ruiz-Torres et al., 1999), which also favours arterial matrix remodelling. Plausibly, lower circulating 25(OH)D coupled with advanced age and other risk factors such

as smoking may act in concord to cause epigenetic silencing of the SOST gene, thus leading to susceptibility to aortic disease development older patients (Golledge, Biros, Bingley, Iyer, & Krishna, 2016; Krishna, Dear, Craig, Norman, & Golledge, 2013; Krishna, Dear, Norman, & Golledge, 2010; Krishna SM, 2014).

VSMCs are abundantly present within the aorta and play a crucial role in its function and integrity (Michel, Jondeau, & Milewicz, 2018). Decreased SOST protein and mRNA expression following dietary restriction of vitamin D underscore the importance of vitamin D in modulating SOST levels within the aorta. In agreement with these findings, previous reports have shown that vitamin D deficiency is associated with lower SOST in human (Jorde et al., 2019; Pirgon et al., 2016). Circulating levels of SOST have been inversely associated with the severity of aortic calcification and cardiovascular outcomes in dialysis patients (Yang et al., 2015) which suggests a role of Wnt/β-catenin signalling plays in vascular inflammation and remodelling.

Cellular experiments demonstrated a dose-dependent upregulation of SOST which was coupled with downregulation of *CTNNB1* following incubation with 1α,25$(OH)_2$D3. Similarly, reduced SOST in response to the VDD diet resulted in upregulation of aortic *Ctnnb1* within the SRA of mice, also confirmed by upregulated expression of GSK-3α/β within the aortic wall. A number of studies have demonstrated a significant role for the Wnt signalling pathway in vascular function (Naito, Shiojima, & Komuro, 2010; Reis & Liebner, 2013). Notably, the study from our group which demonstrated that SOST deficiency promotes expression of several genes that are implicated in the pathogenesis of aortic aneurysm and atherosclerosis (Krishna et al., 2017). Expression of VDR has been suggested to have the ability to directly repress β-catenin activation resulting in deactivation Wnt/β-catenin signalling (Shah et al., 2006). Alternatively, it is also possible that repressive effects of 1α,25$(OH)_2$D3 could have been mediated on Wnt/β-catenin signalling, thus reducing *CTNNB1* expression without direct effects of SOST, at least in *in vitro* studies.

Incubation of 1α,25$(OH)_2$D3 with VSMCs resulted in downregulation of pro-inflammatory and matrix remodelling markers. High serum concentrations of OPN were previously reported to be associated with a number CVD, including AAA (Golledge et al., 2007). OPN promotes inflammation (Denhardt, Noda, O'Regan, Pavlin, & Berman, 2001), and is also involved in the activation of proteolytic pathways including an increase of pro-MMP-9 and MMP-2 activity

(Lai et al., 2006). OPN deficiency has been shown to limit AAA development in the AngII-infused mouse model, a disease well-characterised by SRA wall inflammation and remodelling (Bruemmer et al., 2003). Findings from this study are therefore consistent with investigations from our laboratory and elsewhere which have previously showed that OPN is implicated in the development of AAA, and plays a significant role in aortic inflammation and matrix remodelling (Filis et al., 2014; Koole et al., 2012; Krishna et al., 2012; Moran et al., 2005). In addition, incubation of 1α,25$(OH)_2$D3 with VSMCs resulted in lower expression of *MAPK13* which is consistent with previous investigations showing that MAPK13 signalling is associated with aneurysmal development through upregulation of MMPs and inflammation (Martorell et al., 2016; Ortega et al., 2019; Wang et al., 2018). Nonetheless, although OPG has been shown to be positively associated with AAA formation (Moran et al., 2010; Moran et al., 2005), findings from this study did not find a difference in *OPG* gene expression between the VDD and the VDS groups nor was OPG downregulated following incubation of 1α,25$(OH)_2$D3 with human VSMCs. However, VSMCs used in this experiment were not exposed to AngII or any pro-inflammation stimulant. A previous report showed that OPG promoted an inflammation in human aortic VSMCs, including upregulation of cathepsin S, MMP-2 and MMP-9 following incubation with AngII (Moran, Jose, E. Biros, & Golledge, 2014). While expression of MMPs, including MMP-9 would require incubation with an inflammatory stimulant with cells, MMP-2 is constitutively expressed in VSMCs; and thus its expression was investigated in this study (Crowther, Goodall, Jones, Bell, & Thompson, 2000).

Previous *in vitro* experiments have tended to use supra-physiological concentrations of 1α,25$(OH)_2$D3 in the ranges of 1nM-100 nM (i.e. ~100-1000-folds higher than circulating levels of 1α,25$(OH)_2$D) (Lips, 2007). Selected doses of 1α,25$(OH)_2$D3 for cellular experiments were based on previous investigations examining the effects of 1α,25$(OH)_2$D3 on SOST expression in osteoblastic cells (Wijenayaka et al., 2016; Wijenayaka et al., 2015). While SOST was previously known to be exclusively expressed in bones (Jorde et al., 2019; Veverka et al., 2009; Weivoda & Oursler, 2014), expression of *SOST* was recently reported within the aortic tissue, albeit with an unknown function (Balemans et al., 2001; Brunkow et al., 2001; Rush et al., 2009). In order to confirm whether cultured human aortic VSMCs responded to 1α,25$(OH)_2$D3 treatment doses in this experiment, a number of key genes that are implicated the regulation of vitamin D metabolism were assessed. Significant upregulation of *VDR*, *OCN* and *CYP24A1*; and downregulation of *CYP27B1* confirmed that effects observed within human

aortic VSMCs in this study were in response to 1α,25(OH)2D3 effects (Christakos et al., 2016). Upregulated expression of *CYP27B1* is indicative of the ability for the tissue to locally metabolise bioavailable 25(OH)D while *VDR* expression shows receptor activation in response to 1α,25$(OH)_2$D (Christakos et al., 2016; Dusso et al., 2005).

These findings should be interpreted in light of several strengths and limitations. A noteworthy strength of this study was the design of *in vitro* experiments and *in vivo* studies in mice to demonstrated the effects of vitamin D on SOST. VSMCs play a paramount role in the function and integrity of the aorta (Gomez & Owens, 2012; Lacolley, Regnault, Nicoletti, Li, & Michel, 2012; Mao et al., 2015); therefore, i*n vitro* effects of 1α,25$(OH)_2$D on VSMCs strongly suggested an important role of vitamin D on VSMCs function. While it is possible that different cells may respond differently to 1α,25(OH)2D3 effects, *in vitro* experiments focused on VSMCs due to the paramount role played by these cells in arterial remodelling and AAA formation. Future experiments may be focused on investigating and replicating effects of 1α,25$(OH)_2$D in other cell types that are known to contribute to aortic wall remodelling and matrix degradation. The role of dietary restriction of vitamin D on circulating SOST and aortic SOST in otherwise unmanipulated *ApoE*$^{-/-}$ mice is a novel finding in this study with potential to be investigated in the presence of AAA disease. Therefore, it would be important to assess effects dietary restriction of vitamin D on AAA development in this mouse model.

In conclusion, an important and novel finding from this study was that SOST expression was upregulated in VSMCs following incubation with increasing doses of 1α,25$(OH)_2$D3 whereas the VDD diet reduced SOST in *ApoE*$^{-/-}$ mice. Data from this chapter demonstrated that 1α,25$(OH)_2$D3 promoted expression of SOST in VSMCs in a dose dependent manner. Within *ApoE*$^{-/-}$ mice, dietary restriction of vitamin D deficiency resulted in reduced circulating SOST and decreased SRA SOST expression at protein and mRNA levels. Collectively, these findings suggested that deficiency of vitamin D coupled with resultant decrease in SOST promotes expression of markers that are implicated in aortic wall inflammation and matrix remodelling. Effects of vitamin D deficiency appear to be mediated via changes in reduced SOST and increased OPN, which promotes ECM degradation. Given the importance of inflammation and arterial remodelling in the pathogenesis of AAA, it would be interesting to examine whether deficiency of vitamin D and consequent lower SOST promote formation of severe AAAs within an experimental mouse model of AAA. In light of these findings, the following chapter

will examine effects of dietary restriction of vitamin D on AAA formation using the AngII-infused mouse model.

CHAPTER 3 HIGHLIGHTS

What is Known?

- 1α,25(OH)$_2$D3 (the active metabolite of vitamin D3) has been shown to induce SOST expression in osteoclasts.
- SOST is a negative regulator of Wnt/β-catenin signalling.
- Downregulation of SOST was recently shown to increase infiltration of macrophages, remodelling of elastin and collagen ECM within AngII infused *ApoE*$^{-/-}$ mice.
- Circulating 25(OH)D concentration is the best indicator of vitamin D status.

What New Information Does This Chapter Add?

- 1α,25(OH)$_2$D increased *SOST* expression and downregulated activation of Wnt/β-catenin signalling in human aortic VSMCs.
- 1α,25(OH)$_2$D increased expression of contractile phenotype markers and reduced expression of pro-inflammatory genes related to AAA formation in human aortic VSMCs.
- Dietary restriction of vitamin D resulted in depletion of 25(OH)D levels and reduced circulating SOST and SRA SOST protein as well as SRA *Sost* gene expression in the *ApoE*$^{-/-}$ mice.
- Dietary restriction of vitamin D resulted in upregulation of pro-inflammatory genes within the SRA of *ApoE*$^{-/-}$ mice.

CHAPTER 4:

EFFECT OF VITAMIN D DEFICIENCY ON ANGIOTENSIN II-INDUCED ABDOMINAL AORTIC ANEURYSM IN THE APOLIPOPROTEIN E-NULL MOUSE

4.1. INTRODUCTION

AAA is a focal enlargement of the aorta characterised by degradation and weakening of the aortic wall. AAA is thought to be a multifactorial disease; however, current knowledge of exact pathophysiological mechanisms remains incomplete. Presently, no drug therapies that have been demonstrated to prevent AAA development or limit AAA growth and rupture (Golledge et al., 2019; Golledge, Norman, Murphy, & Ronald L. Dalman, 2017; Lindeman & Matsumura, 2019). There is therefore great interests in identifying drug therapies that can effectively limit AAA growth and rupture.

Data from experimental and epidemiologic studies support an association between vitamin D deficiency and arterial diseases such as CAD, PAD and atherosclerosis (Nsengiyumva et al., 2015; Pilz, Verheyen, Grubler, Tomaschitz, & Marz, 2016). The relevance of vitamin D in AAA however has only recently become of interest. Reduced circulating concentration of 25(OH)D is associated with human aortic aneurysms in a number of cross-sectional studies (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). AAA patients with lowest plasma levels of 25(OH)D were greater than five times likely to have an AAA larger than >4cm compared with those with the highest range of 25(OH)D levels. Contrary to this evidence, it is reported that the prevalence of AAA is highest in Australasia where levels of ultraviolet exposure (and thus likely vitamin D) are very high (Li et al., 2013; Sampson et al., 2014). Excess vitamin D is believed to have potential pathological effects such as inhibiting production of elastin (an important aortic matrix protein) by VSMCs (Hinek et al., 1991; Norman et al., 1995; Norman et al., 2002). Further experimental research is therefore needed to resolve the importance of vitamin D in AAA pathogenesis.

Expression of VDR within cardiac and arterial cells suggests that vitamin D plays a role within the cardiovascular system (Al Mheid, Patel, Tangpricha, & Quyyumi, 2013; Christakos et al., 2016; Norman & Powell, 2014; Nsengiyumva et al., 2015; Pilz et al., 2016; Scragg et al., 2017). A recent study showed that administration of the VDR agonist 1α,25(OH)$_2$D3 inhibited aortic aneurysm in a mouse model of AAA (Martorell et al., 2016). In addition, administration of the vitamin D analogue, paricalcitol, was reported to attenuate inflammation, by reducing infiltration of T-cell ($CD3^+$) and T-helper cell ($CD4^+$) within the aortic wall of AAA patients (Nieuwland et al., 2016). Vitamin D deficiency is clinically managed by cholecalciferol supplementation with an aim to raise 25(OH)D (Bischoff-Ferrari, Giovannucci, Willett,

Dietrich, & Dawson-Hughes, 2006; Holick et al., 2011; Kennel, Drake, & Hurley, 2010; Kleerekoper et al., 2011). Vitamin D plays an important role in the control of bone mineralisation (Goltzman, 2015). The findings from the preceding chapter (Chapter 3) demonstrated that 1α,25$(OH)_2$D3 upregulated *SOST* expression in cultured human aortic VSMCs. It was further shown that within the SRA of *ApoE*$^{-/-}$ mice, dietary restriction of vitamin D resulted in reduced SOST and downstream activation of *Wnt*/β-catenin pathway. The expression of *SOST* is reportedly reduced in experimental mice and human aortic aneurysms (Krishna et al., 2017; Towler, 2017). Mice in which *SOST* was transgenically overexpressed were protected from AAA development following Ang-II infusion (Krishna et al., 2017). Mechanistically, it was proposed that these effects were due to the ability of *SOST* to block signalling via the canonical 'wingless-type mouse mammary virus integration site', or *Wnt*/β-catenin pathway (Krishna et al., 2017). However, it remains unknown whether dietary deficiency of vitamin D promotes AAA in this experimental mouse model.

The following work examined the hypothesis that vitamin D deficiency promotes AAA growth. The aim of the study was to determine if dietary-induced deficiency of vitamin D promoted AAA in the *ApoE*$^{-/-}$, Ang-II mouse model AAA.

***Specific hypotheses of this study were*:**

1. Dietary deficiency of vitamin D promotes AAA development in AngII infused *ApoE*$^{-/-}$ mice.

These hypotheses were assessed with the following specific aims:

1. To determine whether dietary deficiency of vitamin D augments AngII-induced dilatation of the SRA in AngII infused *ApoE*$^{-/-}$ mice.
2. To determine whether dietary deficiency of vitamin D promotes atherosclerosis in AngII-induced dilatation of the SRA in AngII infused *ApoE*$^{-/-}$ mice.
3. To determine whether dietary deficiency of vitamin D increases SBP in Ang-II infused *ApoE*$^{-/-}$ mice.
4. To examine if dietary deficiency of vitamin D promotes inflammation and aortic wall matrix remodelling in AngII-induced infused *ApoE*$^{-/-}$ mice.

4.2. METHODS AND MATERIALS

4.2.1. Sample size determination

The sample size calculation was performed using GPower 3.1.9.2 software version. The primary outcome for this study was the SRA diameter serially measured by ultrasound. The intervention in this study was dietary restriction of vitamin D which was postulated to result in lower circulating 25(OH)D; and hence larger AAAs. Therefore, sample size computation was based on a recent study by (Smriti M Krishna et al., 2015) in which randomisation of *ApoE*$^{-/-}$ mice (n=29) to intervention or placebo after 28 days of AngII infusion resulted in in larger AAAs for the intervention group [1.30±0.21 (mean±SD)] compared with the SRA diameter for controls mice [1.15±0.16 (mean±SD)] which was similarly measured by ultrasound. Assuming a similar trend (80% power; α: 0.05), an estimated total of 48 mice was required (n=24 mice/group). This sample accounted for possible drop-outs in anticipation of mortality (15-20 %) due to AAA rupture following AngII infusion.

4.2.1. Experimental design

The James Cook University/Animal Ethics Committee approved all animal studies described in this Chapter (JCU/AEC no: 1970; Appendix 1A).

All mice were initially maintained on standard chow that contained 3000 IU/kg of cholecalciferol. At 13 weeks old, mice (n=24/ group) were randomly allocated to a VDD or VDS diets using Microsoft excel random number generator function (Microsoft EXCEL, 2016). The two groups were maintained on their respective diets for two months (8 weeks) prior to induction of AAA by AngII infusion. Dietary formulations were provided by Speciality Feeds (WA, Australia). Nutrient content of the feed was balanced such that essential vitamins and minerals, fats, and proteins were comparable between the diets, with exception of cholecalciferol. Cholecalciferol concentration in the VDS diet was 2200 IU/kg of feed, while non-traceable in the VDD diet (**Table 3.2**). At the end of the two-month dietary supplementation, AAA was induced via AngII infusion for 28 days at a flow rate of 1µg/kg/min. At the end of the study, mice were euthanized by CO_2 asphyxiation, followed by dissection and tissue samples collection. A detailed experimental design flow-chart is illustrated in **Figure 4.1.**

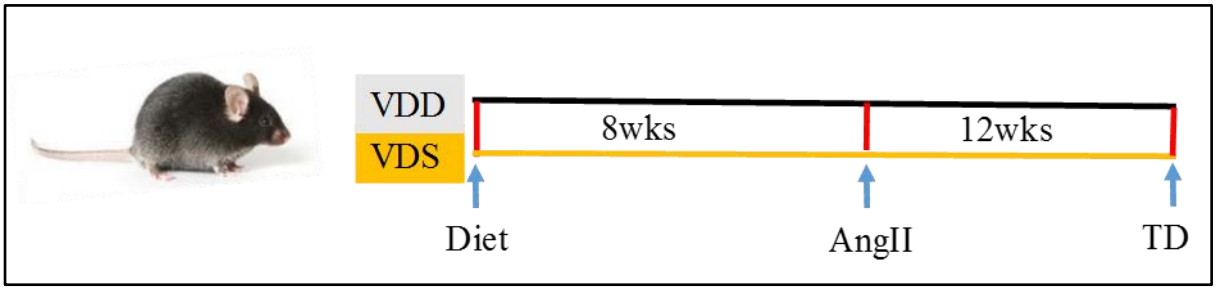


Figure 4.1: Study Design

Apolipoprotein E-null (*ApoE*$^{-/-}$) mice (n=49) were randomly allocated to either a vitamin D deficient (VDD; 0 IU/kg of feed; n=25) or a vitamin D sufficient (VDS; 2200 IU/kg of feed) diet (n=24). Three of the 24 mice allocated to the VDS diet were euthanized within the first three days due to due to unrelated health problems; leaving 21 mice. After 8 weeks, aortic aneurysm was induced by infusion of angiotensin II (AngII), 1µg/kg/min lasting for 28 days while the groups were maintained on their respective diets. Supra-renal aortic diameter, peripheral blood collection and blood pressure were performed at baseline (before diet introduction), at day 0 (before AngII infusion) and at day 14 and 28 post AngII infusion. After 28 days of AngII infusion (total duration was 12 weeks for the experiment), terminal dissection (TD) was performed and samples were collected.

4.2.2. Non-invasive tail-cuff blood pressure assessment

SBP, DBP and heart rate were measured in mice at baseline, prior to starting AngII infusion, at day 14 days post-AngII infusion, and at day 28 post-AngII infusion. Measurements were performed using a computerized non-invasive tail-cuff system (CODA Monitor, Kent Scientific) as described in Chapter 2 (Section 2.6).

4.2.3. Ultrasound monitoring

SRA diameter *in vivo* was measured by ultrasound as described in Chapter 2 (Section 2.5.1), first at baseline (day 0, before AngII) and then at 14-day intervals following commencement of AngII infusion.

4.2.4. Morphometric analysis

Measurement of SRA diameter and characterisation of aneurysm in aortas harvested from surviving mice at the completion of the study period was performed as described in Chapter 2 (Section 2.5.2.). Gross morphology of the SRA (aneurysm type) was classified and compared between the two groups according to Daugherty, et al. (2001) (Daugherty et al., 2001). The inter-assessor rate on classification of aneurysms was achieved by 100% concordance as described in Chapter 2 (Section 2.5.2.).

4.2.5. Plasma analyses

Blood collection was performed by tail bleeding at baseline (before diet) at day 0 (before AngII infusion) and at 14 day intervals after AngII infusion. At the end-point, during terminal dissection, blood was collected by cardiac puncture. The blood was collected into heparin-coated tubes (BD Microtainer) and plasma samples prepared for 25(OH)D and SOST assay as described in Chapter 2 (Sections 2.7).

4.2.6. Protein assay by ELISA in suprarenal aortic tissue

SRA segments were cleared of OCT and homogenized in the presence of the radio-immunoprecipitation assay buffer (1 x RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl; 2 mM EDTA, 1% TritonX-100, 0.1% SDS, and 0.1% sodium deoxycholate) supplemented with protease inhibitors and phosphatase inhibitor. Mouse SRA SOST protein expression was assayed using a Quantikine® ELISA (R&D systems®) as described in Chapter 2, (Sections 2.10.3). Mouse SRA phospho-GSK-3α/β (S21/S9) levels were determined using DuoSet® IC ELISA in accordance (R&D systems®) as described in Chapter 2 (Sections 2.10.4)

4.2.7. Quantification of atherosclerotic lesion area

Aortic arch samples were prepared for *en face* Sudan IV staining as described in Chapter 2 (Section 2.7). The presence and severity of intimal surface atherosclerotic plaque was compared between arch samples obtained from mice on VDD (n=13) and VDS (n=17) by digital image analysis as described in Chapter 3 (Section 2.8).

4.3. Statistical analysis

D'Agostino and Pearson test was used to test the normality of the data. Results were expressed as median and IQR for non-normally distributed data; and as mean ± SEM for normally distributed data. For non-normally distributed data, comparisons were made using Mann-Whitney U-test or Kruskal-Wallis test followed by Dunn's multiple comparisons test. Normally distributed data were compared using ANOVA followed by Bonferroni's multiple comparisons test. Mortality rate was tested using a survival Kaplan Meier curve. Differences were considered to be statistically significant at $P < 0.05$. Repeated measures ANOVA were analysed using GraphPad Prism software All data were performed using GraphPad Prism (Version 8.4.2) software (GraphPad Software, Inc., USA).

4.3. RESULTS

4.3.1. Dietary restriction of vitamin D promoted development of larger AAAs that were prone to rupture.

Two days after dietary allocation, three mice from the VDS group were euthanized due to ill-health unrelated to the experiment and thus excluded from the study. Mice administered VDD diet exhibited a greater rate of SRA expansion over the intervention period compared to control mice receiving VDS diet (P=0.034; **Figure 4.2.A**). Aortic rupture occurred in 48% of mice administered VDD diet (12 out of 25; arch rupture in 4 mice and SRA rupture in 8 mice). In contrast, 19% of mice (4 out of 21) receiving VDS diet died of aortic rupture (arch rupture in 2 mice and SRA rupture in 2 mice). The incidence of aortic rupture was greater in mice allocated VDD diet compared to VDS diet as determined by Kaplan Meier analysis and log rank test (P=0.029; **Figure 4.2.B**).

Morphometric analysis of aortas harvested from surviving mice at the end of the study demonstrated a median maximum and interquartile range [IQR] for SRA diameter significantly larger in VDD mice compared to VDS control mice (2.36 [2.08-3.10] mm vs 1.83 [1.28-2.53] mm, respectively; P=0.039; **Figure 4.4.A**). Gross morphology of the SRA (aneurysm types) was classified and compared between the dietary groups (**Figure 4.4.B.**). Four types of aneurysms were identified in each of the dietary groups. Two type I aortas were identified in the VDD group compared to five in the VDS group. Type II aneurysms were identified in five versus two aortas from the VDS and VDD group, respectively. Four of the more severe type

III aneurysms were identified in the VDD group compared to only two in the VDS group. Finally, two aortas from the VDD group were classified as exhibiting the most severe type IV aneurysm compared to one aorta from VDS group.

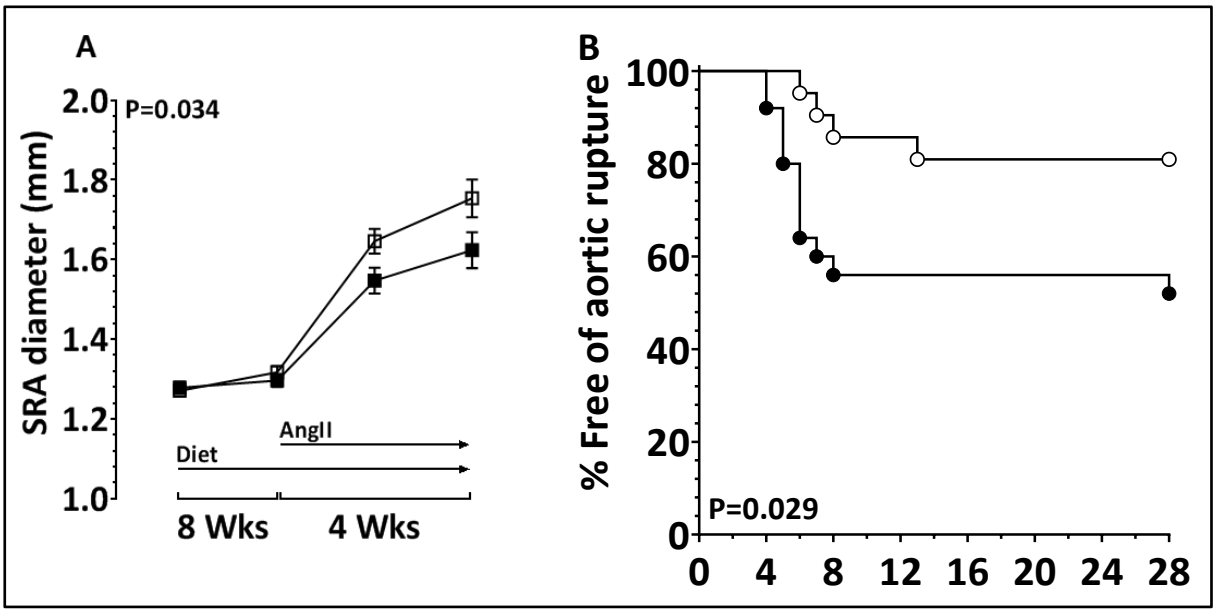


Figure 4.2 Effect of vitamin D dietary supplementation on AAA development and rupture in angiotensin-II infused apolipoprotein E-null mice

Both groups of mice received their respective diets for 8 weeks, followed by AAA induction by AngII-infusion (1µg/kg/min) for a period of 4 weeks during which time the diets were continued. **A.** SRA expansion indicating aneurysmal development was assessed by ultrasound in both groups. Mice on the VDD diet developed larger aneurysms compared to those on the VDS diet (compared with repeated measures ANOVA using GraphPad Prism software). **B.** In mice allocated VDD diet (n=25), a total of 12 mice (48%) mice had aortic rupture. In mice receiving VDS diet (n=21), four mice (19%) died of aortic rupture. By Kaplan Meier analysis and log rank test, the incidence of aortic rupture was greater in mice allocated VDD diet than those receiving VDS diet.

4.3.2. Dietary restriction of vitamin D resulted in depletion of 25(OH)D and reduced SOST in angiotensin-II infused Apolipoprotein E-null mice

This experiment aimed to confirm whether dietary restriction of vitamin D affected the pathways involved in vitamin D metabolism and Wnt/β-catenin signalling within the AngII infused *ApoE*$^{-/-}$ mice. After completion the study, comprising 8 weeks of dietary intervention and 4 weeks of AngII infusion (i.e. total duration of 12 weeks), median [IQR] plasma concentrations of 25(OH)D in mice receiving VDS diet was 26.90 [24.41-31.1] nmol/L, compared to a non-detectable 25(OH)D concentration in VDD diet-fed mice (P<0.001; **Figure 4.5A)**. At the same time point, plasma SOST was significantly lower in mice fed the VDD diet compared to baseline (P=0.008) and to mice receiving VDS diet (P=0.003; **Figure 4.5B**). Importantly, an inverse correlation was demonstrated between plasma SOST levels and AAA diameter as measured by ultrasound (*r*=-0.789**;** P<0.001; **Figure 4.5C**). SOST protein levels within the SRA of VDD mice were significantly reduced compared with the VDS group (P=0.028 **Figure 4.5D**). Conversely, p-GSK-3α/β protein was higher within the SRA of VDD mice compared with VDS controls (P<0.001; **Figure 4.5E**).

AngII-infused *ApoE*$^{-/-}$ + VDS Diet (n=17)

AngII-infused *ApoE*$^{-/-}$ + VDD Diet (n=13)

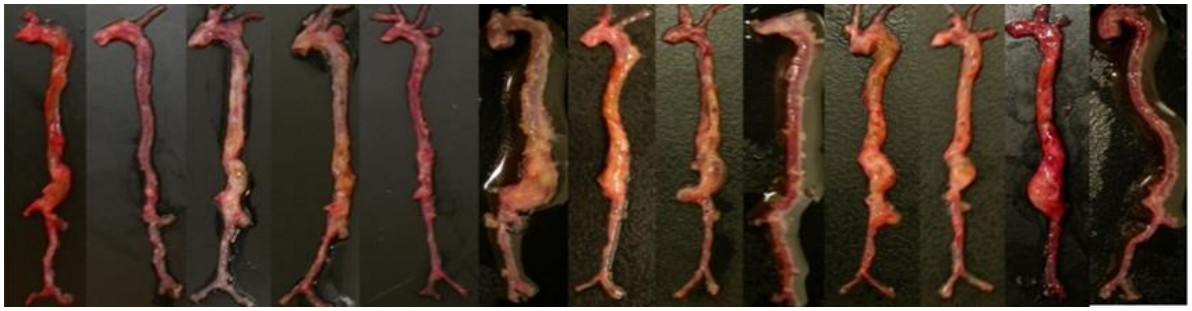


Figure 3: Effect of dietary restriction of vitamin D on aortic dilatation in angiotensin-II infused apolipoprotein E-null mice

Shown are gross morphology of aortas harvested from ApoE-/- mice that were supplemented with vitamin D sufficient diet (VDS) and vitamin D deficient diet (VDD) for 8 weeks, followed by 28 days of AngII infusion. Only aortas eased from surviving mice during terminal dissection are shown.

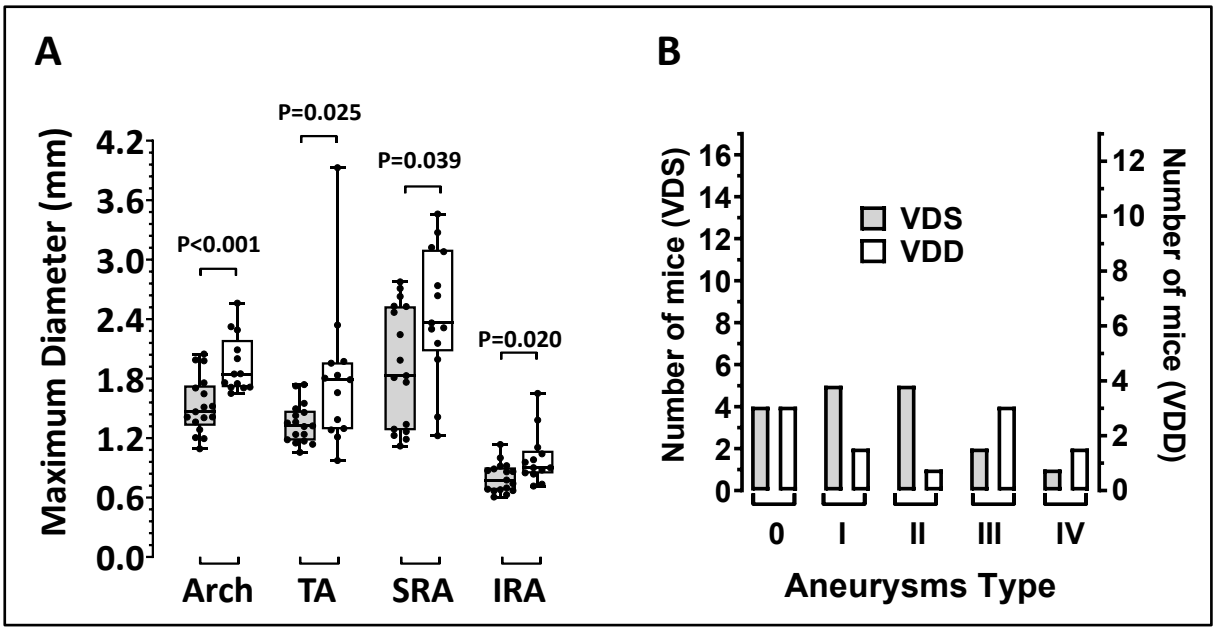


Figure 4.4: Morphometric analysis of aortas harvested during terminal experiment and effect of Vitamin D diet supplementation on the incidence of specific forms of aneurysms

Types of aneurysms were scored and classified according to Daugherty, et al. (2001) (Daugherty et al., 2001) classification. Accordingly, type 0 were defined as aortas with no dilation in any given segment. Type I aneurysms were defined as dilated lumen in the supra-renal region of the aorta with no apparent thrombus. Type II aneurysms were defined as remodelled tissue in the supra-renal region that contained evident thrombus. Type III was defined as a pronounced bulbous form of type II that contains thrombus. Type IV aneurysm were classified as multiple aneurysms containing thrombus, some overlapping, in the suprarenal area of the aorta or other areas of the aortas. Whole aortas tissue were categorized independently by two blinded observers with a 100% concordance in the designation between the two observers. Only surviving mice aortas are shown: VDS (grey boxes); VDD (open boxes)

Furthermore, relative gene expression analysis performed on SRA segments recovered from surviving mice showed that dietary restriction of vitamin D modulated expression of genes implicated in vitamin D metabolism and Wnt/β-catenin signalling. Mice receiving VDD diet had significantly higher expression of *Cyp27b1* (P=0.009) and *Ocn* (P=0.029) compared with the VDS controls. However, there was no significant difference in the expression of *Vdr* and *Opg* (**Table 4.1**). Mice receiving the VDD diet had significantly lower expression of *Sost* (P=0.029) while expression of *Ctnnb1* was significantly higher (P=0.040). However, there was no significant difference in the expression of or *Dkk-1* between the groups (**Table 4.1**).

4.3.3. Dietary restriction of vitamin D promoted atherosclerosis formation within the aortic arch and high blood pressure

Overall, the SBP and DBP were significantly higher in mice fed the VDD diet compared with those marinated on the VDS diet (P<0.001; **Figure 4.6A**). Similarly, DBP was higher in the VDD group compared with the VDS group at these time points (P<0.001; **Figure 4.6B**). In order to assess whether dietary deficiency of vitamin D had effects on aortic arch plaque formation, Sudan IV staining was performed. Overall, the median (inter-quartile range) Sudan IV stained area was significantly greater in the VDD group compared with the VDS group (**P<0.001; Figure 4.6C&D**).

4.3.4. Dietary restriction of vitamin D promoted inflammation and aortic wall matrix remodelling.

Inflammation and aortic matrix degradation, namely depletion of collagen and elastin fibres, are common features of aneurysmal tissue (Isenburg, Simionescu, Starcher, & Vyavahare, 2007). Effects of dietary restriction of vitamin D on expression of pro-inflammatory genes were investigated within the SRA of mice. Mice fed the VDD diet showed significant upregulation of *Opn* (P =0.040), *Mcp-1* (P=0.029) and *Agtr1* (P=0.004) **(Table 4.1).** However, there was no difference in the expression of *Opg* between the two groups. Assessment of genes implicated in matrix remodelling showed that there was significant upregulation of MMP-2 (P=0.021) and MMP-9 (P<0.001) **(Table 4.1).**

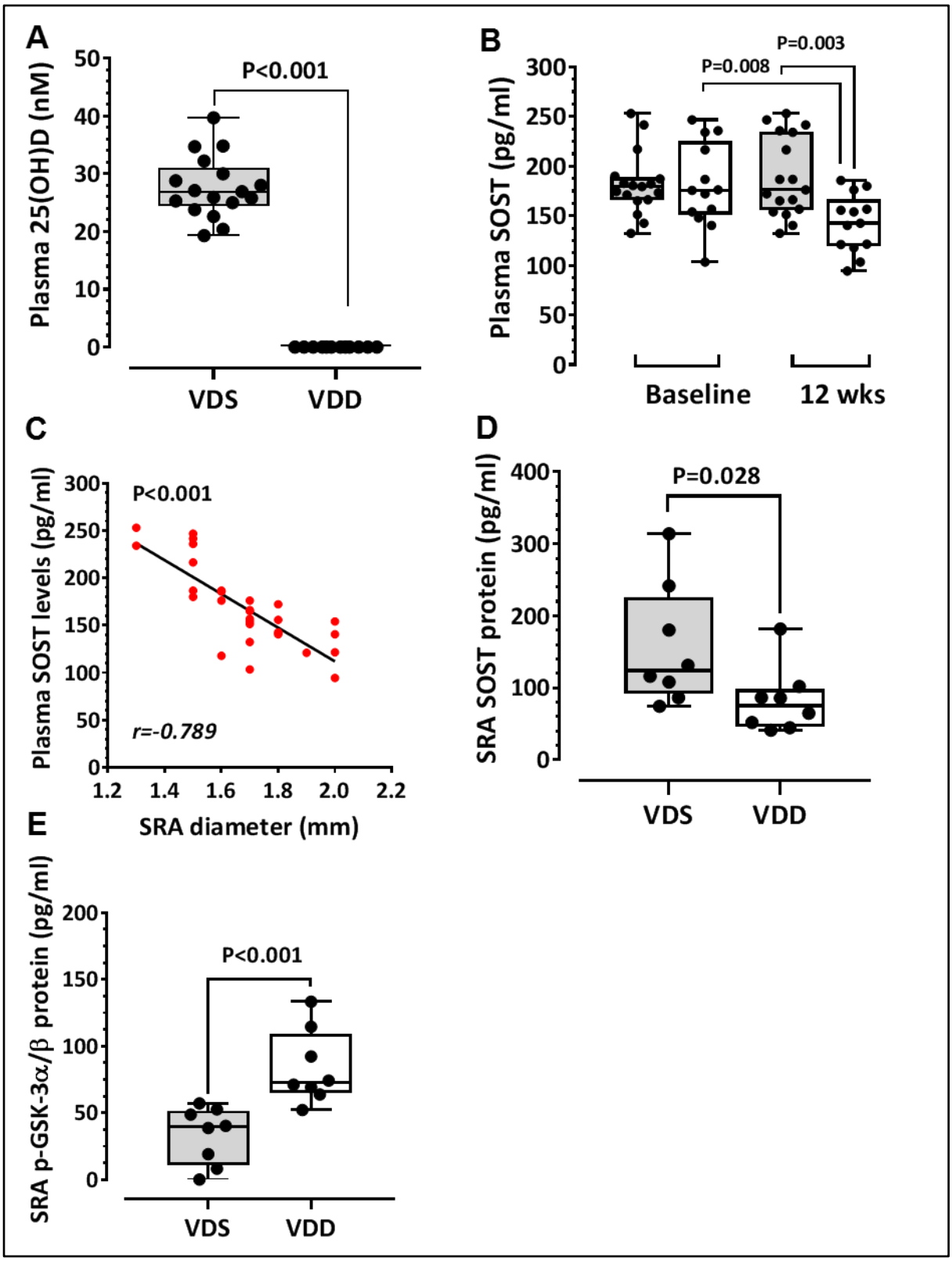


Figure 4.5: Effect of dietary restriction of vitamin D on circulating levels of 25(OH)D and SOST in angiotensin-II infused apolipoprotein E-null mice

Mice were fed either the VDS or VDD diet for 8 weeks, followed by AngII infusion for 4 weeks during which time mice were maintained on their respective diets. **A.** Median [IQR]

plasma concentrations of 25(OH)D in mice receiving the VDD diet were significantly lower compared to mice that were fed the VDS diet. **B.** Plasma SOST concentrations were significantly lower in VDD mice (white) compared to VDS mice (grey) at study end (P=0.003). **C.** Inverse correlation between plasma SOST and AAA diameter measured by ultrasound at the end of the experiment (r=-0.789; **P<0.001**). **D.** Reduced SOST protein levels in the VDD group compared with the VDS group (**P=0.028**). **E.** Higher p-GSK-3α/β protein within the SRA of mice receiving the VDD diet compared to the VDS diet (**P<0.001**). Data expressed as median and interquartile range with maximum and minimum data points (whiskers) for concentration (pg/ml). P-value calculated for difference between groups by Mann-Whitney U test and within groups by Wilcoxon paired test. **Abbreviations:** 25(OH)D: 25-hydroxyvitamin D; VDD: vitamin D deficient; VDS: vitamin D sufficient; SOST: sclerostin; wks: weeks; SRA: suprarenal aorta; p-GSK-3α/β: phosphorylated-glycogen synthase kinase-3α/β.

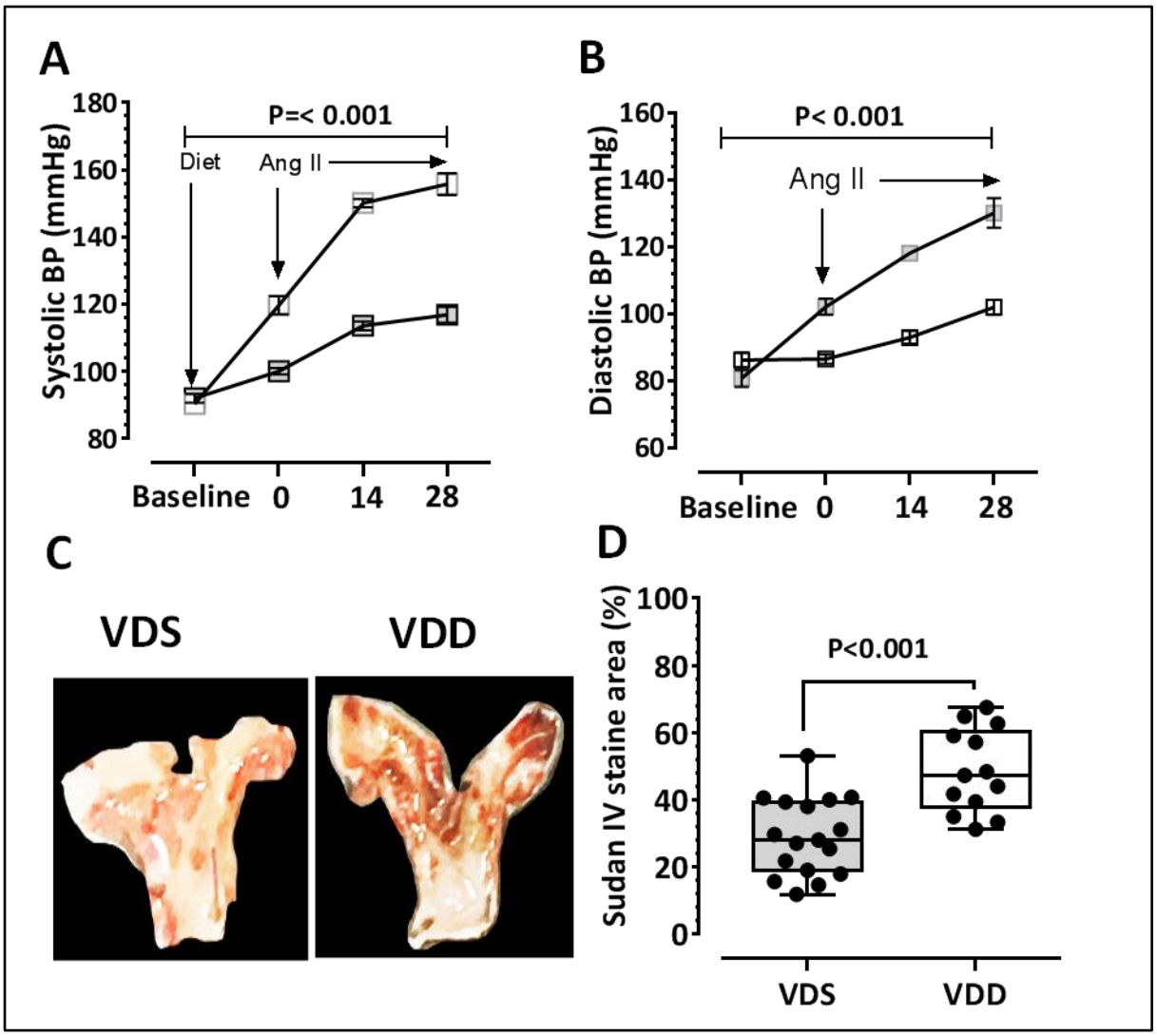


Figure 4.5: Dietary restriction of vitamin D promoted high blood pressure and aortic arch atherosclerosis

A. Systolic blood pressure (SBP) was higher in the VDD group compared to the VDs group. **B.** Similarly, diastolic blood pressure (DBP) was higher within the VDD group compared with the VDS group (compared using repeated measures ANOVA). **C**. Atherosclerotic plaque formation deposits (Red deposits indicate Sudan IV positive staining) was higher in the VDD group compared with the VDS group.

Table 5: Effects of dietary restriction of vitamin D on suprarenal aortic gene expression in angiotensin-II apolipoprotein E-null mice				
Gene	**VDS (n=8)**	**VDD (n=7)**	**Expression**	**P**
Sost	2.25 [1.27-4.11]	1.22 [0.99-1.35]	↓	**0.029**
Opg	2.49 [0.82-3.73]	2.01 [0.74-4.44]	↔	0.999
Opn	8.35 [6.08-19-36]	29.51 [9.05-59.09]	↑	**0.040**
Mmp-9	1.45 [0.028-3.76]	13.95 [9.07-21.98]	↑	**0.001**
Mmp-2	0.62 [0.09-1.82]	6.54 [1.61-9.38]	↑	**0.021**
Vdr	0.71 [0.29-1.32]	0.30 [0.12-0.58]	↔	0.152
Cyp27b1	0.81 [0.46-1.87]	2.40 [1.72-3.51]	↑	**0.009**
Dkk-1	0.91 [0.44-2.07]	0.46 [0.36-0.97]	↔	0.281
Ocn	0.93 [0.53-1.84]	2.35 [1.39-3.43]	↑	**0.029**
Ctnnb1	1.60 [0.60-4.37]	4.35 [3.11-8.84]	↑	**0.040**
Agtr1	0.71[0.35-0.88]	1.13 [0.92-1.84]	↑	**0.004**
Mcp-1	0.30[0.23-0.45]	0.52 [0.43-0.75]	↑	**0.029**

Shown are relative gene expression as median (inter-quartile range) relative to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression. Samples in this study were randomly selected using random generated numbers. Inter-group comparison was analysed using Mann-Whitney U test. Abbreviations: CCF: cholecalciferol; *Sost*: sclerostin; *Opg*: osteoprotegerin; *Opn*: osteopontin; *Vdr*: vitamin D receptor; *Cyp27b1*: gene encoding for 25-hydroxvitamin D3 hydrolase; *Dkk*: *Wnt* inhibitor Dickkopf-1; *Ocn*: osteocalcin (bone-Gla matrix); *Ctnnb1:* gene encoding for β-catenin; *Mmp-2*/*9*: matrix metalloproteinase 2/9; *Agtr1*: angiotensin-II receptor 1; *Mcp-1*: monocyte chemoattractant protein-1.

4.4. DISCUSSION

The main findings from this study were that dietary restriction of vitamin D promoted development of larger AAAs which were prone to rupture. Within AngII-induced mice, dietary restriction of vitamin D resulted in reduced circulating SOST, reduced SRA wall SOST protein and SRA *Sost* gene expression. Mechanistically, effects of vitamin D on SOST and downstream effects on Wnt/β-catenin signalling appear to be the link between vitamin D and AAA. Findings from this study built on the data presented in Chapter 3 to demonstrate that low SOST resulting from deficiency of vitamin D, in turn, promoted AAA development. These findings are supportive of a recent study whereby it was demonstrated that upregulation of SOST through transgenic breeding or intraperitoneal injection of human recombinant SOST (hrSOST) was protective against development of aortic aneurysms and atherosclerosis (Krishna et al., 2017).

The present study provides the first evidence that effects of vitamin D on bone mineralisation proteins, particularly SOST, play an important role in AAA development. Indeed, the role of vitamin D in skeletal health is well-documented and clinical evidence attests that vitamin D limits fracture risks in patients with osteoporosis (Holick et al., 2011). Cellular experimental studies have already shown that vitamin D upregulate SOST secretion and SOST gene expression in osteoblasts (Wijenayaka et al., 2016; Wijenayaka et al., 2015). Depletion of plasma 25(OH)D resulted in reduced circulating SOST and aortic SOST expression which is in agreement with the preceding chapter. Given the role of $1\alpha,25(OH)_2D3$ in upregulation of SOST and maintenance of contractile phenotypes within VSMCs, findings from this study indicate that the VDD diet had adverse effects on the aortic wall homeostasis (Krishna et al., 2017). Downstream effects of reduced aortic *Sost* were demonstrated by upregulation of CTNNB1 in mice SRA wall. However, the difference in aortic expression of another Wnt competitive inhibitor (Jho et al., 2002; Larriba et al., 2013; Nusse & Clevers, 2017; Sankaralingam et al., 2014), *Dkk-1*, was not significant. These findings therefore underscore the importance of SOST and its effects on the Wnt/βcatenin signalling during AAA development as previously reported (Krishna et al., 2017).

Lower levels of 25(OH)D have been linked to the presence of CVD, including occlusive arterial disease such as PAD and CAD (Nsengiyumva et al., 2015; Pilz et al., 2016). However, the relevance of vitamin D in AAA development remains relatively new. Previous epidemiologic studies have reported that lower levels of 25(OH)D are associated with presence of aortic

aneurysms, independent of traditional cardiovascular risk factors (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). Wong *et al* (2013) showed that the increase in AAA diameter was inversely associated with 25(OH)D levels in a dose-dependent manner. Dietary restriction of vitamin D, in this study, resulted in markedly lower circulating 25(OH)D (non-traceable) VDD mice and promoted larger AAAs, following AngII infusion. The implication of these findings suggests that patients who are vitamin D deficient may be prone to developing larger, rupture-prone AAAs. Furthermore, these findings are consistent with previous reports demonstrating that AAAs that are expanding at a faster rate are more likely to rupture (Aggarwal et al., 2011). However, from the current data it is not immediately clear how expansion rate of AAA in mice was dose-dependently related to circulating levels of 25(OH)D at all-time points. Nonetheless, a clear inverse correlation between circulating levels of SOST and AAA diameter was demonstrated. Moreover, two recent studies (Martorell et al., 2016; Nieuwland et al., 2016) respectively reported that administration of calcitriol or paricalcitol, which are vitamin D analogues, had potential to attenuate a number of pathways that are implicated in AAA development. Administration of calcitriol was reported to lower incidences of aortic rupture within the AngII-infused mouse model by limiting macrophage infiltration and downregulation of *Mmp-2* and *Mmp-9* expression within the SRA wall. In a small cohort of patients comprising patients who were given paricalcitol (n=11) as intervention and untreated controls (n=11), it was concluded that daily administration of 1μg for 2-4 weeks reduced of inflammatory markers which was characterised by aortic biopsies (Nieuwland et al., 2016). However, it remains inconclusively understood whether long-term intervention could have attenuated of AAA growth in these patients. Nevertheless, vitamin D deficiency is clinically managed through cholecalciferol supplementation in order to increase circulating 25(OH)D (Kennel et al., 2010). Findings from this study therefore suggest that lower levels of 25(OH)D could explain the development of larger AAA post-AngII infusion in mice maintained on the VDD diet.

The role of vitamin D in skeletal health is well-documented. Vitamin D deficiency has been associated with osteoporosis and increased risk of fractures in older adults and one potential mechanism for this association could be the effect of vitamin D on the expression of bone proteins (Holick et al., 2011). Incubation of osteoblasts with $1\alpha,25(OH)_2D_3$ has been reported to induce *SOST* expression (Wijenayaka et al., 2016). Ergocalciferol administration has previously been reported to increase circulating SOST concentrations in patients with osteoporosis (Sankaralingam et al., 2014). In the current study, a VDD diet resulted in

reduction in plasma SOST concentration, aortic SOST protein levels and aortic *Sost gene* expression. Aortic concentrations of p-GSK-3α/β, which is indicative of Wnt/β-catenin signalling activity, were also upregulated mice receiving the VDD diet. Phosphorylation of the GSK-3α/β leads to stabilization of β-catenin complex and consequent translocation to the nucleus (Nusse & Clevers, 2017). The Wnt/β-catenin signalling has been implicated in stimulating the activation of an inflammatory cascade in a number of autoimmune diseases (Beurel, Grieco, & Jope, 2015). In line with these findings, a VDD diet led to increased aortic expression of the *Wnt* pathway-signalling gene *Ctnnb1*. These responses were plausibly attributable to downregulation of SOST expression within the aortic wall. Indeed, vitamin D associated effects on *Wnt*/β-catenin signalling, including downregulation of β-catenin have been previously described, most notably in cancer cells (Larriba et al., 2007; Palmer et al., 2001; Shah et al., 2006). It was recently reported that SOST upregulation in a mouse model reduced phosphorylation of GSK-3β and consequent β-catenin accumulation in SRA tissue (Krishna et al., 2017). Implicitly, these findings suggest that supplementation of higher doses of vitamin D may be an interesting therapy for AAA patients. It is therefore conceivably possible that higher doses of vitamin D will contribute to upregulation of SOST and *Wnt*/βcatenin signalling; and thus, inhibition of AAA progression.

Chronic inflammation is a driving factor for AAA development and progression (Golledge et al., 2007; Krishna et al., 2010; Krishna et al., 2016). In this study, pro-inflammatory genes, including OPN, AGTR1, MCP-1 were upregulated within the group of mice maintained of the VDD diet. Expression of these genes has been previously implicated in the pathogenesis of AAA (Bruemmer et al., 2003; Filis et al., 2014; Golledge et al., 2007; Li et al., 2016). In particular, plasma concentrations of OPN have been reported to be increased in patients with AAA (Golledge et al., 2007). It was also demonstrated that increase in circulating OPN was positively correlated with AAA growth (Golledge et al., 2007). Within the AngII-infused mouse model, deficiency of OPN was reported to protect against AAA formation (Bruemmer et al., 2003). In turn, the current study showed that VDD diet resulted in upregulation of SRA expression of *Opn*. The effect of vitamin D deficiency on the expression of these pro-inflammatory genes may, at least in part, explain greater expansion of AAA in mice that were maintained on the VDD diet.

Degradation of the ECM has been strongly implicated in AAA pathogenesis (Galis & Khatri, 2002; Krishna et al., 2017; Krishna et al., 2015; Moran et al., 2014). In particular,

overexpression of MMP-2 and MMP-9 has been consistently associated with AAA formation and progression (Krishna et al., 2017; Krishna et al., 2015; Moran et al., 2005). In this study, dietary deficiency of vitamin D resulted in higher aortic expression of *Mmp-2* and *Mmp-9* within the SRA compared to controls. These findings are consistent with those from a recent publication in which it was shown that parenteral administration of 1α,25(OH)$_2$D3 (1 μg/kg) downregulated aortic *Mmp-2* and *Mmp-9* and upregulated endogenous tissue inhibitor of *Mmp-1* expression in AngII-infused *ApoE*$^{-/-}$ mice (Martorell et al., 2016). Furthermore, VDR expression has also been reported to downregulate *MMP-2* and *MMP-9* activity and expression in cultured human VSMCs (Aoshima et al., 2012; Britt et al., 2015), although no significant upregulation of VDR was observed in this study.

Physiologically, extrarenal expression of *Cyp27b1* is indicative of the ability for the tissue to locally metabolise bioavailable 25(OH)D (Christakos et al., 2016; Dusso et al., 2005). 1α,25(OH)$_2$D3 targets its nuclear receptor *Vdr* expression resulting in downregulation or upregulation of targeted genes (Christakos et al., 2016). Almost all genomic effects of 1α,25(OH)$_2$D are mediated through activation of VDR to elicit gene expression within the target cell (Christakos et al., 2016). This study showed that gene expression of *Cyp27b1* was downregulated within the SRA tissue in response to the VDD diet. However, VDR expression was not significantly different between the groups. It is plausible that 25(OH)D levels in the VDS group were insufficient to elicit VDR activity within the SRA wall. Nonetheless, findings suggest that bioavailability and conversion of 25(OH)D to 1α,25(OH)$_2$D was locally reduced in these mice. Urbonavicius *et al* (2010) previously reported that VDR protein expression was downregulated in tissue samples from AAA patients and was correlated with AAA size.

Hypertension has been reported to be a risk factor for AAA diagnosis and rupture (Kent et al., 2010; Tang et al., 2016). SBP has previously been reported to rise in mice receiving AngII-infusion although it is not thought to be responsible for the induction of AAA in this model (Ayabe et al., 2006; Cassis et al., 2009; Kawada, Imai, Karber, Welch, & Wilcox, 2002; Krishna et al., 2015). Consistent with previous findings, a VDD diet promoted higher SBP in *ApoE*$^{-/-}$ mice. Vitamin D has been reported to suppress renin expression (Ajabshir, Asif, & Nayer, 2014) and administration of CCF (25000 IU/week) has been reported to attenuate hypertension in patients (Carrara et al., 2014). However, given that AngII is not thought to induce AAA via increases in SBP, it is unlikely that the link between vitamin D deficiency and SBP is responsible for the findings in this study. Similarly, dietary restriction of vitamin D

resulted increased atherosclerotic plaque build-up within the aortic arch of mice. These findings are consistent with a recently reported studies, which demonstrated that *ApoE*$^{-/-}$ mice with low SOST were prone to atherosclerosis development (Costa, Bilezikian, & Lewiecki, 2014; Krishna et al., 2017). Additionally, there is a great body of literature to support the role of low vitamin D deficiency in atherogenesis (Bennett & Lavie, 2017).

Strengths of this study include the use the AngII-infused, *ApoE*$^{-/-}$ mouse model, the use of dietary intervention and measurement of 25(OH)D. The AngII-infused, *ApoE*$^{-/-}$ mouse model has been shown to have several similarities with human AAA pathology, including medial degeneration;, inflammation; thrombus and atherosclerosis (Daugherty & Cassis, 2004; Kawada et al., 2002; Trollope et al., 2011). While vitamin D toxicity is extremely rare (Holick, 2007; Marcinowska-Suchowierska, Kupisz-Urbańska, Łukaszkiewicz, Płudowski, & Jones, 2018), the use of dietary intervention with a well-balanced formulation was a very important step to gauge actual levels of 25(OH)D in these mice. Furthermore, circulating 25(OH)D were measured using a highly sensitive mass spectrometry assay in a validated laboratory (Clarke et al., 2013; Holick, 2009; Kleerekoper et al., 2011). One major limitation of this study, plausibly inherent to most animal studies, is the difficult to extrapolate these findings to human studies. While depletion of 25(OH)D within this mouse model was an important step to investigate a causal role of vitamin in AAA development, at least experimentally, total depletion of 25(OH)D is a less likely presentation in clinical settings. The reason for total depletion of 25(OH)D in these mice can be explained by the fact that the only source of vitamin D for these mice was from the diet chow since mice were otherwise totally restricted from sun exposure. Indeed, severe vitamin D deficiency is a common presentation in institutionalised elderly who have limited sun exposure (Fahrleitner-Pammer et al., 2005; Fahrleitner et al., 2002). However, epidemiologic data do not suggest that 25(OH)D levels in AAA patients can be categorised a deficient (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). The levels in AAA patients are at best deemed suboptimal or insufficient in accordance with current vitamin D status categorisation (Holick et al., 2011). Therefore, this raises a question as to whether increasing 25(OH)D to optimal levels (i.e. $\geq$ 75 nM) could attenuate AAA progression.

In conclusion, the findings from this study demonstrated that dietary deficiency of vitamin D promoted formation of larger AAA that were prone to rupture. Mice receiving the VDD diet had depleted levels of 25(OH)D which was accompanied by significantly lower SOST and activated *Wnt*/β-catenin signalling within the SRA. These findings are in agreement with the

previous study demonstrating that SOST deficiency promoted formation of AAA and atherosclerosis in a similar experimental mouse model. Furthermore, this study showed that VDD diet resulted in increased inflammatory markers and atherosclerosis within the mice. It remains unknown however, whether increasing circulating 25(OH)D to optimal levels could limit progression of pre-established AAA. Thus, in light of these findings the following chapter will examine whether vitamin D supplementation in the form of CCF can limit progression of pre-established AAA within this experimental mouse model.

CHAPTER 4 HIGHLIGHTS

What is Known?

- A number of osteogenic proteins have been previously implicated in AAA development.
- Recently, it was demonstrated that reduced SOST was associated with AAA development within an experimental mouse model.
- The preceding chapter showed that 1α,25$(OH)_2$D3 induced SOST expression in human aortic VSMCs while dietary restriction of vitamin D reduced SOST in *ApoE*$^{-/-}$ mice.
- Dietary restriction of vitamin D was associated with increased expression of pro-inflammatory genes and matrix remodelling genes.

What New Information Does This Chapter Add?

- Dietary restriction of vitamin D resulted in formation of larger AAAs that were prone to rupture mouse model.
- Dietary restriction of vitamin D resulted in high blood pressure and atherosclerosis development in a mouse model of AAA.
- Changes in AAA severity appeared to be mediated via changes in genes involved in ECM remodelling, particularly SOST.

CHAPTER 5:

EFFECTS OF CHOLECALCIFEROL SUPPLEMENTATION ON PROGRESSION OF PRE-ESTABLISHED ABDOMINAL AORTIC ANEURYSM IN THE ANGIOTENSIN-II INFUSED APOLIPOPROTEIN E-NULL MOUSE

5.1. INTRODUCTION

AAA is characterized by progressive degradation and weakening of the aortic wall integrity. The lack of pharmacological agents to limit or slow AAA progression remains a challenging factor for medical management of patients with AAA. Finding pharmacological agents with potential to halt or slow AAA expansion is therefore of urgent necessity.

Preceding chapters (Chapter 3 & 4) demonstrated that incubation of $1\alpha,25(OH)_2D_3$ with VCMCs promoted SOST expression while restriction of CCF intake resulted in decreased circulating SOST and SRA *Sost* expression. Vitamin D deficiency is clinically managed by CCF supplementation with an aim to raise circulating 25(OH)D levels (Bischoff-Ferrari et al., 2006; Holick et al., 2011; Kennel et al., 2010; Kleerekoper et al., 2011). While epidemiological studies have shown that patients with aortic aneurysm have lower circulating 25(OH)D; these studies did not conclusively indicate that AAA patients are within the category currently defined as vitamin D deficiency (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). In particular, Wong *et al* (2013) reported that patients within the lowest quartile of 25(OH)D levels were five times more likely to have AAAs >40 mm compared with those with the highest range of 25(OH)D levels. However, a question that remains unaddressed was whether increasing 25(OH)D levels could slow or limit expansion of AAA.

Currently, no pre-clinical studies have investigated the effects of CCF supplementation in animal models of AAA. Low circulating concentrations of 25(OH)D have been associated with presence of human aortic aneurysm in a number of cross-sectional studies (Demir et al., 2012; Van De Luijtgaarden et al., 2012; Wong et al., 2013). Contrary to this evidence, it has been reported that the prevalence of AAA is highest in Australasia where levels of ultraviolet exposure (and thus likely vitamin D) are very high (Li et al., 2013; Sampson et al., 2014). Excess vitamin D has also been reported to have potential pathological effects such as inhibiting production of elastin (an important aortic matrix protein) by VSMCs (Hinek et al., 1991; Norman et al., 1995; Norman et al., 2002). AAAs are detected when they are still small and medical management is needed to slow the growth of an established AAA rather than prevent its formation as investigated in the previous study.

While AAA was more common in men with lower circulating 25(OH)D, these levels were not in the deficient range as currently defined (Holick et al., 2011). This study sorted to assess the

effect of up-titration of 25(OH)D levels in Ang-II infused *ApoE*$^{-/-}$ mice maintained on normal laboratory chow diet in order to simulate clinical presentations. The study hypothesized that increasing the concentration of circulating 25(OH)D levels to >75 nM by CCF supplementation would limit AAA growth. Therefore, this study aimed to investigate whether increasing circulating 25(OH)D levels using CCF could attenuate AAA growth in *ApoE*$^{-/-}$ AngII-infused mice with pre-established AAA.

***Specifically, the primary hypothesis of this study were that*:**

1. Up-titration of 25(OH)D by CCF supplementation attenuates growth and rupture of pre-established AAA in Ang-II infused *ApoE*$^{-/-}$ mice.

Secondary hypotheses

1. Using a previously defined equation for repletion of circulating 25(OH)D, a *loading dose* of CCF can be used to achieve high (above sufficient) levels of 25(OH)D (i.e. ≥ 75 nM).
2. CCF supplementation and consequent up-titration of circulating plasma 25(OH)D increase SOST in Ang-II infused, *ApoE*$^{-/-}$ mice.

This study had the following specific aims:

1. To examine if CCF supplementation attenuates growth and rupture of pre-established AAA in Ang-II infused *ApoE*$^{-/-}$ mice.
2. To assess whether a *loading dose* of CCF can be used to increase circulating 25(OH)D levels (i.e. ≥ 75 nM) in Ang-II infused *ApoE*$^{-/-}$ mice.
3. To examine whether CCF supplementation and consequent up-titration of circulating 25(OH)D increase SOST in Ang-II infused, *ApoE*$^{-/-}$ mice.

5.2. MATERIALS AND METHODS

5.2.1. Sample size determination

Sample size calculation was performed using GPower 3.1.9.2 software version for t-test in this study. Sample size computation was based on a recent study by Seto et al (Seto et al., 2014). In this study, ApoE-/- mice (n=18) were randomised to intervention (Aliskiren) or placebo after establishment of AAA for 28 days. Subsequent treatment with a higher dose of at Aliskiren (50 mg/kg/day) resulted in significant decrease in aortic diameter, measured by repeated ultrasound for a further 28 days, from 1.07±0.12 mm to 1.06±0.9 mm (mean±SD). Assuming a similar trend (80% power; α: 0.05), an estimate sample size of 17 mice were required per group for this study. Therefore, a total 34 *ApoE*$^{-/-}$ mice was required. Sample size estimation accounted for a potential drop-outs due to AAA rupture following AngII infusion prior to randomisation. All animal experiments described in this chapter received approval (AEC#: A2354; **Appendix 1B**) from the JCU/AEC.

5.2.2. Cholecalciferol Loading Dose Estimation.

The dose of CCF that can rapidly and safely correct 25(OH)D levels in mice remains undefined (van Groningen et al., 2010). Preceding chapters (Chapter 3 & 4) showed that *ApoE*$^{-/-}$ mice that were maintained on a normal laboratory chow diet (i.e. 3000 IU/kg of feed) had insufficient plasma levels of 25(OH)D. The present study attempted to achieve up-titration of plasma 25(OH)D levels that are comparable to what is currently defined as optimal levels in human (i.e.25(OH)D levels ≥75nM). Therefore, a formula suggested by Groningen et al (van Groningen et al., 2010) for calculating a loading dose of CCF intended to achieve plasma 25(OH)D levels ≥ 75 nM was used in this study. Briefly, the loading dose of CCF (IU) = 40 × (75−baseline 25(OH)D) × body weight). For practicality, baseline plasma 25(OH)D were assumed to be approximately the median plasma 25(OH)D levels observed in mice in the previous experiments (Chapter 4). It was proposed that the average AAA patient weighs approximately 80 kg. Therefore, the human equivalent loading dose (IU) was computed as 40 x (75-35.0) x 80 which equals to 128,000 IU (i.e. 3,200 µg/kg) of CCF (van Groningen et al., 2010). The mouse equivalent dose of human equivalent dose was calculated as follows 12.3 x 3,200= 39,360 µg/kg (15,744,000 IU), as proposed by Rockville *et al* (2005). In order to rapidly and safely achieve optimal plasma 25(OH)D levels (i.e. 75nmol/L), the loading dose was given

over five weeks (i.e. 7,872 µg/kg/week). For instance, assuming that an experimental mouse weighed 30g, a CCF dose of 236.2 µg/week was estimated per mouse (i.e. 9446 IU/week). It was assumed that plasma 25(OH)D levels ≥75 nM would be achieved in the circulation within 5 weeks of supplementation (van Groningen et al., 2010). The actual dose was determined according to individual mouse's weight prior to oral gavage.

5.2.3. Experimental Design

A detailed flow diagram of the experimental design is shown in **Figure 6.1**. In this study, 12-13 weeks old *ApoE*$^{-/-}$ mice (n=32) were maintained on the normal laboratory chow (3000 IU/kg/feed). AAA was induced by Ang-II infusion (1µg/kg/min) in all mice. SRA diameter was assessed using ultrasound at baseline (i.e. day 14-post AngII infusion). On day 14, surviving mice were stratified into two groups (intervention and control) with equivalent SRA diameter size (**Figure 5.3**). The intervention group was subjected to oral gavage administration of the loading dose of CCF (OsteVit-D®). The control group was supplemented with 0.1% carboxymethylcellulose (Sigma) as a vehicle by oral gavage. Oral gavage was performed weekly at the same time of the day and under same conditions (i.e. day 14, day 21, day 28, day 35, day 42 and day 56). Mice were fasted for 12 hours by removing the food tray. Mice were then monitored for 2 hours after oral gavage for any abdominal distention and docility. It was postulated that plasma 25(OH)D levels would be increased to ≥75 nM in CCF supplemented mice while the levels in control mice would remain unchanged.

In this experiment, AAA was induced by AngII-infusion for the first two weeks. After the two weeks, mice were administered with either a weekly dose of 7,872 µg/kg/week of CCF or 0.1% CMC. The duration of the osmotic pumps at the proposed delivery rate is usually 28 days (Krishna et al., 2017; Krishna et al., 2015). Therefore, after 28 days, a second pump with a lower flow rate of AngII (0.5µg/kg/min) was inserted to maintain AAA growth. Assessment of the SRA diameter was performed every 14 days before the oral gavage.

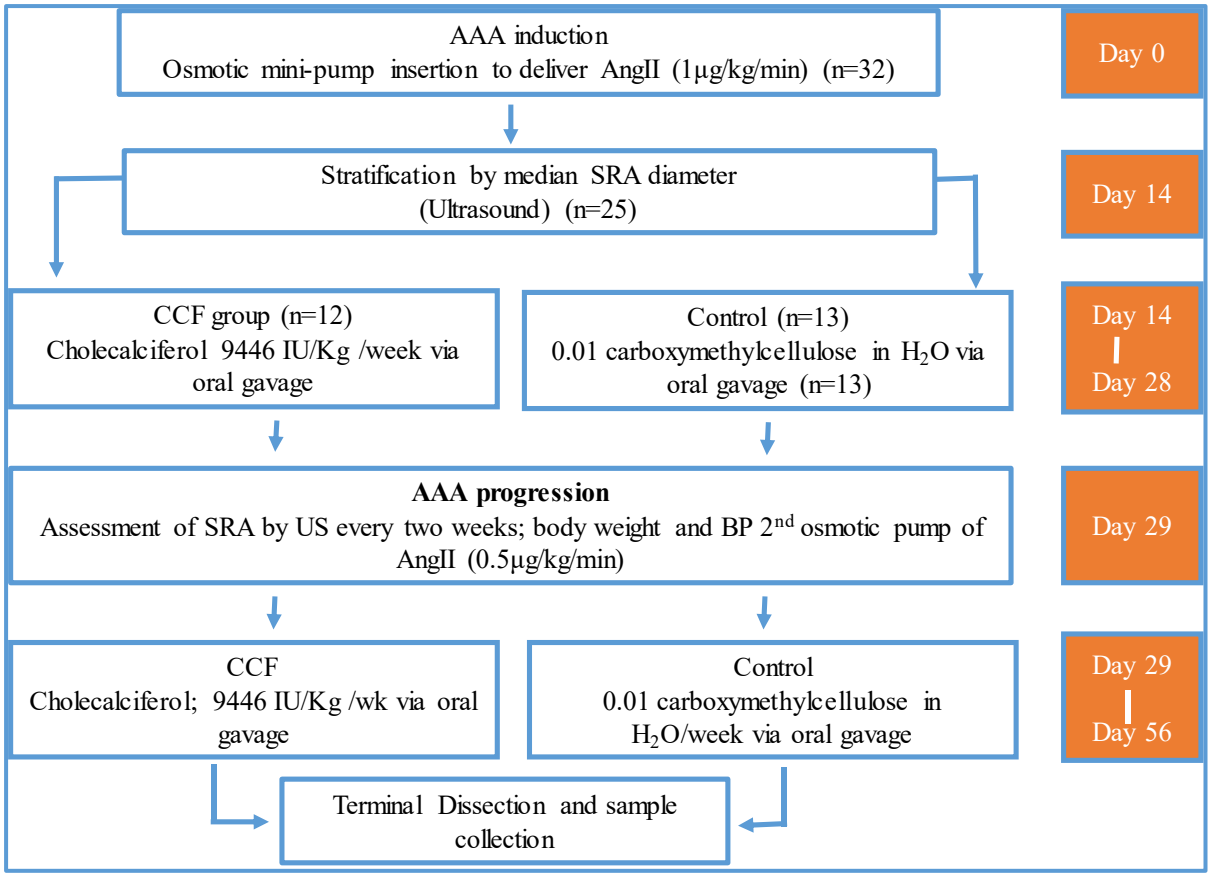


Figure 5.6: Experimental Flow Diagram

Infusion of full dose of AngII (1µg/kg/min) for 28 days. After 14 days of AngII infusion surviving mice were stratified by median SRA diameter, assessed by ultrasonography, into two groups with equivalent SRA diameter. The intervention group was supplemented with 9446 IU/Kg of cholecalciferol (CCF) per week. The control group was given 0.1% of carboxymethylcellulose (CMC) as a vehicle. At the end of 28 days, an additional low dose of AngII (0.5 µg/kg/min) was given to mice by inserting a second osmotic mini-pump. AAA progression was then followed up with fortnightly ultrasound for 42 days (in total 56 days of intervention). All mice were maintained on the normal laboratory chow during the experiment. Blood collection and blood pressure measurement were performed at several time points.

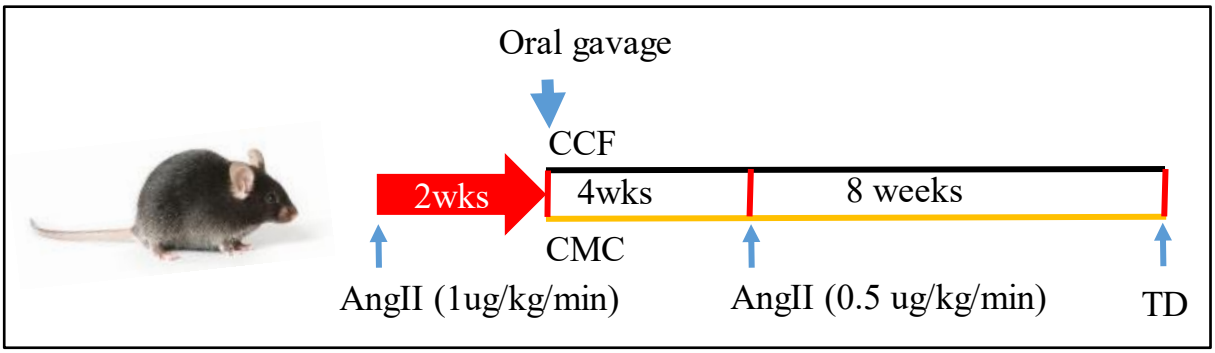


Figure 5.2: Study Deign

ApoE$^{-/-}$ mice (n=32) were infused with AngII (1µg/kg/min) lasting for 28 days. After 14 days, mice were stratified into two groups with equivalent median SRA diameter. One group was subjected to weekly oral gavage of cholecalciferol (CCF; 9446 IU/Kg) while the other groups was subjected to 0.1% of carboxymethyl cellulose (CMC) at the same time points. After 28 days, AngII infusion was continued for an additional 28 days at a lower rate of (0.5 µg/kg/min). Total experimental duration of 56 days or 8 weeks (including 6 weeks of oral gavage).

5.2.4. Ultrasound monitoring

In this mouse model, SRA expansion and aortic ruptures are usually established within 14 days of AngII infusion (Manning et al., 2002; Rateri et al., 2011). SRA diameter *in vivo* (primary outcome) was measured by ultrasound as described in Chapter 2 (Section 2.5.1), first at baseline (day 0, before AngII) and then at 14-days intervals following commencement of AngII infusion.

5.2.5. Morphometric analysis

Morphometric analysis of aortas harvested from surviving mice at the completion of the study period was performed as described in Chapter 2 (Section 2.5.2.). Characterisation of gross morphology of aneurysm types was performed as reported by Daugherty, *et al.* (2001) (Daugherty et al., 2001). A 100% concordance was achieved within the inter-assessor rating for classification of aneurysms as described in Chapter 2 (Section 2.5.2).

5.2.6. Plasma analyses

Blood collection was performed by tail bleeding in Chapter 2 (Section 2.4.1) at baseline, before oral gavage, at day 14, at day 42 and at day 56. At the end-point, during terminal dissection, blood was collected by cardiac puncture. The blood was collected into heparin-coated tubes (BD Microtainer) and plasma samples prepared for 25(OH)D and SOST assay as described in Chapter 2 (Sections 2.4.1 and 2.7).

5.2.7. Suprarenal aortic tissue protein assay by ELISA

SRA segments were cleared of OCT and homogenized in the presence of the radio-immunoprecipitation assay buffer (1 x RIPA buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl; 2 mM EDTA, 1% TritonX-100, 0.1% SDS, and 0.1% sodium deoxycholate) supplemented with protease inhibitors and phosphatase inhibitor. Mouse SRA SOST protein expression was assayed using a Quantikine® ELISA (R&D systems®) as described in Chapter 3 (Sections 2.10.1). Mouse SRA phospho-GSK-3α/β (S21/S9) levels were determined using DuoSet® IC ELISA in accordance (R&D systems®) as described in Chapter 3 (Sections 2.10.4).

5.2.8. Quantification of atherosclerotic lesion area

Aortic arch samples were prepared for *en face* Sudan IV staining as described in Chapter 2 (Section 3.3). The presence and severity of intimal surface atherosclerotic plaque was compared between arch samples obtained from mice on CCF (n=11) and control (n=7) by digital image analysis as described in Chapter 2 (Section 2.8).

5.2.9. Histology & Immunohistochemistry

Hematoxylin and eosin (H&E), Elastin Van Giessen (EVG) and picrosirius red staining were performed as described in Chapter 2 (Sections 2.9.1; 2.9.2; 2.9.3). Staining was performed on 5 µM serial cryostat sections cut from the SRA of the CCF group (n=11) and control mice (n=7). Semi-quantitative assessment of staining area was performed using digital image analysis as described in Chapter 2 (Sections 2.9.1; 2.9.2; 2.9.3).

5.3. Statistical analysis

The D'Agostino and Pearson test was used to test the normality of the data. Results were expressed as median and interquartile range (IQR) for non-normally distributed data and as mean ± SEM for normally distributed data. For non-normally distributed data, comparisons were made using Mann-Whitney U-test. Normally distributed data were compared using ANOVA followed by Bonferroni's multiple comparisons test. Repeated measures were analysed using repeated measures ANOVA. All data analyses were performed using GraphPad Prism 8.4.2 software (GraphPad Software, Inc., USA). Differences were considered statistically significant at $P < 0.05$.

5.4. RESULTS

5.4.1. Cholecalciferol supplementation increased circulating levels of 25(OH)D and SOST in angiotensin-II infused apolipoprotein E-null mice.

The dose of CCF to be administered was estimated in order to achieve relatively high circulating 25(OH)D concentrations of >75 nmol/L (van Groningen et al., 2010). After the first 2 weeks, mice were allocated to intervention (CCF administration; 9446 IU/kg/week oral gavage; n=12) and control (0.1% carboxymethyl cellulose oral gavage; n=13) groups (**Figure 5.3**). Both groups were maintained on a similar diet. The experiment continued for a further 6 weeks during which time AngII infusion was continued. Baseline median [IQR] plasma 25(OH)D was similar in both groups (CCF: 17.26 [13.47-21.52 nmol/L] vs control: 19.61 [16.57-23.15] nmol/L; P=0.242). Within 4 weeks of CCF supplementation, the CCF group had already achieved the median [IQR] plasma levels of 25(OH)D 75.77[59.79- 95.13] nmol/L. Overall, mice receiving CCF exhibited a time-dependent increase in plasma levels of 25(OH)D at concentrations significantly higher than controls (P<0.001; **Figure 5.4A**).

Median [IQR] plasma SOST was similar in both groups at baseline (CCF: 182.80 [167.70-216.30] nmol/L vs control: 182.40 [160.80-212.30] nmol/L; P=0.832]. At the end of the 8-week experiment, median plasma SOST was significantly higher in mice administered CCF compared to baseline (P=0.001) and to control mice (P=0.001; **Figure 5.4B**). In attempt to establish the relationship between circulating SOST and AAA diameter at the end of the study, it was found that AAA size was negatively correlated with SOST levels. An inverse correlation

was demonstrated between circulating SOST concentrations and maximum SRA diameter measured by ultrasound after 56 days (r=-0.653, P=0.003; **Figure 5.4C**). In contrast, SRA concentration of p-GSK-3α/β was lower within the CCF group compared to the control group (P=0.003, **Figure 5.4D**). Finally, SRA protein concentration of SOST was also higher in mice receiving CCF compared to controls (P=0.012, **Figure 5.4E**).

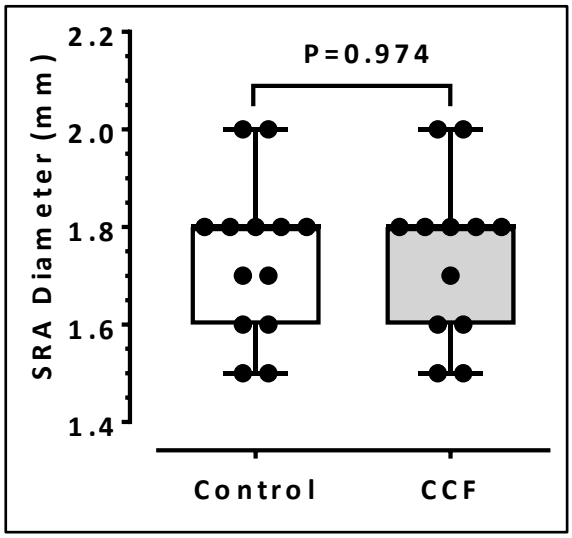


Figure 5.3: Stratification of mice into intervention and control groups following suprarenal aortic diameter assessment

After 14 days (two weeks) of AngII infusion, SRA diameter was assessed using ultrasound and mice were stratified into two groups of equivalent SRA diameter (**P=0.974**). One group was then allocated intervention (cholecalciferol; CCF) while the other group was maintained of carboxymethyl cellulose (control) for the following 42 days (6 weeks).

In this study, relative gene expression analysis was performed on SRA segments recovered from mice at the end of the experiment. Effects of CCF supplementation on expression of genes that are implicated in vitamin D metabolism and Wnt/β-catenin signalling was demonstrated. Mice receiving CCF had significantly higher expression of *Vdr* (P=0.027), *Sost* (P=0.035) and *Dkk-1* (P=0.004) and compared with controls. Conversely, mice that were administered with CCF had significantly lower SRA expression of *Cyp27b1* (P<0.001) and *Ctnnb1* (P=0.015). However, there was no significant difference in the expression of *Opg and Ocn* between the two groups (**Table 5.1**).

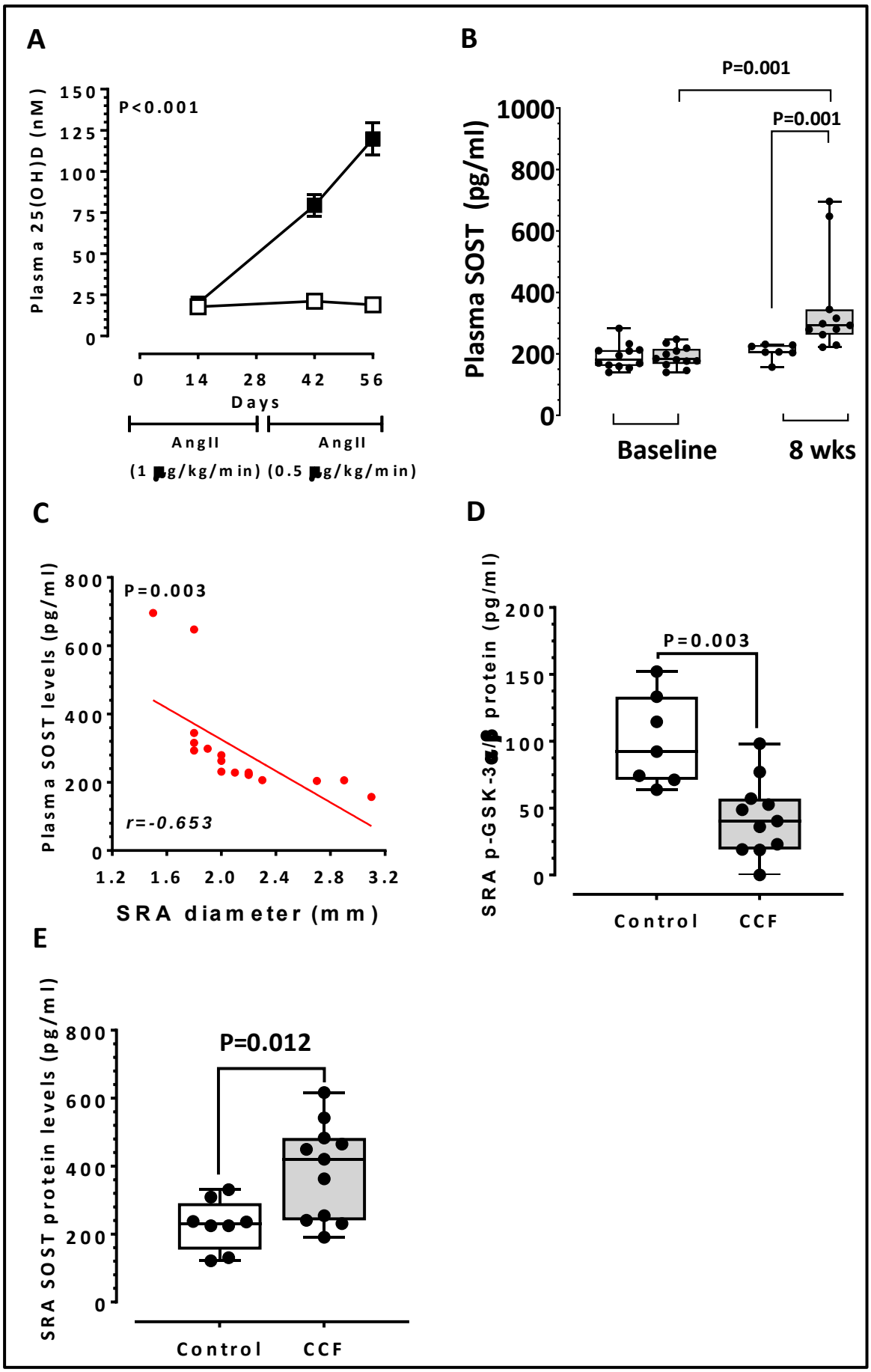

A
Plasma 25(OH)D (nM)
150
125
100
75
50
25
0
P<0.001
0
14
28
42
56
Days
AngII
(1 µg/kg/min)
AngII
(0.5 µg/kg/min)
B
P=0.001
P=0.001
Plasma SOST (pg/ml)
1000
800
600
400
200
0
Baseline
8 wks
C
Plasma SOST levels (pg/ml)
800
600
400
200
0
P=0.003
r=-0.653
1.2
1.6
2.0
2.4
2.8
3.2
SRA diameter (mm)
D
SRA p-GSK-3α/β protein (pg/ml)
200
150
100
50
0
P=0.003
Control
CCF
E
SRA SOST protein levels (pg/ml)
800
600
400
200
0
P=0.012
Control
CCF

Figure 5.4: Effects of cholecalciferol supplementation on 25(OH)D and SOST in angiotensin-II infused apolipoprotein E-null mice. AAA was first induced in mice through AngII-infusion for the first 2 weeks before being stratified into groups with equivalent SRA diameter. Mice were then supplemented with either the cholecalciferol (9446 IU/kg/week) or 0.1% carboxymethyl cellulose (Control) by oral gavage for the next 6 weeks. Both groups were maintained on a similar diets throughout the experiment. **A.** Overall, plasma concentrations of 25(OH)D in mice receiving CCF were significantly higher compared to control mice. **B.** Plasma Epi-25(OH)D3 remained negligible (*) in control mice but the levels rose significant higher between week 4 and week 8 in mice supplemented with CCF. **C.** Plasma SOST concentrations were significantly higher in mice supplemented with CCF compared with control mice at the end of study. **D.** There was an inverse correlation between plasma SOST and AAA diameter measured by ultrasound at the end of the experiment (r=-0.789). **E.** Aortic SOST protein levels were significantly increased in the CCF mice compared to controls. **E.** Aortic protein levels of p-GSK-3α/β were higher within the SRA of mice receiving CCF compared with control. **F.** Data expressed as median and interquartile range with maximum and minimum data points (whiskers). Data were compared between groups with a Mann-Whitney U test; and within the same group, data were compared with Wilcoxon paired test (**B & C**). **Abbreviations:** 25(OH)D: 25-hydroxyvitamin D; CCF: cholecalciferol; Epi-25(OH)D3: C-3 epimer of 25-hydroxyvitamin D(3); SOST: sclerostin; wks: weeks; SRA: suprarenal aorta; p-GSK-3α/β: phosphorylated-glycogen synthase kinase-3α/β.

5.4.2. Cholecalciferol limited growth and rupture of pre-established AAAs in angiotensin-II infused in Apolipoprotein-null mice

AAA was firstly induced in mice (n=32) through infusion of AngII. After 14 days following initial AngII infusion, 7 mice died from aortic rupture, leaving a total of 25 mice. Mice were then stratified into two groups with equivalent SRA diameter (**Figure 5.3**). One group was allocated to intervention (CCF administration; 9446 IU/kg/week; n=12) and the control group which was allocated to 0.1% CMC (carboxymethyl cellulose; n=13). The SRA dilatation was monitored fortnightly by ultrasound and a time-dependent increase in maximum SRA diameter in response to AngII was demonstrated by Repeated measures ANOVA. Overall, mice supplemented with CCF exhibited significantly smaller increases in SRA diameter compared to controls (P<0.001; **Figure 5.5A**). Furthermore, supplementation with CCF significantly limited rupture of pre-established AAA in the AngII-infused *ApoE*$^{-/-}$ mice (P=0.031; **Figure**

5.5B). Specifically, it was found that 6 out of 13 control mice (46%) exhibited aortic rupture compared to only one mouse of 12 mice (8%) administered CCF (**Figure 5.5B**).

Morphometric analysis of aortas at completion of the study (excluding ruptures) confirmed significantly smaller median [IQR] maximum SRA diameter in mice receiving CCF compared to controls (P=0.036; **Figure 5.7A)**. Gross morphology of the SRA (aneurysm types) was classified and compared between the dietary groups (**Figure 5.7B**).

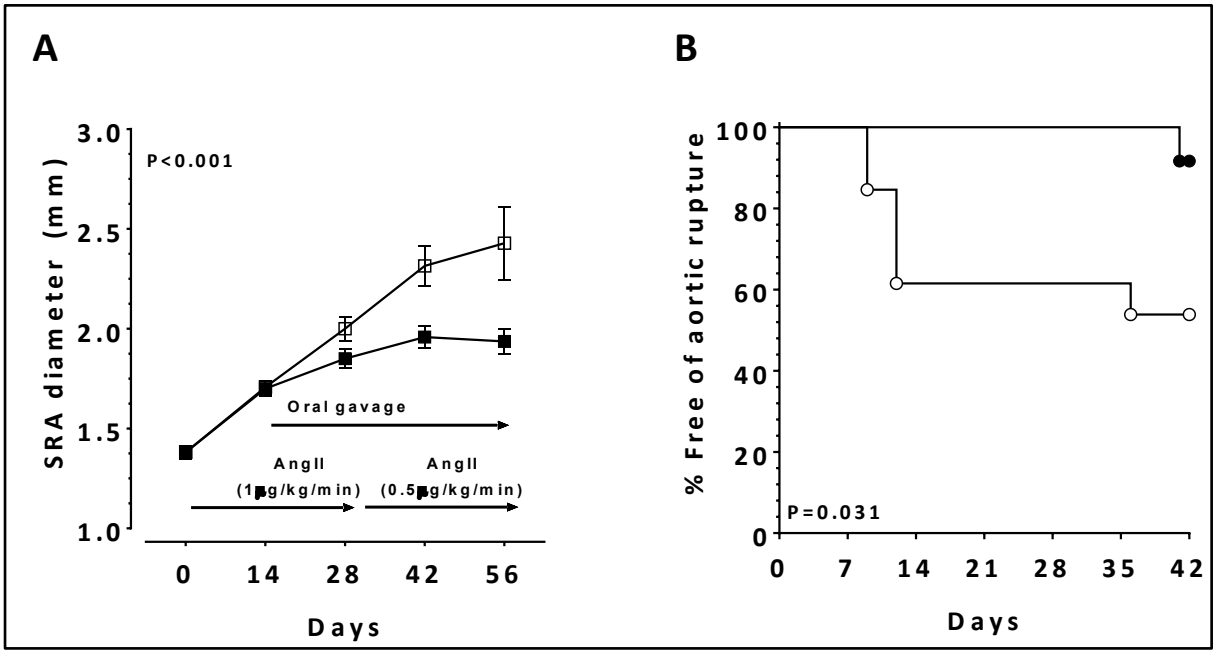
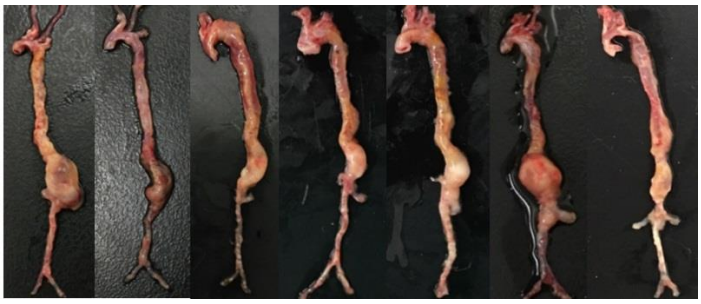


Figure 5.5: Effects of cholecalciferol supplementation on AAA progression and rupture.

One group was allocated to CCF as intervention (9446 IU/kg/week; n=12) and the control group which was allocated to 0.1% CMC (carboxymethyl cellulose; n=13). The SRA diameter was monitored fortnightly by ultrasound and a time-dependent in maximum SRA diameter in response to AngII was assessed by repeated measures ANOVA (the P-value relates to interaction between time and treatment). **A**. Mice supplemented with CCF exhibited significantly smaller increases in SRA diameter compared to controls.

AngII-infused *ApoE*$^{-/-}$ + carboxymethyl cellulose (0.1%) (n=7)



AngII-infused *ApoE*$^{-/-}$ + cholecalciferol (9446 IU/kg/week) (n=11)

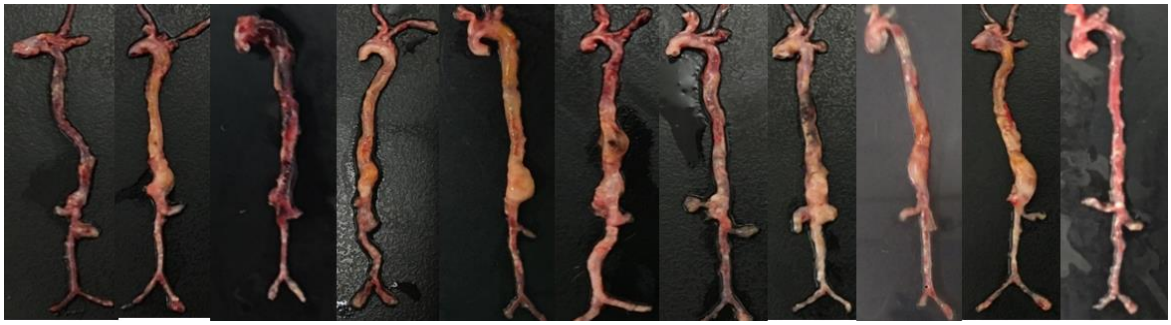


Figure 5.6: Effect of cholecalciferol supplementation on AAA progression in angiotensin-II infused apolipoprotein E-null mice

Shown are gross morphology of aortas harvested from *ApoE*$^{-/-}$ mice subjected to 56 days (8 weeks) of AngII infusion and supplemented with carboxymethyl cellulose (0.1%) or cholecalciferol (9446 IU/kg/week) by oral gavage for 42 days (6 weeks). Only aortas excised from surviving mice during terminal dissection are shown.

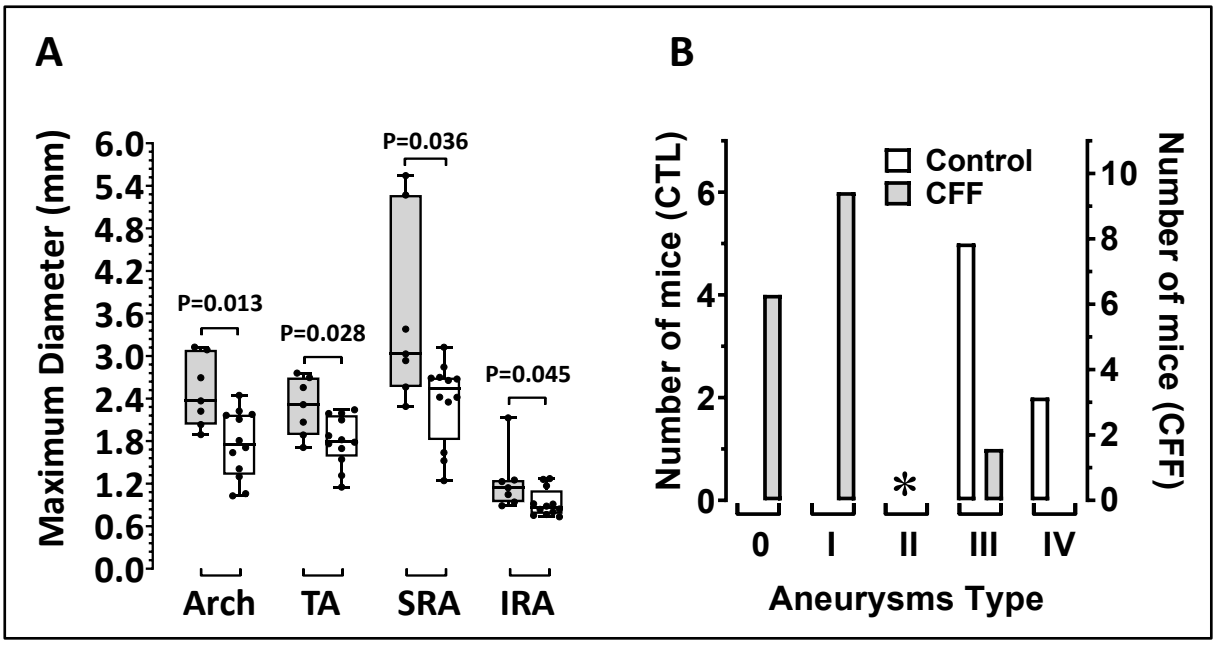


Figure 5.7: Morphometric analysis of aortas harvested during terminal experiment and effect of cholecalciferol supplementation on the incidence of specific forms of aneurysms on

Morphometric analysis showed that all areas of the aorta were significantly smaller in diameter compared with controls. **B.** Types of aneurysms were scored and classified according to Daugherty, et al. (2001) (Daugherty et al., 2001) classification. Accordingly, type 0 were defined as aortas with no dilation in any given segment. Type I aneurysms were defined as dilated lumen in the supra-renal region of the aorta with no apparent thrombus. Type II aneurysms were defined as remodelled tissue in the supra-renal region that contained evident thrombus. Type III was defined as a pronounced bulbous form of type II that contains thrombus. Type IV aneurysm were classified as multiple aneurysms containing thrombus, some overlapping, in the suprarenal area of the aorta or other areas of the aorta. Whole aortas tissue were categorized independently by two observers, one of them was blinded to the labelling of groups. There was a 100% concordance in the classification of the aortas between the two observers. Only surviving mice aortas are shown: CCF (grey boxes); Control (open boxes). *Denotes no entry on figure 6.6B. **Abbreviations:** TA: thoracic aorta; SRA: suprarenal aorta; IRA: infrarenal aorta; CCF: cholecalciferol.

5.4.3. Cholecalciferol supplementation reduced inflammation and degradation of elastin and collagen within the suprarenal aortic wall of angiotensin-II infused Apolipoprotein E-null mice

Inflammation and aortic matrix degradation are common features of aneurysmal tissue (Isenburg et al., 2007). Common features of aneurysmal tissue include depletion of collagen and elastin fibres, presence of inflammatory cells infiltrates and reduced medial VSMCs (Isenburg et al., 2007). Effects of CCF supplementation on the degradation of collagen and elastin fibres were characterised in the SRA of mice in both groups. Mice supplemented with CCF had significantly higher SRA collagen content (P=0.015) and elastin fibres content (P=0.033) compared with control mice (**Figure 5.8 A&B**).

Similarly, effects of on expression of pro-inflammatory genes was compared within the SRAs of mice maintained on CCF and controls. Mice supplemented with CCF showed significant downregulation of *Opn* (P =0.035), *Mcp-1* (P=0.035) and *Agtr1* (P=0.002). Assessment of genes implicated in matrix remodelling showed that there was significant downregulation of *Mmp-2* (P=0.035) and *Mmp-9* (P=0.020) **(Table 5.1)**.

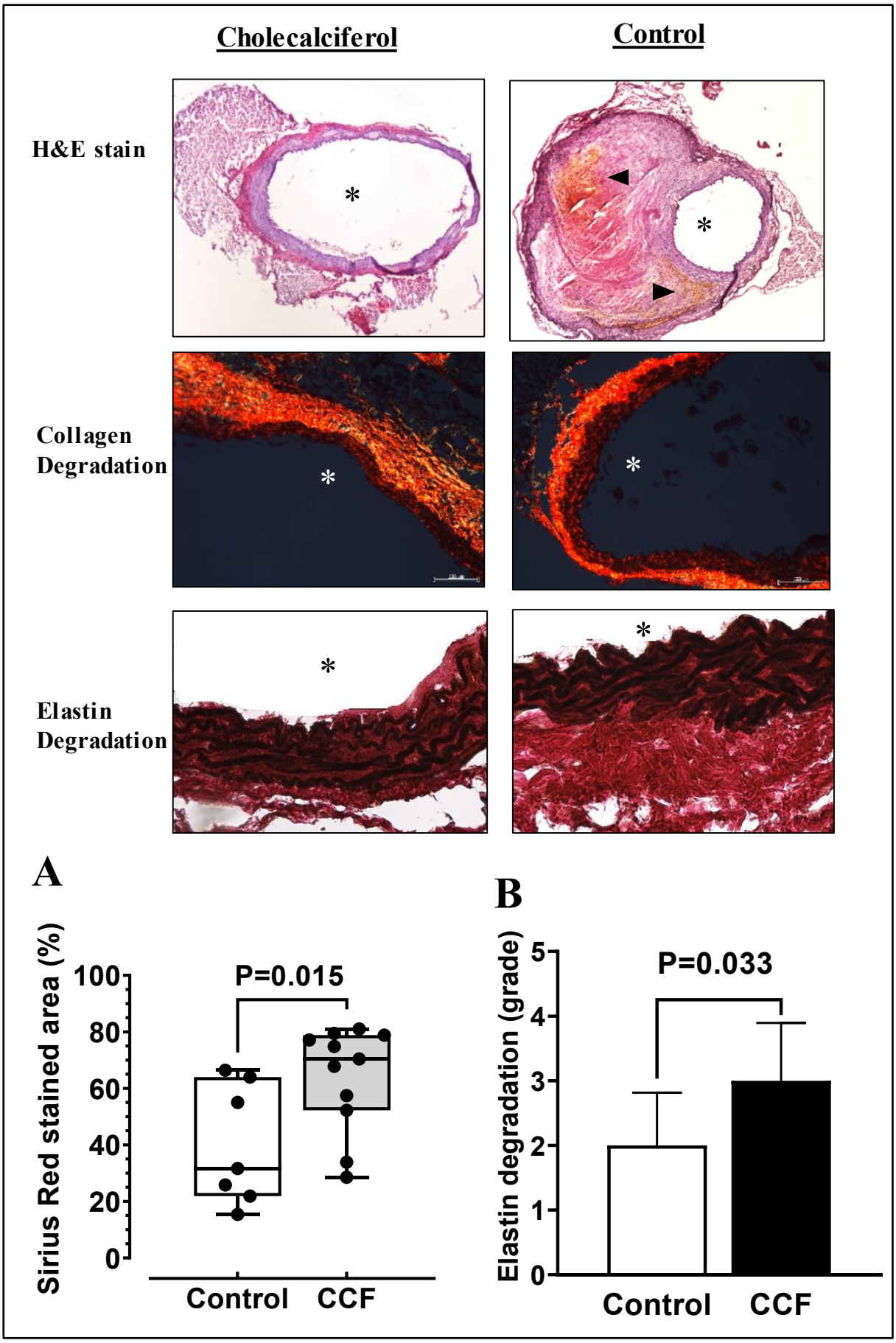

Cholecalciferol
Control
H&E stain
Collagen Degradation
Elastin Degradation
A
Sirius Red stained area (%)
100
80
60
40
20
0
P=0.015
Control
CCF
B
Elastin degradation (grade)
5
4
3
2
1
0
P=0.033
Control
CCF

Figure 5.8: Effect of cholecalciferol supplementation on the aortic wall gross morphology, collagen and elastin fibres within the suprarenal aortas harvested from angiotensin-II infused apolipoprotein E-null mice

Representative images presented denote Hematoxylin and eosin (H&E), picrosirius red and Elastin Verhoeff–Van Gieson (EVG) stained suprarenal aortic (SRA) sections for the CCF (* denotes lumen). Gross assessment of H&E images showed that CCF supplemented mice had a relatively intact aortic wall with less dissection (false lumen) and accumulation of inflammatory cells within the aortic wall (►) compared with the contol group. Picrosirius red stained collagen images were photographed using polarization microscopy (yellow or green). CCF supplemented mice showed increased collagen birefringence under polarization. Comparison of medial elastin filament breaks (black structures) within Verhoeff–Van Gieson (EVG)–stained sections of suprarenal aortas. The CCF supplemented mice had higher collagen and elastin content within the SRA wall compared with control mice (**A & B**). **A.** Quantification of polarization images for collagen content expressed as a percentage (%) of the total suprarenal aorta section area (n= 11 for CCF and 7 for control). **B.** Quantification graph showing elastin filament degradation (n= 11 for CCF and 7 for control). Aortic wall elastin filament degradation was graded based on the degree of breaks in elastin filaments (graded on a scale of 1–4) as described in Chapter 2 (Section 2.9.2). Data are shown as median and interquartile ranges.

Table 6: Effects of cholecalciferol supplementation on gene expression within the suprarenal aorta of apolipoprotein E-null mice infused with angiotensin-II

Gene	Control (n=7)	CCF (n=11)	Expression	P
Sost	1.05 [0.55-1.91]	2.71 [1.53-5.97]	↑	**0.035**
Opg	1.10 [0.97-1.49]	1.19 [0.95-1.46]	↔	0.724
Opn	57.96 [35.14-82.32]	24.07 [13.68-48.24]	↓	**0.035**
Mmp-9	12.49 [8.452-34.06]	7.36 [5.05-9.20]	↓	**0.020**
Mmp-2	5.47 [2.94-13.68]	2.27 [1.86-3.56]	↓	**0.035**
Vdr	0.72 [0.21-0.97]	1.30 [0.75-2.23]	↑	**0.027**
Cyp27b1	1.33 [1.27-2.41]	0.77 [0.43-1.04]	↓	**<0.001**
Dkk-1	0.76 [0.51-0.95]	1.81 [1.16-2.27]	↑	**<0.001**
Ocn	1.17 [0.97-1.88]	1.22 [0.97-2.57]	↔	0.930
Ctnnb1	4.59 [3.66-8.28]	2.12 [1.79-4.02]	↓	**0.015**
Agtr1	1.95[1.39-2.44]	0.76[0.28-1.09]	↓	**0.002**
Mcp-1	1.05[0.86-1.46]	0.85[0.51-0.99]	↓	**0.035**

Shown are relative gene expression as median (inter-quartile range) relative to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression. Inter-group comparison was analysed using Mann-Whitney U test. Abbreviations: CCF: cholecalciferol; *Sost*: sclerostin; *Opg*: osteoprotegerin; *Opn*: osteopontin; *Vdr*: vitamin D receptor; *Cyp27b1*: gene encoding for 25-hydroxvitamin D3 hydrolase; *Dkk*: *Wnt* inhibitor Dickkopf-1; *Ocn*: osteocalcin (bone-Gla matrix); *Ctnnb1:* gene encoding for β-catenin; *Mmp-2*/*9*: matrix metalloproteinase 2/9; *Agtr1*: angiotensin-II receptor 1; *Mcp-1*: monocyte chemoattractant protein-1.

5.5. DISUSSION

The main findings from this study were that supplementation of CCF attenuated progression and rupture of pre-established AAA within the AngII-infused *ApoE*$^{-/-}$ mouse model. Circulating levels of 25(OH)D levels were raised to >75 nmol/L (considered optimal vitamin D status) following CCF administration while these levels remained low within control mice. The present study demonstrated that supplementation of CCF by oral gavage resulted in increased plasma levels of 25(OH)D which was accompanied with increased levels of circulating SOST and aortic SOST expression. Preceding findings (Chapter 3 & 4) showed that low levels of plasma 25(OH)D coupled with reduced SOST promoted development of AAAs which were more likely to rupture. In this study, however, up-titration of 25(OH)D to >75nM was associated with slow growth of AAA and reduced rupture rate in AngII-infused *ApoE*$^{-/-}$ mice with pre-established AAA. Recently, it was reported that transgenic upregulation and parenteral administration of SOST protected against experimental AAA development (Krishna et al., 2017). The present study shows that CCF supplementation resulted in increased plasma 25(OH)D levels and SOST, which in turn, attenuated the progression of pre-established AAA within the AngII-infused mouse model.

Association between low circulating 25(OH)D and CVD pathologies such as PAD, CAD and atherosclerosis has been reported, although the findings have been conflicting (Nsengiyumva et al., 2015; Pilz et al., 2016). Vitamin D in the form of CCF or ergocalciferol is a nutraceutical that is widely available as an over the counter supplement, although higher doses require a physician's prescription. In clinical settings, vitamin D deficiency is conservatively managed by CCF or ergocalciferol supplementation with an intention to raise circulating levels of 25(OH)D (Bischoff-Ferrari et al., 2006; Holick et al., 2011; Kennel et al., 2010; Kleerekoper et al., 2011). Achieving >75 nM of serum 25(OH)D concentration has been postulated to be beneficial in multiple health outcomes (Bischoff-Ferrari et al., 2006; Holick et al., 2011; Kennel et al., 2010; Kleerekoper et al., 2011). It has been suggested that CCF is more efficacious at raising circulating levels of 25(OH)D levels compared with ergocalciferol (Tripkovic et al., 2012); and thus a preferred choice for 25(OH)D replenishment for this study. Guidelines from the American endocrinology society suggest that vitamin D supplementation should be best maintained at weekly doses interval (Rosen et al., 2012). Weekly doses appear to be more efficacious in correcting circulating levels of 25(OH)D compared with daily, monthly, yearly or single doses supplementation of CCF (Rosen et al., 2012). Given that

25(OH)D levels associated with AAA presence are not definitively within the deficiency range (i.e. <50 nmol/L), this study postulated that raising 25(OH)D levels to ≥75 nM could confer protective effects against AAA progression. Findings from this study demonstrated that increasing plasma 25(OH)D levels slowed AAA growth. In addition, this was accompanied by limited aortic rupture incidences suggesting that vitamin D supplementation played a role in attenuation of pre-established AAA within the mice that were supplemented with CCF. In previous studies, it was shown that administration of 1α,25(OH)$_2$D3, a VDR agonist, has been reported to limit AAA development in a mouse model, and paricalcitol administration has been reported to limit inflammation in human AAA (Martorell et al., 2016; Nieuwland et al., 2016).

Mechanistic studies have previously suggested that vitamin D is multilevel repressor of Wnt/β-catenin signalling (Larriba et al., 2013). It was recently reported that transgenic SOST downregulation and recombinant SOST administration resulted in hyper-phosphorylation of GSK-3β and accumulation of the β-catenin complex, indicating involvement Wnt/β-catenin signalling in AAA formation (Krishna et al., 2017). Nonetheless, it has been suggested that exogenous administration of SOST or overexpression of SOST could promote osteoporosis (Costa et al., 2014). However, total inhibition of SOST was also shown to promote inflammation, aortic aneurysms and atherosclerosis (Krishna et al., 2017; Wehmeyer et al., 2016). Interestingly, ergocalciferol administration has previously been reported to increase circulating SOST concentrations in patients with osteoporosis (Sankaralingam et al., 2014). Emerging evidence suggests that vitamin D limits fracture risks in osteoporotic patients (Holick et al., 2011) while recent findings showed that vitamin D upregulated in osteoblasts (Sutherland, Geoghegan, Yu, Winkler, & Latham, 2004; Tartaglione et al., 2017; Wijenayaka et al., 2015; Wijenayaka et al., 2015). Consistent with these findings, data in Chapter 3 demonstrated that 1α,25(OH)$_2$D3 upregulated expression of SOST in VSMCs. This suggests that consequent increase of bioavailable 1α,25(OH)$_2$D3 due to CCF supplementation resulted in higher SOST expression within VSMCs, particularly within the SRA wall. Moreover, Chapter 3 of this thesis showed that 1α,25(OH)$_2$D3 promotes *SOST* expression in human aortic VSMCs while dietary deficiency of vitamin D resulted in reduced circulating and SRA wall SOST. It is therefore plausible that increased of circulating 25(OH)D levels were locally metabolised by SRA VSMCs, resulting in upregulation of SRA *Sost* expression as demonstrated by SRA upregulation of CYP27B1 (Christakos et al., 2016; Mitsuhashi, Morris, & Ives, 1991). Moreover, findings from this study further showed that *CYP27B1* was

downregulated in the SRA tissue in response to CCF supplementation. Collectively, these findings suggested that bioavailability and conversion of circulating 25(OH)D to 1α,25$(OH)_2$D were locally increases in these mice. CCF supplementation resulted in upregulation of VDR suggesting genomic effects of 1α,25$(OH)_2$D to elicit gene expression within the SRA wall cells (Christakos et al., 2016).

VSMCs play an important role that involves matrix synthesis and structural integrity of the aortic wall. Collagen and elastin are structural proteins that control arterial distension and elasticity within the aortic wall (Isenburg et al., 2007). Key histological features of AAA-diseases tissues include evident depletion of elastin and collagen matrix fires (Krishna et al., 2017; Norman et al., 1995). Indeed, matrix degradation has been consistently characterized within the *ApoE*$^{-/-}$, AngII-infused mouse model (Krishna et al., 2017; Krishna et al., 2015; Moran et al., 2014). The present study demonstrated that CCF supplementation protected against proteolytic destruction of the aortic wall as evidenced by SRA elastin and collagen fibers and *Mmp-2* and *Mmp-9* expression within the SRA of mice that were supplemented with CCF. Specifically, this study showed integrity of both elastin and collagen fibres was higher in mice that were supplemented with CCF compared with controls. Furthermore, lower expression of *Mmp-2* and *Mmp-9* was demonstrated in CCF supplemented mice. This is consistent with previous studies advocating that these genes are associated with AAA formation and progression in both in human AAA and animal models. These findings are also in agreement with a recently publication by Martorell *et al* (2016) which reported that administration of 1α,25$(OH)_2$D3 (1 μg/kg) downregulated SRA MMP-2 and MMP-9 and upregulated endogenous tissue inhibitor of metalloproteinases-1 (TIMP-1) expression in *ApoE*$^{-/-}$ AngII infused mice. Consistently, it has been shown that demonstrated that VDR activation downregulated MMP2 and MMP-9 in cultured human VSMCs (Aoshima et al., 2012; Britt et al., 2015). In addition, Chapter 3 of this thesis showed that *in vitro* incubation of 1α,25$(OH)_2$D3 with VSMCs resulted in upregulated expression of contractile phenotype markers suggesting a homeostatic maintenance of SRA VSMCs by locally bioavailable 1α,25$(OH)_2$D3. Interestingly, AAA is prevalent in elderly persons aged >65 years which coincides with the half-life of elastin (at ~70 years) and decreased cutaneous absorption of sunlight vitamin D (Holick, 2007; Norman et al., 1995), suggesting that lower 25(OH)D levels could insidiously contribute to AAA expansion.

Interpretation of these findings is prone to a number of strengths and limitations. Experimental design of this study aimed to partially simulate clinical settings where patients present with small established AAAs. Most previous rodent AAA studies have focused on the ability of interventions to limit AAA development (Golledge, Norman, et al., 2017); however, in the current study the ability of CCF supplementation to limit growth of *pre-**established*** AAAs was investigated. The loading dose of CCF required to rapidly achieve >75 nmol/L (defined as optimal 25(OH)D (Bischoff-Ferrari et al., 2006; Holick et al., 2011) was estimated using a previously proposed formula (van Groningen et al., 2010). Furthermore, circulating 25(OH)D were measured using a highly sensitive mass spectrometry assay in a validated laboratory (Clarke et al., 2013; Holick, 2009; Kleerekoper et al., 2011). It is noteworthy to mention; given that baseline 25(OH)D were similar after 14-days of AAA induction, it is unlikely that AAA is causative of low 25(OH)D which has been a limitation of previous epidemiologic findings. Nonetheless, circulating PTH levels were not measured in this study. Vitamin D is an important regulator of PTH, and high PTH levels are deleterious to cardiovascular function (Bischoff-Ferrari et al., 2006; Christakos et al., 2016; Norman & Powell, 2014). Indeed, optimal 25(OH)D levels have been previously defined on the basis of maximal suppression of PTH secretion (Bischoff-Ferrari et al., 2006). While renal *CYP27B1* expression is tightly regulated PTH and FGF23, it is still unknown whether similar mechanisms are involved extra skeletal control of this enzyme (Christakos et al., 2016).

In conclusion, this study examined the efficacy of CCF supplementation in limiting the growth of ***pre-established*** AAA in the presence of continuous AngII infusion. Findings from this study showed that CCF supplementation attenuated AAA progression and limited aortic rupture rate in this experimental mouse model. CCF supplementation resulted in achievement of higher circulating 25(OH)D levels (≥ 75 nmol/L) which are currently defined as optimal levels (Holick et al., 2011). CCF supplementation also resulted in increased circulating SOST, SRA SOST protein and SRA *Sost* gene expression. Mice supplemented with CCF manifested lower expression of pro-inflammatory markers and matrix remodelling markers. Taken together, these findings also confirm that effects of CCF supplementation on SOST and Wnt/β-catenin signalling may plausibly explain the mechanism by which vitamin D limits AAA progression. CCF supplementation may be an attractive therapy in clinical settings where patients present with already established AAA. Further research is therefore needed to examine the ability to translate these findings to AAA patients.

CHAPTER 5 HIGHLIGHTS

What is Known?

- Vitamin D deficiency is clinically managed through CCF supplementation in order to raise circulating levels of 25(OH)D.
- Optimal vitamin D status has been suggested to be 25(OH)D levels ≥ 75 nmol/L for multiple health outcomes.
- Chapters 3 &4 showed dietary deficiency of vitamin D promoted expression of genes involved in AAA inflammation and aortic wall matrix remodelling.
- Chapter 4 showed that dietary restriction of vitamin D promoted formation of larger AAAs which were more prone rupture in the *ApoE*$^{-/-}$, Ang-II infused mouse model.
- Chapters 3 & 4 demonstrated that these effects are mediated through changes in circulating SOST and aortic SOST expression.

What New Information Does This Chapter Add?

- Using a previously defined formula for 25(OH)D up-titration, a human equivalent dose was calculated and defined for the *loading dose* of CCF.
- Dietary restriction of vitamin D resulted in higher elastin and collagen degradation.
- CCF supplementation and resultant increase in plasma 25(OH)D levels were associated with slower growth of AAA and lower rupture rate of pre-established AAA in the ApoE$^{-/-}$, Ang-II infused mouse model.
- Effects of CCF supplementation appeared to be mediated via changes in expression of genes involved in ECM remodelling, particularly the increase of SOST.

CHAPTER 6:

GENERAL DISCUSSION

6.1. Discussion of Findings

In the present thesis, the effects of vitamin D on AAA development, growth and rupture were investigated *in vivo* using the *ApoE*$^{-/-}$ mouse model in which AAA was induced by AngII infusion. The thesis also investigated the effect of vitamin D on human aortic VSMCs *in vitro*. This thesis had 4 aims: (1) to examine whether the active metabolite of vitamin D, 1α,25(OH)$_2$D3, increased *in vitro SOST* expression in VSMCs; (2) to investigate whether dietary-induced vitamin D deficiency reduced circulating levels of SOST in *ApoE*$^{-/-}$ mice; (3) to investigate if dietary-induced deficiency of vitamin D promoted AAA development and rupture; and (4) to examine if up-titration of circulating 25(OH)D levels through CCF supplementation limited progression of pre-established AAA.

The main findings of this thesis were that within the AngII-induced mouse model, diet-induced vitamin D deficiency promoted development of large AAAs which were prone to rupture; and that CCF supplementation to achieve high circulating concentrations of 25(OH)D limited growth and rupture of pre-established AAAs. These effects appeared to be linked to changes in circulating and aortic expression of SOST, along with effects on the *Wnt*/β-catenin signalling pathway within the aorta. These findings support those from a previous study, which suggested that transgenic upregulation and parenteral administration of hrSOST protected against AAA development in the same mouse model through competitive inhibition of the *Wnt*/β-catenin signalling pathway (Krishna et al., 2017; Towler, 2017)

Guidelines recommend achieving circulating 25(OH)D concentrations of >75 nmol/L as optimal levels (Al Mheid et al., 2013; Bischoff-Ferrari et al., 2006; Holick et al., 2011; Kennel et al., 2010; Kleerekoper et al., 2011; van Groningen et al., 2010). In a previous community screening study in older men it was reported that men with plasma concentrations of 25(OH)D in the lowest quartile were more than five times as likely to have an AAA measuring >40 mm compared with men with 25(OH)D concentrations in the upper quartile (Wong et al., 2013). In keeping with those clinical findings, in Chapter 4 of this thesis, *ApoE*$^{-/-}$ mice with no detectable plasma 25(OH)D were predisposed to development of larger AAA which were more likely to rupture during AngII-infusion. While AAA diagnosis has been associated with lower circulating concentrations of 25(OH)D, most patients that develop AAA do not have 25(OH)D

concentrations within the deficient range of <50 nmol/L (Van De Luijtgaarden et al., 2012; Wong et al., 2013). In Chapter 5 of this thesis, raising 25(OH)D concentrations to ≥75 nmol/L limited growth and rupture of pre-established AAA within the AngII-mouse model. Supplementation of mice with CCF was associated with upregulation of the aortic expression of the *VDR* and downregulation of *CYP27B1*. Findings were similar within an *in vitro* experiment involving VSMCs in which incubation of cells with 1α,25$(OH)_2$D3 led to a dose-dependent upregulation of *VDR* and *CYP24A1* while *CYP27B1* was dose-dependently downregulated.

The role of vitamin D in skeletal health is well-documented. Vitamin D deficiency has been associated with osteoporosis and increased risk of fractures in older adults and one potential mechanism for this association could be the effect of vitamin D on the expression of bone proteins (Holick et al., 2011). Incubation of osteoblasts with 1α,25$(OH)_2D_3$ has been reported to induce *SOST* expression (Wijenayaka et al., 2015). Ergocalciferol administration has previously been reported to increase circulating SOST concentrations in patients with osteoporosis (Sankaralingam et al., 2014). Current findings demonstrated that incubation of VSMCs with 1α,25$(OH)_2$D3 led to a dose-dependent increase in *SOST* expression. In mice experiments, a VDD diet led to a reduction in plasma SOST concentration, aortic SOST protein levels and aortic *SOST* gene expression while supplementation of CCF in mice with established AAAs increased plasma and aortic SOST. Aortic concentrations of p-GSK-3α/β, which is indicative of Wnt/β-catenin signalling activity, were also lower in mice receiving CCF. Phosphorylation of the GSK-3α/β leads to stabilization of β-catenin complex and consequent translocation to the nucleus. The Wnt/β-catenin signalling has been implicated in stimulating the activation of an inflammatory cascade in a number of autoimmune diseases (Beurel et al., 2015). Additionally, findings from this thesis showed that CCF administration upregulated aortic expression of *Dkk-1*, another *Wnt* pathway inhibitor (Larriba et al., 2013; Nusse & Clevers, 2017; Sankaralingam et al., 2014). In line with these findings, a VDD diet led to increased aortic expression of the *Wnt* signalling gene *Ctnnb1* whereas CCF supplementation led to decreased aortic expression of *Ctnnb1*. *In vitro* experiments showed that *CTNNB1* was dose-dependently downregulated in response to 1α,25$(OH)_2$D3 administration, suggesting inhibition of *Wnt*/β-catenin signalling. This was associated with downregulation of AGTR1 and MCP-1 as well as upregulation of key contractile markers in VSMCs (Lino Cardenas et al., 2018). These responses were plausibly attributable to upregulation in SOST expression.

Indeed, vitamin D associated effects on *Wnt*/β-catenin signalling, including downregulation of β-catenin have been previously described, most notably in cancer cells (Larriba et al., 2007; Palmer et al., 2001; Shah et al., 2006). It was recently reported that SOST upregulation in a mouse model reduced phosphorylation of GSK-3β and consequent β-catenin accumulation in SRA tissue (Krishna et al., 2017). These findings suggest that CCF supplementation upregulates SOST and inhibits experimental AAA progression by inhibiting *Wnt*/βcatenin signalling.

Degradation of the ECM has been strongly implicated in AAA pathogenesis (Galis & Khatri, 2002; Krishna et al., 2017; Krishna et al., 2015; Moran et al., 2014). In particular, overexpression of MMP-2 and MMP-9 has been consistently associated with AAA formation and progression in this experimental mouse model (Krishna et al., 2017; Krishna et al., 2015; Moran et al., 2005). In the current thesis, mice administered a VDD diet had greater aortic expression of *Mmp-2* and *Mmp-9* compared to controls. Similarly, supplementation with CCF in mice with established AAA led to downregulation of aortic *Mmp-2* and *Mmp-9* expression compared to controls. Taken together, these findings suggest that an increase in circulating 25(OH)D concentrations via CCF supplementation limited proteolytic degradation within the aorta. These findings are consistent with data from a recent publication, in which it was reported that parenteral administration of 1α,25$(OH)_2$D3 (1 μg/kg) downregulated aortic *Mmp-2* and *Mmp-9* and upregulated endogenous tissue inhibitor of *Mmp-1* expression in AngII-infused *ApoE*$^{-/-}$ mice (Martorell et al., 2016). In addition, VDR activation has been reported to downregulate *MMP-2* and *MMP-9* activity and expression in cultured human VSMCs (Aoshima et al., 2012; Britt et al., 2015). OPN, while initially described as a bone protein, has been strongly implicated in AAA development (Bruemmer et al., 2003; Filis et al., 2014; Golledge et al., 2007; Li et al., 2016). It has previously been reported that plasma concentrations of OPN are increased in patients with AAA and positively correlated with AAA growth rates (Golledge et al., 2007). Within the AngII infused mouse model, it has been reported that OPN deficiency protects against AAA formation (Bruemmer et al., 2003). Findings from this thesis suggest that a VDD diet upregulated aortic expression of *Opn*, while supplementation with CCF downregulated *Opn* in comparison to controls. *In vitro* incubation of VSMCs with 1α,25$(OH)_2$D3 dose-dependently downregulated *OPN* expression. The effect of CCF supplementation on MMP-2, MMP-9 and OPN may, at least in part, explain its ability to limit AAA progression.

Traditionally, osteogenic markers of bone mineralisation such as OCN, OPN and OPG have been exclusively known to play a role in bone health with no known function in arterial health (Huang, Yang, Shao, & Li, 2007). Emerging evidence however, suggests increased OPN and OPG are associated with AAA pathology in both human and animal models, particularly the AngII infused *ApoE*$^{-/-}$ mice (Golledge et al., 2007; Golledge, Tsao, Dalman, & Norman, 2008; Moran et al., 2014). Recently, it was reported that increased circulating levels of OCN were associated with severe abdominal aortic calcification and acute aortic dissection in patients (Vianello et al., 2017). In this thesis, up-titration of 25(OH)D levels by CCF supplementation resulted in downregulation of OPN without changes on OPG and OCN which could be used as markers for calcification (Jono, Nishizawa, Shioi, & Morii, 1998). Experimental studies have suggested that high circulating 1α,25$(OH)_2$D3(≥ 100 mol/L) could induce calcification and upregulation of osteogenic markers, including OPN in VSMCs (Jono, Nishizawa, Shioi, & Morii, 1998). However, similar expression of *Opg and Ocn* between CCF supplemented mice and controls provides confidence that no calcification due to high CFF. Conversion of 25(OH)D to 1α,25$(OH)_2$D3 is tightly regulated physiologically (Christakos et al., 2016); and thus CCF supplementation is deemed safe even at higher doses.

Hypertension is understood to be a risk factor for AAA development and rupture (Kent et al., 2010; Tang et al., 2016). In patients with AAA, it recommended that blood pressure be controlled along with other traditional CVD risk factors (Golledge & Powell, 2007; Lindeman & Matsumura, 2019). A recent meta-analysis reported that hypertension increased the risk of developing AAA by 66% (Kobeissi et al., 2019). However, another meta-analysis previously suggested that hypertension may not be associated with AAA expansion (Takagi & Umemoto, 2017). SBP has previously been reported to rise in mice receiving AngII-infusion, although it is not thought to be responsible for the induction of AAA in this experimental model (Ayabe et al., 2006; Cassis et al., 2009; Kawada et al., 2002; Krishna et al., 2015). A VDD diet promoted higher SBP in *ApoE*$^{-/-}$ mice. Vitamin D has been reported to suppress renin expression (Ajabshir et al., 2014) and administration of CCF (25000 IU/week) has been reported to attenuate hypertension in patients (Carrara et al., 2014). In addition, findings from previous studies have reported that circulating 25(OH)D is may be associated with lower blood pressure (Kunutsor et al., 2014; Vimaleswaran et al., 2014), although trials with vitamin D supplementation have yielded conflicting outcomes (Beveridge et al., 2015). However, given

that AngII is not thought to induce AAA via increases in SBP it is unlikely that the link between vitamin D and SBP is responsible for AAA formation and progression in accordance with findings from this thesis (Chapter 4 & 5). Indeed, non-AngII targeted agents do not promote aneurysms *ApoE-/-* mice; and it has previously been established that blood pressure elevation is not responsible for the ability of AngII to promote aneurysm development in this mice model (Cassis et al., 2009). It can therefore be deduced that the blood pressure changes are not responsible for the effects of lower 25(OH)D in these studies. Moreover, calcium channel blockers, such as amlodipine, diltiazem and nifedipine, have been previously shown to limit experimental AAA within the angiotensin II model independent of any effects on blood pressure (Chen et al., 2013; Miao et al., 2015; Mieth et al., 2013); and therefore, administration of these agents will not advance understanding of the effect of vitamin D deficiency. On this basis, it may not be possible to isolate the effects of blood pressure from vitamin D deficiency since agents such as calcium channel blocking drugs have been shown to limit AAA development in this model independent of effects on blood pressure.

6.2. Strengths and Limitations

A number of strengths and limitations of studies in this thesis should be acknowledged. Strengths of the current the studies include the use of multiple mouse experiments designed to examine the link between vitamin D and AAA, in addition to an *in vitro* study. Most previous rodent AAA studies have focused on the ability of interventions to limit AAA development; however, in Chapter 5 of this thesis the ability of CCF supplementation to limit growth of *established* AAAs was also investigated (Golledge, Norman, et al., 2017). The design of animal experiment in Chapter 5 aimed to partially simulate the clinical settings where patients present with small established AAAs. The loading dose of CCF required to rapidly achieve >75 nmol/L [(defined as optimal 25(OH)D (Bischoff-Ferrari et al., 2006; Holick et al., 2011)] was estimated using a formula proposed by van Groningen *et al* (2010). In patients presenting with vitamin D deficiency, a loading dose is required in order to raise circulating 25(OH)D levels as rapidly as possible (>75nM) (Kennel et al., 2010) before placing patients on a maintenance dose. Furthermore, circulating 25(OH)D were measured using a highly sensitive mass spectrometry assay in a validated laboratory (Clarke et al., 2013; Holick, 2009; Kleerekoper et al., 2011). Oral supplementation of CCF has been previously shown to increase production of vitamin D epimers in mice (Ghaly et al., 2019); therefore, the LC-MS/MS technique was an excellent modality to discriminate between actual circulating plasma 25(OH)D and the C3-epimers (Al-

Zohily et al., 2020). Some of the limitations in findings presented in this thesis include the use of one mouse model since animal experiments were limited to the AngII-infused *ApoE*$^{-/-}$ mouse model. In addition, while it is possible that different cell types might respond to 1α,25(OH)2D3 effects differently, experiments in Chapter 3 focused on VSMCs effects due to the paramount role of these cells in arterial remodelling and AAA formation. It also remains uncertain what the precise mechanism involved in the protective effect of CCF administration against AAA development and progression. Characterisation of inflammatory cells infiltrates is another hallmark feature of AAA-diseased tissue (Isenburg et al., 2007); however attempts to characterise infiltration of macrophages within the SRA tissue using macrophages/monocytes-2 (MOMA-2) antibodies were not successful, and should be acknowledged as a limitation. Finally, although the AngII-infused *ApoE*$^{-/-}$ mouse model shares several common pathological features with human AAA, direct translatability of findings from mice studies to human studies remains challenging (Patelis et al., 2017; Rush et al., 2009; Senemaud et al., 2017; Trollope et al., 2011) .

Furthermore, circulating levels of PTH were not measured in mice studies presented in this thesis. Physiologically, PTH is an important regulator of vitamin D since optimal circulating 25(OH)D is reflective of suppression of PTH levels (Bischoff-Ferrari et al., 2006; Christakos et al., 2016; Jones, 2013; Norman & Powell, 2014). PTH is also recognized as a negative regulator for SOST expression (Keller & Kneissel, 2005; Wijenayaka et al., 2015). Also, as a regulator of bone remodelling and ion homeostasis, PTH has been shown to reduce *SOST* expression in bone osteocytes and vascular VSMCs (Gooi et al., 2010; Keller & Kneissel, 2005; Silvestrini et al., 2007; Song, Fiaschi-Taesch, & Bisello, 2009). Several studies have shown that chronic or intermittent administration of PTH may be associated with downregulation of *SOST* in osteocytes (Bellido et al., 2005; Jilka, O'Brien, Bartell, Weinstein, & Manolagas, 2010; Lavi-Moshayoff, Wasserman, Meir, Silver, & Naveh-Many, 2010). Chronically excessive circulating PTH (hyperparathyroidism) has been associated with presence and worsening CVD events and CVD risk factors, including hypertension, vascular calcification and PAD (Garcia, Clemens, Fagin, Finkielman, & Pirola, 1998; Ishikawa et al., 2000; Song et al., 2009). Although no studies have investigated the effects of circulating PTH on aortic aneurysm, Demir *et al* (2012) previously reported an increase in PTH levels among TAD patients compared with controls. However, it can be inferred that the indicator for this was the lower circulating levels of circulating 25(OH)D in these patients. It was previously reported

that PTH inhibited *SOST* expression via a non-direct mechanism which suggested a transcriptional regulatory pathway such as that implicated in the Wnt/β-catenin signalling (Brandenburg et al., 2015; Keller & Kneissel, 2005).

6.3. Conclusions and Future Directions

In conclusion, findings from these studies suggested that within an experimental mouse model of AAA, dietary vitamin D deficiency promoted formation of larger AAAs that were more prone to rupture. CCF supplementation limited growth and rupture of established AAAs within the same model. Vitamin D appears to act via changes in SOST and OPN, which limit ECM degradation promoted by MMP-2 and MMP-9. Further research is needed to examine the ability to translate these findings to AAA patients. Therefore, important additions to the current literature include ability of vitamin D to induce SOST in VSMCs, ability of VDD to promote development and rupture of AAAs, and more importantly, the ability of CCF supplementation and consequent 25(OH)D up-titration to attenuate growth and rupture of pre-established AAAs in an experimental mousse model of AAA.

Clearly, AAA is multifaceted diseases, which encompasses a broad spectrum of potential areas for research, including pre-clinical and clinical research. Given that most human AAAs are discovered when they are still small, it is hoped that suitable pharmacological candidates will be found to assist with medical management of AAA patients with the ultimate goal of delaying the need for AAA surgical repair and potential AAA rupture. Accordingly, a number of future directions are proposed in response to the limitations deduced from this thesis. It remains unknown whether SOST could be inducible by vitamin D in other cells that are well-known to be involved in AAA pathogenesis. It would therefore be interesting to investigate whether these findings are repeatable in other cell lines such as monocytes, endothelial cells, and mast cells, particularly for the ability to induce SOST expression from these cells.

A previous study demonstrated that exogenous administration of SOST was able to limit AAA development (Krishna et al., 2017) while Chapter 5 of this thesis showed that CCF supplementation upregulated both *Sost* and *Dkk-1* expression within the SRA of mice. The findings showing that CCF supplementation upregulated *Dkk*-1 are potentially hypothesis generating and deserve further investigations. For instance, it would be interesting, at least

experimentally, to investigate whether exogenous administration of recombinant human Dkk-1 has any therapeutic potential in pre-clinical studies such as mice and cellular experiments. Additionally, it would be worthwhile to investigate whether direct abrogation or modulation of β-catenin could confer therapeutic benefits with regard to attenuation of experimental AAA. Encouragingly, recent advances in the development of small molecules designed to directly bind to β-catenin could make these experiments possible (Cui, Zhou, Zhang, Qu, & Ke, 2018; Hwang et al., 2016). So far, five small molecules and two short peptides have been reportedly assessed for their ability to directly target β-catenin and thereby inhibiting Wnt/β-catenin signalling which is understood to be of experimental interests (Cui et al., 2018). Renewed interests, particularly in cancer research are already exploring these investigations (Cui et al., 2018; Hwang et al., 2016). Nonetheless, clinical translatability of pre-clinical investigations with these agents would remain to be seen in vascular clinical research, especially in AAA clinical trials.

Finally, it would be worthwhile to confirm whether observed lower circulating 25(OH)D levels are also associated with lower circulating SOST in epidemiological studies of AAA patients. Such investigations can be achieved as well by using retrospective cohorts. Finally, the findings from this thesis pave a solid foundation for translation into clinical trials. In particular, the design of animal studies presented in Chapter 5 can be directly translated into human clinical trials for patients presenting with small AAAs. Follow-up parameters in these patients should include measurement of aortic diameter and assessment of vitamin D metabolites as well as assessment of molecular pathways involved in vitamin D metabolism. Vitamin D in the form of CCF is an inexpensive nutraceutical with negligible side effects. Therefore, it is a good candidate for proof-of concept clinical trials.

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APPENDICES

APPENDIX A: JCU/AEC Approval forms

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