The Anti-inflammatory Effects of Testosterone on Atherosclerosis

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A Thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy

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Candidate Declaration

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Date	September 2020
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Dedication

For my mum

I may not always seem it, but I will be forever grateful for the love and support you have given me my whole life. You inspired and encouraged me to pursue science, so this thesis is dedicated to you.

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Abbreviations

ADT	Androgen deprivation therapy
ApoE	Apolipoprotein E
AR	Androgen receptor
ARKO	Androgen receptor knock out mouse
CVD	Cardiovascular disease
CAD	Coronary artery disease
CHD	Coronary heart disease
CD	Cluster of differentiation
cDNA	Complimentary DNA
CRP	C-reactive protein
CSF	Colony stimulating factor
CIMT	Carotid intimamedia thickness
Ct	Cycle threshold
CBA	Cytometric bead array
DAPI	4',6-diam idino-2-phenylindole
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
ECM	Extracellular matrix
EDTA	Ethylenediam inetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
eNOS	Endothelial nitric oxide synthase
FBS	FBS Foetal bovine serum
GnRH	Gonadotropin releasing hormone
HbA_{1c}	Glycated haemoglobin
HAEC	Human aortic endothelial cells
HDL	High-density lipoprotein
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
lg	Immunoglobulin
IL	Interleukin
IMT	Intimamedia thickness
iNOS	Inducible nitric oxide synthase

LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MCP1	Monocyte chemoattraction protein 1
M-CSF	Macrophage colony-stimulating factor
MRI	Magnetic resonance imaging
MetS	Metabolic syndrome
mRNA	Messenger ribonucleic acid
MI	Myocardial infarction
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
ORO	Oil red O
oxLDL	Oxidised low-density lipoprotein
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol myrisate acetate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
SHBG	Sex hormone binding globulin
SMC	Smooth muscle cell
Tfm	Testicular feminised mouse
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TTh	Testosterone replacement therapy
T2D	Type 2 diabetes
VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very low-density lipoprotein

VSMC	Vascular smooth muscle cells
vWF	Von Willebrand Factor
WC	Waist circumference

Abstract

Testosterone deficiency is prevalent in men with type 2 diabetes (T2D) and is associated with greatly elevated risk of cardiovascular mortality. Testosterone replacement therapy (TTh) has beneficial effects on surrogate markers and risk factors of atherosclerosis, including inflammation, cholesterol and insulin resistance, improving survival in men with T2D. The underlying mechanisms of this action remain poorly understood. Inflammation is a central feature to both T2D and atherosclerosis and is driven by monocyte/macrophages, placing these immune cells at the crossroads of disease pathology. The recruitment of immune cells to atherosclerosis-prone areas of the vasculature is influenced by many factors, including the inflammatory status of the circulating monocytes and activation of the vascular endothelium by numerous proatherogenic stimuli. Macrophages are involved in all stages of atherosclerotic plaque development and are central to disease progression through the expression of inflammatory cytokines, chemokines and lipid accumulation. Their interaction with vascular cells and recruited immune cells within the developing plaque can amplify the inflammatory activation of the vascular wall, lead to vascular remodeling, promote apoptosis within the atheroma and ultimately contributes to the destabilisation and rupture of the atherosclerotic lesion.

Using combined *in vivo* and *in vitro* approaches, the aim of this thesis was to investigate whether testosterone treatment improves cardiovascular risk in men by reducing inflammation associated with the development and progression of atherosclerosis. The clinical study investigated the influence of testosterone therapy on monocyte inflammatory markers and atherosclerosis risk factors in a randomised double-blinded placebo-controlled clinical trial consisting of 65 men with poorly controlled T2D and hypogonadism. Orchidectomised $ApoE^{-/-}$ mouse as a model of testosterone deficiency and atherosclerosis, respectively, was used to assess the effects of androgen status and testosterone treatment on inflammatory mechanisms of atheroma formation. Additionally, *in vitro* studies investigated the influence of testosterone on inflammatory markers in cultured human monocytes and macrophages.

Testosterone treatment in orchidectomised $ApoE^{-/-}$ mice significantly reduced lipid accumulation in the aortic root compared to testosterone deficient orchidectomised littermates, which was associated with reduced monocyte/macrophage infiltration and a localised reduction of ICAM-1 at the site of the atherosclerotic plaque. Furthermore, systemic circulating ICAM-1, indicative of disease extent, was also reduced with testosterone treatment. This may be due to a downregulation in pro-inflammatory cytokine TNFα, a potent inducer of ICAM-1, as the clinical study revealed that gene expression of this key inflammatory mediator was significantly reduced over time in monocytes from patients receiving TTh and

significantly reduced compared to placebo-treated patient monocytes after six months. These findings importantly indicate for the first time that testosterone influences monocyte inflammatory activation in men with T2D by altering expression of TNFα as a potential mechanism to protect against atherosclerotic plaque development in hypogonadism. This effect of testosterone was independent of atherosclerosis risk factors including serum lipids, HbA_{1c} and body composition as they were not altered following six months of TTh. Testosterone also did not significantly improve lesion stability as collagen content, smooth muscle migration, fibrous cap thickness, and MMP expression was not affected in the lesion of $ApoE^{-/-}$ mice. While these *in vivo* studies suggest anti-inflammatory actions of testosterone on mechanisms of atherogenesis and monocyte/macrophage function, *in vitro* testosterone treatment had no effect on the inflammatory profile of human monocytes and macrophages in response to pro-inflammatory stimulation or on macrophage subset phenotype thus leaving the specific cellular pathways of action to be elucidated.

This thesis has demonstrated that testosterone reduces aortic atherosclerosis indicated by decreased lipid deposition and reduced monocyte/macrophage infiltration into atherosclerotic lesions. This is likely modulated through local anti-inflammatory actions at the vessel wall, as opposed to systemically, and potentially via a testosterone-induced reduction of monocyte inflammatory activation by TNFα in diabetic men. Risk factors for atherosclerosis were unaffected by testosterone treatment both in hypogonadal men with T2D and $ApoE^{-/-}$ mice again suggesting beneficial actions in these models may be confined to plaque localised effects. Overall, this indicates a local anti-atherogenic mechanism at the vessel wall by which testosterone may protect against the development and progression of atherosclerosis to reduce cardiovascular disease in men.

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1. General Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in the Western world accounting for more than a 30% of deaths globally and 27% in the UK (Allender *et al.*, 2020). CVD comprises all diseases of the heart and circulatory system, the three significant areas being coronary heart diseases (myocardial infarction (MI), angina, heart failure and coronary death), cerebrovascular diseases (stroke, transient ischaemic attacks) and peripheral vascular diseases (intermittent claudication, gangrene) (Glaudemans *et al.*, 2010). Healthcare costs relating to CVD are estimated at £9 billion annually. CVD's cost to the UK economy (including premature death, disability and informal costs) are estimated to be £19 billion each year (Allender *et al.*, 2020). Although government legislation has led to a significant decrease in smoking related CVD deaths over the last decade, a continued growth in incidence of obesity and type 2 diabetes (T2D) facilitates CVD as the leading cause of mortality and morbidity globally (Mensah *et al.*, 2017, Mc Namara *et al.*, 2019, Virani *et al.*, 2020).

Arteries within the circulatory system are, simplistically, a thick muscular tube lined by a smooth tissue surface to allow ease of blood flow through them. The muscle element of them can control constriction and dilation of the vessel for systemic control of vascular parameters such as blood flow and pressure (Figure 1.1.A). This is opposed to the venous circulatory system which is composed of thinner and less muscular vessels as veins do not function primarily in a contractile manner and are not subject to the high pressures of systole, as arteries are. Veins have valves to prevent backflow and rely on skeletal muscle contractions to aid predominantly unidirectional blood flow. Veins have a thick outer layer of connective tissue, the tunica adventitia, the middle layer is the tunica media and consists of smooth muscle, and the interior is lined with endothelial cells called tunica intima (Figure 1.1B).

Similarly, arteries consist of three distinct layers. The outermost layer is known as the tunica adventitia and is composed of collagen fibres and elastic tissue that anchors arteries to surrounding tissues as the arterial vasculature travels through the body. Located within the tunica adventitia is the tunica media layer which consists of smooth muscle, elastic tissue, and collagen fibres to give the vessel strength to withstand high pressures of systole. The innermost layer, which is in direct contact with

the flow of blood, is the tunica intima which is primarily made up of endothelial cells in addition to a supporting layer of elastin rich collagen. The hollow internal cavity in which the blood flows is the lumen (Figure 1.1A). Venous diseases generally fall into one of two categories: venous thrombosis, or blood clot; and venous insufficiency, or inadequate blood return to the heart (Beckman, 2002). Arterial disease includes aneurysms and atherosclerosis, causing stenosis and occlusion of the affected vessel.

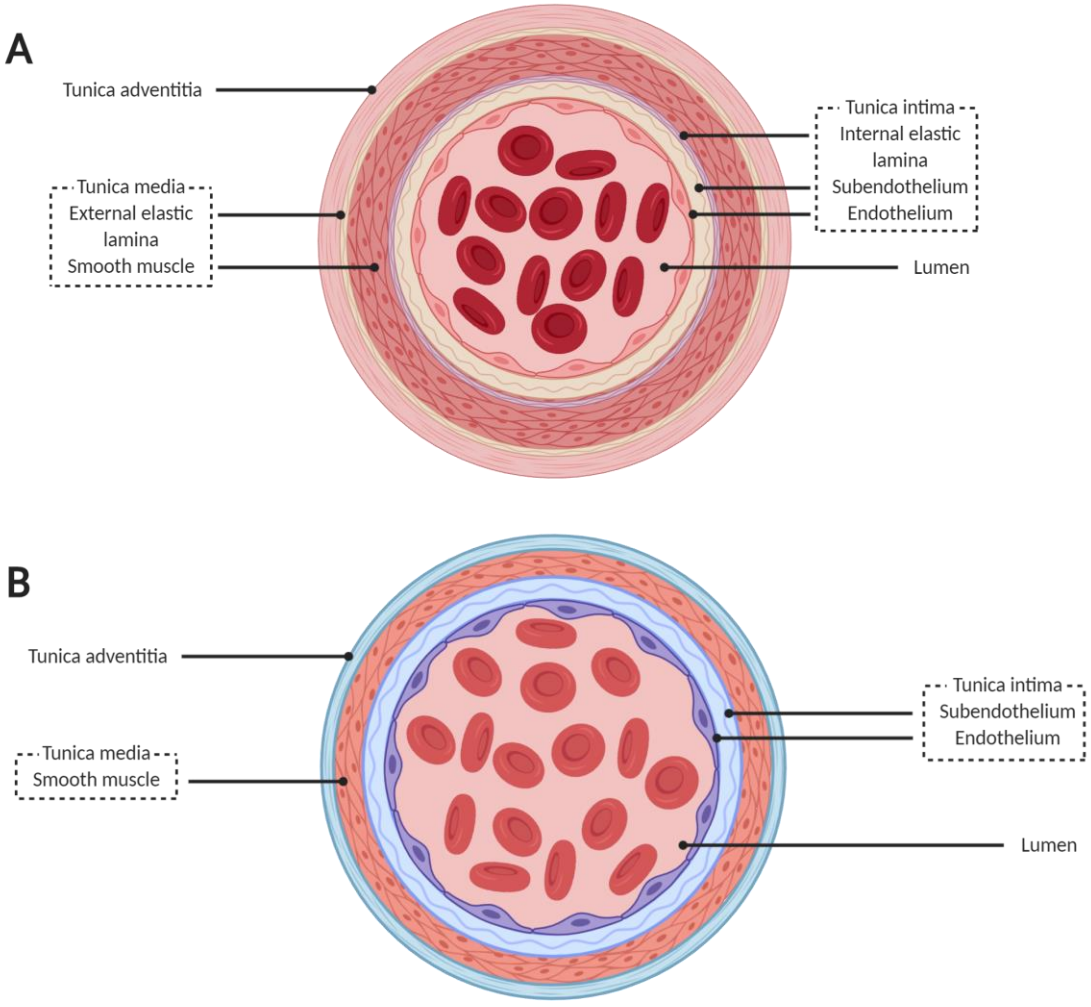


Figure 1.1 Classification and structure of arteries and veins. The wall of an artery consists of three layers. The innermost layer, the tunica intima is simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibers. The middle layer, the tunica media, is primarily smooth muscle and is usually the thickest layer. Smooth muscle provides support for the vessel and changes vessel diameter to regulate blood flow and pressure. The outermost layer, which attaches the vessel to the surrounding tissue, is the tunica adventitia. This layer is connective tissue with varying amounts of elastic and collagenous fibers. The walls of veins have the same main three layers as the arteries. Although all the layers are present, there is less smooth muscle and connective tissue. This makes the walls of veins thinner than those of arteries and withstand less pressure than arteries.

1.1.1 Atherosclerosis

Atherosclerosis is the most common underlying cause of CVD and can be considered the cellular and molecular pathogenic underpinnings to the systemic consequences of arterial CVD (Zhou *et al.*, 2019, Townsend *et al.*, 2016). Beginning in the first decade of life and progressing over time, atherosclerosis typically manifests clinically in middle and late adulthood (Hansson and Libby, 2006, Hong, 2010). Several major modifiable risk factors are known to contribute to the pathogenesis of atherosclerosis including (but not limited to) T2D, cigarette smoking (active and passive), obesity, and physical inactivity. Additional non-modifiable risk factors for atherosclerosis are evident, including a family history of premature coronary heart disease (CHD) (genetic predisposition), history of hypertension, age and biological sex. Of particular note is the sex difference, with men being more than twice as likely to suffer from CVD than women of a similar age (Hung *et al.*, 1990, Dannenberg *et al.*, 1989, Expert Panel on Detection, 2001), a relationship that exists across different geographical locations and socio-economic background (Allender *et al.*, 2020).

The vascular endothelium is the interface between the bloodstream and the vessel wall. Endothelial cell dysfunction encompasses maladaptive changes in the functional phenotype of endothelial cells which is believed to be the initiating step in the development of atherosclerosis (Gimbrone and García-Cardeña, 2013). Vascular endothelial cells are responsive to a variety of stimuli, both local and systemic, which can modify and alter the structural and functional properties of the endothelium. Shear stress has emerged as an essential feature of atherogenesis. Shear stress is the biomechanical force that is determined by blood flow, vessel geometry and fluid viscosity (Resnick *et al.*, 2003). This fluid drag force acting on the vessel wall is sensed by mechano-receptors and the subsequent intracellular signals resulting in changes in vascular behaviour and causing modulation of cellular structure and function (Cunningham and Gotlieb, 2005, Azuma *et al.*, 2000). Disturbed blood flow activates the transcription factor NF-κB in endothelial cells contributing to endothelial dysfunction (Nagel *et al.*, 1999). The activation of the NF-κB pathway leads to the upregulation of pro-inflammatory and pro-atherogenic adhesion molecules, growth factors and cytokines such as intercellular adhesion molecule 1 (ICAM-1), E-selectin, platelet-derived growth factor (PDGF)-BB, interleukin (IL)-1α (Dardik *et al.*, 2005, Yun

et al., 2002), monocyte chemotactic protein-1 (MCP-1) (Hsiai *et al.*, 2003), and the vasoconstrictor endothelin-1, all of which are implicated in the pathogeneses of atherosclerosis (Ziegler *et al.*, 1998).

The process of atherogenesis differs in humans and mice, with vascular smooth muscle cell (VSMC) migration and formation of an intimal thickening and a neointima preceding fatty streak formation in humans. VSMCs play the key role in this process. Smooth muscle cells (SMC) originate from various sources, including media SMCs, endothelial cells, adventitial fibroblasts, circulating stem cells and neural crest cells. All of which in the intima are subject to phenotype modulation. VSMCs show remarkable plasticity in response to vascular injury, inflammation, and lipoprotein accumulation during disease progression via reprogramming gene expression and a shift to a proliferative, pro-migratory, and activated phenotype.

Following endothelial dysfunctions, serum lipoproteins accumulate in the subendothelial space at sites of disturbed blood flow, such as the aortic arch, and areas where endothelial dysfunction persists. Disruption to the endothelium causes increased vascular permeability, which broadly refers to structural and functional changes in the vessel wall whereby the passage of lipoproteins into the intima may occur (Egawa *et al.*, 2013). Low-density lipoprotein (LDL) cholesterol accumulation in the arteries indicative of atherosclerosis correlates with classical cardiovascular risk factors such as hypertension, and metabolic dysregulation in obesity and T2D, therefore, linking these common comorbidities.

This process is followed by intracellular accumulation of lipids by migrated cells, both recruited immune cells and migrated resident arterial wall cells, such as VSMCs that alter their phenotype to acquire the ability for phagocytosis. Circulating monocytes are recruited to sites of vascular inflammation, predominantly by chemokines, and adhere to the activated vascular endothelium in a process mediated by the upregulated adhesion molecules at sites of vascular dysfunction. Cell adhesion plays a critical role in initiating and sustaining an effective immune response against foreign pathogens (Vestweber, 2007). This initial adhesive interaction is termed tethering (capture) and is a transient interaction that slows the progress of leukocytes through the artery as they roll across the vascular endothelial cells allowing closer

cellular communication between the two (Ley, 2008) (Figure 1.2). These early low-affinity interactions are subsequently strengthened as a result of pro-inflammatory cytokine adhesion molecule activation on the leukocytes. Firmly adhered monocytes migrate into the intima through spaces between adjacent endothelial cells. Several mechanisms play a role in transendothelial migration, including binding, intracellular signalling cascades, disruption of adherens junctions, and targeted recycling of platelet/endothelial cell adhesion molecule (Muller, 2011). Macrophage colony-stimulating factor (M-CSF) produced by activated endothelial and SMCs is released into the vascular intima layer, which stimulates monocyte differentiation into macrophages (Rajavashisth *et al.*, 1990). Macrophages in atherosclerotic lesions actively participate in lipoprotein ingestion, particularly of LDL which has been oxidised (oxLDL) in the growing inflammatory vascular environment. oxLDL is recognised by scavenger receptors, predominantly CD36 and CD68 on the macrophage cell surface, initiating intracellular signaling to activate phagocytosis (de Gaetano et al., 2016). Engulfed oxLDL is then hydrolysed into free cholesterol and fatty acids (Maxfield and Tabas, 2005, Pluddemann *et al.*, 2007). Subsequently, free cholesterol will undergo re-esterification, forming cholesteryl esters that are stored in the macrophage cytoplasm as lipid droplets transforming macrophages into foam cells (Lusis, 2000, Brown et al., 1980). SMCs also adapt to fluctuating environmental cues and adopt a macrophage-like phenotype and make up the other type of foam cell following lipid phagocytosis. The accumulation of foam cells is a key characteristic of early atherosclerotic plaques known as fatty streaks or lesions (Figure 1.3B). Foam cells are pro-inflammatory, secreting cytokines (IL-1β and TNFα) and chemokines (CX_3CL1, CCL2 and IL-8) that further propagate the recruitment of monocytes to the atherosclerotic lesion (Shirai et al., 2015, Tacke et al., 2007) and can eventually undergo apoptosis, forming the lesion's necrotic core (Figure 1.3C) (Seimon and Tabas, 2009).

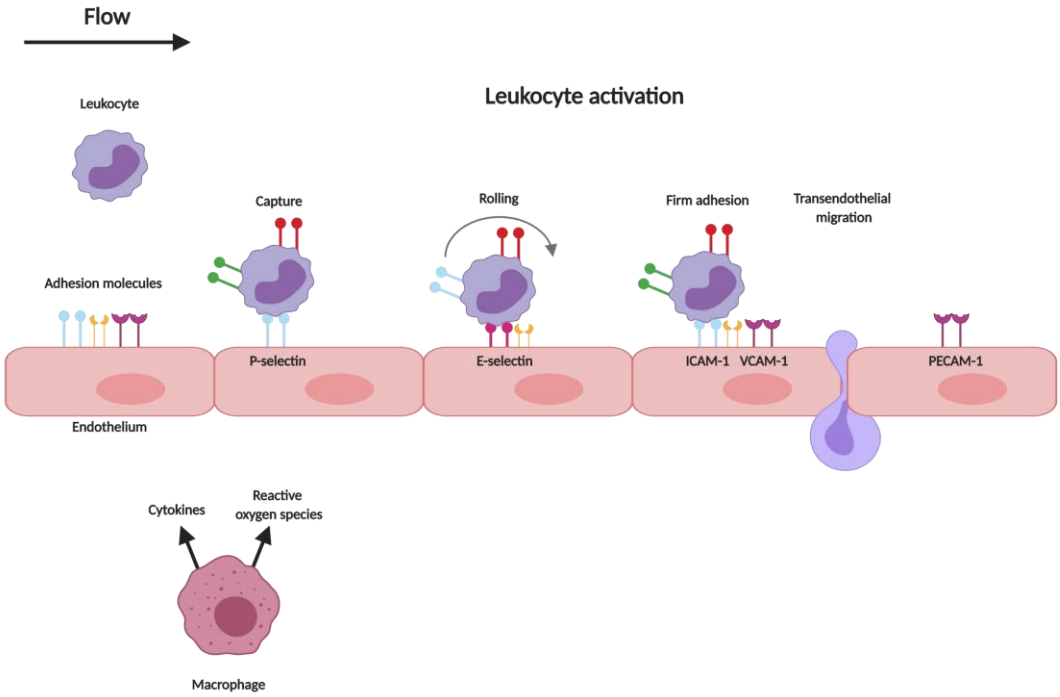


Figure 1.2 Multistep adhesion cascade and the major molecular contributors to leukocyte recruitment during an inflammatory response. Following endothelial cell activation and the increased expression of P- and E-selectins, low-affinity adhesive interactions (capture and rolling) are elicited that subsequently lead to leukocyte activation, followed by firm adhesion and transendothelial migration. Adapted from Granger and Senchenkova (2010).

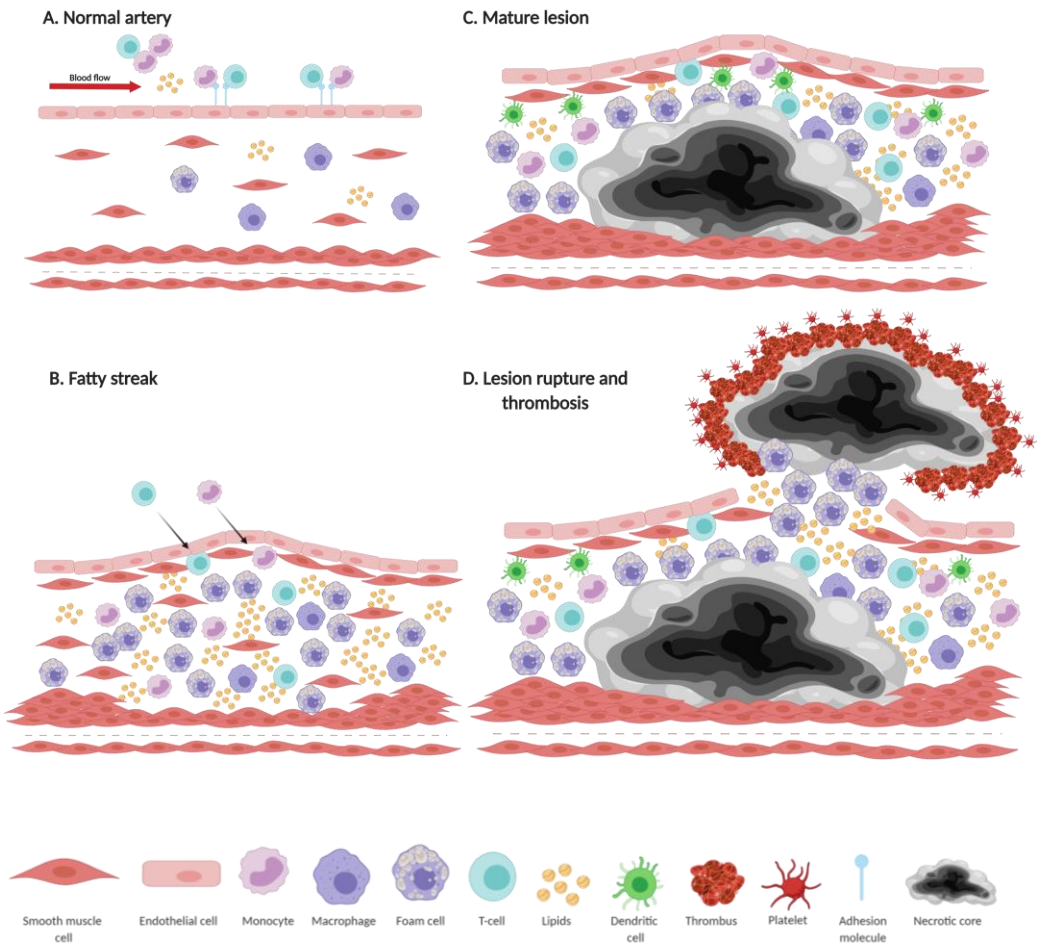


Figure 1.3 The stages of atherosclerosis. The development of an atherosclerotic lesion occurs in multiple steps. A: Activation of the endothelium due to lipid accumulation leads to recruitment and adhesion of leukocytes. B: Pro-inflammatory mediators expressed in the activated vascular wall promote leukocyte maturation and release of inflammatory mediators such as cytokines, chemokines and adhesion molecules. These mediators recruit additional leukocytes to the activated endothelium and develops the fatty streak. Smooth muscle cells migrate from the tunica media to form a protective cap over the fatty streak. Continuous deposition of extracellular proteins results in narrowing the arteries. C: Fatty streaks develop into mature plaques. As the plaque size increases, oxygen levels diminish, leading to a central necrotic core and neovascularization within the plaque, which may account for leakage of blood components. The protective fibrous cap thins, due to continued inflammation and becomes prone to rupture. D: Eventually, secretion of proteases and cytokines by cells in the affected lesion leads to matrix degradation with plaque eruption and triggering of the coagulation cascade that causes acute complications of atherosclerosis such as occlusion and ischemia. Adapted from Hansson and Libby (2006).

Cytokines and growth factors secreted by foam cells and macrophages in the intima induce dedifferentiation and migration of SMCs from the tunica media into the intima where they produce collagen-rich matrix (Libby *et al.*, 2002). This additional matrix deposition and SMCs can form a fibrous cap over the plaque providing structural stability and reducing vulnerability to rupture. However, a fibrous cap is not always established in atherogenesis; it depends on environmental cues to induce the phenotypic alterations that allow migration of SMCs. SMCs also contribute to atheroma progression by releasing pro-inflammatory cytokines, further promoting the pro-inflammatory cycle of endothelial dysfunction, leukocyte recruitment and cellular inflammatory activation (Cai *et al.*, 2004).

Vascular remodelling can occur in arteries to maintain constant blood flow despite increases in atherosclerotic lesion size. Remodelling can either be inward or outward and can be hypertrophic (thickening of the vascular wall), eutrophic (constant wall thickness), or hypotrophic (thinning of the vascular wall) (Mulvany *et al.*, 1996). This is typically observed in large central elastic arteries and is characterised by increased vessel diameter and thickened intimal and medial layers of the vascular wall (outward hypertrophic remodelling) (O'Rourke and Hashimoto, 2007). Vascular remodelling is believed to be driven by matrix metalloproteinases (MMPs) that degrade extracellular matrix (ECM) macromolecules that lends to the structural integrity of the lesion and strength of the lesion's fibrous cap (Johnson, 2017, Watanabe and Ikeda, 2004, Chávez-Sánchez *et al.*, 2014). Macromolecules composing the ECM include collagen, elastin, and glycoproteins. The ECM also participates in several key events such as cell migration and proliferation, lipoprotein retention and thrombosis (Katsuda and Kaji, 2003). Cellular sources of MMPs within human atherosclerotic plaques include endothelial cells, vascular SMCs, and macrophages in response to stimulation by growth factors (Dollery *et al.*, 1995, George, 2000), cytokines (Galis *et al.*, 1994, Galis *et al.*, 1995) and oxLDL (Rajagopalan *et al.*, 1996). In mature plaques, vascular SMCs can become deprived of matrix-dependant, cell-cell and cell-matrix survival signals, leading to apoptosis which further contributes to necrotic core formation (Katsuda and Kaji, 2003).

Degradation of the protective structural components of the lesion may result in plaque rupture and the exposure of thrombotic factors to reactive clotting agents in

the blood, thus triggering thrombus formation in the lumen (Figure 1.3D). Thrombi can occlude the artery lumen or break free and travel in the circulation to block a narrower vessel downstream of the plaque origins. Depending on the location of the blockage, severe and often fatal clinical complications occur such as type 1 myocardial infarction (MI) (coronary arteries), stroke (carotid and cerebral arteries), peripheral artery disease (PAD) (femoral and popliteal arteries) cardiovascular death (Figure 1.4) (Libby *et al.*, 2002, Libby *et al.*, 2009). Lesion rupture accounts for around two thirds of type 1 MIs, while the remainder are caused when the endothelial is disrupted over the lesion termed plaque erosion and eruption of calcified nodules.

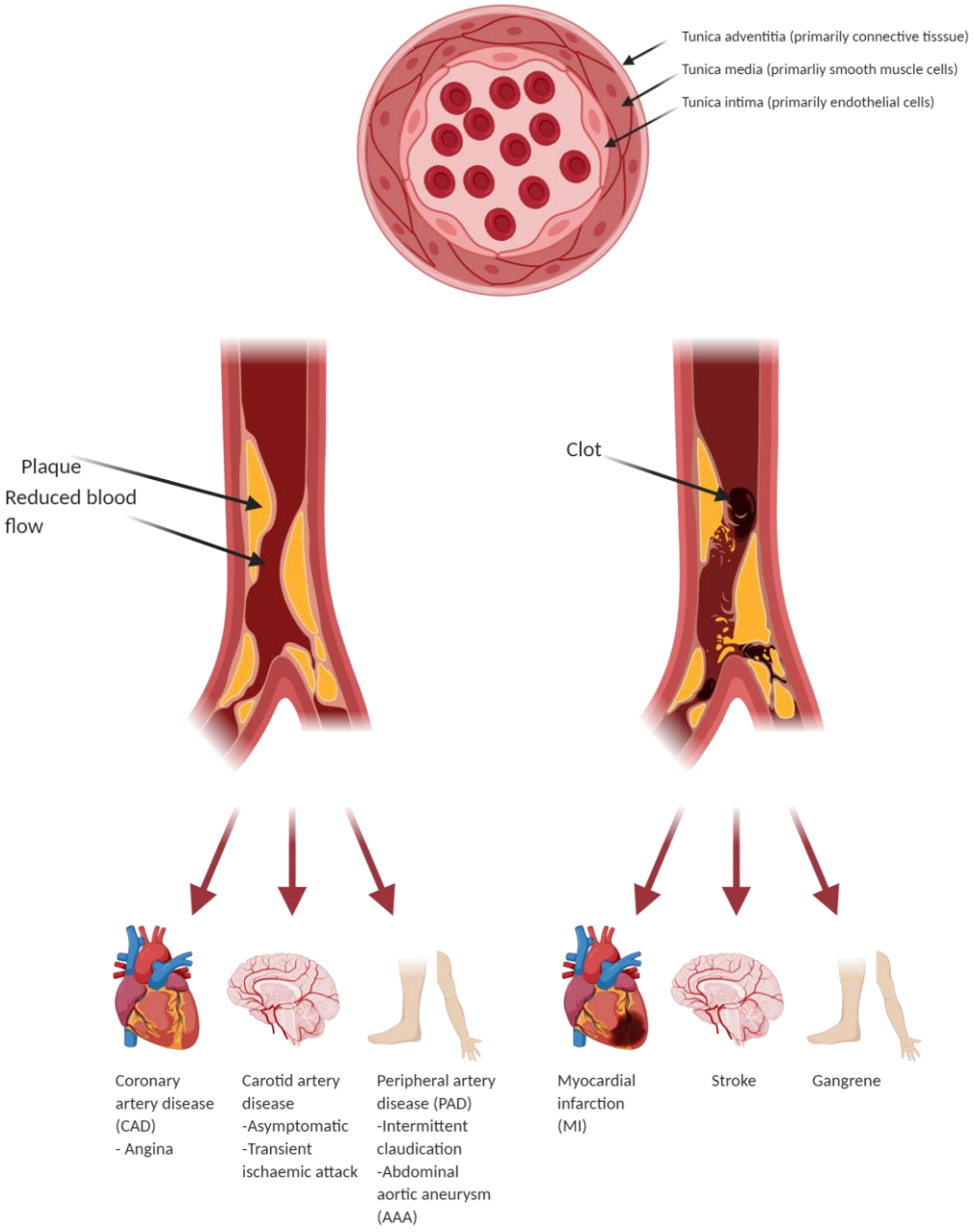


Figure 1.4 Artery section showing stenosis and blockage with associated clinical complications. (A) Cross section of a normal artery. Arteries are made up of multiple distinctive layers: the intima, the inner layer lined by endothelial cells, the media, a layer of smooth muscle and the adventitia, connective tissue anchoring arteries to nearby tissues. (B) As leukocytes and lipids accumulate in the vessel wall during atherosclerosis, the resulting lesions narrows the lumen and restricts blood flow. Clinical complications can occur as a result of restricted blood flow depending on the vascular territory of the lesion. Common sites of atherosclerosis include the abdominal aorta, coronary arteries, popliteal arteries, and carotid arteries. (C) Artery occlusion from thrombus formation can cause severe and often fatal consequences. Adapted from Kelly (2010).

1.1.2 Biological sex differences in cardiovascular disease

CVD is the leading cause of death of both men and women globally. However, there are substantial sex differences in the prevalence and burden of CVD, with men being more than twice as likely to suffer from CVD as women of a similar age (Allender *et al.*, 2020). Sex-related differences have been found in the presentation, prevalence, and clinical outcomes of CVD in many studies. For both men and women, CHD is the largest contributor to CVD morbidity and mortality. The prevalence of CHD is higher in men than women within each age stratum until after 75 years of age, which may contribute to the perception that heart disease is a predominantly male disease (Mosca *et al.*, 2011). This finding is consistent over different socio-economic backgrounds, establishing male sex as an important risk factor (Allender *et al.*, 2020, Levine *et al.*, 2010, Jones, 2010b). Indeed, compared to women, men present with ST-segment elevation myocardial infarction more often and have a higher prevalence of CAD (Sanchis-Gomar *et al.*, 2016).

This difference in sex-related risk is thought to be associated with sex hormones. The initial reasoning was, as men develop CVD more frequently and earlier than their female counterparts, oestrogens are cardioprotective in females, and testosterone is detrimental to the cardiovascular system in males. Epidemiological studies have suggested that premenopausal females have reduced incidence of CVD compared to age-matched males, and the incidence and severity of CVD increases post-menopause (Wake and Yoshiyama, 2009, Iorga *et al.*, 2017). The lower incidence of CVD in women during reproductive age is attributed at least in part to the protective effects of oestrogen (Iorga *et al.*, 2017) with oestrogens reported to improve vascular function and reduce atherosclerosis (Mendelsohn and Karas, 2005, Mendelsohn, 2002, Arnal *et al.*, 2010, Wu *et al.*, 2011, Meyer and Barton, 2009). It is unclear whether an overall poor state of health causes testosterone deficiency, or vice versa. Evidence suggests that a normal physiologic level of testosterone is beneficial, and deficiency is associated with unfavourable outcomes such as increased cardiovascular events.

1.2 Testosterone

Testosterone is the predominant androgen in males, is responsible for developing and maintaining masculine characteristics in reproductive tissues (secondary sexual characteristics, directing sexual differentiation, male phenotype and fertility) and contributes to the anabolic status of somatic tissues. Testosterone, together with its potent metabolite, dihydrotestosterone (DHT), are the principal androgens in the circulation of mature male mammals. Testosterone is synthesised primarily in the Leydig cells in the testes of males, and to a lesser extent in the ovaries of females, with a small amount also produced in the adrenal glands, from the precursory substances dehydroepiandrosterone (DHEA) and androstenedione. Testosterone is biosynthesised in several steps from cholesterol and is converted in the liver to inactive metabolites (Figure 1.5).

Sex Hormone Binding Globulin (SHBG) is the major sex hormone carrier protein in serum. Under physiological conditions, approximately 70% of testosterone is bound to SHBG with high affinity, about 20–30% is weakly bound to albumin, and the remaining 1-2% is free/unbound (Vermeulen and Verdonck, 1968, Dunn *et al.*, 1981).

The classical biological effects of androgens are primarily mediated by binding to the cytoplasmic androgen receptor (AR), following free diffusion across the target cell membrane, then dimerising with a similarly activated AR and translocating to the nucleus to exert transcriptional effects on target genes. Testosterone can also act via the amplification pathway, where it is converted to its more potent metabolite dihydrotestosterone (DHT). DHT potentiates the effects of testosterone action as it binds to the AR with higher affinity and has a slower dissociation rate from the receptor complex, amplifying the effects of testosterone. Alternatively, in the diversification pathway testosterone is converted to 17β-estradiol by cytoplasmic aromatase which then acts upon oestrogen receptors, eliciting diverse effects to AR activation (Rommerts, 2004).

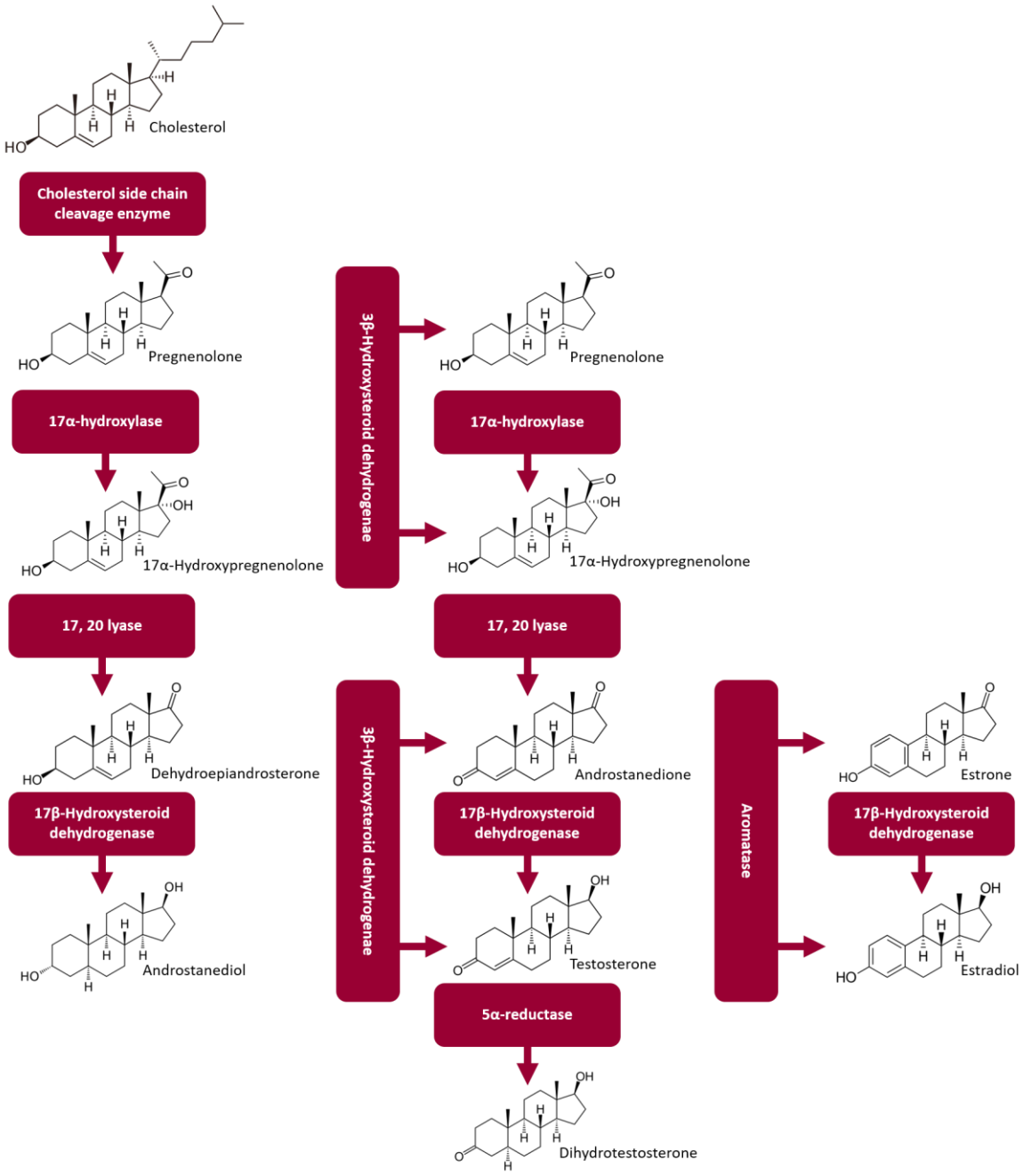


Figure 1.5 The testosterone synthesis pathway. Steroid hormones are derived from cholesterol and are converted by a highly regulated and complex pathway of multiple enzymes which determine the end product.

1.3 Cardiovascular effects of testosterone

Testosterone has received a 'bad reputation' regarding CVD, due to athletes and bodybuilders abusing testosterone and other anabolic steroids with reports of sharply increased risk of high blood pressure, MI, and stroke. It is well established that total, and bioavailable testosterone in men decreases with age, and the age-associated decline may be related to the increased prevalence of CVD and comorbidities. In fact, testosterone deficiency has been reported in population studies to be associated with an increase in all-cause mortality, and this is accounted for mainly by CVD (Khaw *et al.*, 2007, Vikan *et al.*, 2009, Araujo *et al.*, 2011).

Testosterone deficiency is highly prevalent in men with established CVD (Hak *et al.*, 2002, Morris and Channer, 2012, Jones, 2010b). Epidemiological studies have established a negative correlation between serum testosterone levels and CVD outcomes. Low testosterone levels associated with ageing, and related co-morbidities are directly linked with CVD and particularly atherosclerosis (Epstein *et al.*, 2012, Vikan *et al.*, 2009, English *et al.*, 2000a, Makinen *et al.*, 2005, Saigal *et al.*, 2007). It has become increasingly apparent that age-related androgen decline is associated with the pathophysiology of multiple diseases, in particular diseases of cardiovascular and metabolic consequence (Lunenfeld and Nieschlag, 2007, Saigal *et al.*, 2007, Keating *et al.*, 2006).

A decline in circulating testosterone levels is known as hypogonadism, which refers to the diminished functional activity of the gonads that may result in reduced levels of androgens being produced. Age-related reduced testosterone is often referred to as late-onset hypogonadism (LOH), defined as ageing men presenting with serum testosterone levels less than 11nmol/L combined with decreased libido, decreased spontaneous erections and erectile dysfunction by the European Male Aging Study (Wu *et al.*, 2010). The term 'LOH' was renamed and is appropriately now referred to as functional hypogonadism, characterised by borderline low testosterone levels mainly secondary to age-related comorbidities and metabolic derangements such as MetS. Testosterone deficiency is low serum testosterone levels with the absence of other syndromes.

Orchidectomised hypogonadal testicular cancer survivors are also demonstrated to have an increased risk of impaired metabolic function and cardiovascular risk (Bogefors *et al.*, 2017), thus supporting a crucial role of testosterone in atheroprotection, noted by a science advisory from the American Heart Association (Levine *et al.*, 2010). Low levels of endogenous androgens have been related to an increased risk of aortic atherosclerosis (Svartberg *et al.*, 2006) and peripheral arterial disease (Tivesten *et al.*, 2007).

Androgen-deprivation therapy (ADT) is a widely used treatment for prostate carcinoma and has been associated with increased risk of cardiovascular events, including MI and CV mortality (Keating *et al.*, 2006, Tsai *et al.*, 2007, Saigal *et al.*, 2007, D'Amico *et al.*, 2007, D'Amico *et al.*, 2008). More recent evidence from multiple observational studies also suggests that men treated with ADT are at increased risk of cardiovascular events, including MI and stroke (Zhao *et al.*, 2014, Bosco *et al.*, 2015, Keating *et al.*, 2012, Nguyen *et al.*, 2015).

To date there have been few testosterone replacement therapy (TTh) studies published which have been adequately powered to assess whether there is an increase, decrease or no effect on the incidence of major adverse cardiovascular events (MACE). Individual trials have not reported an increase in MACE where testosterone was replaced within physiological levels. Meta‐analysis of 39 randomised controlled trials (RCT) and ten observational studies also did not identify any significant association of TTh with MACE (Alexander *et al.*, 2017). The TRAVERSE trial is a large (6000 participants), ongoing, long-term (five years) randomised blinded placebo-controlled trial of transdermal testosterone gel versus a placebo gel powered to assess MACE. Furthermore, the secondary outcome of the trial is assessing cardiovascular safety. The trial is the first randomised placebo-controlled trial to assess cardiovascular events and safety of this scale and duration and will provide insight into the cardiovascular safety of TTh long-term. The trial commenced in 2018; however, and with a planned intervention duration of five years, it will likely be a decade before its findings are published. Still, RCTs will never be long-term enough (12-years) to adequately reflect the long-term actions of TTh and therefore real-world evidence (RWE) may be more appropriate for assessing cardiovascular safety long-

term. Furthermore, RWE has shown no adverse CV effects on TTh long-term (Haider *et al.*, 2016, Shores *et al.*, 2012, Baillargeon *et al.*, 2014, Saad *et al.*, 2008, Saad *et al.*, 2013, Saad *et al.*, 2016, Traish *et al.*, 2014, Traish *et al.*, 2017, Yassin *et al.*, 2016).

1.3.1.1 The association between low testosterone and risk factors of cardiovascular disease

Low testosterone is associated with a cluster of cardiovascular risk factors including visceral obesity, insulin resistance, dyslipidemia and hypertension, all of which are key components of the metabolic syndrome (MetS) and which strongly predict the later development of CVD (Jones, 2010b). MetS is a clustering of medical conditions that include abdominal adiposity, hypertension, hyperglycaemia, elevated serum triglycerides, and low serum HDL-cholesterol. MetS is associated with the risk of developing T2D, and both are associated with elevated CVD risk. T2D is due to insufficient insulin production from beta or insulin resistance, usually due to obesity and genetic factors. The aforementioned cardiovascular risk factors are also linked with T2D, which is the most prevalent cause of morbidity and mortality in patients with T2D.

Low testosterone levels are highly prevalent in men with T2D (Dhindsa *et al.*, 2004, Ding *et al.*, 2006, Kapoor *et al.*, 2007a, Corona *et al.*, 2006, Corona *et al.*, 2009). Low testosterone levels are linked to the development of a number of the clinical characteristics associated with T2D and MetS, and total testosterone is negatively correlated to insulin levels and insulin resistance in men (Haffner *et al.*, 1994, Simon *et al.*, 1997). Cross-sectional epidemiological studies have also reported an association between low testosterone and T2D (Kapoor *et al.*, 2007a, Ding *et al.*, 2006, Dhindsa *et al.*, 2004). Systematic reviews and meta-analyses support that endogenous total, and free testosterone is lower in men with MetS compared to men without MetS (Brand *et al.*, 2011, Corona *et al.*, 2011). Furthermore, longitudinal studies demonstrate a similar association with a low testosterone concentration is independently predicting the future development of insulin resistance, MetS and T2D (Oh *et al.*, 2002, Haffner *et al.*, 1996, Stellato *et al.*, 2000, Laaksonen *et al.*, 2004, Kupelian *et al.*, 2006, Rodriguez *et al.*, 2007, Selvin *et al.*, 2007). Insulin resistance is the major biochemical defect in men

with MetS or T2D and is considered to be a cardiovascular risk factor that promotes hyperglycaemia, dyslipidemia, hypertension, and endothelial dysfunction (Kelly and Jones, 2013a). The notion that insulin resistance promotes atherosclerosis is supported by a study that demonstrated that it is an independent predictor of coronary artery calcium, a surrogate marker of subclinical coronary artery disease (CAD) (Lee *et al.*, 2009). Coronary artery calcification positively correlates with the total coronary atherosclerosis load and the risk of cardiovascular events (Budoff *et al.*, 2006). Furthermore, testosterone is negatively correlated with calcified plaque volume (Budoff *et al.*, 2017).

Abdominal and subcutaneous adiposity is negatively correlated with low testosterone and reduced lean muscle mass in males (Blouin *et al.*, 2008, Corona *et al.*, 2009). Furthermore, central fat deposits have a high degree of aromatase activity (Vermeulen *et al.*, 2002), an enzyme that metabolises testosterone to estradiol and obese men have higher levels of circulating estradiol than non-obese men (Ding *et al.*, 2006). Obesity is linked with cardiovascular risk factors such as impaired glycaemic control, reduced insulin sensitivity and dyslipidemia (Kapoor *et al.*, 2006, Hackett *et al.*, 2013). A bidirectional relationship between testosterone and obesity underpins this association indicated by the hypogonadal–obesity cycle hypothesis (Kelly and Jones, 2015). The hypogonadal–obesity cycle hypothesis suggests that adipocytes metabolise testosterone to estradiol via aromatase. This facilitates fatty acid uptake and triglyceride storage resulting in increased fat stores and further increasing aromatase activity and driving the cycle forward (Kelly and Jones, 2015). Adipose tissue is the largest endocrine 'organ' and secretes pro-inflammatory adipocytokines such as TNFα, IL-6 and adiponectin (Jones, 2010b). Meta-analyses also suggests that weight loss as a result of diet, exercise or bariatric surgery can significantly increase testosterone levels in men and the increase in testosterone is proportional to the amount of weight lost (Corona *et al.*, 2013).

Obesity frequently co-exists with dyslipidemia that contributes to, and is a consequence of, dysfunctional lipid metabolism and leads to increased adipose deposition, obesity and therefore increased cardiovascular risk. Studies have shown mixed results of the association between low testosterone and serum lipids. Some

studies have shown low testosterone is associated with a pro-atherogenic lipid profile (Haidar *et al.*, 2007), characterised by elevated triglyceride and LDL-cholesterol (Haffner *et al.*, 1993, Barrett-Connor and Khaw, 1988, Simon *et al.*, 1997, Barud *et al.*, 2002, Wu and von Eckardstein, 2003, Dockery *et al.*, 2003, Nishiyama *et al.*, 2005, Braga-Basaria *et al.*, 2006, Yannucci *et al.*, 2006) and decreased HDL (Simon *et al.*, 1997, Van Pottelbergh *et al.*, 2003, Stanworth *et al.*, 2011). However, a few cross-sectional studies have found no association between serum lipid measurements and endogenous testosterone (Kiel *et al.*, 1989, Denti *et al.*, 2000).

1.3.1.2 The association between low testosterone and inflammation

Low testosterone levels are inversely associated with pro-inflammatory biomarkers such as C-reactive protein (CRP), macrophage inflammatory protein 1-α, macrophage inflammatory protein 1-β, and TNFα (Shores *et al.*, 2006, Laughlin *et al.*, 2008). CRP is a sensitive marker of inflammation produced by the liver and is correlated with CHD and deaths from other causes (Khaw *et al.*, 2007, Shores *et al.*, 2006). Circulating pro-inflammatory cytokines are particularly evident in patients with low testosterone levels and obesity (Tivesten *et al.*, 2009). Furthermore, adipokines mediate insulin resistance (Haring *et al.*, 2010) and the significant adipokines include TNFα, IL-1β, IL-6, IL-8, IL-10 and MCP-1 (Smith *et al.*, 2005), all of which play a crucial role in the development of atherosclerosis (Araujo *et al.*, 2007). This relationship is supported by the finding that ADT is associated with increased levels of pro-inflammatory factors and decreased levels of anti-inflammatory cytokines (Maggio *et al.*, 2005, Maggio *et al.*, 2006).

1.3.2 Cardiovascular effects of testosterone replacement

TTh is increasingly prescribed for men with clinical hypogonadism and low testosterone levels. The main target of TTh is to replace levels to within the physiological range and to reverse hypogonadal symptoms, primarily indicated by sexual function measures. Benefits of TTh include increased libido and energy levels, beneficial effects on bone density, strength and muscle mass. However, questions regarding the cardiovascular safety of TTh still persist following the abuse of steroids by athletes. It is important to recognise that the effects of testosterone will depend on

normalisation of levels for an individual as benefits may not occur if there is undertreatment or overtreatment. Supraphysiological levels of testosterone have been shown to be associated with adverse cardiovascular consequences and can cause myocardial stiffening and hypertrophy (Calof *et al.*, 2005, Tambo *et al.*, 2016). Whereas most RCTs using TTh that have normalised testosterone levels have not only shown an improvement in sexual function but also an improvement in risk factors associated with CVD, including insulin resistance, glycaemic control, visceral adiposity and lipid profiles (Table 1.1).

Studies have reported that TTh in obese men reduces weight, BMI, waist circumference and fat mass (Rebuffé-Scrive *et al.*, 1991, Mårin *et al.*, 1992a, Mårin *et al.*, 1992b, Mårin *et al.*, 1995, Saad *et al.*, 2007, Agledahl *et al.*, 2008). Waist circumference was reduced following nine months of TTh in two cohorts of elderly men and a cohort of men with MetS and T2D following 52 weeks of TTh. The Moscow study also reported significant decreases in waist circumference but also weight and BMI following 30 weeks of TTh in men with hypogonadism (Kalinchenko *et al.*, 2010). Furthermore, the TIMES 2 study showed TTh decreased waist circumference and reduced percentage of body fat over 12 months in hypogonadal men with MetS and/or T2D (Jones *et al.*, 2011). Other studies investigating TTh on parameters of obesity have also reported improved body weight, visceral obesity, body fat, BMI and waist circumference in men with MetS, T2D and/or hypogonadism (Kapoor *et al.*, 2006, Boyanov *et al.*, 2003, Svartberg *et al.*, 2008)

TTh increased insulin sensitivity after only three months of treatment and was maintained for at least 12 months (Kapoor *et al.*, 2006, Kalinchenko *et al.*, 2010, Aversa *et al.*, 2010, Jones *et al.*, 2011, Hackett *et al.*, 2014a, Dhindsa *et al.*, 2016). The majority of these studies, however, reported no change in HbA_{1c} (universal measurement for glycaemic control) with only two reporting a small but significant reduction (Kapoor *et al.*, 2006, Heufelder *et al.*, 2009). While many of these studies failed to demonstrate an overall change in HbA_{1c}, they had not exclusively been performed on men with poor control and were not powered to detect changes in glycaemic control. Therefore, no clear conclusion can be made regarding the effect of

testosterone on glycaemic control until a more extensive definitive study with HbA_{1c} as a primary endpoint in hypogonadal men with uncontrolled diabetes is undertaken.

TTh has had mixed effects on serum lipid profiles, with some studies showing no effects at all (Isidori *et al.*, 2005, Huo *et al.*, 2016), whilst others have shown that TTh reduces total cholesterol and LDL-cholesterol by 5-14% from baseline (Malkin *et al.*, 2004a, Malkin *et al.*, 2004b, Kapoor *et al.*, 2006, Jones *et al.*, 2011, Hackett *et al.*, 2014a, Cornoldi *et al.*, 2010, Huo *et al.*, 2016, Isidori *et al.*, 2005) in men with CVD, metabolic syndrome (MetS) and T2D. The effects of TTh on HDL-cholesterol have been even less consistent with varying reports of increasing and decreasing levels or no change at all (Isidori *et al.*, 2005, Huo *et al.*, 2016, Jones and Saad, 2009). TTh has been shown to have no significant effects on triglycerides (Huo *et al.*, 2016, Jones *et al.*, 2011). Jones *et al.* (2011) demonstrated that lipoprotein (a), a strong predictor for the future development of atherosclerosis, significantly decreased after six months of TTh in the overall study population. Moreover, the subgroup of patients with MetS showed significant reductions of lipoprotein a from baseline with TTh versus placebo after six months, reducing CVD risk.

The effects of TTh on systemic inflammation are somewhat inconsistent, a number of trials have recorded suppression of TNFα (Malkin *et al.*, 2004a, Kalinchenko *et al.*, 2010, Malkin *et al.*, 2004b), in contrast, others have not (Pugh *et al.*, 2005). Similarly, serum IL-1β and CRP decreased with TTh in one study (Kalinchenko *et al.*, 2010) but not in another (Dhindsa *et al.*, 2016). Kalinchenko *et al.* (2010) also reported no change in serum IL-6 or IL-10 with TTh. However, another study has reported an increase in serum IL-10 in response to TTh (Malkin *et al.*, 2004a). It is postulated that testosterone has immunomodulatory actions and may reduce systemic inflammation that correlates with atherosclerosis *in vivo*.

	Low endogenous testosterone	Exogenous testosterone replacement
Obesity	Adiponectin ↑ Adipogenesis ↑ Lipolysis ↓	Lean body mass ↑ Body fat ↓ Waist circumference ↓
Dyslipidemia	Total cholesterol ↑ LDL-cholesterol ↑ HDL-cholesterol ↓	Total cholesterol ↓ LDL-cholesterol ↓ HDL-cholesterol ↑
Glucose metabolism	Insulin levels ↑ Insulin resistance ↑ Type 2 diabetes ↑	Insulin sensitivity ↑ Blood glucose ↓ HbA_{1c} ↓
Hypertension	Systolic BP ↑ Diastolic BP ↑	Systolic BP ↓ Diastolic BP ↓
Inflammation	IL-1β ↑ IL-6 ↑ IL-10 ↓ TNFα ↑ CRP ↑	IL-1β ↓ IL-6 ↓ IL-10 ↑ TNFα ↓ CRP ↓

Table 1.1 Summary of effects of testosterone therapy on cardiovascular risk factors. LDL: low-density lipoprotein; HDL: high-density lipoprotein; HbA_{1c}: glycated hemoglobin; BP: blood pressure; TNFα: tumor necrosis factor-α; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-10: interleukin-10; CRP: C-reactive protein; ↑increase; ↓: decrease.

1.4 The effects of testosterone on atherosclerosis

Clarifying the relationship of testosterone with preclinical atherosclerosis could elucidate pathways by which androgens are associated with cardiovascular events and mortality. Carotid intima-media thickness (CIMT) is considered a surrogate marker of preclinical atherosclerosis and is known to predict future vascular events, including stroke and MI (Lorenz *et al.*, 2007). CIMT is a quantitative measure of the extent of carotid atherosclerotic vascular disease and can be measured using external ultrasound to monitor ongoing disease progression and regression in clinical trials (O'Leary and Bots, 2010). The test measures the thickness of the inner two layers of the carotid artery, the intima and media, to detect any thickening when patients are still asymptomatic (Darabian *et al.*, 2013, Naqvi and Lee, 2014). Carotid plaque is also a marker of atherosclerosis and represents a more advanced stage of atherogenesis (Inaba *et al.*, 2012). The degree of atherosclerosis in the carotid artery as assessed by CIMT and plaque scores, the number of ultrasound-detected plaques in the carotid

artery, have been found to be inversely associated with testosterone levels in some studies (Demirbag *et al.*, 2005, Fukui *et al.*, 2003, Muller *et al.*, 2004, Svartberg *et al.*, 2006). Higher normal testosterone levels are also shown to be associated with an improved CIMT and lower occurrence of carotid plaque while higher DHT is also associated with a lower prevalence of carotid plaque in men with pre-existing CAD (Chan *et al.*, 2015).

A study by Vikan *et al.* (2009) investigated the prospective association between testosterone levels and the progression of CIMT and plaque area in men from 1994 to 2001. They also performed a cross-sectional study of 2,290 men from the population in 2001. They found an inverse association between testosterone levels and total carotid plaque area after adjusting for age, systolic blood pressure, smoking and use of lipid-lowering drugs. However, they failed to find any prospective association between testosterone levels and the progression of carotid atherosclerosis.

Soisson *et al.* (2012) investigated whether an inverse correlation between plasma testosterone levels and CIMT could be mediated or modified by traditional cardiovascular risk factors as well as inflammatory status in a population-based cohort study of 354 men aged 65 and over. They reported that analysis with and without adjustment for CVD risk factors showed that CIMT was inversely and significantly correlated with total and bioavailable testosterone levels. Additionally, low bioavailable testosterone combined with low-grade inflammation, assessed by elevated CRP levels, was associated with elevated CIMT. Tsujimura *et al.* (2012) measured CIMT and assessed several metabolic factors in middle-aged healthy Japanese men to clarify the relationship between testosterone and atherosclerosis. Although they found the association between total serum testosterone and CIMT was not significant, they suggested that low bioavailable serum testosterone was an influencing and independent risk factor for CIMT. Svartberg *et al.* (2006) investigated the relationship between testosterone levels and CIMT and found an inverse association between total testosterone levels and atherosclerosis independent of age, CVD risk factors and lifestyle but not independent of BMI. Kwon *et al.* (2014) investigated the relationships between testosterone, MetS, and mean CIMT in ageing men. They suggested that CAD, late-onset hypogonadism, and MetS are not separate

diseases in ageing men and that low-grade inflammation is one possible mechanism behind the relationship between testosterone and CIMT. They found that CIMT, testosterone, and MetS were significantly and independently inversely correlated with each other in men aged between 55 and 58 years.

TTh has also improved vascular dysfunction and reduced inflammation associated with atherosclerosis in hypogonadal men (Malkin *et al.*, 2004a, Malkin *et al.*, 2004b). A RCT evaluated the effect of testosterone on CIMT over 12 months and reported an improvement; however, the study was underpowered (Mathur *et al.*, 2009). Another trial evaluating TTh observed a reduction in CIMT in the treated group and a reduction in CRP over 24 months in men with MetS and hypogonadism (Aversa *et al.*, 2010). However, an adequately powered RCT (TEAAM trial) that studied the effect of TTh over three years did not observe any changes in either CIMT or coronary artery calcium scores (Basaria *et al.*, 2015). A 12 month RCT of hypogonadal men >65 years examined the effect of TTh on coronary artery plaque volume (Budoff *et al.*, 2017). They reported that TTh was associated with a greater increase in noncalcified plaque volume; however, there was no change in the coronary artery calcium score between the groups. Analysis of plaque components found that testosterone therapy significantly increased the fibrous plaque volume compared to placebo. Post-mortem studies have revealed that atherosclerotic lesions associated with coronary thrombosis, sudden death, and rupture typically have less fibrous tissue (Davies *et al.*, 1993b, Davies and Thomas, 1984, Burke *et al.*, 1997, Cheruvu *et al.*, 2007). The changes in noncalcified coronary artery plaque volume, however, were not associated with changes in the levels of total testosterone following TTh. Short-term TTh may promote a more stable plaque that may initially increase in plaque volume. An increase in plaque volume may not be associated with an increased risk of MACEs as the lesion content, stabilisation, and risk of rupture may be more relevant to clinical outcomes. Animal studies have become invaluable in atherosclerosis research due to the difficulties in investigating lesion composition and stability in humans. Animals studies of testosterone treatment compliment clinical research and the value of these investigations may add mechanistic detail and clinical relevance to TTh in men.

1.5 The Y chromosome and atherosclerosis

The human Y chromosome has been shown to exert pleiotropic effects on atherosclerosis susceptibility. Analysis of the male-specific region of the Y chromosome by Bloomer *et al.* (2014) showed that one of its common European lineages, haplogroup I, was associated with increased risk of CAD compared with other haplogroups. This association was independent of conventional cardiovascular risk factors (Bloomer *et al.*, 2013). Furthermore, phylogenetic analysis of 129133 Y chromosomes revealed that haplogroup I1 is associated with an increased risk of CAD and is the only common UK lineage of the Y chromosome associated with CAD in white British men (Eales *et al.*, 2019). Specifically, haplogroup I1 genetic variants show an overlap with transcription start site and enhancer chromatin states in epigenomes from cells and tissues relevant to CAD. Transcriptome-wide analysis of human macrophages also revealed that a majority of autosomal gene sets showing differential expression between haplogroup I and other MSY lineages map to immune and inflammatory pathways (Bloomer *et al.*, 2013). Collectively, this data suggests that the MSY genetically regulates susceptibility to CAD, possibly through modulation of the immune response. This adds to an expanding body of evidence from experimental animal models and human studies suggesting that genetic variation within the Y chromosome plays an important role in male health (Prokop and Deschepper, 2015).

1.6 Animal models of atherosclerosis

Animal models of atherosclerosis are essential to improve the understanding of the molecular mechanisms behind atherosclerotic plaque formation and progression as well as allow assessment of novel pharmacological treatments for atherosclerosis. Typically, animal models of atherosclerosis are based on accelerated plaque formation due to a cholesterol-rich/Western-type diet (pro-atherogenic), manipulation of genes involved in the cholesterol metabolism, and the introduction of additional risk factors for atherosclerosis, such as diabetes (Emini-Veseli *et al.*, 2017).

1.6.1 Mouse models of atherosclerosis

Murine models have become the predominant species to study atherosclerosis *in vivo* due to rapid reproduction, ease of genetic manipulation and induction of atherosclerosis by genetic manipulation and/or dietary intervention over relatively short periods of time (Tannock and King, 2010, Bond and Jackson, 2011, Getz and Reardon, 2012, VanderLaan *et al.*, 2004, Schwartz *et al.*, 2007). However, non-transgenic mice are markedly resistant to the development of atherosclerosis as their lipid profile differs significantly than that of humans and therefore genetic manipulation of their lipid metabolism and dietary intervention is necessary (Getz and Reardon, 2012, Meir and Leitersdorf, 2004).

The mouse genome can be relatively easily altered to over-express or delete genes of interest and has allowed the development of murine models of atherosclerosis (Daugherty, 2002). C57BL/6 mice have been determined to be the most susceptible to dietary-induced atherosclerosis, although this is limited to only small lesions in the aortic root, and was utilised for genetically manipulated models (Daugherty, 2002). The Apolipoprotein E (ApoE) deficient ($ApoE^{-/-}$) and LDL-receptor knockout ($LDLr^{-/-}$) mice are the most well-characterised strains and are the two most popular strains for atherosclerosis research and are both backcrossed to the C57BL/6 genetic background.

Mice transport the majority of cholesterol in HDL-like particles and only have low levels of atherogenic LDL and VLDL. The LDL receptor is essential for lipoprotein clearance in the liver and uptake into macrophages in peripheral tissues and mice deficient in the receptor ($LDLr^{-/-}$ mice) have significantly higher plasma levels of cholesterol. ApoE plays a key protective role in atherosclerosis functioning as a ligand for receptors that clear chylomicron remnant lipoprotein particles and VLDL remnants. ApoE is synthesised mainly in the liver and facilitates hepatic uptake of lipoproteins and stimulates cholesterol efflux from macrophages, maintaining overall plasma cholesterol homeostasis. Deficiency in ApoE ($ApoE^{-/-}$ mice) leads to increased plasma levels of total cholesterol, mostly in the VLDL and chylomicron fractions (Piedrahita *et al.*, 1992) which are increased four-fold by a pro-atherogenic diet (Plump *et al.*, 1992,

Plump and Breslow, 1995). ApoE$^{-/-}$ mice develop progressive lesions more characteristic of those observed in humans and on a pro-atherogenic diet, ApoE-/- mice develop plaques more rapidly and with a more advanced phenotype as compared to LDLr$^{-/-}$ mice, making them the more popular choice for studying experimental atherosclerosis (Silvestre-Roig *et al.*, 2014). Although the lipid profile of LDLr$^{-/-}$ mice, with a higher percentage of cholesterol carried in IDL/LDL particles, more closely resembles that in dyslipidemic humans (Emini-Veseli *et al.*, 2017).

ApoE is synthesised by monocytes and macrophages and is thought to have local effects on cholesterol homeostasis and on inflammatory reactions in atherosclerotic vessels. ApoE specifically expressed in macrophages regulates cholesterol efflux from foam cells and ApoE$^{-/-}$ macrophages effluxed significantly less cholesterol than wildtype macrophages. (Yancey *et al.*, 2007, Zanotti *et al.*, 2011). Furthermore, increased atherosclerosis has been observed in healthy mice reconstituted with ApoE$^{-/-}$ macrophages, independently of changes in the plasma lipoprotein profile (Fazio *et al.*, 1997, Linton *et al.*, 1995). Macrophage-derived ApoE is also internalised by endothelial cells and enhances endothelial NO production by disrupting the inhibitory interaction of eNOS. These results establish a novel mechanism by which ApoE modulates endothelial cell function.

1.6.2 Testosterone and animal studies of atherosclerosis

While clinical studies assessing the effects of testosterone on atherosclerosis are limited in number and lack mechanistic detail, animal models of atherosclerosis and testosterone deficiency have allowed a more direct investigation of the relationship. Several animal models demonstrate that castration in mice (Nettleship *et al.*, 2007a, Casquero *et al.*, 2006) and rabbits (Alexandersen *et al.*, 1999; Bruck *et al.*, 1997; Larsen *et al.*, 1993) results in increased atherosclerosis when fed a pro-atherogenic diet.

Low endogenous testosterone levels in the testicular feminised (Tfm) mouse and surgically castrated XY littermates fed a pro-atherogenic diet was associated with a pro-atherogenic serum lipid profile and fatty streak formation within the aortic root (Nettleship *et al.*, 2007a). Observations from this study suggest that diet-induced fatty

streak formation is a consequence of low endogenous testosterone levels in these mice. This study also demonstrated that this effect of testosterone is independent of a functional AR, since Tfm mice have a non-functional AR and fatty streaks within the aortic root were also observed in surgically castrated male mice (functional AR). Casquero *et al.* (2006) also demonstrated that diet-induced atherosclerosis is more significant in castrated mice and that lesion area in the aortic root was increased by up to 100% compared to intact sham-operated controls.

Testosterone treatment has been demonstrated to reduce aortic lipid accumulation and fatty streak formation in male mice (Nettleship *et al.*, 2007a, Kelly *et al.*, 2012) and rabbits (Larsen *et al.*, 1993, Bruck *et al.*, 1997, Alexandersen *et al.*, 1999, Qiu *et al.*, 2010) when fed a pro-atherogenic diet. These animal studies show beneficial effects of androgen supplementation on atherogenesis in male animals and display evidence for AR-dependent and independent actions (Nettleship *et al.*, 2007a, Nathan *et al.*, 2001, Bruck *et al.*, 1997, Alexandersen *et al.*, 1999). A study using AR knockout (ARKO) male mice and wild-type littermates with an $ApoE^{-/-}$ background observed that physiological testosterone replacement reduced the atherosclerotic lesion area and presence of a necrotic core compared with placebo-treated mice. Although not as extensively as that observed in testosterone-treated orchidectomised wild-type controls (Bourghardt *et al.*, 2010). Contrary to these studies von Dehn *et al.* (2001) reported a decrease in fatty streak formation in the aortic root and ascending aorta after testosterone suppression by administration of the gonadotropin-releasing hormone (GnRH) antagonist. Treatment with testosterone led to small but significant increases in serum cholesterol levels and lesion area. The reason for this discrepancy is unclear; however, many studies provide evidence for a protective role for testosterone against atherogenesis in male animal models.

Serum cytokines TNFα and IL-6 have been reported to be elevated in the Tfm mouse model compared to XY littermates, suggesting that low endogenous testosterone and non-functional AR may influence selective cytokines known to be involved in atherogenesis (Kelly *et al.*, 2012). Serum IL-6 levels were reduced following testosterone treatment in the Tfm mice, suggesting that testosterone has systemic anti-inflammatory actions independent of the AR (Kelly *et al.*, 2012). This may be of

importance as IL-6 has been identified as an independent risk factor for CAD (Yudkin *et al.*, 2000, Luc *et al.*, 2003). Bourghardt *et al.* (2010), however, did not report an effect of testosterone treatment on IL-6 in ARKO mice on the $ApoE^{-/-}$ background. This could be attributed to the extent of the disease progression as Kelly *et al.* (2012) investigated early stages of atherogenesis, where subtle changes may be more apparent when compared to the complex wide-spread plaque formation in the ARKO $ApoE^{-/-}$ mice.

Testosterone treatment reduced the levels of serum TNFα, IL-6, sICAM-1 and MMP2 in castrated male rabbits fed a high-cholesterol diet, while the presence of AR blocker flutamide impeded this effect. Furthermore, testosterone treatment also reduced the plaque area and the aortic intimal thickness, whereas the fibrous cap thickness and collagen contents increased, thus increasing the stability of atheromatous plaque. However, the presence of AR blocker flutamide increased the plaque area and aortic intimal thickness and decreased the thickness of the fibrous cap and collagen contents in castrated rabbits. Suggesting that testosterone treatment plays a vital role in the inhibition of atherosclerosis via the AR (Li *et al.*, 2008).

While most studies investigating the effects of testosterone on atherosclerosis demonstrate an atheroprotective role, the specific actions of testosterone on inflammatory mechanisms in the plaque are still unclear. Further investigations are required to explain these underlying mechanisms and the potential effects of testosterone on atherogenesis. Understanding the influence of testosterone on atherogenesis may further explain some of the CVD benefits of TTh seen clinically and may ultimately lead to a step-change in the therapeutic management of men with increased cardiovascular risk.

1.7 Aims and objectives

Evidence from clinical and animal studies investigating the association between testosterone levels and atherosclerosis highlights the potential benefits of TTh and the notion that testosterone has anti-inflammatory properties is generally well accepted. Many of the increasingly prevalent pathological conditions such as obesity, MetS and T2D that are involved in the atherosclerotic process are interlinked and have shared chronic inflammatory aetiology, suggesting that testosterone therapy has potentially wide-ranging health benefits. Specific actions of testosterone on the chronic inflammatory pathogenesis of atherosclerosis development and progression in the vasculature are complex and not entirely understood.

The aim of this research was to test the hypothesis that 'testosterone treatment reduces inflammation associated with the development of atherosclerosis and improves atherosclerotic burden and cardiovascular risk factors'.

1.7.1 Specific aims

Using a randomised placebo-controlled clinical trial of TTh in hypogonadal men with T2D, an animal model of atherosclerosis and *in vitro* investigations, this research specifically aims to:

- Investigate the effects of testosterone of atheroma formation and composition.
- Investigate the anti-inflammatory effects of testosterone on localised atherosclerotic lesion inflammation and cells implicated in atherogenesis.
- Investigate the effects of testosterone on risk factors of atherosclerosis, including systemic inflammation, glucose regulation and obesity.

2. Materials and Methods

2.1 *In vivo* model of atherosclerosis

2.1.1 The ApoE$^{-/-}$ mouse

The ApoE knockout mouse model was developed in 1992 by homologous recombination of mouse embryonic stem cells, whereby the ApoE gene was disrupted in mouse embryonic stem cells derived from 129/Ola mice and injected into C57BL/6 blastocysts (Piedrahita *et al.*, 1992). Resultant chimaeras were backcrossed for ten generations and inbred to homozygosity. Mice that are homozygous for the ApoE knockout are hypercholesterolemic (~400mg/dL) while being fed standard chow diet. Spontaneous atherosclerotic lesions are detected from 3-4 months of age, mostly in the proximal aorta with lesions at the origins of the coronary artery and also in the pulmonary artery (Reddick *et al.*, 1994). Lesion size increases with age and progresses to a more typical advanced stage of atherosclerotic lesions defined histologically by reduced lipid accumulation, fibrotic conversion of necrotic areas and reduction of stability and disappearance of the fibrous cap (Rosenfeld *et al.*, 2000). The breeding colony of ApoE$^{-/-}$ mice (strain C57BL/6J-A) used in this study were obtained from Charles River Laboratories, UK.

2.1.2 Animal husbandry

ApoE$^{-/-}$ mice were bred in sterile barrier conditions at The University of Sheffield Field Laboratories by technical staff, and male ApoE$^{-/-}$ were transferred to the holding room upon weaning (3 weeks old), where they were maintained for the duration of the experiment. Females not required for breeding were sacrificed via a UK Home Office-approved schedule 1 method once weaned. All animals used for the experimental procedures were maintained in cages containing up to 5 animals, on a twelve-hour light/dark cycle in a temperature (between 19 and 23°C) and humidity controlled (55 ± 10%) environment. All procedures were carried out under the jurisdiction of UK Home Office personal and project licenses (project license number P3714F016, personal license number I1EDE3DC6), governed by the Animals Scientific Procedures Act 1986.

2.1.3 Experimental treatments

Animals were randomly assigned to specific diet and treatment groups after weaning until desired group numbers were reached (Table 2.1).

Species	Subsets	Treatment	n=()
Standard chow	Sham Orchidectomy	N/A	5
	Orchidectomy	N/A	9
High fat 'Western' diet	Sham Orchidectomy	10µL intramuscular saline injection	15
	Orchidectomy	10µL intramuscular saline injection	15
	Orchidectomy	10µL intramuscular sustanon® injection	15

Table 2.1 Animal groups. Animals were randomly assigned to specific diet and treatment groups after weaning until desired group numbers were reached. Sustanon® (20mg/mL testosterone propionate, 40mg/mL testosterone phenylpropionate, 40mg/mL testosterone isocaproate; equivalent to 74mg/mL testosterone)

2.1.3.1 Surgical procedures

2.1.3.1.1 Surgical bilateral orchidectomy

Seven-week old mice were prepared for surgery using 5% inhaled isoflurane (Zoetis, UK), administered via an induction chamber, and then maintained on 1-3% isoflurane for the duration of the procedure. The unrestrained mouse was placed in a supine position on to a clean heat-pad overlaid with a sterile surgical towel with its tail pointing towards the investigator. The scrotum plucked clean and the skin aseptically prepared with 70% ethanol. A small incision of around 5mm was made through the skin in the midline of the scrotum using a sterile No 22 scalpel (Swann-Morten Ltd, UK) the subcutaneous connective tissue was separated so that the testes lying in their intramuscular sacs were visible. If necessary, pressure was placed on the lower abdomen to push the testes down into the scrotum. A small incision was made along the exposed tunica of each of the testes, and the testes, vas deferens and fat pad were squeezed through the incision and clamped with forceps. An absorbable suture was

used, and a single ligature was placed around each vas deferens and blood vessel which were severed distal to the ligature using a shearing motion with dissecting forceps and the testes removed. The remaining vas deferens and fat-pad were pushed back into the sac, and the skin incision was closed with a resorbable 5-0 sutures (Ethicon Ltd, UK). The respiratory rate was visually monitored carefully throughout the surgical procedure. All animals then received an analgesic agent, 50µL subcutaneous injection of 5mg/mL meloxicam (Metacam, Boehringer Ingelhein, Germany) to ensure post-operative pain relief. Animals were then placed in a warm recovery chamber and allowed to regain full consciousness and movement before being returned to their cages.

2.1.3.1.2 Sham orchidectomy

Seven week-old mice were prepared for surgery as described in section 2.2.3.1.1. A small incision of around 5mm was made through the skin in the midline of the scrotum. Using a sterile No 22 scalpel (Swann-Morten Ltd, UK) the subcutaneous connective tissue was separated so that the testes lying in their intramuscular sacs were observed. The area was probed for approximately 5 minutes, in order to mimic the same tissue disruption and time taken under general anaesthesia in these animals as in the orchidectomised animals. The skin incision was closed with resorbable 5-0 sutures (Ethicon Ltd, UK). The respiratory rate was monitored carefully throughout the surgical procedure. All animals then received an analgesic agent, 50µL subcutaneous injection of 5mg/mL meloxicam to ensure post-operative pain relief. Animals were then placed in a warm recovery chamber and allowed to regain full consciousness and movement before being returned to their cages.

2.1.3.2 Atheroma promotion

At age eight weeks, ApoE$^{-/-}$ mice either continued on a normal chow diet or were placed on a high fat "Western" diet (Special Diet Services, UK), for a period of 17 weeks, ad libitum. Prior to receiving a "Western" diet, animals received a normal chow diet. Normal chow diet composition % (wt/wt) simple sugars 7%, fat 3%, polysaccharide 50%, protein 15% (w/w). Energy content 3.5 kcal/g (fat 3% kcal, protein 15% kcal, carbohydrate 82% kcal). Western diet composition g% (wt/wt): Sucrose

33.9%, milk fat 20%, Casein 19.5%, Maltodextrin 10%, Corn starch 5%. Cellulose 5%, Corn oil 1%, Calcium Carbonate 0.4%, L-cystine 0.3%, Choline Bitartrate 0.2%, Cholesterol 0.15%, Antioxidant 0.01%, AIN-76-mx 3.5%, AIN-76A-VX 1%. Energy content 4.63 kcal/g (fat 42% kcal, protein 15% kcal, carbohydrate 43% kcal).

2.1.3.3 Testosterone treatment

Mice that underwent testosterone or saline treatment received this via intramuscular injections. Animals were anesthetised using 5% inhaled isoflourane, administered via an induction chamber, and then maintained on 1-3% isoflourane for the duration of the procedure. The injection site of hind leg was gently wiped clean using a 70% ethanol and a sterile 0.5mL 30G needle (Beckett Dickinson, UK) was introduced at a right angle to the skin surface into the centre of the muscle mass and 10µL of either 100mg/mL (50mg/kg) testosterone esters (Sustanon®100 (testosterone propionate 20mg/mL, testosterone phenylpropionate 40mg/mL, and testosterone isocaproate 40mg/mL), Organon Laboratories Ltd., Cambridge, UK) or physiological saline was injected. Previous studies have determined the half-life of Sustanon®100 to be around 6 days in mice of the same background strain and of similar weight. Animals were then returned to cages. Mice were injected once fortnightly, from eight weeks of age, alternating the leg injected to minimise discomfort or irritation. Animals were carefully monitored for the duration of the study and were weighed on a weekly basis.

2.1.3.4 Blood glucose measurement

Blood glucose was measured at eight (baseline), 17 (mid-point) and 25 weeks old (end-point). Mice were fasted for four hours before blood was collected from the tail vein and measured using an Accu-Check blood glucose meter and Accu-Check blood glucose test strips (Roche, UK).

2.1.4 Collection of animal tissues

At the end of the 17 week-long treatment period (25 weeks old) mice were killed by cardiac puncture whilst the mouse was under deep terminal anaesthesia but whilst the heart was still beating. Blood samples were taken from the ventricle, which was accessed through the diaphragm. Blood was withdrawn slowly to prevent the

heart collapsing. The left ventricle was injected with 10mL of cold saline to flush blood from the vasculature.

2.1.4.1 Serum collection

Whole blood for serum measurements was collected via cardiac puncture using a 2mL syringe (Beckett Dickinson, UK), collected into 1.5mL Eppendorf tubes and allowed to clot for a minimum of 30 minutes at room temperature. Whole blood was then centrifuged at 800 *g* for 10 minutes at room temperature and the serum removed and frozen in 75µL aliquots containing 2µL protease inhibitor cocktail (Sigma Aldrich, UK) at -80°C, until analysis. All analyses were carried out on non-pooled serum, and samples only underwent one freeze-thaw cycle to maintain sample integrity.

2.1.4.2 Tissue collection

For aortic dissection, the chest wall was removed to expose heart and lungs and the lungs removed to expose the heart. The heart, with the thoracic aorta attached was carefully dissected free by blunt dissection until the entire heart and thoracic aorta was free from the back of the chest wall.

The basal half of the ventricles was cut on a plane formed by drawing a line between the tips of the atria, and the tissue removed. Dissection at this angle resulted in a final tissue orientation that allowed true cross sections of the aortic root to be obtained, by compensating for the angle at which the aorta leaves the heart. The ascending aorta and aortic arch was then removed and any extraneous tissue was trimmed, taking care not to remove any tissue from the heart or aorta, and fixed in 4% paraformaldehyde at 4°C for subsequent *en face* evaluation. The upper half of the heart, with the aortic root and initial section of the aortic arch still attached, was placed in a PVC cryogenic tissue mold measuring 13mm x 5mm (Fisher Scientific, UK) and embedded in optimum cutting temperature (OCT) compound (Bright Cryo-M-Bed; Bright Instrument Company Ltd., UK), with the aorta centered and facing upwards. Hearts were then frozen in liquid nitrogen-cooled isopentane. Tissues were individually processed for histological, gene and protein analysis and were archived for future analysis. Analyses were made on individual samples.

2.1.4.3 Cryosectioning

OCT embedded mouse hearts were removed from -80°C storage and left at -20°C for 30 minutes to equilibrate. Starting at the apex and moving towards the base of the heart, transverse 10µm sections were taken through the tissue using a Leica CM1950 UV cryostat at -20°C. Unstained sections were examined on an Olympus CH2 microscope to check for aortic positioning and sections were discarded until the early appearance of the aortic valve leaflets became visible. Once the appropriate region was located, 10µm sections through the aortic sinus (approximately 50 per mouse) were collected onto positively charged slides (Leica, UK) for histological analysis or uncharged slides (Thermo Scientific, UK) for laser capture microdissection until the 3-valve cusps or the characteristic architecture of the aortic wall disappeared. Slides were stored at -80°C for later histological, gene and protein analysis.

2.1.5 Analysis

2.1.5.1 Measurement of serum hormones via ELISA

2.1.5.1.1 Testosterone

Total testosterone levels in the serum of the ApoE$^{-/-}$ mice were analysed using a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding (cat number: EIA1559) (DRG Diagnostics, Germany). The microtiter wells were pre-coated with a monoclonal antibody directed towards a unique antigenic site on the testosterone molecule.

2.1.5.1.2 Estradiol

Estradiol levels in the serum of the ApoE$^{-/-}$ mice were analysed using a solid phase ELISA, based on the principle of competitive binding (cat number: EIA2693) (DRG Diagnostics, Germany). The microtiter wells are pre-coated with a polyclonal antibody directed towards an antigenic site of the estradiol molecule.

2.1.5.1.3 Insulin

Insulin levels in the serum of the ApoE$^{-/-}$ mice was analysed using a solid phase ELISA, based on the principle of competitive binding (cat number: EIA2935) (DRG Diagnostics, Germany). The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the insulin molecule.

2.1.5.1.4 Serum hormone ELISAs procedure

Test samples were incubated in the well coated with the anti-hormone antibody conjugated with Biotin. After incubation the unbound conjugate was washed off. During the second incubation step Streptavidin Peroxidase Enzyme Complex binds to the biotin-anti-hormone antibody. The amount of bound HRP complex is proportional to the concentration of target hormone in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of target hormone in the test sample.

All reagents and multiwell plates in the hormone ELISA kits were allowed to equilibrate to room temperature following 4°C storage. Twenty-five microlitres of hormone standards; testosterone: 0, 0.2, 0.5, 1, 2, 6, and 16ng/mL, estradiol: 0, 25, 100, 250, 500, 1000, 2000pg/mL and insulin: 0, 6.25, 12.5, 25, 50 and 100µIU/mL and test serum were dispensed into the appropriate wells in duplicate, followed by the addition of 200µL of enzyme conjugate to each well. The plate was mixed for 10 seconds on a plate shaker and allowed to incubate at room temperature for 60 minutes for testosterone and estradiol and 30 minutes for insulin. Following incubation, the wells were emptied by inversion and 400µL/well of wash solution was added. The wash solution was emptied from all wells and the plate was blotted onto clean absorbent paper to ensure complete removal of residual droplets. This wash procedure was repeated 3 times.

The substrate solution (200µL) for testosterone and estradiol was then added to each well and the plate incubated at room temperature for 15 minutes before the addition of 100µL of stop solution (0.5M H_2S0_4) to terminate the enzymatic reaction. For insulin, the enzyme complex (50µL) was added and incubated for 15 minutes at

room temperature followed by the addition of 50µL of stop solution (0.5M H_2S0_4) to terminate the enzymatic reaction. The absorbance of the well contents for each hormone ELISA was determined using a CLARIOstar reader (BMG Labtesch, Germany) at 450±10nm within 10 minutes of stop solution addition.

Serum testosterone concentrations were calculated from the standard curve in ng/mL using Microsoft Excel (Microsoft, USA) and converted into the more widely applied SI units (nmol/L) via multiplication by the testosterone conversion factor (3.467). Similarly, serum estradiol concentrations were calculated from the standard curve in pg/mL using Microsoft Excel (Microsoft, USA) and converted into the more widely applied SI units (pmol/L) via multiplication by the testosterone conversion factor (3.671).

2.1.5.2 Measurement of serum lipids

Total cholesterol, high-density lipoprotein-cholesterol (HDL-C) and triglycerides were measured using a VITR0S®5, 1 FS high capacity chemistry system (Orthoclinical 186 Diagnostics, UK) with parallel processing by Emilia Bettell at the Department of Clinical Chemistry, Sheffield Children's Hospital. The VITROS® chemistry technology was performed on the MicroSlide, an entire integrated test environment on a thin piece of layered film. Mouse serum was thawed at 4°C and diluted two-fold with 7% BSA (VITROS®). Seventy microlitres of sample was transferred to a loading cup and placed in a rack in the VITROS®5, 1 FS analyser. All three lipid measurements were obtained from each sample and values were adjusted for dilution factors. The principles underlying the three measurements are outlined below.

2.1.5.2.1.1 Cholesterol

Cholesterol was measured enzymatically in a series of coupled reactions that hydrolyse cholesteryl esters and oxidize the 3-OH group of cholesterol. Five and half microlitres of sample was transferred to the microslide which is coated with Triton X-100 to aid sample the dissociation of the cholesterol and cholesterol esters from lipoproteins. Free cholesterol is then oxidised, forming cholestenone and hydrogen peroxide. One of the reaction by-products, hydrogen peroxide oxidises a leuco dye and

is measured quantitatively in a peroxidase catalyzed reaction that produces a colour (Allain *et al.*, 1974). The colourimetric density of the dye that is formed is measured by reflectance spectrophotometry (VITROS®) and the colour intensity is proportional to cholesterol concentration.

2.1.5.2.1.2 Triglycerides

Triglycerides are measured enzymatically in a series of coupled reactions in which triglycerides are hydrolysed to produce glycerol and free fatty acids. 5.5µL of sample was transferred to the microslide which is coated with Triton X-100 to aid sample distribution and dissociation of triglycerides from lipoproteins. The triglyceride molecules are hydrolysed by lipase to generate glycerol and fatty acids. Glycerol diffuses into the underlying layer where it is phosphorylated by glycerol kinase, to L-α-glycerophosphate, in the presence of ATP and $MgCl_2$ on the slide. L-α-glycerophosphate is then oxidised to dihydroxyacetone phosphate and hydrogen peroxide by L-α-glycerophosphate oxidase. Hydrogen peroxide oxidises a leuco dye and is measured quantitatively in a peroxidase catalysed reaction that produces a colour (Spayd *et al.*, 1978).

The colourimetric density of the dye that is formed is measured by reflectance spectrophotometry and the colour intensity is proportional to triglyceride concentration.

2.1.5.2.1.3 High density lipoprotein cholesterol (HDL-cholesterol)

HDL-cholesterol is measured enzymatically in a series of coupled reactions in which HDL-derived cholesterol esters are hydrolyzed to cholesterol and fatty acids. Ten microlitres of sample was transferred to the multi-layered microslide and non-HDL is precipitated using phosphotungstic acid (PTA) and magnesium chloride within the microslide. Emulgen B-66 aids the selective dissociation of cholesterol and cholesterol esters from the HDL lipoprotein complexes in the sample. Cholesterol esters hydrolase in the underlying layer of the slide and catalyses the hydrolysis of HDL-derived cholesterol esters to cholesterol and fatty acids. Free cholesterol is then oxidised forming cholestenone and hydrogen peroxide, which oxidises a leuco dye and is

measured quantitatively in a peroxidase catalyzed reaction that produces a colour (Allain *et al.*, 1974, Burstein *et al.*, 1970). The colourmetric density of the dye that is formed is measured by reflectance spectrophotometry and is proportional to the HDL concentration of the sample.

2.1.5.2.1.4 Low density lipoprotein cholesterol (LDL-cholesterol)

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol using the Friedewald equation (Friedewald *et al.*, 1972). As very low density lipoprotein (VLDL) carries the majority of the circulating triglycerides, VLDL-cholesterol can be estimated from the measured total triglycerides divided by 2.2 (Warnick *et al.*, 1990).

LDL-C is then calculated as: **LDL-cholesterol = TC - (measured HDL-cholesterol + estimated VLDL-cholesterol)**

2.1.5.3 Measurement of serum cytokines by Cytometric Bead Array

Becton Dickinson (BD™) Cytometric Bead Array (CBA) is a flow cytometry application that allows quantification of multiple proteins simultaneously. CBA combines the principles of ELISA and flow cytometry.

Capture beads contain unique amounts of a single red dye that have unique spectral properties distinguishable by flow cytometric analysis, allowing analysis of samples. The distinct bead populations are coated with antibodies directed against a particular soluble protein target. The targets are captured to the beads and detected with a secondary fluorescent antibody against the analyte of interest, which forms a sandwich complex. Using flow cytometry for excitation and subsequent detection of emission of the fluorochromes conjugated to the detection antibody, which is different to the emission wavelength of the bead dye, analytes can be measured quantitatively against a range of standards with known concentration. Multiple distinct bead sets can be added to an individual sample to detect multiple targets simultaneously, allowing for small sample volumes to be analysed for multiple analytes. In the present study the BD™ CBA soluble proteins, targeted against IL-1β (Cat No.:560232), IL-6 (Cat No.:558301), IL-10 (Cat No.:558300) MCP-1 (Cat No.:558342), TNFα (Cat No.:558299),

IFNγ (Cat No.:558296), E-selectin (Cat No.:560384) and L-selectin (Cat No.:560385) (BD, UK) were analysed.

All reagents and 96-well assay plates were supplied by Becton Dickinson (BD, UK). All sample preparation and analysis was performed by Susan Clark as part of the University of Sheffield core research facilities service.

All reagent working solutions were made up fresh on the day and stored at 4°C until use. Serum samples were thawed at 4°C. A standard curve of known concentrations of each target was produced by serially diluting the standards. 96-well reaction plates were pre-wet with 100µL of wash buffer added to each well, followed by immediate removal. Capture bead working solution was vortexed directly before use and 25µL was added to each assay well. Twenty-five microlitres of sample or known standard was added to each assay well. The plate was then mixed for 5 minutes on a shaker at 500rpm and then incubated at room temperature for 1 hour. Twenty-five microlitres of PE detection reagent working solution was added to each assay well. The plates were then mixed on a shaker at 500rpm and incubated at room temperature for 2 hours. Assay wells were drained and the beads were resuspended in 150µL of wash buffer per well, followed by shaking for 5 minutes. The assay plate was measured on the BD FACS Array™ flow cytometer and analysed using FCAP Array™ software (Figure 2.1).

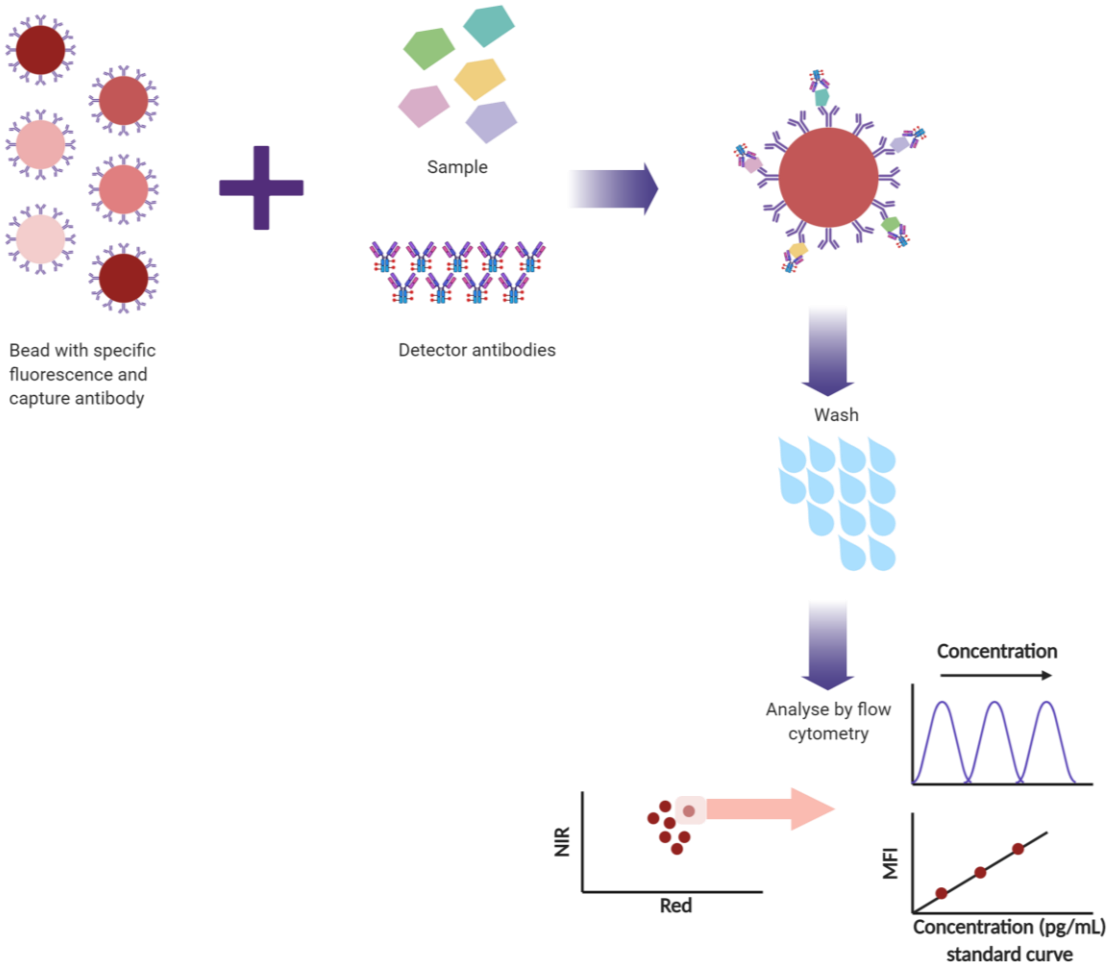


Figure 2.1 Cytometric Bead Array (CBA) principle. Each capture bead in the array has a unique fluorescence intensity and is coated with a capture antibody specific for a single analyte. A combination of different beads is mixed with a sample or standard and a mixture of detection antibodies that are conjugated to a reporter molecule (PE). Following incubation and subsequent washing, the samples are acquired on a flow cytometer. The FCAP Array analysis software gates each individual bead population and determines the median fluorescence intensity (MFI) for each analyte in the array. From this a standard curve can be generated and sample concentrations were calculated relative to this. (Adapted from bdbiosciences.com)

2.1.5.4 Analysis of adhesion molecules by ELISA

The mouse VCAM-1 and ICAM-1 solid-phase sandwich ELISAs were designed to measure the amount of the target bound between a matched antibody pair. The target-specific antibody was pre-coated in the wells of the supplied microplate. Samples, standards, or controls were then added into these wells and bound to the immobilized (capture) antibody. The sandwich was formed by the addition of the second (detector) antibody, a substrate solution is added that reacts with the enzyme-antibody-target complex to produce measurable signal. The intensity of the signal is directly proportional to the concentration of target present in the serum.

All reagents and multiwell plates in the VCAM-1 ELISA kit (Cat No.: EMVCAM1) and ICAM-1 ELISA (Cat No.: EMICAM1ALPHA) (Invitrogen, USA) were allowed to equilibrate to room temperature following 4°C storage. For VCAM-1 100µL of standards (0, 2.05, 5.12, 12.8, 32, 80, 200 and 500pg/mL) and test serum (diluted 1:400 in assay diluent) were dispensed into the appropriate wells in duplicate, wells were covered and incubated for 2.5 hours at room temperature with gentle shaking. For ICAM-1 100µL of standards (0, 24.69, 74.07, 222.2, 666.7, 2000 and 6000pg/mL) and test serum (diluted 1:1 in assay diluent) were dispensed into the appropriate wells in duplicate, wells were covered and incubated for 2.5 hours at room temperature with gentle shaking.

Following incubation, the wells were emptied by inversion and washed with 300µL/well of wash buffer. The wash solution was emptied from all wells and the plate was blotted onto clean absorbent paper to ensure complete removal of residual droplets. This wash procedure was repeated 4 times. 100µL of biotinylated antibody was then added to each well and the plate incubated at room temperature for 1 hour with gentle shaking. Following incubation, the wells were emptied by inversion and washed with 300µL/well of wash buffer. The wash solution was emptied from all wells and the plate was blotted onto clean absorbent paper to ensure complete removal of residual droplets. This wash procedure was repeated 4 times before the addition of 100µL of Streptavadin-HRP solution to each well, which was incubated for 45 minutes at room temperature with gentle shaking. Following incubation, the wells were emptied by inversion and washed with 300µL/well of wash buffer. The wash solution

was emptied from all wells and the plate was blotted onto clean absorbent paper to ensure complete removal of residual droplets and repeated 4 times. 100µL/well of TMB substrate was added and incubated for 30 minutes at room temperature in the dark with gentle shaking. The plate was evaluated within 30 minutes of stopping the reaction. The absorbance was measured on an CLARIOstar reader (BMG Labtesch, Germany) at 450nm.

2.1.5.5 Quantification of aortic lipid deposition using oil red O staining

Oil red O (ORO) is a lysochrome (fat-soluble dye) used for staining neutral triglycerides and lipids in pathological tissue. Lysochromes stain by preferential solubility, lipids will take up the dye and appear deep red, whilst phospholipids appear pink.

In the present study, ORO was used for the detection and localisation of lipid deposition within the aortic root of previously frozen heart sections. Prior to staining of the aortic root with ORO (Sigma-Aldrich, UK), the stock solution; 1g ORO powder was dissolved in 200mL isopropyl alcohol (Fisher Scientific, UK), thoroughly mixed and the working solution (3:2 stock solution:double distilled water) filtered through student grade filter paper (Whatman, UK) at room temperature was prepared. The working solution required use within two hours of preparation.

Frozen sections, six-nine per animal distributed evenly throughout the aortic root (approximately every 8^{th} serial section) were air dried for 30 minutes at room temperature. The sections were fixed in 10% formalin (Leica, UK) for 45 minutes on the bench then rinsed with tap water. Sections were dipped in 60% isopropyl alcohol for 30 seconds and stained for 1 hour with ORO. Slides were then briefly rinsed in 60% isopropyl alcohol until the non-lipid areas of the sections appeared colourless (approximately 5-10 seconds), and further washed in tap water. Slides were then dipped in Harris's haematoxylin (20% v/v) (Leica, UK) for 1 minute to counterstain the nuclei, and then washed well in tap water to blue the counterstain and remove any excess stain. Finally, slides were mounted using 60°C heated glycerol gelatin (Sigma-Aldrich, UK).

Following ORO staining, the sections of aortic root were digitally photographed using an Olympus BX60 research microscope and cellSens software (Olympus, Japan). Quantification of the lipid stained areas was performed with computer-assisted morphometry, using Image J software (National Institute of Health, USA). Analysis was performed by manually outlining the outer and inner medial areas of the aortic root, followed by outlining the lesions from the internal elastic lamina to the luminal edge. The lipid-stained areas were expressed as a percentage of the medial area. Measurements were taken from 6 sections per mouse and median values calculated.

Lesion thickness and area were measured using Image J software (National Institute of Health, USA) by manually outlining the lesions from the internal elastic lamina to the luminal edge. Measurements were taken from 6 sections per mouse and median values calculated.

2.1.5.6 Quantification of aortic collagen content using Masson's trichrome staining

Following fixation (see section 2.1.5.5) Masson's Trichrome (Bio-Optica, Miller&Miller (Chemicals) Ltd, Hainault UK) was used for collagen visualisation. Sections were stained with Weigert's iron hematoxylin for 10 minutes and briefly washed with distilled water. The sections were then stained with Ponceau acid fuchsin for 4 minutes and washed in distilled water and were treated with phosphomolybdic acid for 10 min. Then, sections were stained with Masson's aniline blue for 5 minutes. Sections were washed in distilled water, dehydrated in IMS (3 x 5 minutes), cleared in SubX (Leica Microsystems, Milton Keynes UK) (3 x 5 minutes) and mounted in Pertex (Leica Microsystems, Milton Keynes UK). Collagen content and necrotic core area were measured/assessed using Image J software (National Institute of Health, USA). Collagen content analysis was performed by manually outlining the outer and inner medial areas of the aortic root, followed by outlining the lesions from the internal elastic lamina to the luminal edge. The collagen-stained areas were expressed as a percentage of the medial area. Measurements were taken from 6 sections per mouse and median values calculated.

2.1.5.7 Analysis of lesion composition by immunohistochemistry

Aortic root samples were further investigated by immunohistochemistry for a visual indication of lesion composition. Sections double stained for targets, and lesion areas matched for comparison. Antibodies targeting vWF were used to identify the endothelial lining of the aorta, anti-MOMA2, a mouse-specific monocyte/macrophage marker, used to identify these cells in the vessel wall and αSMA, used to identify smooth muscle cells. Antibodies targeting E-selectin, ICAM-1 and VCAM-1 were used to identify localised inflammation within the aortic root. Antibodies targeting MMP9, 10 and 13 were used to assess lesion stability. Isotype controls were used to check non-specific background signal (Table 2.2).

The fixation method, blocking, antibody dilution, and incubation period was optimised to give the strongest specific antigen staining with the lowest non-specific binding for each antibody (Table 2.2). Once this was established, the same procedure was repeatedly utilised for immunostaining.

Sections were removed from -80°C storage and allowed to air dry for 30 min. During this time, sections were encircled with a wax hydrophobic pen (Vector Labs Inc, UK) to form a barrier around the sample. Sections were then fixed by submerging in ice-cold acetone for 5 min followed by 10 min air drying. Blocking with 5% goat serum for 30 min at room temperature in a humidified chamber was followed by incubation with 75µL (enough to completely cover the section) of pre-optimised concentrations of primary antibodies overnight in a humidified chamber at 4°C. Unbound antibody was then removed by 3 x 5 min washes in PBS. 75µL of pre-optimised concentrations of appropriate secondary antibody was applied to the sections and incubated for 1 hour at room temperature in the dark and in a humidified chamber. The wash procedure was repeated, and sections were completely covered with Sudan black solution for 15 minutes at room temperature. To make the Sudan black solution, 0.3% w/v of Sudan black B powder was dissolved in 70% v/v ethanol with continuous stirring at room temperature in the dark for 2h. The resulting solution was filtered through student grade filter paper (Whatman, UK), and stored at 4°C until use. Following Sudan black staining to block autofluorescence, sections were then washed briefly with PBS, 8 times in succession to remove excess Sudan black stain and mounted in Vectashield

mounting medium for fluorescence (Vector Labs Inc, UK) containing DAPI to stain the nuclei. Sections were covered with coverslips and were sealed at the edges with nail varnish to prevent tissue drying. Negative controls omitting the primary antibody were included to determine nonspecific binding of the secondary antibodies and isotype controls were included to determine nonspecific staining of the primary antibodies. Sections were examined and images captured using a Zeiss 800 laser scanning confocal microscope and ZEN 2 (Blue Edition) software (Carl Zeiss Ltd, UK). Quantification of the positively stained areas was performed with computer-assisted morphometry, using Image J software (National Institute of Health, USA). Analysis was performed by manually outlining the outer and inner medial areas of the aortic root, followed by outlining the lesions from the internal elastic lamina to the luminal edge and taking the fluorescence intensity. The positively stained areas were expressed as a percentage of the medial area. Fibrous cap thickness was measured using ZEN 2 (Blue Edition) software (Carl Zeiss Ltd, UK).

Antibody	Isotype	Company	Dilution	Secondary
MOMA2	IgG2b (rat monoclonal)	Abcam (ab33451)	1:25-1:200 **1:50**	Goat anti-rat Alexa Fluor 488
vWf	IgG (rabbit polyclonal)	Abcam (ab9378)	1:50-1:500 **1:100**	Goat anti-rabbit Alexa Fluor 594
E-selectin	IgG2b (rat monoclonal)	Abcam (ab2497)	1:25-1:200 **1:50**	Goat anti-rat Alexa Fluor 488
αSMA	IgG (rabbit polyclonal)	Abcam (ab32575)	1:50-1:200 **1:100**	Goat anti-rabbit Alexa Fluor 594
ICAM-1	IgG2b (rat monoclonal)	Abcam (ab119871)	1:25-1:200 **1:50**	Goat anti-rat Alexa Fluor 488
VCAM-1	IgG (rabbit polyclonal)	Abcam (ab134047)	1:12.5-1:100 **1:25**	Goat anti-rabbit Alexa Fluor 594
MMP9	IgG (rabbit polyclonal)	Abcam (ab38898)	1:250-1:2000 **1:1000**	Goat anti-rabbit Alexa Fluor 488
MMP10	IgG (rabbit polyclonal)	Abcam (ab59437)	1:100-1:500 **1:200**	Goat anti-rabbit Alexa Fluor 488
MMP13	IgG (rabbit polyclonal)	Abcam (ab84594)	1:200-1:100 **1:400**	Goat anti-rabbit Alexa Fluor 488
Isotype control	IgG2b (rat monoclonal)	Abcam (ab18541)	**1:50**	Goat anti-rat Alexa Fluor 488
Isotype control	IgG (rabbit polyclonal)	Abcam (ab172730)	**1:25**	Goat anti-rabbit Alexa Fluor 594

Table 2.2 Antibodies used for immunohistochemistry. Summary of the antibodies used for target protein detection in mouse aortic root sections following optimisation of methods. The range of antibody dilutions tested is shown, with the selected optimum dilution in bold.

2.1.5.8 Analysis of lesion inflammation by qRT-PCR

2.1.5.8.1 Laser capture microdissection

Laser-capture microdissection (LCM) is a method to procure subpopulations of tissue cells under direct microscopic visualisation. LCM technology harvests cells of interest directly to give histologically pure enriched cell populations.

Frozen sections, six-nine per animal distributed evenly throughout the aortic root (approximately every 8th serial section) were fixed in 75% industrial methylated spirit (IMS) (Fisher, UK) for 30 seconds, washed in double distilled H_2O and stained with Toludine Blue O (TBO) solution (0.1% w/v TBO (Sigma Aldrich, UK) dissolved in phosphate buffered saline (Giboco, UK). Sections were dehydrated in 70%, 95% and 100% IMS for 30 seconds each and dipped in xylene (Leica, UK) for 5 minutes. Sections were allowed to air dry for 20 minutes.

LCM was performed using the PixCell II laser-capture microdissection system (Arcturus Engineering, Mountain View, CA, USA) and Arcturus® CapSure™ Macro LCM Caps (Applied Biosystems, UK). The air dried, TBO stained section was overlaid with the CapSure cap, which consists of a thermoplastic film mounted on a transparent cap. A focused infrared laser was fired through the cap causing the film to melt and adhere to the endothelial layer covering the lesion and on a separate CapSure cap, the lesion. The LCM system was set to the following parameters: 7.5µm spot size and ~100 mW power. Lesion-specific cells and endothelial cells were selected for capture using a 20× objective. After microdissection, the film was removed from the cap using sterile tweezers and transferred to a sterile 0.5 ml Eppendorf tube for RNA extraction.

2.1.5.8.2 RNA isolation

Total RNA was extracted from the isolated cells using the Arcturus® PicoPure™ RNA isolation kit (Applied Biosystems, UK), 50µL of extraction buffer was added to the tube containing the CapSure cap film and incubated at 42°C for 30 minutes. Meanwhile an RNA extraction column was pre-conditioned by incubating with 250µL conditioning buffer for 5 minutes, prior to a centrifugation step at 16,000*g* for 2 minutes, and the flow through discarded. Following the incubation step, 50µL 70% ethanol was added to

the sample and gently pipetted up and down to mix. The sample was transferred to the pre-conditioned column and centrifuged at 100 *g* for 2 minutes to allow the RNA to bind to the column, followed by centrifugation at 16,000 *g* for 1 minute, and the flow through discarded. 100µL of wash buffer 1 was applied to the column which was then centrifuged at 8,000 *g* for 1 minute. One hundred microlitres of wash buffer 2 was applied and centrifuged at 8,000 *g* for 1 minute. A further 100µL wash buffer 2 was added to the column and centrifuged at 16,000 *g* for 2 minutes. Finally the column was placed in a fresh sterile 1.5mL collection tube, 11µL of elution solution was added and the column left to incubate at for 1 minute to allow the release of the RNA from the column matrix prior to centrifugation at 1,000 *g* for 1 minute, followed by centrifugation at 16,000 *g* for 2 minutes. The quantity and quality of the RNA were determined using a NanoDrop 1000 spectrophotometer (Thermoscientific, UK). All RNA samples were stored under sterile conditions at −80°C for future analysis.

2.1.5.8.3 cDNA synthesis

For preamplification of cDNA the RT^2 PreAMP cDNA Synthesis Kit (Qiagen, UK) was used. The RT^2 PreAMP cDNA Synthesis Kit is designed for preamplification of cDNA from nanogram amounts of RNA (1–100 ng) specifically for analysis using RT^2 Profiler PCR Arrays.

The genomic DNA elimination mix for each RNA sample was prepared in a sterile PCR tube according to Table 2.3 mixed gently by pipetting up and down and then briefly centrifuged. The genomic DNA elimination mix was incubated at 42°C for 5 minutes, then placed immediately on ice for at least 1 minute. 10µL of the reverse-transcription mix (prepared according to table 2.3) was added to each tube containing the genomic DNA elimination mix, gently pipetted to mix and briefly centrifuged to remove air bubbles. The RNA mixture was incubated at 42°C for 30 minutes and the reaction stopped by a further incubation at 95°C for 5 minutes. The reaction was placed on ice and proceeded to the preamplification step. Five microlitres of the cDNA synthesis reaction was added to a sterile PCR tube containing 20µL of the preamplification mix (prepared according to table 2.4), gently pipetted to mix and briefly centrifuged to remove air bubbles. The reaction was placed in the real-time

cycler, programmed according to table 2.5 and then placed immediately on ice. Two microlitres of side reaction reducer was added to each pre-amplified reaction, gently pipetted to mix and briefly centrifuged to remove air bubbles. The reaction was incubated at 37°C for 15 minutes followed by heat inactivation at 95°C for 5 minutes. Eighty-four microlitres nuclease-free water was added to each reaction, mixed well and stored on ice prior to real-time PCR or long-term storage at -20°C.

Component	Amount for 1 RNA sample
RNA	1-100ng
Buffer GE	2µL
RNase-free water	Variable
Total volume	**10µL**

Table 2.3 Genomic DNA elimination mix.

Component	Amount for 1 RNA sample
5x Buffer BC3	4µL
Control P2	1µL
cDNA synthesis enzyme mix	1µL
RNase inhibitor	1µL
RNase-free water	3µL
Total volume	**10µL**

Table 2.4 Reverse transcription mix.

Component	Amount for 1 RNA sample
RT^2 PreAMP PCR Mastermix	12.5µL
RT^2 PreAMP Target Primer Mix	7.5µL
Total volume	**20µL**

Table 2.5 Preamplification mix.

Cycles	Duration	Temperature
1	10 minutes	95°C
12	15 seconds	95°C
	2 minutes	60°C
Hold		4°C

Table 2.6 Cycling conditions for preamplification.

2.1.5.8.4 qRT-PCR using SYBR® Green methodology

qRT-PCR is a molecular biology technique which follows amplification of a targeted gene in real time and allows quantification of gene expression. qRT-PCR detects fluorescence produced by a reporter molecule which increases as the reaction progresses due to accumulation of PCR product with each amplification cycle. SYBR® Green is a green fluorescent cyanine dye that has high affinity for double-stranded DNA, however, unbound dye has very little fluorescence, therefore, the amount of fluorescence is proportional to the amount of double stranded DNA. The measured fluorescence is expressed as an amplification plot. Melt curve analysis was used to assess whether the assay amplified a single, specific product.

Expression of housekeeping genes (B2M and ACTB) were used as previously determined by Dr Daniel Kelly (Kelly, 2010) and Dr Hanan Bokhamada (Bokhamada, 2014) were used to normalise the mean C_T values of all sample targets to account for variations in the starting amounts of total RNA in each sample. The real-time PCR mastermix was prepared according to table 2.7 and 25µL added to each well containing the pre-optimised primer mix (Cat No.: 330171) (Qiagen, UK). For a number of targets (Table 2.9). The reaction was placed in the real-time cycler that was programmed according to table 2.8 and the reaction run.

2.1.5.8.5 Relative quantification analysis of qRT-PCR data

Duplicate C_T values from the same biological sample were averaged to give a representative value. Measured C_T values were analysed using the $2^{-\Delta\Delta CT}$ method to determine the relative gene expression normalised against housekeeping genes and

the mean of the control (Livak and Schmittgen, 2001). ΔC_T values were calculated using the following equation:

$$\Delta C_T = C_T \text{ (mean target)} - C_T \text{ (mean housekeeping)}$$

Target gene expression was normalised to untreated controls and calculated as follows:

$$\Delta\Delta CT = \text{mean } \Delta CT \text{ (treatment group)} - \text{mean } \Delta CT \text{ (mean untreated control)}$$

$$\text{Relative expression} = 2^{-\Delta\Delta CT}$$

Median relative gene expression is presented graphically as relative fold-change from the calibrator control value.

Assay format	Amount for 1 RNA sample
2 x RT[2] SYBR Green Mastermix	10µL
Nuclease-free water	7.5µL
cDNA reaction	2.5µL
Total volume	**20µL**

Table 2.7 Real-time PCR components mix.

Cycles	Duration	Temperature
1	10 minutes	95°C
50	15 seconds	95°C
	1 minute	60°C

Table 2.8 Cycling conditions.

Lesion	Endothelial layer
B2M (housekeeping gene)	GAPDH (housekeeping gene)
ACTB (housekeeping gene)	RPL3A1 (housekeeping gene)
ICAM-1	VCAM1
IL-1β	ICAM1
IL-6	CX3CL1
IL-10	IL-6
TNFα	PECAM1
CCL2	MMP9
CX3CL1	MMP13
MMP9	MMP10
MMP10	SELP
MMP13	NOS3

Table 2.9 Summary table of custom qRT-PCR array (Qiagen, UK) (Catalog No.:330231) used for mRNA analysis of the lesion-specific tissue (CLAM35533) and the endothelial cell layer (CLAM35530) isolated from the aortic root of ApoE$^{-/-}$ mice from the testosterone treatment *in vivo* study.

2.2 *In vitro* investigation of the role of testosterone on monocytes/macrophages and endothelial cells

2.2.1 Cell Culture

All cell culture procedures were carried out under sterile conditions in a laminar flow hood using sterile equipment. All cell lines were tested regularly for mycoplasma contamination using the MycoAlert™ mycoplasma detection kit (Lonza) and were all tested negative throughout the study.

2.2.1.1 THP-1 culture

THP-1 is a human monocytic cell line derived from the peripheral blood of a 1-year old male with acute monocytic leukaemia. THP-1 cells are widely used as an appropriate model of human blood monocytes, as they express many distinct monocytic markers and characteristics, which are maintained over time in culture (Auwerx, 1991, Tsuchiya *et al.*, 1980, Chanput *et al.*, 2014, Bosshart and Heinzelmann, 2016). The THP-1 cell line can also be successfully employed as an alternative model for primary macrophages, since THP-1 cells exhibit changes consistent with those observed in primary macrophages (Daigneault *et al.*, 2010, Auwerx, 1991). Phorbol 12-myristate 13-acetate (PMA) induces differentiation of THP-1 cells to macrophage-like cells (Tsuchiya *et al.*, 1982). THP-1 cells have become one of most widely used cell lines to investigate the function and regulation of monocytes and macrophages in the cardiovascular system (Qin, 2012). THP-1 cells also express the androgen receptor and thus can be utilised to study effects of testosterone treatment (Huang *et al.*, 2014).

The cell line was obtained from the European collection of cell cultures (ECACC, UK) as an unknown passage. THP-1 cells were seeded in suspension and were cultured in RPMI 1640 media with 2mM glutamine (GIBCO, UK), supplemented with 10% charcoal stripped foetal calf serum (FCS) and 2mM penicillin/streptomycin (GIBCO, UK). Media were changed approximately every 48 hours.

2.2.1.1.1 Preparation of charcoal stripped media

Treatment of FCS with charcoal stripping removes any unknown lipid-related elements in the serum, such as hormones, without affecting the amino acid, glucose and salt content (Sikora *et al.*, 2016). In brief, FCS was incubated with 20g/L dextran-coated charcoal (Sigma Aldrich, UK) overnight at 4˚C shaking. After centrifugation (20,000 *g* for 20 min) the resulting supernatant was filtered through a 0.2µm filter (Fisher, UK) and added to RPMI media in place of untreated FCS.

2.2.1.2 Sub-culture of cell cultures

THP-1 cultures were maintained up to 70-90% confluence before passaging. The cell suspension was removed to sterile tubes and centrifuged for 5 minutes at 200 *g*. After removing the supernatant, the cells were resuspended in 1mL of fresh medium. 20µL of this cell suspension was added to an equal volume of 0.4% w/v trypan blue solution (Gibco, UK), cells were counted using the automated Countess II FL Automated Cell Counter, (Life Technologies, UK). Viable cells were then further diluted to working densities and used in the experimental procedures, subcultured or cryopreserved.

2.2.2 Experimental conditions for testing the effect of testosterone on the inflammatory profile in human monocytes

Testosterone (Sigma-Aldrich, UK) was dissolved in molecular grade ethanol (Sigma-Aldrich, UK) at an initial dilution of 100mg/mL and stored at 4°C until use. The testosterone solution was further diluted in serum-free medium to working concentrations, via an initial 1:1000 dilution in ethanol. Cells were seeded into 6 well plates (NUNC™), at an initial density of $3x10^5$ cells/mL in complete medium containing testosterone at working concentrations of 10, 30, 50 and 100nmol/L and cultured for 24-48 hours at 37°C. Ethanol, at a volume equal to that of the diluted working solution of testosterone was used as the vehicle control. Serum-free medium was used as control and all experiments repeated at least three times for each condition.

2.2.2.1 Experimental conditions for investigating the effect of testosterone on the inflammatory profile in human macrophages

Cells were seeded into 6 well plates (NUNC™), at a density of $3x10^5$ cells/mL in 2mL of complete medium containing and differentiated with 10ng/mL phorbol 12-myristate-13-acetate (PMA) for 48 hours. Following initial differentiation, cells were incubated with 10ng/mL LPS (Sigma Aldrich, Poole UK) and 20ng/mL recombinant IFN-γ (Peprotech, UK) in fresh serum-free medium for 24 hours to induce an M1 phenotype activation state. M2a differentiation was achieved by the combined treatment with 25ng/mL of recombinant human cytokines IL-4 and IL-13 (Peprotech, UK) for 24 hours in fresh serum free medium. After differentiation cells were treated with testosterone at working concentrations of 10, 30, 50 and 100nM/mL and cultured for 24-48 hours at 37°C. Ethanol, at a volume equal to that of the diluted working solution of testosterone was used as the vehicle control.

2.2.3 Experimental conditions for investigating the effects of high glucose on the inflammatory profile in human monocytes

Cells were seeded into $25cm^2$ cell culture flasks (NUNC™), at an initial density of $3x10^5$ cells/mL in 10mL of complete medium containing 5, 11.1, 25mM/L of glucose or an osmolality control (25mM/L mannitol) and cultured for 24 hours at 37°C. All experiments were repeated at least three times for each condition.

2.2.4 Experimental conditions for investigating the effects of testosterone on fatty acid loaded human macrophages

A stock solution of 100mg/mL oleic acid and 100mg/mL palmitic acid in DMSO was used to create a 30mM stock fatty acid stock solution (20mM oleic acid/10mM palmitic acid, 2:1) in basic culture medium containing 1% fatty acid free-BSA. The stock solution was further diluted in basic culture medium containing 1% fatty acid free-BSA to obtain a 2mM final concentration.

THP-1 cells were seeded into 6 well plates (NUNC™), at a density of $3x10^5$ cells/mL in 2mL of complete medium containing and differentiated with 10ng/mL PMA for 48 hours. Following initial differentiation, cells were incubated with 0, 0.25, 0.5, 1.5

and 2mM fatty acids and co-treated with testosterone at working concentrations of 10, 30, 50 and 100nM/mL and cultured for 24 hours at 37°C. Ethanol, at a volume equal to that of the diluted working solution of testosterone was used as the vehicle control.

Cells were fixed in 10% v/v formalin (Leica, UK) for 30 minutes on the bench then rinsed with tap water. Cells were incubated in 60% v/v isopropyl alcohol for 30 seconds and stained for 1 hour with ORO (see section 2.2.4.8) and further washed in tap water. Cells were then counterstained with Hoechst for 30 minutes to counterstain the nuclei, and then washed well in tap water. Cells were imaged and counted using the Cytation5 (Biotek, USA). Finally, the ORO was dissolved in isopropanol for 15 minutes and the absorbance read on a CLARIOstar reader (BMG Labtesch, Germany) at 450±10nm.

2.2.5 Molecular investigation of gene expression in human monocytes/macrophages

2.2.5.1 RNA extraction

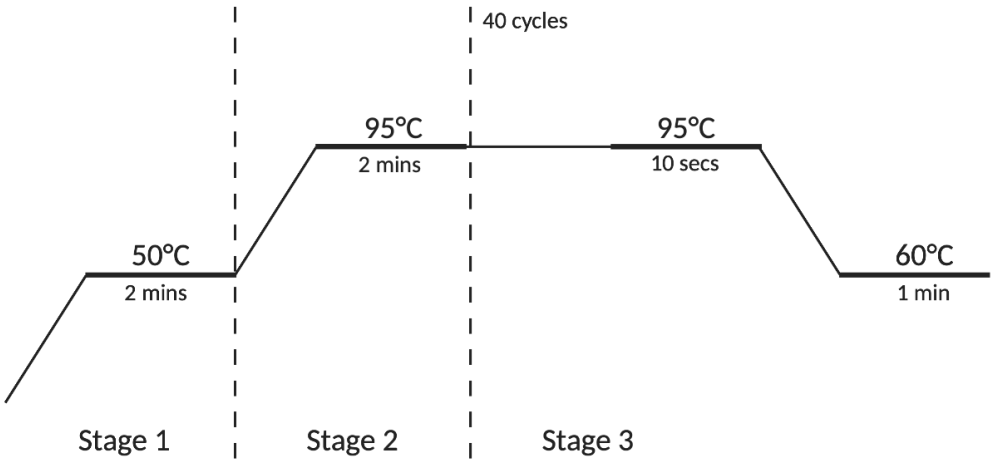
Total RNA was isolated from experimental THP-1 cells with TRI Reagent™ Solution (Invitrogen Life Technologies). One millilitre TRI reagent was added to cells and left to stand at room temperature for 10 minutes causing lysis of the cell membranes. Chloroform (Fisher, UK) was added at a volume of 200µL per 1mL of Tri-reagent used, samples were briefly vortexed and left to stand for 10 minutes at room temperature, until separation had occurred. Centrifugation at 12,000 g for 15 minutes at 4°C was performed to allow separation of protein, DNA and RNA layers. The upper aqueous phase containing the RNA was collected and 500µL isopropanol (Sigma-Aldrich, UK) was added to the sample and mixed by pipetting. Samples were stored at -80°C overnight to allow the RNA to precipitate, after which samples were centrifuged for a further 20 minutes at 12,000 *g*. Cell pellets were washed in 70% v/v molecular grade ethanol (Sigma-Aldrich, UK) in distilled water, centrifuged at 12,000 *g* for 5 minutes before being allowed to airdry for 10 minutes and resuspended in 30µL RNAse free water and store at -80°C until cDNA synthesis.

2.2.5.2 cDNA synthesis

Prior to carrying out quantitative real-time polymerase chain reaction (qRT-PCR), RNA was reverse-transcribed to cDNA using the Precision nanoScript2 Reverse Transcription Kit (Pimerdesign Ltd, UK). RNA samples were incubated with an Oligo-dT primer and random nonamer primer at 60°C for 5 minutes and immediately put in ice. 10µL of a reverse transcriptase mastermix was added to the RNA and incubated at 42°C for 20 minutes followed by a heat inactivation step at 75°C for 10 minutes. cDNA was stored at -20°C until use.

2.2.5.2.1 qRT-PCR using SYBR® Green methodology

Expression of housekeeping genes were used to normalise the mean C_T values of all sample targets to account for variations in the starting amounts of total RNA in each sample. A volume of 5µL of cDNA sample was added in duplicate into a 96-well PCR plate. qRT-PCR mastermix (10µL Precision®PLUS Master Mix with ROX (Primerdesign, UK), 2µL primer (Primerdesign, UK), 3µL RNase free water per well) (see table 2.10 for targets) was prepared and 15µL was added to each well. Plates were sealed using an adhesive film before being run on the QuantStudio™ 3 (Applied Biosystems, UK) using the following cycle:



2.2.5.2.2 Relative quantification analysis of qRT-PCR data

See section 2.1.5.8.5

Gene symbol	Gene name	Catalog number
ACTB	Beta-actin	HK-SY-hu-600-ACTB
B2M	Beta-2-microglobulin	HK-SY-hu-600-B2M
IL-1β	Interleukin 1 beta	HK-SY-hu-600-IL-1β
IL-6	Interleukin 6	HK-SY-hu-600-IL-6
IL-10	Interleukin 10	HK-SY-hu-600-IL-10
TNFα	Tumour necrosis factor alpha	HK-SY-hu-600- TNFα
CCL2	Monocyte chemoattractant protein 1	HK-SY-hu-600-CCL2
SCARB1	Scavenger receptor class B type 1	HK-SY-hu-600-SCARB1
TLR2	Toll receptor 2	HK-SY-hu-600-TLR2
TLR4	Toll receptor 4	HK-SY-hu-600-TLR4

Table 2.10 Summary table of qRT-PCR primer targets from Primerdesign Ltd, UK used for mRNA analysis of the monocytes and macrophages at different phenotypic stages treated *in vitro* with testosterone.

2.3 The effect of testosterone replacement in male patients with hypogonadism and type 2 diabetes - results from the randomised double-blind placebo-controlled crossover study

2.3.1 Patients

A randomised double-blind placebo-controlled crossover study was performed at the Centre for Diabetes and Endocrinology, Barnsley NHS Foundation Trust Hospital, Barnsley, UK led by Professor T Hugh Jones. The study included a total of 65 male patients, over the age of 40 years with T2D and hypogonadism. The Derby Research Ethics Committee approved the protocol and the study authorised by the MHRA (25951/0003/001-0001), patients provided their written consent. Inclusion criteria were males with T2D with HbA_{1c} (glycated haemoglobin) up to 9.5% and hypogonadism, defined as the total testosterone level <12nM/L and bioavailable testosterone <4nM/L with symptoms of hypogonadism according to the ADAM questionnaire (androgen deficiency on the aging male). Hypogonadal men were referred to the andrology clinic where androgen replacement therapy was deemed appropriate by a consultant endocrinologist. Patients were excluded at the recruitment stage if they had any inflammatory disease or infection with elevation of C-reactive protein 10 mg/L, were already on hormone therapy or had any contraindication to testosterone therapy such as elevation of prostate-specific antigen (PSA) beyond the age-adjusted normal range.

2.3.1.1 Randomisation and drug treatment

The trial was 12 months in duration in which patients had two treatment phases, Phase 1: patients were randomly assigned by a computer-generated algorithm to either placebo (n=32) or treatment (n=33) arm for 6 months of testosterone therapy. The patients on the treatment arm received testosterone undecanoate (Nebido) (1g in 4mL oily base) (Bayer-Schering, Germany) at 0, 6, 18, 30 and 42 weeks. The patients on the placebo arm received placebo solution at 0, 6, 18 weeks. The treatment was unblinded at 24 weeks and those on placebo received testosterone undecanoate from then onwards at 24, 30, 42 weeks. Treatments were prepared in identical coded syringes in a separate clinical room not in the presence of the patient

or assessing doctor. Treatment was administered via slow (2 minute) intramuscular injections. The six weekly followed by 3-monthly injection regime is pragmatic, commonly used, and consistent with recommendations on the British National Formulary (recommended injection interval 10—14 weeks). 12-13 week administration has been shown to be biologically effective, to achieve testosterone levels in the normal range, and to minimize the risk of testosterone accumulation (Schubert *et al.*, 2004, Rhoden and Morgentaler, 2004).

2.3.1.2 Sample collection and preparation

40mL of peripheral blood was collected in a tube containing Ethylenediaminetetraacetic acid (EDTA) (1.8mg per 1mL of blood) at 0, 3 and 6 months in Phase 1 of the study in treated and placebo subjects to compare response to treatment longitudinally (versus baseline) and cross-sectionally compared to placebo. In phase 2, blood was collected from testosterone treated patients who moved from the placebo arm of the study at the end of phase 1, 3- and 6-months post-treatment to increase sample number for longitudinal analysis. Whole blood was collected and stored for subsequent analysis or processed for monocyte isolation within 4 hours. Blood collection was done between 9am-10.30am and patients fasted for 12 hours prior to collection. All treatments and blood collection were performed by the staff of Barnsley NHS Foundation Trust Hospital, Barnsley, UK.

2.3.1.2.1 Peripheral blood mononuclear cell isolation

Whole blood was transferred into 2x50mL centrifuge tubes, diluted with an equal volume of physiological saline and overlaid onto Ficoll-Paque (GE Healthcare, UK) in a 2:1 ratio. Samples were centrifuged at 800 *g* for 35 minutes with the brake setting off, at room temperature and the PBMC was layer carefully collected and washed twice with PBS. Using the Dynabeads® FlowComp™ Human CD14 Isolation kit (Invitrogen, USA); magnetic beads were resuspended in a glass vial by vortexing for >30 seconds. 300μL of the bead suspension was added to a 1.5mL centrifuge tube and washed 1mL of isolation buffer (PBS (Ca^{2+} and Mg^{2+} free) 0.1% BSA and 2mM EDTA, pH 7.4) and resuspended by repeated pipetting. The tube was placed in the DynaMag™-5 for 1 minute and the supernatant discarded. The beads were resuspended again in

1mL isolation buffer and stored on ice. The isolated PBMCs were resuspended in 500µL isolation buffer and 50µL FlowComp™ Human CD14 Antibody was added, mixed well and incubated on ice for 15 minutes. The cells were washed with 2mL isolation buffer and centrifuged for 10 minutes at 350 *g* at 4˚C. The cells were resuspended in 4mL isolation buffer with 300µL pre-washed Dynabeads® and incubated for 15 minutes at 4°C on a roller. The tube was placed in the magnet for 2 minutes and the supernatant discarded. 1mL of release buffer (modified biotin in 0.1% BSA and 2mM EDTA) was added to the tube containing the bead-bound cells, resuspended by gentle pipetting and incubated for 10 minutes at 4°C on a roller. To release the cells, 1mL of isolation buffer was added to the cell suspension and pipetted gently. Finally, the cell suspension was centrifuged for 10 minutes at 350 *g* at 4˚C, supernatant discarded and cell pellet stored at -80˚C until further use.

2.3.1.3 Molecular investigation of gene expression in human monocytes isolated from patients by real-time reverse transcription polymerase chain reaction (qRT-PCR)

2.3.1.3.1 RNA isolation

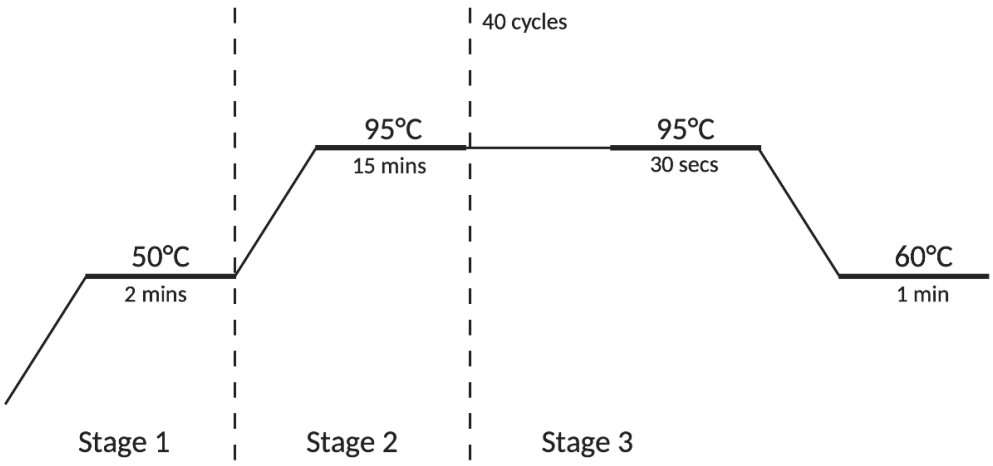
To isolate total RNA from cells, the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) was used according to the manufacturer's instructions. In order to avoid any RNase activity, RNase-free water and RNase-free reaction tubes were used during the procedure. Briefly, total RNA was extracted by adding 350µL of lysis buffer containing 1% β-mercaptoethanol. An equal volume of 70% ethanol was added and mixed with the lysates. The mixture was then transferred to the mini spin column, centrifuged for 25 seconds at 13000 rpm following two washing steps (wash solutions 1 and 2). Finally, the RNA was eluted with 30µl RNase-free water.

2.3.1.3.2 cDNA synthesis

Prior to carrying out quantitative real-time polymerase chain reaction (qRT-PCR), RNA was reverse-transcribed to cDNA using the Precision nanoScript2 Reverse Transcription Kit (Pimerdesign Ltd, UK) (see section 2.2.5.2).

2.3.1.3.3 qRT-PCR using SYBR® Green methodology

See section 2.2.5.3. A volume of 5µL of cDNA sample was added in duplicate into a 96-well PCR plate. qRT-PCR mastermix (10µL KiCqStart® SYBR® Green qPCR ReadyMix™ containing ROX™ (Sigma Aldrich, UK), 2µL primer (See table 2.11 for targets), 3µL RNase free water) was prepared and 15µL was added to each well. Plates were sealed using an adhesive film before being run on the Agilent Mx3000P QPCR System (Agilent, USA) using the following cycle:



2.3.1.3.4 Relative quantification analysis of qRT-PCR data

See section 2.1.5.8.5

Gene symbol	Gene name	Catalog number
B2M	Beta-2-microglobulin	NM_004048
IL-1β	Interleukin 1 beta	NM_000576
IL-6	Interleukin 6	NM_000600
IL-10	Interleukin 10	NM_000572
TNFα	Tumour necrosis factor alpha	NM_000594
CCL2	Monocyte chemoattractant protein 1	NM_002982
ICAM-1	Intracellular adhesion molecule 1	NM_000201
SCARB1	Scavenger receptor class B type 1	NM_001082959
IRF3	Interferon regulatory factor 3	NM_001197122
TLR2	Toll receptor 2	NM_003264
TLR4	Toll receptor 4	NM_003266

Table 2.11 Summary table of qRT-PCR primer targets used from Qiagen, UK for mRNA analysis of the monocytes isolated from patients from the clinical trial: testosterone replacement in male patients with hypogonadism and T2D.

2.3.1.4 Determination of pro- and anti-inflammatory biomarkers in patients by Cytometric Bead Array

In the present study the BD™ Cyotmetric Bead Array analysis was used, and antibodies targeted against MCP-1, VCAM-1, ICAM-1, and E-selectin. All reagents and 96-well assay plates were supplied by Becton Dickinson (BD, UK). All sample preparation and analysis were performed by with Susan Clark as part of the University of Sheffield core research facilities service. (See section 2.1.5.3).

2.3.2 Determination of biochemical parameters

HbA_{1c}, serum lipids, serum testosterone, serum SHBG, serum estradiol and CRP were determined at Barnsley Hospital NHS Foundation Trust as part of routine blood tests. Methods are summarised in table 2.12.

Test	Subtype	Manufacturer	Method	Sample type
HbA_{1c}		Launch	Akray 8180 Cation exchange chromatography	EDTA whole blood
Lipids	Triglycerides	Siemens Advia	Glycerol kinase with GPO	Serum gel
	Cholesterol	Siemens Advia	Cholesterol oxidase	Serum gel
	HDL cholesterol	Siemens Advia	Direct via elimination of non-HDL cholesterol	Serum gel
	LDL cholesterol	N/A	Friedwald calculation	Serum gel
Testosterone		Siemens Centaur	Chemiluminescent immunoassay. Competitive with analogue	Serum gel
SHBG		Siemens Centaur	Chemiluminescent immunoassay. Two site sandwich	Serum gel
CRP		Siemens Advia	Latex agglutination turbidimetric	Serum gel
Estradiol		Siemens Centaur	Chemiluminescent immunoassay. Competitive with analogue	Serum gel

Table 2.12 Summary of biochemical tests carried out by Barnsley Hospital NHS Foundation Trust for the clinical trial: testosterone replacement in male patients with hypogonadism and T2D.

2.4 Statistical analysis

Data is presented as the median unless otherwise stated. Experimental groups were assessed for normality and equal variance by the Shapiro–Wilk test. Appropriate statistical analyses were selected to establish significant differences between groups.

Where three or more samples were compared a one-way ANOVA followed by Tukey's post hoc was used to establish the significance of any differences. For two group comparisons of equal variance a student's *t test* was used. If the data was non-normal, of unequal variance, or sample size was considered too small for parametric analyses, a non-parametric Kruskal-Wallis test was applied to the original data for multiple groups, followed by Conover-Inman's post hoc test. For two group comparisons a Mann Whitney U test was used. Significance was accepted at $P<0.05$.

Multivariant analysis using SPSS statistics (UK) software was performed on all parameters and measurements obtained from the clinical study.

3. The effects of testosterone on parameters of atherosclerosis

3.1 Introduction

Clinical studies have reported that the degree of atherosclerosis in the carotid artery as assessed by CIMT and lesion scores are inversely associated with testosterone levels (Demirbag *et al.*, 2005, Fukui *et al.*, 2003, Muller *et al.*, 2004, Svartberg *et al.*, 2006). Physiological testosterone levels in the higher quartile of the range are associated with an improved CIMT and lower occurrence of carotid lesions (Chan *et al.*, 2015). Furthermore, CIMT has been shown to be inversely and significantly correlated with total and bioavailable testosterone levels (Soisson *et al.*, 2012, Svartberg *et al.*, 2006).

Results from male animal models support that testosterone confers a beneficial effect on atherogenesis. Animal studies have observed that testosterone deficiency promotes the development of lipid streak formation, the first stage of the atherosclerotic lesion, in the aortae of animals fed a pro-atherogenic diet (Nettleship *et al.*, 2007a, Kelly *et al.*, 2012, Larsen *et al.*, 1993, Bruck *et al.*, 1997, Alexandersen *et al.*, 1999). Furthermore, testosterone treatment reduces atherosclerosis development in both male ApoE$^{-/-}$ mice (Elhage *et al.*, 1997, McRobb *et al.*, 2009) and LDLr$^{-/-}$ mice (Nathan *et al.*, 2001), as well as other rodent models of atherosclerosis and testosterone deficiency (Larsen *et al.*, 1993, Bruck *et al.*, 1997, Alexandersen *et al.*, 1999, Nettleship *et al.*, 2007a, Kelly *et al.*, 2012). Possible anti-atherogenic actions include beneficial effects on serum lipoprotein levels and anti-inflammatory activity (Wu and von Eckardstein, 2003, Elhage *et al.*, 1997, Nathan *et al.*, 2001, Jones and Saad, 2009, Kelly *et al.*, 2012), however, effects of testosterone directly on plaque specific mechanisms in the development and progression of atherosclerosis are not yet known. Enhancement of reverse cholesterol transport and HDL-induced cholesterol efflux from macrophages by up-regulation of SCARB1 may be one mechanism by which testosterone protects from atherosclerosis (Langer *et al.*, 2002).

Post-mortem studies have discovered that atherosclerotic lesions associated with coronary thrombosis, sudden death, and rupture typically have less fibrous tissue, a larger lipid-laden necrotic core and a high ratio of macrophages to smooth muscle cells (Davies *et al.*, 1993b, Davies and Thomas, 1984, Burke *et al.*, 1997, Cheruvu *et al.*,

2007), whereas, stable lesions do not contain a lipid-rich necrotic core. Macrophages are the dominant cell type within atherosclerotic lesions and actively participate in lipoprotein ingestion and accumulation, giving rise to foam cells filled with lipid droplets. Accumulation of foam cells contributes to lipid storage, and atherosclerotic lesion growth and apoptotic foam cells are chiefly responsible for necrotic core formation in progressing lesions (Seimon and Tabas, 2009). The key role that macrophages play in the pathogenesis of atherosclerosis makes them an attractive target for atherosclerosis therapy. It has been proposed that testosterone may reduce lipid uptake into the arterial wall and have anti‐inflammatory effects directly at the level of the macrophage (Kelly *et al.*, 2012), although evidence in the literature is contradictory with testosterone shown to have both anti and pro-inflammatory effects of macrophages (Corcoran *et al.*, 2010, Gilliver *et al.*, 2006, Becerra-Díaz *et al.*, 2018). Other possible mechanisms of testosterone include inhibiting monocyte/macrophage recruitment to progressing lesions, stimulating cholesterol efflux and diminishing lipid uptake, and taking advantage of macrophage plasticity and the ability to polarise towards pro- or anti-inflammatory phenotypes (Moore *et al.*, 2013, Gilliver *et al.*, 2006). Pro-inflammatory macrophages contribute towards lesion instability as they can break down the ECM through secretion of MMPs.

Some of the key clinical studies investigating TTh show reduced cardiovascular events (Haider *et al.*, 2016, Traish *et al.*, 2017). Many mechanisms by which testosterone may be reducing cardiovascular events have been hypothesised, including slowing lesion progression, or inducing regression, reducing lesion inflammation, improving shear stress by reducing blood pressure and increasing lesion stability and thus preventing rupture. ECM integrity constitutes a critical determinant in the stability of atherosclerotic lesion. Unstable rupture-prone lesions have thin, highly inflamed, and collagen-poor fibrous caps that contain elevated levels of proteases, including MMPs, which weaken lesion caps and promote rupture (Braganza and Bennett, 2001). The stability of an atherosclerotic lesion depends on the interplay of vascular SMCs (VSMCs) and leukocytes. The VSMCs synthesise structurally important collagens that provide stability to the lesion whilst intra-lesion leukocytes tend to release pro-inflammatory factors such as cytokines and MMPs. However,

MMPs also facilitate migration and proliferation VSMCs, promoting fibrous cap stability. In unstable lesions, the balance between these competing factors favours collagen breakdown rather than synthesis.

MMPs are a family of endopeptidases with a range of functions in immunity, tissue repair and other disease processes (Murphy and Knäuper, 1997, Nagase *et al.*, 2006). In healthy tissue, the activity of MMPs is tightly regulated with a delicate balance between activation and inhibition, which is mediated largely by the endogenous tissue inhibitors of metalloproteinases (TIMPs). All cells present in the healthy and diseased blood vessel wall can activate and regulate MMPs in a multistep fashion driven in part by soluble cytokines and cell-cell interactions. MMPs are upregulated in the inflammatory lesion environment, and this upregulation of MMPs can ultimately lead to the structural breakdown and lesion rupture. Foam cells are a prominent source of several MMPs in atheromatous lesions, driven by stimuli such as TNFα. High-level activation of a broad spectrum of MMPs is associated with inflammation that contributes to pathological matrix destruction and lesion rupture.

Inhibiting the activity of specific MMPs or preventing their upregulation could improve lesion stability and induce regression. Only pro-MMP2, TIMP-1 and TIMP-2 have been found in normal human arteries, and no MMP activity was detected by *in situ* zymography (Newby, 2005). Whereas atherosclerotic lesions have increased global MMP activity (Choudhary *et al.*, 2006). MMP9 fragments elastin and its expression has been shown to be significantly higher in unstable atheromatous lesions than in stable fibrous human lesions (Sluijter *et al.*, 2006). MMP9 also induces vascular calcification and is present in calcified carotid endarterectomy samples (Bouvet *et al.*, 2008). Furthermore, histological sections of rabbit and mouse atherosclerotic lesions and the rupture-prone shoulder regions of human atherosclerotic lesions show increased levels of MMP9 (Newby, 2005).

MMP10 has been identified in human atherosclerotic lesions and is associated with vascular calcification and in patients with CV risk, additionally, increased circulating MMP10 in humans is associated with coronary calcium (Coll *et al.*, 2010, Orbe *et al.*, 2007). Purroy *et al.* (2018) also demonstrated *ex vivo* that MMP10 is

released from human atheroma, likely contributing to increased serum MMP10 levels reported in patients with atherosclerosis. Maximal MMP10 secretion was observed in calcified haemorrhagic lesions. MMP10 expression in mice is also restricted to atherosclerotic lesions and $ApoE^{-/-}MMP10^{-/-}$ mice is associated with a substantial reduction of atherosclerosis, decreased macrophage content and lesion calcification (Montero *et al.*, 2006, Purroy *et al.*, 2018).

MMP13 has been detected in human lesions and was co-localised with cleaved collagen. MMP13 can cleave fibrillar type-I and type-III collagen (Nagase *et al.*, 2006, Sukhova *et al.*, 1999). Degradation of fibrillar collagen may decrease the ability of the fibrous cap to withstand mechanical stress. Knockout (Deguchi *et al.*, 2005, Quillard *et al.*, 2014) or selective inhibition (Quillard *et al.*, 2011) of MMP13 in $ApoE^{-/-}$ mice have been shown to increase lesion collagen content, suggesting that MMP13 contributes to lesion instability. In summary, these studies demonstrate that MMPs plays a relevant role in atherogenesis by favouring lesion inflammation, development and complication.

The absence of most MMPs in healthy tissue makes them a potential therapeutic target in atherosclerosis. To date, regulation of atherosclerotic lesion stability and regulation of MMPs by androgens has not been fully elucidated. Some early studies have demonstrated that MMP1 production in human endometrial stromal cells *in vitro* was inhibited by testosterone via androgen receptor signalling (Ishikawa *et al.*, 2007). Dihydrotestosterone treatment on human aortic smooth muscle cells reduced MMP2 activation, leading to an inverse relationship between DHT level, MMP2 activity, and VSMC migration *in vitro* (Mountain *et al.*, 2013).

3.1.1 Summary

Testosterone treatment is implicated as beneficial in preventing the development of atherosclerosis in animal models and reducing cardiovascular events in some clinical studies of hypogonadal men. *In vivo* and *in vitro* studies provide strong evidence that testosterone diminishes atherosclerotic burden by reducing fatty streak formation and lipid accumulation. However, very little is known regarding the effects

of testosterone on lesion composition and stability in the context of this chronic inflammatory disease of the arterial vessels.

3.2 Aims and objectives

The aim of the *in vivo* study was to use a mouse model of atherosclerosis to address the hypothesis that testosterone treatment inhibits atheroma formation, reduces atherosclerotic burden by reducing aortic lipid accumulation and influences lesion complexity by increasing lesion stability. The aim of the *in vitro* study was to use a macrophage model to investigate the hypothesis that 'testosterone reduces lipid accumulation in human macrophages'. Specifically, this study will;

- Determine the effects of low endogenous testosterone on atherosclerotic parameters and lesion stability using the ApoE$^{-/-}$ mouse model.
- Uncover the effects of testosterone treatment on atherosclerotic parameters and lesion stability using the ApoE$^{-/-}$ mouse model fed a pro-atherogenic diet.
- Investigate the effects of testosterone treatment on fatty acid uptake in THP-1 macrophages.

3.3 Experimental design

The ApoE$^{-/-}$ mouse was used as a murine model of atherosclerosis. Initially, mice were orchidectomised to reduce endogenous testosterone levels and fed a standard chow diet to investigate the effects of orchidectomy on fatty streak formation within the aortic root, identified by ORO staining. Secondly, mice were fed a pro-atherogenic diet, and orchidectomised mice were treated with placebo or testosterone to investigate the effects of testosterone treatment on lesion composition, complexity and stability. ORO staining was used again to determine the fatty streak in the aortic root, calculated as lipid deposition within the aortic root. Lesion area and thickness were also determined from ORO stained sections. Masson's trichrome staining was used to identify lesion collagen content within the aortic root and the presence and area of the necrotic core. This study also assessed the expression of MMPs within aortic lesions using immunohistochemistry. Immunohistochemistry was also used to determine monocyte/macrophage and smooth muscle lesion content; smooth muscle

staining was also used to determine the presence and thickness of the protective fibrous cap overlying the lesion.

The THP-1 cell line was used as a human macrophage model. THP-1 macrophages were treated *in vitro* with varying concentrations of fatty acids (0-2mM) and co-cultured with and without 30nmol/L testosterone. Secondly, THP-1 macrophages were treated with varying concentrations of testosterone (0-100nmol/L) and co-cultured with 2mM fatty acids. Fatty acid uptake analysis was performed by using ORO and quantified. The experimental design is summarised in Figure 3.1.

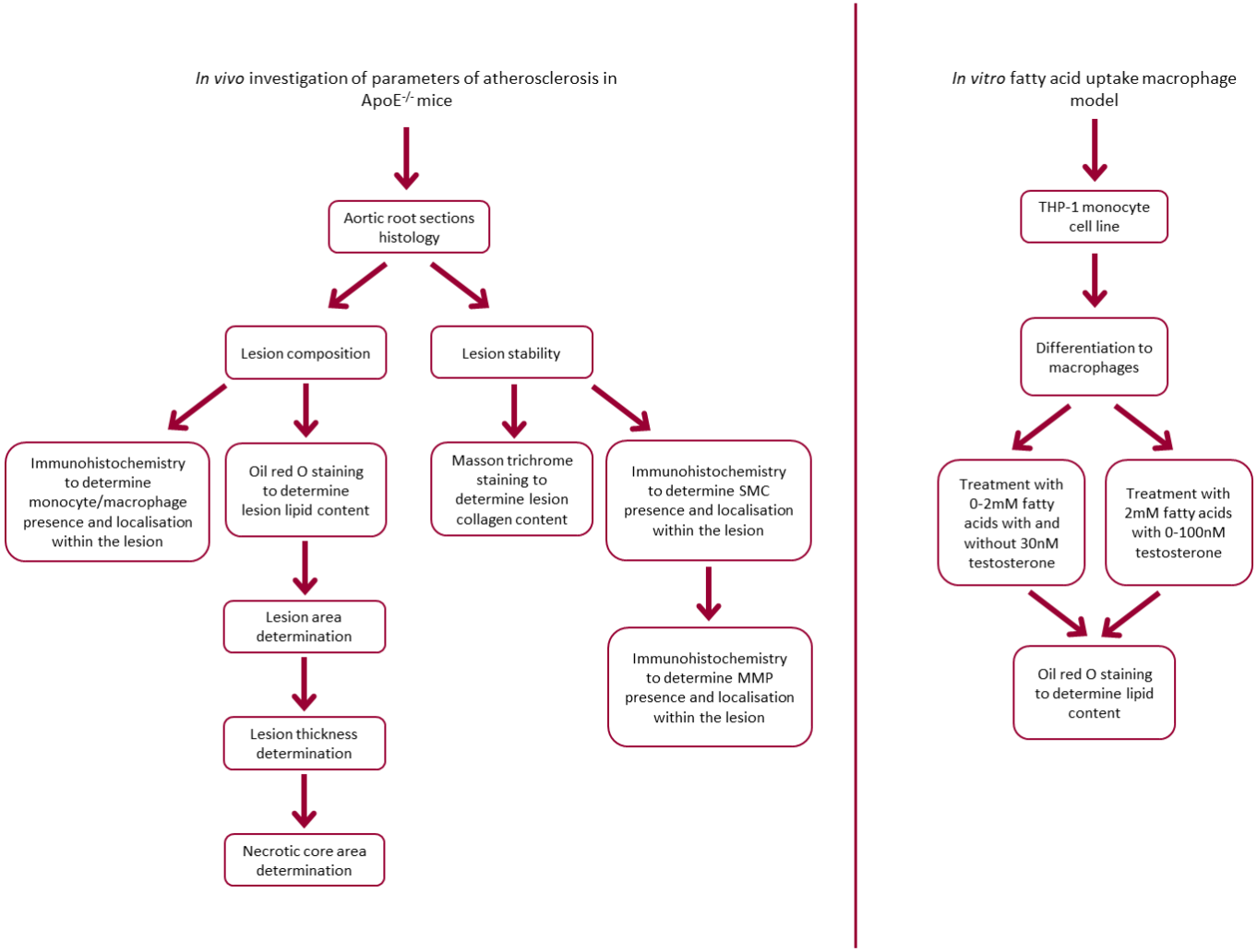


Figure 3.1 Experimental design to investigate the effect of testosterone on parameters of atherosclerosis in ApoE$^{-/-}$ mice, and in human macrophages *in vitro*.

3.4 Results

3.4.1 Serum testosterone measurements

Serum hormone measurements were taken at the end of the experimental period and compared between sham-operated, and orchidectomised mice fed a standard chow diet. The median serum testosterone concentration in sham-operated mice was within the expected physiological range for testosterone (20.63nmol/L). Serum testosterone levels were significantly reduced in the orchidectomised mice (1.57nmol/L) compared to sham-operated mice (Figure 5.11) (P<0.0001) (Figure 3.2).

Fortnightly testosterone replacement was effective in elevating serum testosterone in orchidectomised mice (8.44nmol/L) compared to placebo-treated orchidectomised mice (1.04nmol/L, P<0.001) both fed a high fat 'Western' diet (Figure 5.17). Testosterone replacement did not increase serum testosterone levels to within the expected physiological range (10-30nmol/L); however, they were still comparable to sham-operated mice (7.52nmol/L) (Figure 3.3).

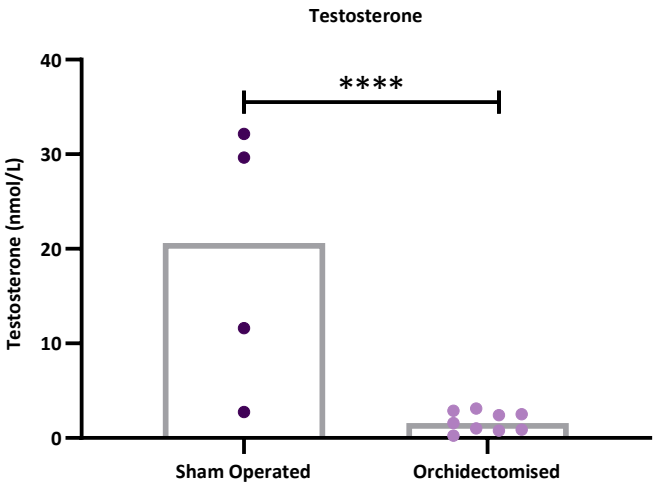


Figure 3.2 Serum total testosterone concentration in ApoE$^{-/-}$ mice fed a standard chow diet. Sham-operated (n=4), and orchidectomised mice (n=9) on a standard chow diet were compared for serum total testosterone concentration at the end of the 17-week experimental period. (****P<0.0001), Mann–Whitney U test.

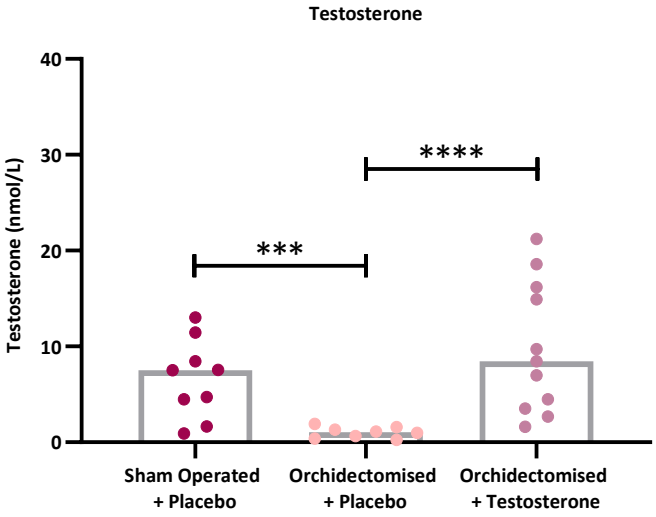


Figure 3.3 Serum total testosterone concentration in ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Sham-operated (n=9) and orchidectomised mice (n=8) receiving placebo and orchidectomised mice receiving testosterone (n=9) were compared for serum total testosterone concentration at the end of the 17-week experimental period. (***P<0.001, ****P<0.0001), Kruskal–Wallis test.

3.4.2 The effect of testosterone on atherosclerotic lesions

3.4.2.1 Lipid deposition in the aortic root of ApoE$^{-/-}$ mice

Analysis of oil red O (ORO) stained sections was performed by manually outlining the outer and inner medial areas of the aortic root, followed by outlining the lesions from the internal elastic lamina to the luminal edge. The lipid-stained areas were expressed as a percentage of the medial area. Measurements were taken from 6 sections per mouse and median values calculated.

ORO staining was detected throughout the aortic root of the orchidectomised ApoE$^{-/-}$ mice and was significantly elevated in the middle of the root in orchidectomised mice compared to sham-operated mice (39.3 vs 24.5%, *P*=0.0317) (Figure 3.4B). There were no differences observed in lipid deposition in orchidectomised mice compared to sham-operated mice fed a standard chow diet (20.84 vs 26.70%, *P*= 0.1986) (Figure 3.4C). This is due to an insufficient sample size; power analysis indicates that n=7 for each group would demonstrate if there are differences between the two groups.

In mice fed a high fat 'Western' diet, placebo-treated orchidectomised mice had significantly higher lipid deposition compared to sham-operated mice (48.00 vs 42.22%, *P*=0.0029). Furthermore, testosterone-treated mice had significantly lower intra-lesion lipid deposition compared to placebo-treated orchidectomised mice (39.90 vs 48.00%, *P*=0.0031) (Figure 3.5).

3.4.2.2 Lesion inflammation and composition analysis by immunohistochemistry

Aortic root sections adjacent to sections containing fatty streaks were selected for immunohistochemical analysis. Monocyte/macrophage presence within the aortic root was detected by positive MOMA2 staining (Figure 3.6). Positive staining for MOMA2 was observed in all lesion regions and located directly below the endothelial layer, which was further identified by positive staining for von Willebrand Factor (vWF). Placebo treated orchidectomised mice had significantly higher macrophage infiltration in the lesion areas compared to placebo sham-operated ApoE$^{-/-}$ mice (55% vs 77% of the lesion region, *P*=0.0016) when normalised to total lesion area.

Furthermore, testosterone treatment in orchidectomised mice reduced macrophage infiltration and was significantly lower than placebo-treated orchidectomised mice (61% vs 77% of the legion region, *P*=0.0171) (Figure 3.6).

3.4.2.3 Atherosclerotic lesion necrotic core area

The necrotic core area was quantified from the Masson's Trichrome sections and was defined as the area within the aortic root lesion that had an absence of cellularity (Figure 3.7A). There were no differences observed between placebo-treated orchidectomised mice and sham-operated mice in the necrotic core area (0.0082 vs 0.0075μm^2, *P*=0.473) (Figure 3.7B) or percentage of the lesion (2.8 vs 2.9%, *P*=0.9684) (Figure 3.7C). The study was sufficiently powered to assess changes in necrotic core area.

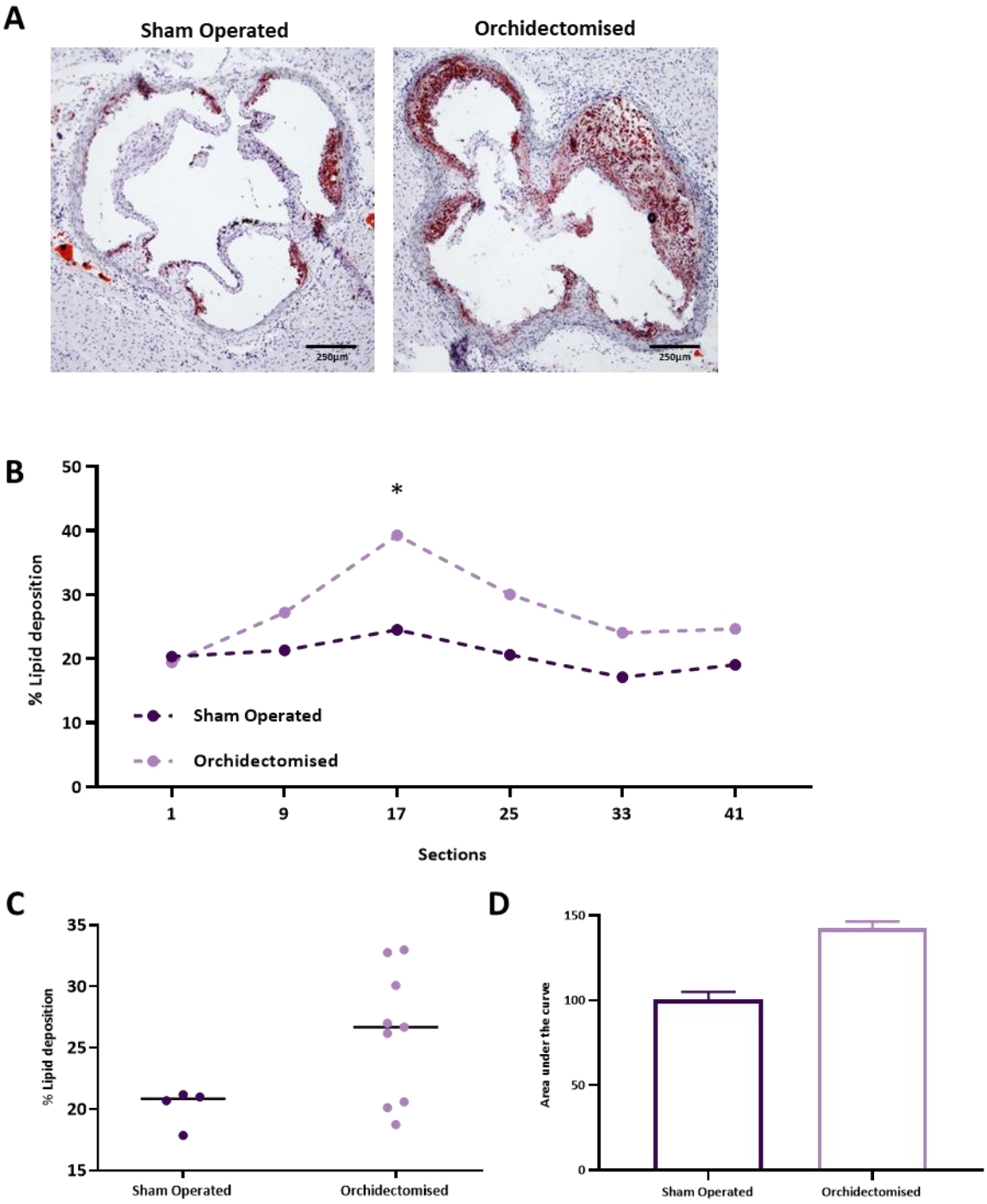


Figure 3.4 Microscopic analysis of the intimal lipid deposition in the aortic roots of ApoE$^{-/-}$ mice fed a standard chow diet. (A) Representative oil red O stained cross sections of the aortic root from sham-operated (n=4), and orchidectomised ApoE$^{-/-}$ (n=7) mice fed a standard chow diet. ORO staining was distinguished from non-specific accumulation of the stain by its cellular location identified by the haematoxylin counterstain compared to large droplets of red dye, respectively. (B) Lipid deposition areas of nine individual cross sections of the aortic root of each mouse. (C) Average lipid deposition in atherosclerotic lesions in the aortic root. (D) The area under the curve. (*P ≤ 0.05), Mann–Whitney U test. Scale bar = 250µm.

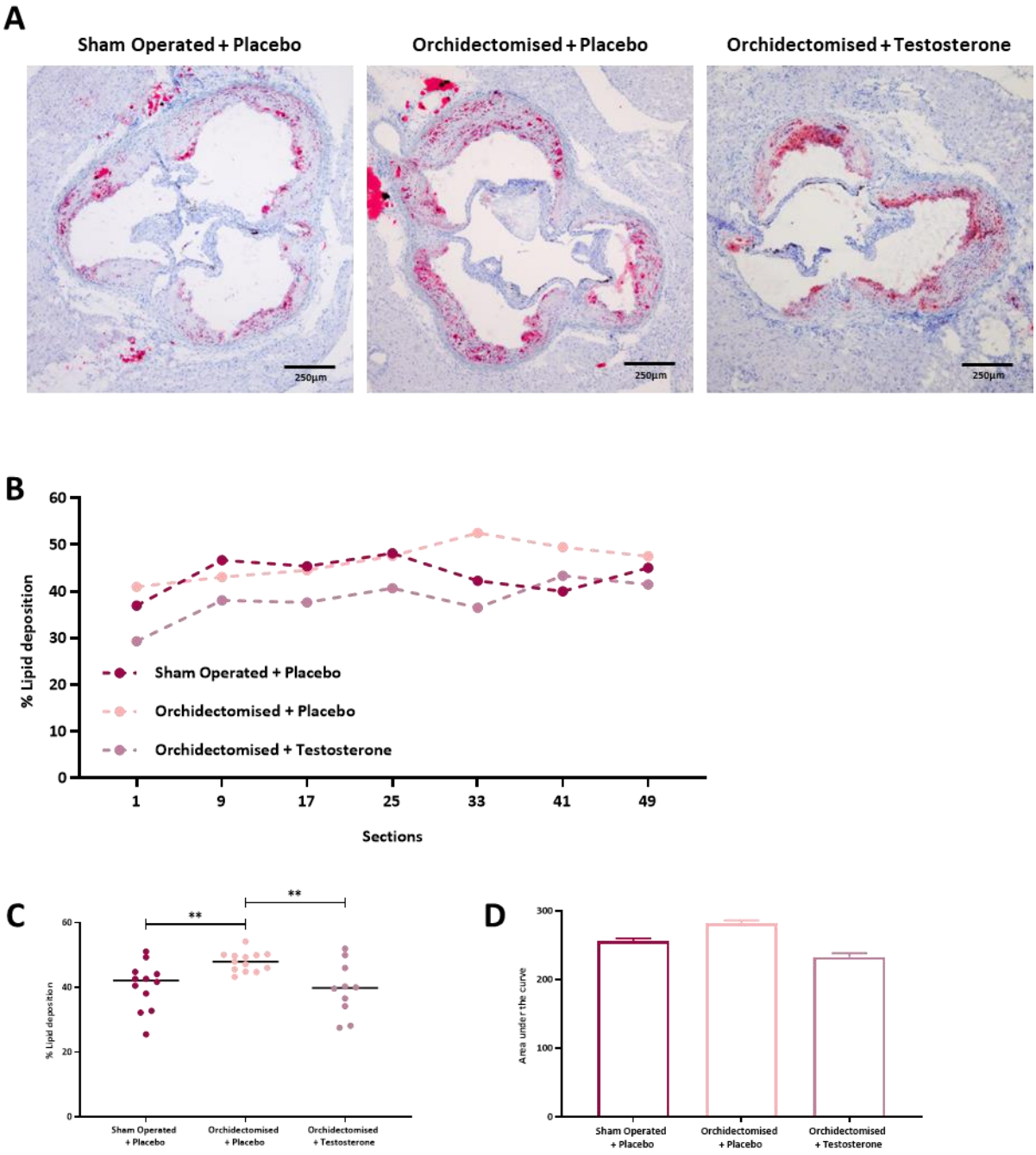


Figure 3.5 Microscopic analysis of the intimal lipid deposition in the aortic roots of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. (A) Representative oil red O stained cross sections of the aortic root from placebo-treated sham-operated (n=12) and orchidectomised (n=13) ApoE$^{-/-}$ mice and orchidectomised ApoE$^{-/-}$ mice treated with testosterone (n=10) fed a high fat 'Western' type diet. (B) Lipid deposition areas of nine individual cross sections of the aortic root of each mouse. (C) Average lipid deposition in atherosclerotic lesions in the aortic root. (D) The area under the curve. Scale bar = 250µm. (**P ≤ 0.01), Kruskal–Wallis test.

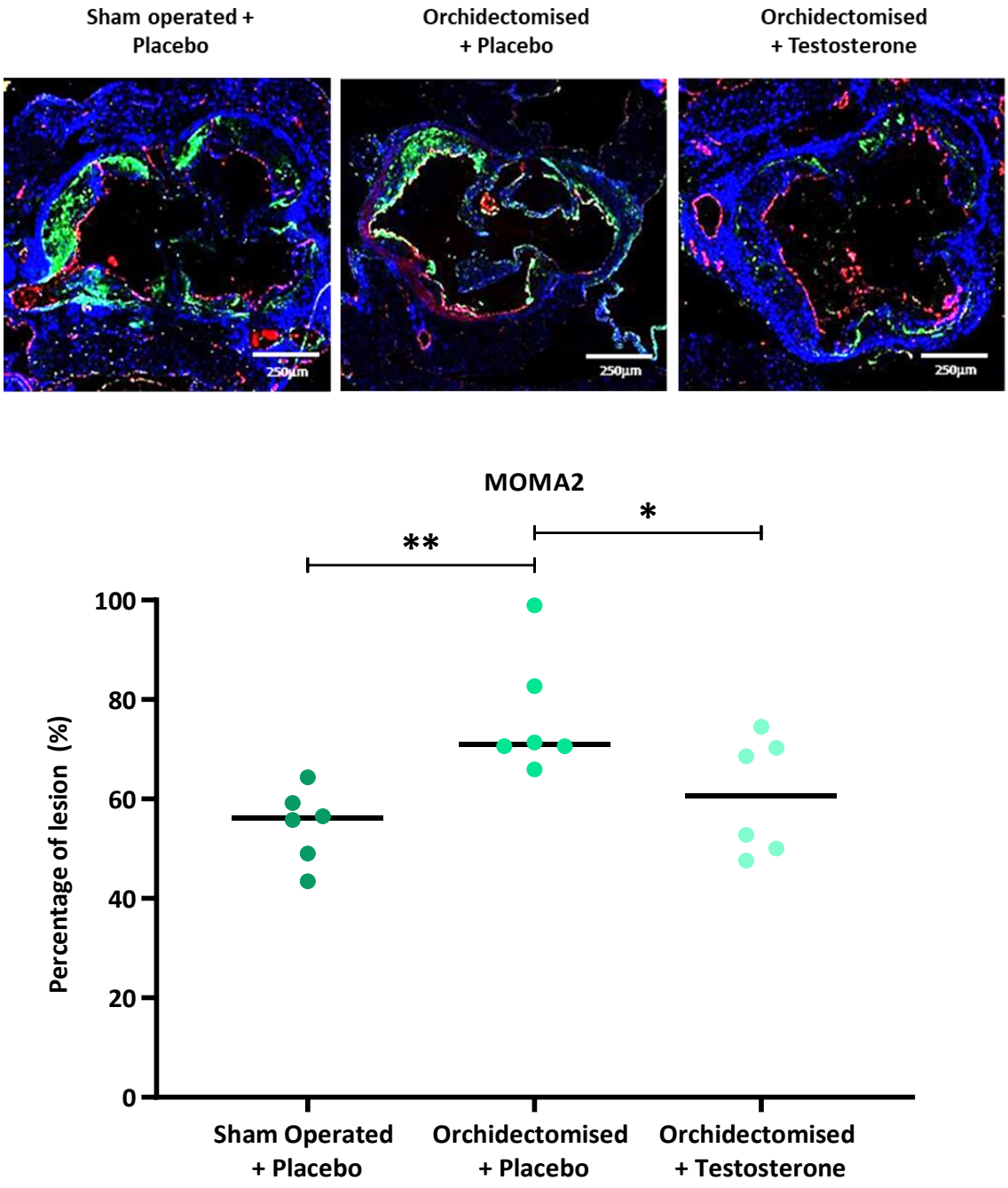


Figure 3.6 Immunohistochemical analysis of fatty streak composition in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone treatment. Aortic root samples were selected from the middle of the aortic root. The endothelial layer was marked by von Willebrand factor (VWF, red) and monocyte/macrophage infiltration was detected adjacent to fatty streaks (MOMA2, green). Cell nuclei were stained with DAPI (blue). Scale bar 250µm. (*P≤ 0.05, **P ≤ 0.01), Kruskal–Wallis test.

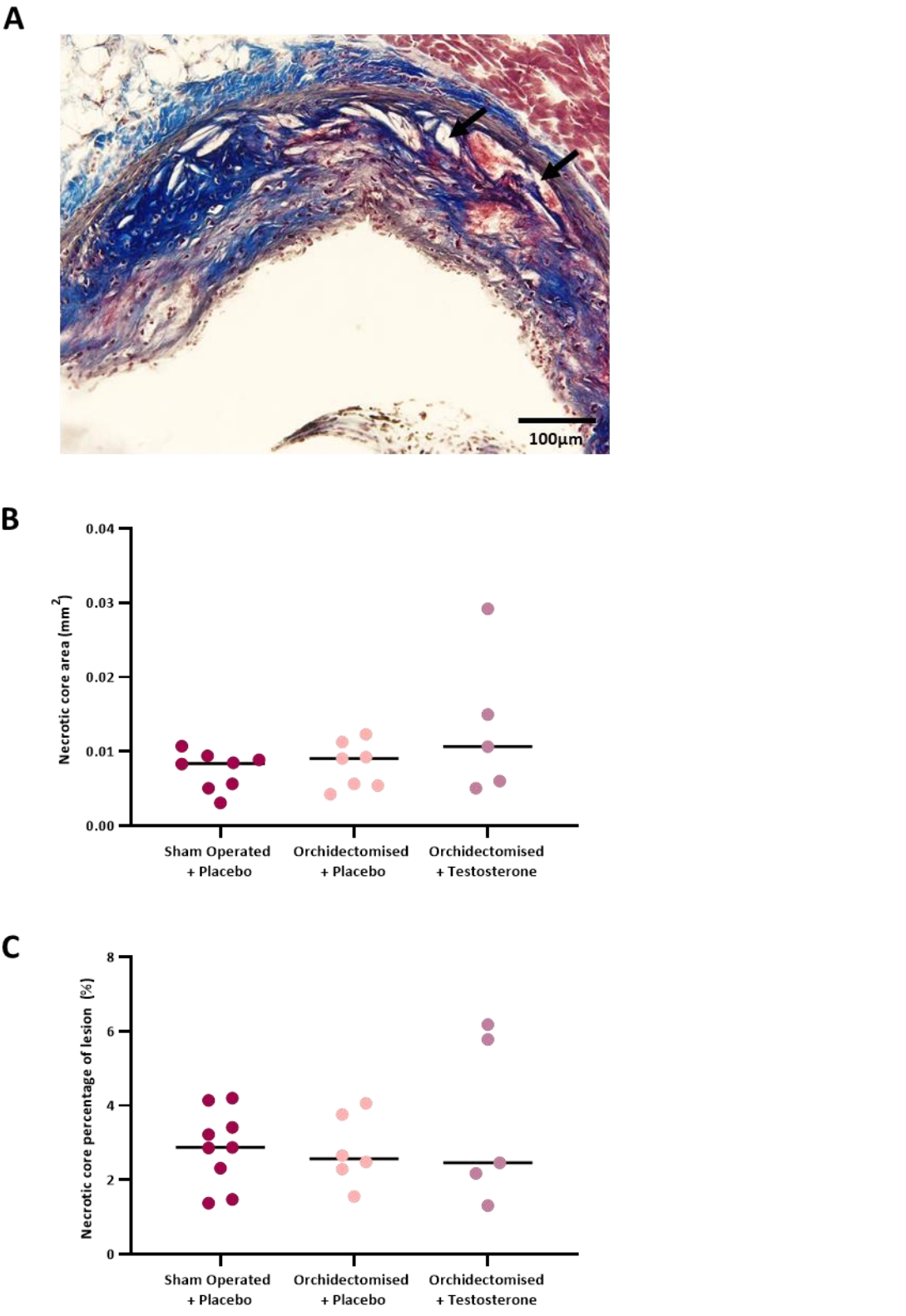


Figure 3.7 Histological analysis of the necrotic core area in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. (A) Representative section of Masson's Trichrome-stained aortic root (arrows indicate necrotic core). Necrotic core defined by an area of acellularity. Scale bar 100µm. (B) Quantification of the total area of the necrotic core (C) and as a percentage of the atherosclerotic lesion.

3.4.2.4 Aortic root-lesion area and thickness of ApoE$^{-/-}$ mice

In the aortic root, there were no significant differences in lesion area in orchidectomised mice compared to sham-operated mice fed a standard chow diet (0.88 vs 0.37mm^2, *P*=0.0112) (Figure 3.8B). This is due to an insufficient sample size; power analysis indicates that n=7 for each group would demonstrate if there are differences between the two groups.

When fed a pro-atherogenic diet, there were no differences in lesion area observed in the placebo-treated orchidectomised mice compared to sham-operated mice and testosterone treated mice (0.40 vs 0.29 vs 0.35mm^2, *P*=0.3223) (Figure 3.9B). The study was sufficiently powered to assess changes in lesion area.

There were no significant differences in maximal lesion thickness between the sham-operated and orchidectomised mice fed a standard chow diet (234.62 vs 349.26µm, *P*=0.1091) (Figure 3.10). This is due to an insufficient sample size; power analysis indicates that n=7 for each group would demonstrate if there are differences between the two groups. Similarly, there was also no significant differences in maximal lesion thickness between the placebo-treated sham-operated and orchidectomised mice and the testosterone-treated orchidectomised mice fed a high fat 'Western' diet (184.30 vs 211.69 vs 202.06µm, *P*= 0.7153) (Figure 3.11). The study was sufficiently powered to assess changes in lesion thickness.

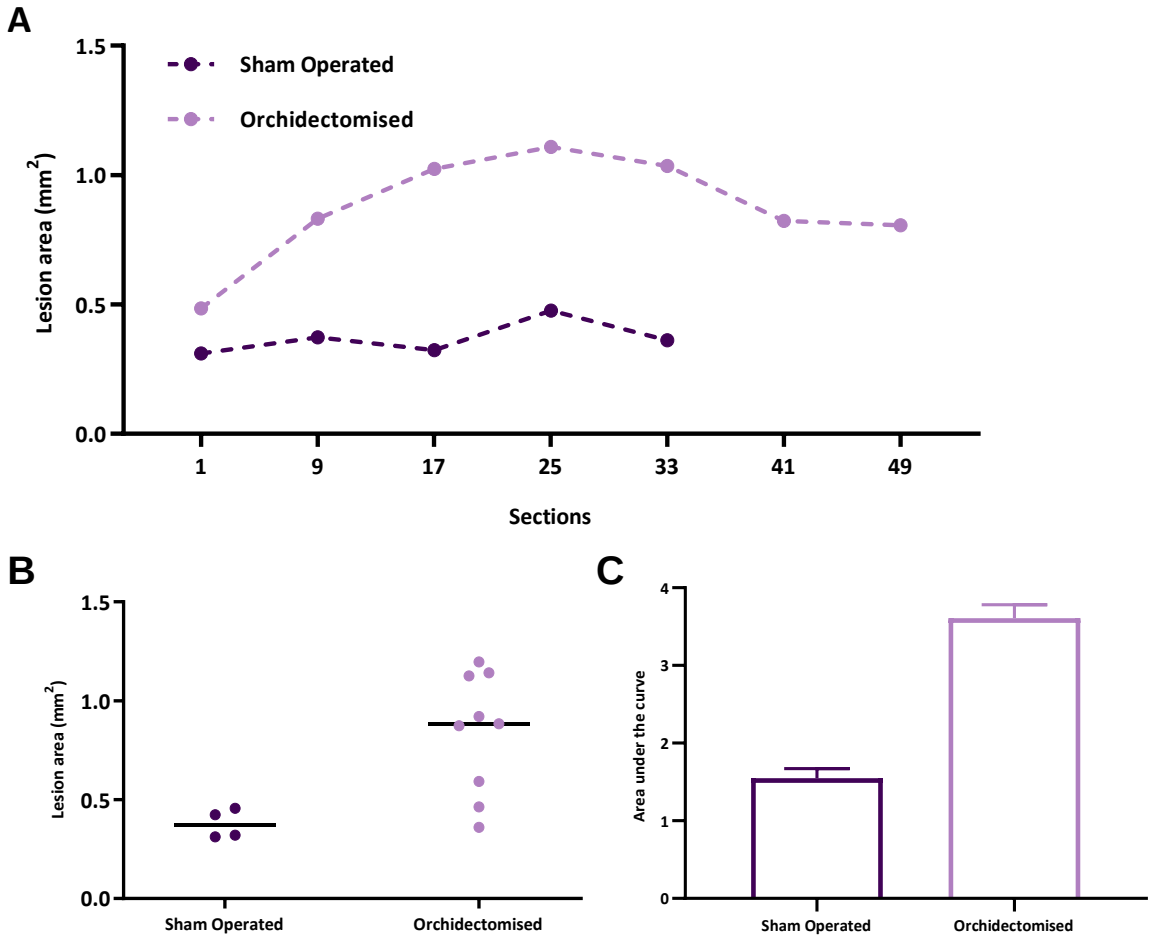


Figure 3.8 Quantitative analysis of the lesion area in the aortic root of ApoE$^{-/-}$ mice fed a standard chow diet. (A) lesion area of nine individual cross sections of the aortic root of each mouse. (B) Average lesion area in atherosclerotic lesions in the aortic root.

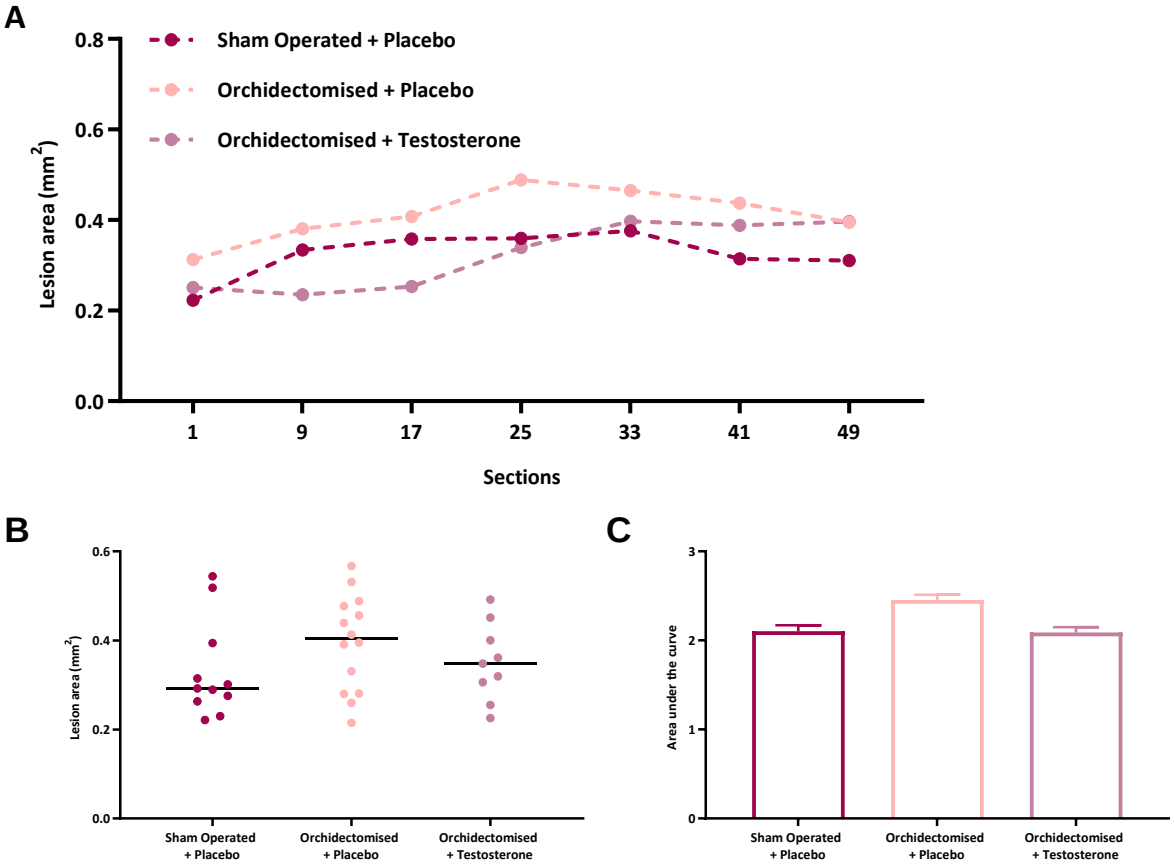


Figure 3.9 Quantitative analysis of the lesion area in the aortic root of ApoE$^{-/-}$ mice fed a high fat 'Western' diet. (A) lesion area of nine individual cross sections of the aortic root of each mouse. (B) Average lesion area in atherosclerotic lesions in the aortic root.

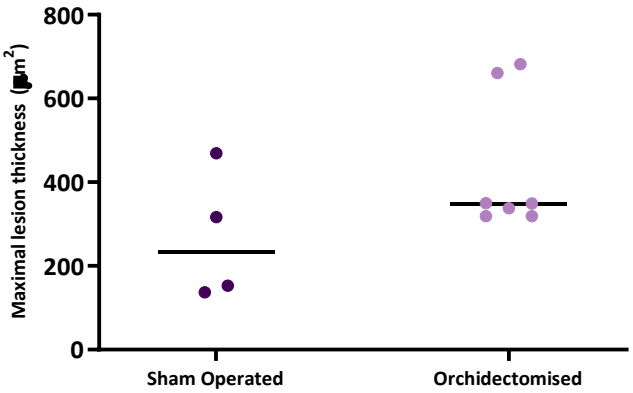


Figure 3.10 Quantitative analysis of the maximal lesion thickness in the aortic root of ApoE$^{-/-}$ mice fed a standard chow diet. ORO stained cross sections were imaged, and the thickness of the lesion was measured at the thickest part of the lesion.

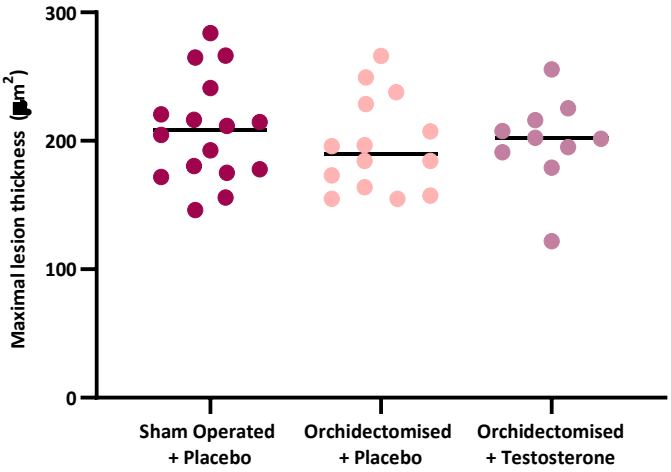


Figure 3.11 Quantitative analysis of the maximal lesion thickness in the aortic root of ApoE$^{-/-}$ mice fed a high fat 'Western' diet. ORO stained cross sections were imaged, and the thickness of the lesion was measured at the thickest part of the lesion.

3.4.3 The effect of testosterone on aortic root lesion stability

3.4.3.1 Fibrous cap thickness of mice fed a high fat 'Western' diet

The thickness of the fibrous cap was quantified from the immunohistochemical staining for αSMA (Figure 3.12A). No differences in fibrous cap thickness were observed between placebo treated orchidectomised mice compared to sham-operated mice and testosterone treated mice (31.4 vs 34.3 vs 41.9μm, *P*=0.2083).

Fibrous cap thickness was also calculated as a percentage of the overall lesion thickness (Figure 3.12C). There were no differences between placebo-treated sham-operated and orchidectomised mice (12.51 vs 13.39%, *P*=0.6878). Similarly, there were also no differences between placebo-treated orchidectomised mice and testosterone-treated orchidectomised mice (13.89 vs 15.62, *P*=0.6878).

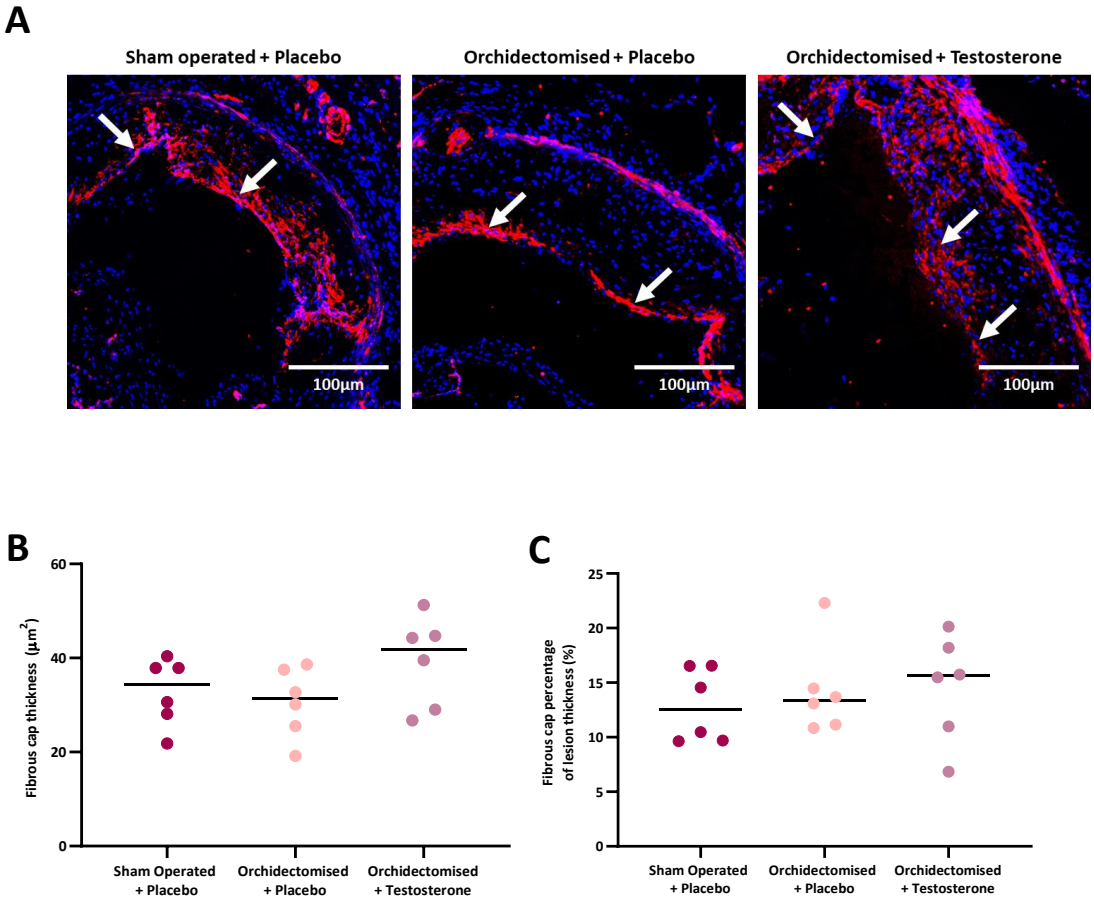


Figure 3.12 Immunohistochemical analysis of fibrous cap thickness in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. (A) Representative section of αSMA-stained (red) aortic root (arrows indicate fibrous cap) Cell nuclei were stained with DAPI (blue). Scale bar 100µm. (B) Quantification of the thickness of the fibrous cap. (C) Percentage of the fibrous cap of the total lesion thickness.

3.4.3.2 Atherosclerotic lesion collagen content

Masson's Trichrome staining was performed, and positive area for collagen (blue) was quantified as a measure of collagen content. There were no significant differences in lesion collagen content between the placebo-treated orchidectomised and sham-operated mice fed a high fat 'Western' diet (48.73 vs 51.23%, *P*=0.4115). Similarly, there were also no differences between testosterone-treated and placebo-treated orchidectomised mice (61.61 vs 48.73%, *P*=0.4115) (Figure 3.13). Power analysis indicates that the study was sufficiently powered to reveal any differences between the treatment groups.

3.4.3.3 MMP expression within the atherosclerotic lesion in the aortic root

3.4.3.3.1 Gene analysis of MMP expression

There were no significant differences in MMP9 and 10 gene expression in the lesion-tissue in placebo-treated orchidectomised mice compared to sham-operated and testosterone-treated mice (Figure 3.14). MMP13, however, was significantly downregulated in the placebo-treated orchidectomised mice compared to sham-operated mice (0.21 vs 2.1-fold, *P*=0.0021). Similarly, MMP13 was upregulated in testosterone-treated mice compared to placebo-treated orchidectomised mice (1.7 vs 0.21-fold, *P*=0.0341) (Figure 3.14).

There were no differences observed in MMP9, 10 and 13 gene expression between treatment groups in the endothelial layer (MMP9: *P*=0.1168, MMP10: *P*=0.4922, MMP13: *P*=0.5945) (Figure 3.15). Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups.

3.4.3.3.2 Analysis by immunohistochemistry

Aortic root sections adjacent to sections containing fatty streaks were selected for immunohistochemical analysis. Positive staining for MMP9, 10 and 13 were observed throughout the lesion in ApoE$^{-/-}$ mice fed a high fat 'Western' diet. There

were no differences observed in MMP9, 10 and 13 expression in placebo-treated orchidectomised mice compared to sham-operated mice and testosterone treated mice (40.7 vs 51.4% vs 48.5, *P*=0.2209) (Figure 3.16, 3.17 and 3.18). Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups.

3.4.3.4 Atherosclerotic lesion smooth muscle content

Aortic root sections adjacent to sections containing fatty streaks were selected for immunohistochemical analysis. Smooth muscle cell presence within the aortic root was detected by positive αSMA staining (Figure 3.20). Positive staining for αSMA was observed in all lesion regions forming the fibrous cap and throughout the lesion. There were no significant differences in lesion positive αSMA staining between the placebo-treated orchidectomised and sham-operated mice and testosterone treated mice fed a high fat 'Western' diet (45.7 vs 44.1% vs 35.8, *P*=0.2359) (Figure 3.19). Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups.

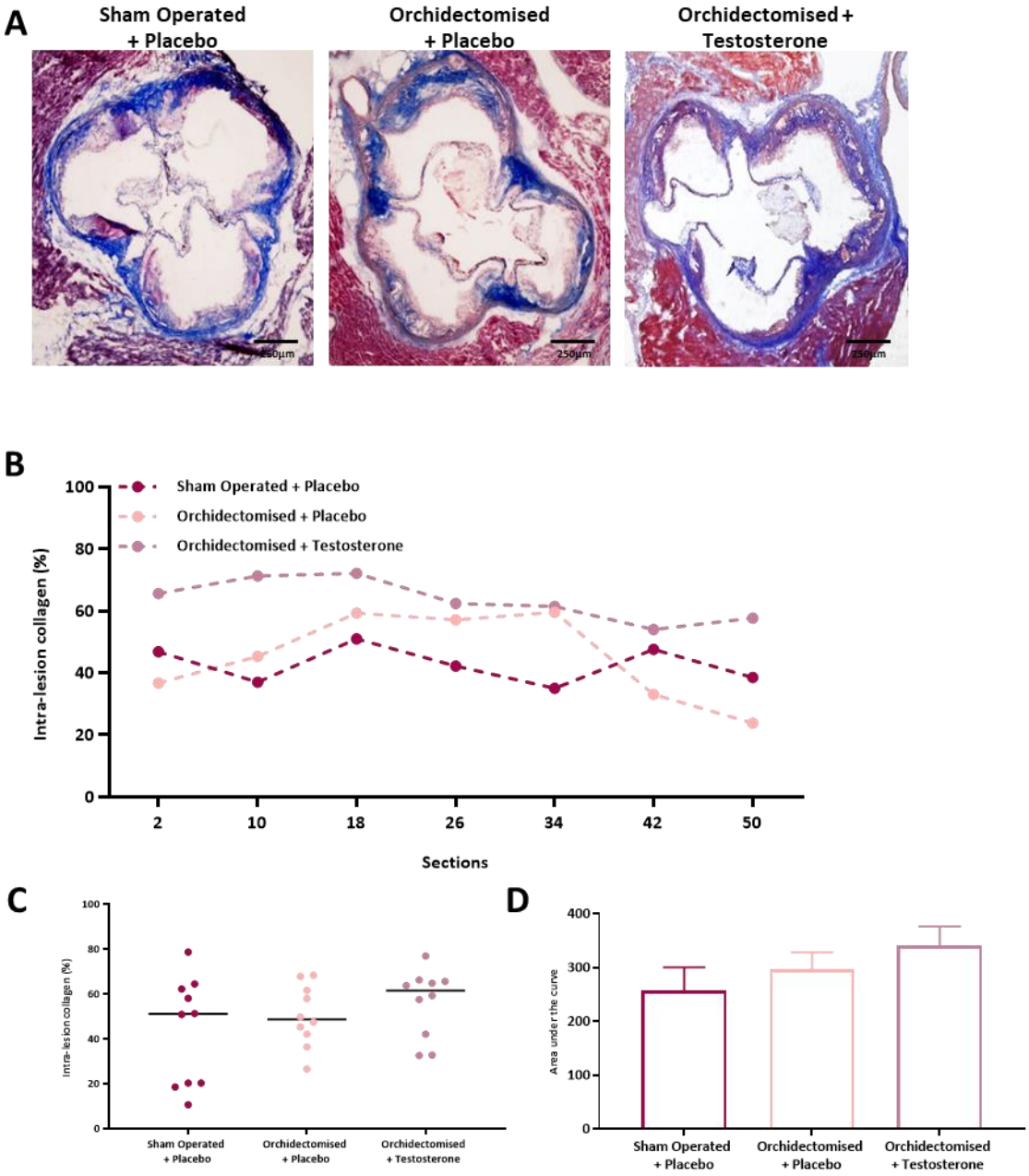


Figure 3.13 Microscopic analysis of the aortic root intra-lesion collagen content of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. (A) Representative Masson's Trichrome stained cross sections of the aortic root from placebo-treated sham-operated (n=10) and orchidectomised ApoE$^{-/-}$ mice (n=10) and orchidectomised mice treated with testosterone (n=10) fed a high fat 'Western' type diet. Collagen fibres are indicated in blue. (B) Collagen content within lesion areas of nine individual cross sections of the aortic root of each mouse. (C) The area under the curve collagen in atherosclerotic lesions in the aortic root. Scale bar = 250µm.

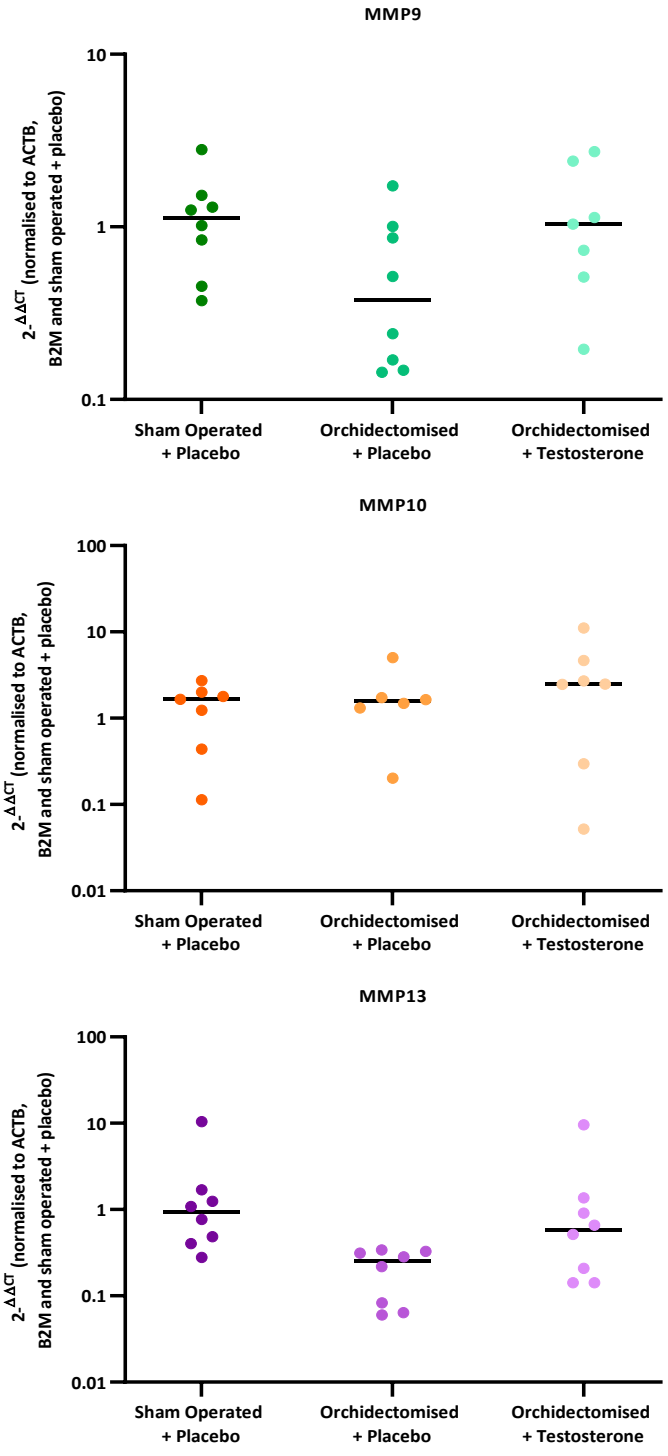


Figure 3.14 The effect of testosterone treatment on gene expression of MMP9, 10 and 13 in lesion-specific tissue of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. Aortic root samples, identified as having atherosclerotic lesions from positive ORO staining, were selected throughout the aortic root. Sham-operated mice (n=8) and orchidectomised receiving placebo (n=8) and orchidectomised receiving testosterone treatment (n=8) were compared for gene expression of MMP9, 10 and 13 which are linked to the pathogenesis of atherosclerosis. (*P≤ 0.05, **P ≤ 0.01), Kruskal–Wallis test.

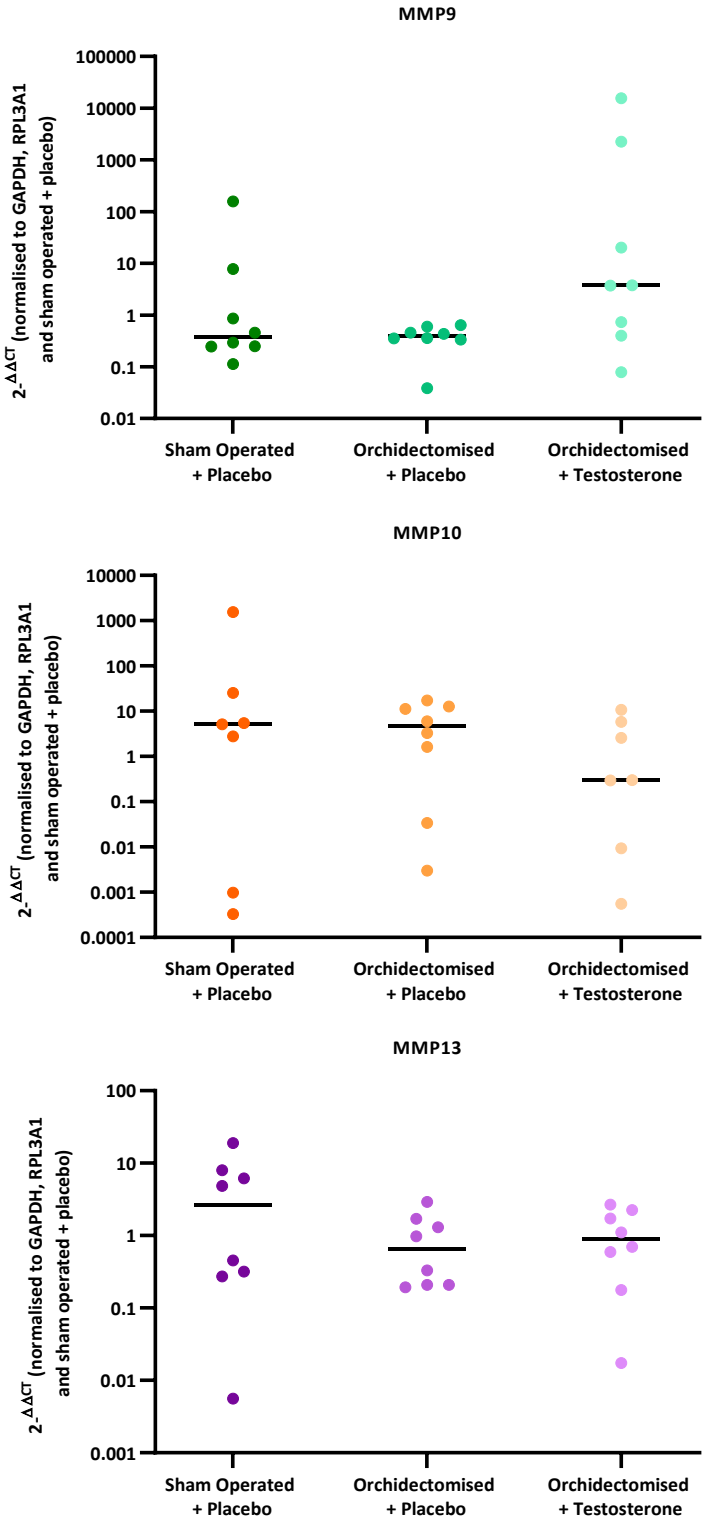
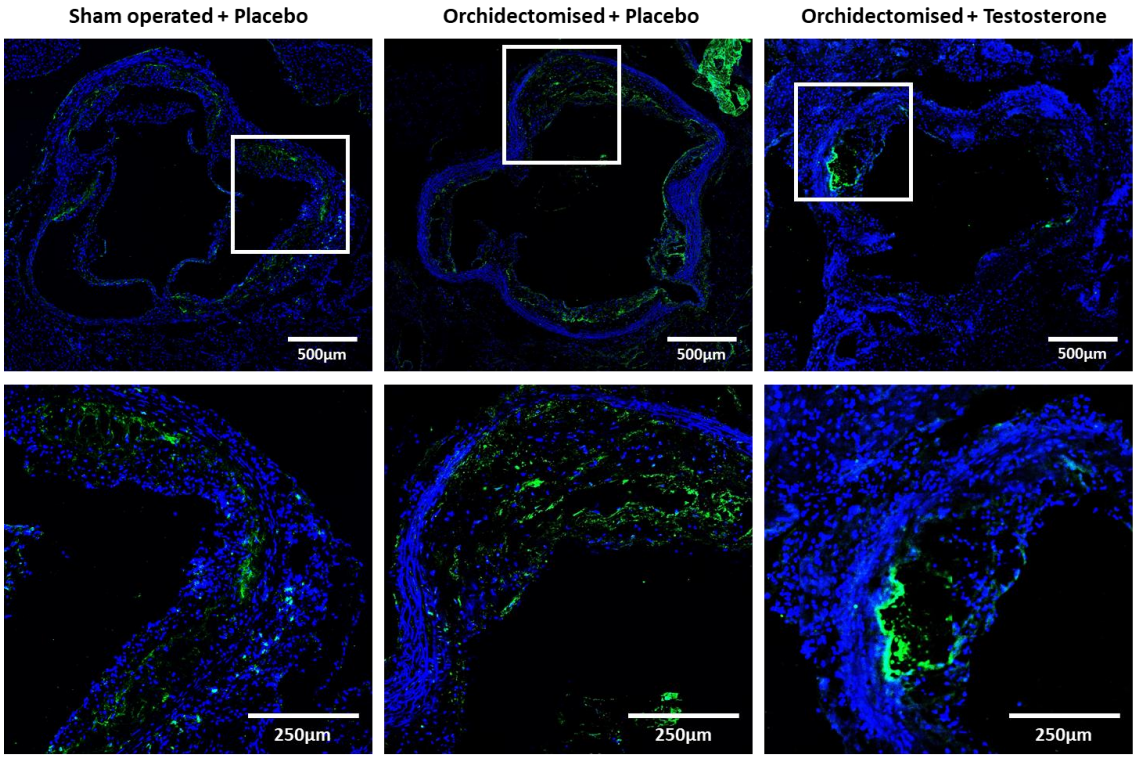


Figure 3.15 The effect of testosterone treatment on gene expression of MMP9, 10 and 13 in endothelial cells lining the top of the fatty streak of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. Aortic root samples, identified as having atherosclerotic lesions from positive ORO staining, were selected throughout the aortic root. Sham-operated mice (n=8) and orchidectomised receiving placebo (n=8) and orchidectomised receiving testosterone treatment (n=8) were compared for gene expression of MMP9, 10 and 13 which are linked to the pathogenesis of atherosclerosis.



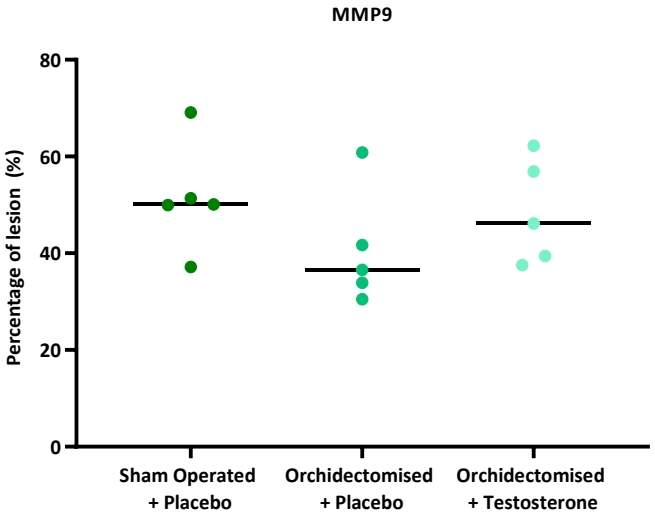


Figure 3.16 Immunohistochemical analysis of MMP9 expression within the fatty streak in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. Aortic root samples were selected from the middle of the aortic root. MMP9 was detected within the lesions of placebo-treated sham-operated and orchidectomised mice and testosterone-treated mice. Immunopositivity was expressed as a percentage of the lesion area. Cell nuclei were stained with DAPI (blue). Scale bars 500 and 250µm.

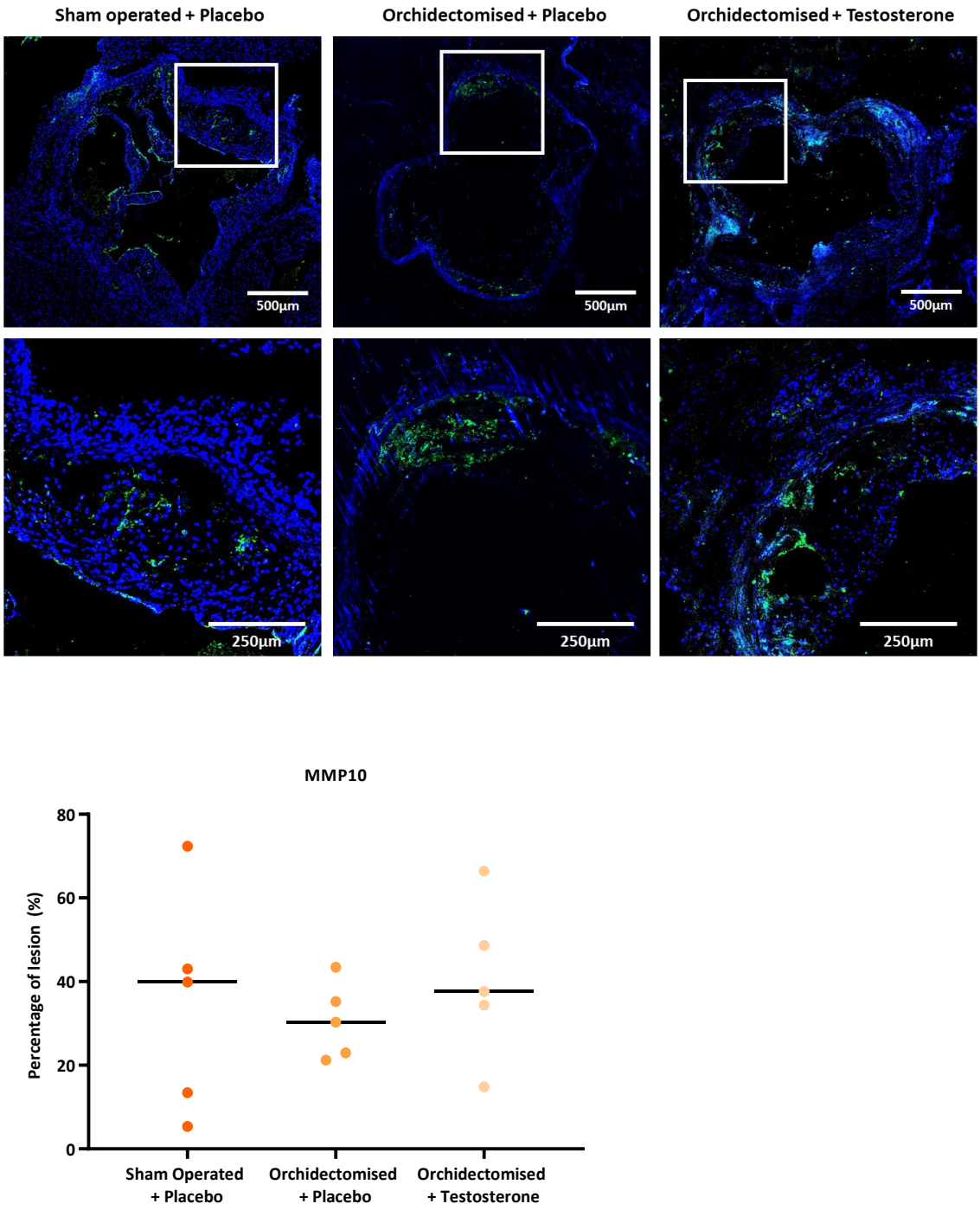
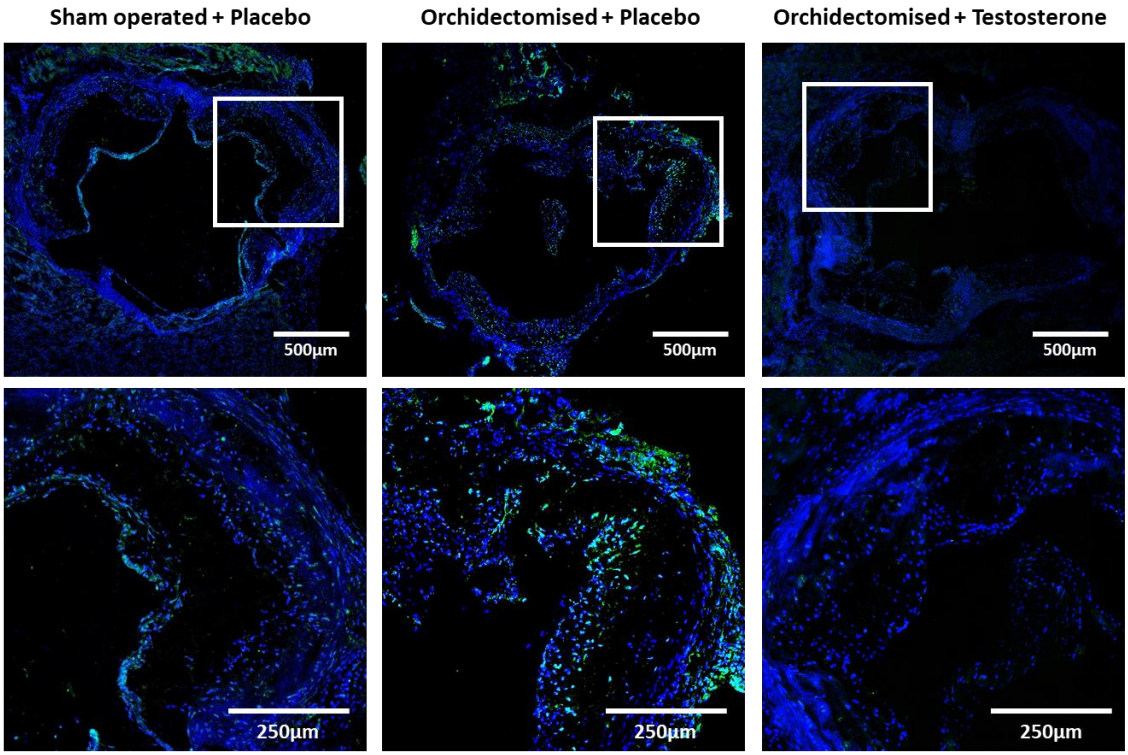


Figure 3.17 Immunohistochemical analysis of MMP10 expression within the fatty streak in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. Aortic root samples were selected from the middle of the aortic root. MMP10 was detected within the lesions of placebo-treated sham-operated and orchidectomised mice and testosterone-treated mice. Immunopositivity was expressed as a percentage of the lesion area. Cell nuclei were stained with DAPI (blue). Scale bars 500 and 250µm.



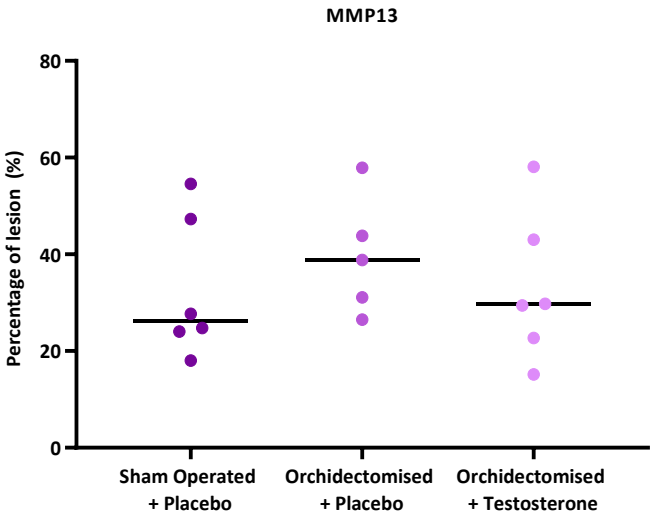
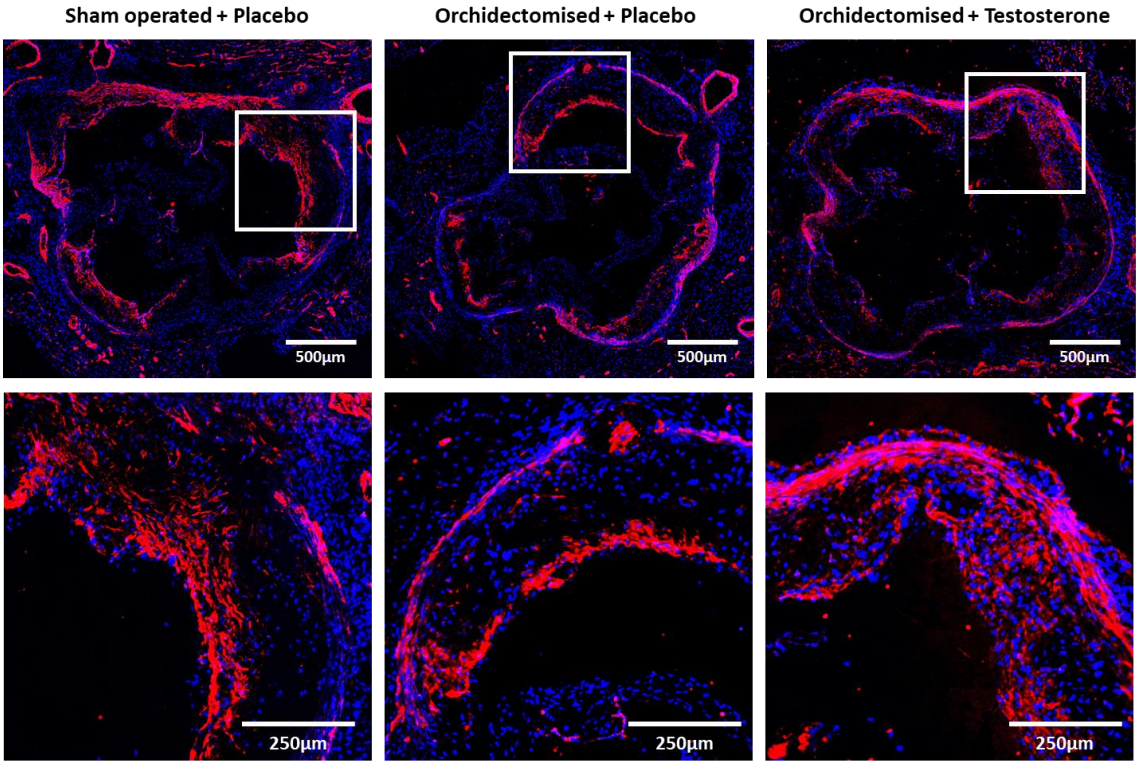


Figure 3.18 Immunohistochemical analysis of MMP13 expression within the fatty streak in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. Aortic root samples were selected from the middle of the aortic root. MMP13 was detected within the lesions of placebo-treated sham-operated and orchidectomised mice and testosterone-treated mice. Immunopositivity was expressed as a percentage of the lesion area. Cell nuclei were stained with DAPI (blue). Scale bars 500 and 250µm.



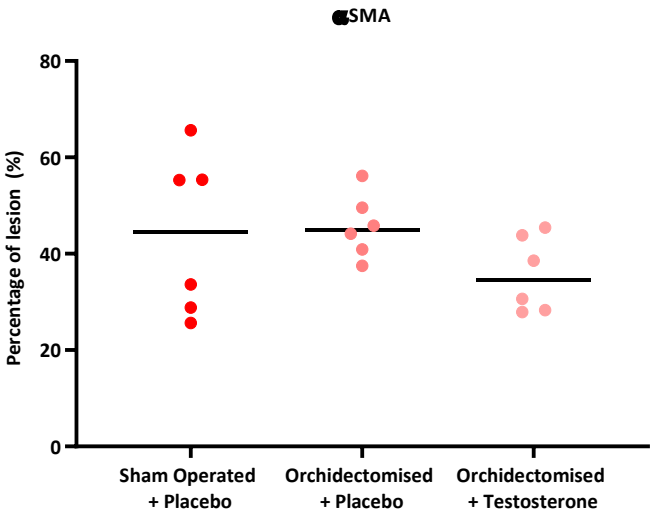


Figure 3.19 Immunohistochemical analysis of alpha-smooth muscle actin (αSMA) expression within the fatty streak in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone replacement. Aortic root samples were selected from the middle of the aortic root. αSMA was detected within the lesions of placebo-treated sham-operated and orchidectomised mice and testosterone-treated mice. Immunopositivity was expressed as a percentage of the lesion area. Cell nuclei were stained with DAPI (blue). Scale bars 500 and 250µm.

3.4.4 The effect of testosterone on fatty acid loaded macrophages

The effects of testosterone on macrophages when co-cultured with 0-2mM of fatty acids were investigated (Figure 3.20). Co-culturing fatty acid loaded macrophages with testosterone did not affect lipid uptake compared to untreated fatty acid loaded macrophages.

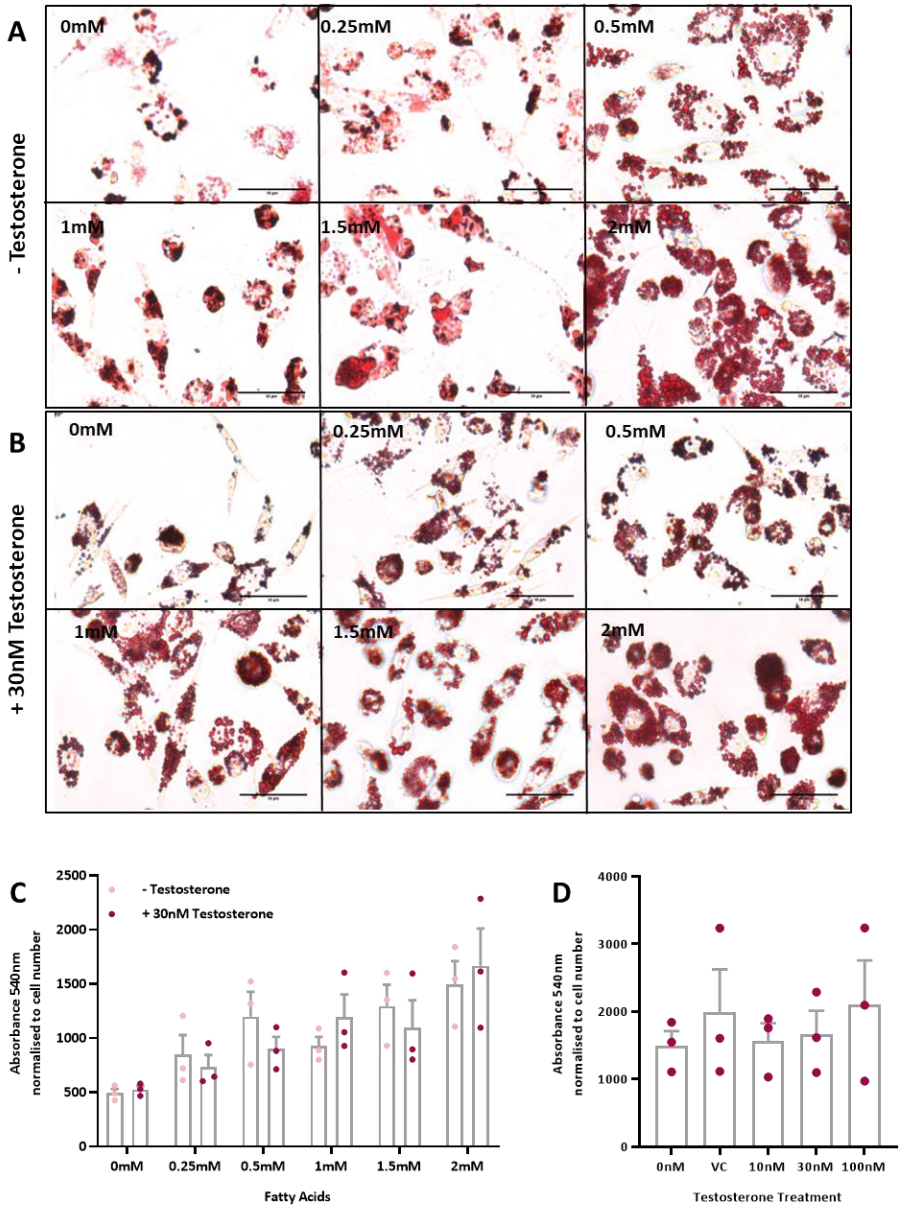


Figure 3.20 Analysis of lipid uptake in differentiated THP-1 macrophages after fatty acid loading. After differentiation THP-1 macrophages were fatty acid loaded (0-2mM) and fixed and stained with oil red-O. (A) Untreated cells were compared with (B) cells co-treated with 30nM testosterone. Scale bar 50µM. Lipid content was measured by quantification of oil red-O staining (absorbance at 540 nm) (n=3) for (C) varying fatty acid concentration treated with and without 30nM testosterone and (D) varying testosterone concentrations 0-100nM treated with 2mM fatty acids.

3.5 Discussion

The present study demonstrates beneficial effects of testosterone treatment in atherogenesis, as lipid deposition in the aortic root was significantly inhibited by testosterone treatment in ApoE$^{-/-}$ mice on a pro-atherogenic diet. Furthermore, this study demonstrated that testosterone deficiency was associated with increased lipid deposition in mice fed a standard chow and a pro-atherogenic diet.

The present study has demonstrated that low endogenous testosterone is associated with increased lesion lipid deposition within the aortic root of ApoE$^{-/-}$ mice fed a standard chow diet and a high fat 'Western' diet. These results concur with previous animal studies (Alexandersen *et al.*, 1999, Li *et al.*, 2008, Li *et al.*, 2013, Kelly *et al.*, 2012, Bruck *et al.*, 1997, Nathan *et al.*, 2001, Nettleship *et al.*, 2007a, Larsen *et al.*, 1993). Alexandersen *et al.* (1999) induced aortic atherosclerosis by feeding sham-operated or orchidectomised male rabbits a pro-atherogenic diet for 35 weeks. They reported a significant increase of 100% of aortic atherosclerosis in orchidectomised rabbits compared to sham-operated rabbits suggesting a protective effect of endogenous testosterone. Furthermore, following daily 80mg oral testosterone undecanoate, or twice weekly 25mg intramuscular injection of testosterone enanthate, aortic lesions were inhibited as indicated via a reduction in aortic lipid content (Alexandersen *et al.* (1999). Li *et al.* (2008) also reported similar results in rabbits. The route of administration and dose was deemed of importance to the extent of the beneficial effects observed with rabbits receiving the intramuscular testosterone having more significant reductions in aortic lipid content compared the rabbits receiving oral testosterone (Alexandersen *et al.*, 1999). This administration effect was considered due to intramuscular testosterone application resulting in initial higher serum testosterone levels as an early consequence compared to the oral testosterone. Twenty-four hours after administration, it is expected that intramuscular testosterone would result in a higher concentration of serum testosterone as it bypasses the initial pass through the liver prior to entering the circulation. Oral testosterone, however, will be partially metabolised by the liver before entering the circulation (Shoskes *et al.*, 2016). Dose and administration route in the present study was based on previous studies in mice of the same mouse strain (Nettleship *et al.*, 2007a). The present study

confirmed previous findings that fortnightly injections of testosterone treatment could return serum testosterone concentrations to within the physiological (10-30nmol/L) range in ApoE$^{-/-}$ mice. However, some mice fell below the normal range of testosterone levels. This may be due to under treatment for ApoE$^{-/-}$ mice, they were heavier and had more adipose tissue compared to mice the dose was based on, although testosterone levels did not correlate with weight and weight gain. However, at the time of sampling, the expected testosterone concentration would be around half of the maximum concentration achieved, as the half-life of the treatment used in this study is around six days.

Nettleship *et al.* (2007a) and Kelly *et al.* (2012) utilised the Tfm mouse model of testosterone deficiency with a non-functional AR. Nettleship *et al.* (2007a) demonstrated that low endogenous testosterone, be it associated with a non-functional AR (Tfm mouse) or a fully functional AR (orchidectomised XY male littermate), is associated with increased lipid deposition, therefore lesion area, within the aortic root. This demonstrates that lesion formation is a consequence of low endogenous testosterone levels and not due to the absence of a functional AR, as aortic lesions were observed in surgically orchidectomised male mice. Kelly *et al.* (2012) added to this by revealing that testosterone treatment in Tfm mice partially reduced lipid deposition and lesion formation in the aortic root, however not to the extent seen in XY littermates suggesting that testosterone protects against early atherogenesis via both AR-dependent and -independent pathways. Bourghardt *et al.* (2010) further demonstrated the protective effects of testosterone treatment on lesion formation in orchidectomised ARKO mice fed a pro-atherogenic diet. Testosterone treated orchidectomised ARKO mice had a significant reduction in lesion area compared to placebo treated controls. However, this was to a lesser extent than that observed in testosterone treated castrated wild-type mice, further supporting AR-dependent and -independent mechanisms in atheroprotection. While the present study did not investigate specific AR actions, similar benefits of endogenous and administered testosterone were observed in mice on standard and western diets.

Multiple studies have examined the association between endogenous testosterone levels and IMT of various major blood vessels as a marker of

atherosclerosis. Testosterone has been shown to correlate negatively with *in vivo* surrogate markers of atherosclerosis, including CIMT and the degree of aortic calcification (Fukui *et al.*, 2003, Muller *et al.*, 2004, Hak *et al.*, 2002, Svartberg *et al.*, 2006, Fu *et al.*, 2008).

The present clinical study measured CIMT as a surrogate marker of atherosclerosis in hypogonadal men with T2D, however, due to the COVID-19 pandemic, results could not be retrieved and analysed from the clinical instrumentation and are therefore not included in this thesis. Studies have demonstrated that CIMT negatively correlates with testosterone levels (Fukui *et al.*, 2003, Fu *et al.*, 2008, Muller *et al.*, 2004, Svartberg *et al.*, 2006, Kwon *et al.*, 2014, Soisson *et al.*, 2012, Sartorato *et al.*, 2007, de Sousa *et al.*, 2010). The association between low testosterone levels and increased IMT was reported in phenotypically different populations, including aging men, men with T2D, obese adolescent females, and patients with classic congenital adrenal hyperplasia, suggesting an association between testosterone levels and IMT (de Sousa *et al.*, 2010, Kwon *et al.*, 2014, Sartorato *et al.*, 2007, Fukui *et al.*, 2003). However, (de Sousa *et al.*, 2010, Kwon *et al.*, 2014) noted a high prevalence of MetS or insulin resistance in their patient population, limiting the scope of interpretation as to whether the apparent atherosclerosis may be caused by the low serum testosterone levels or metabolic disturbances. A four year follow up study reported that men with lower testosterone levels had greater progression of CIMT (Muller *et al.*, 2004). A population-based cross-sectional study of 1482 men aged 25 to 84 years found an inverse association between total testosterone levels and CIMT although this finding was not independent of BMI, suggesting that the relationship between total testosterone level and CIMT is partially mediated by body fat or body fat distribution (Svartberg *et al.*, 2006). Whereas, Fukui *et al.* (2003) reported an inverse association between free testosterone levels and CIMT and in men with T2D. A randomised placebo-controlled study treated patients with MetS and testosterone deficiency with TTh for 12 months and observed an improvement in insulin resistance and CIMT (Aversa *et al.*, 2010). Another study found a similar beneficial effects of TTh on CIMT in obese men, which was reversed after the withdrawal of TTh (Francomano *et al.*, 2014b). These studies have shown that

testosterone deficiency is associated with the presence of increased CIMT and that TTh ameliorates this. It is predicted given the short duration of treatment and follow up that CIMT may not change following TTh. The reduction of CIMT by TTh in these clinical studies may be due to improvements in lesion composition as in the present study we showed that testosterone treatment reduces immune infiltration and lipid accumulation.

Low endogenous testosterone increased the lesion area, and maximal thickness in mice fed a standard chow diet. Atherosclerosis is the main cause of angina, and arterial stenosis in humans and increased lesion thickness causes stenosis. A small RCT reported an improvement on angina as well as CIMT following 12 months of TTh (Mathur *et al.*, 2009). TTh whether it be intramuscular (Malkin *et al.*, 2004b), intravenous (Rosano *et al.*, 1999, Webb *et al.*, 1999) or transdermal (English *et al.*, 2000b, Malkin *et al.*, 2006) had a beneficial anti-ischemic effect in men with CAD. TTh has been shown to improve cardiac ischemia and improved or abolished symptomatic angina chest pain in men with CAD (Lesser, 1946, English *et al.*, 2000b, Malkin *et al.*, 2004b, Mathur *et al.*, 2009). 12 weeks of TTh in elderly men with T2D and CVD reduced the frequency of angina episodes and number of daily ischemic episodes and total ischemic burden (Cornoldi *et al.*, 2010). However, in the present study, testosterone treatment did not influence lesion area or thickness in mice fed a high fat 'Western' diet. Nathan *et al.* (2001) assessed the effects of testosterone on early atherogenesis in orchidectomised $LDLr^{-/-}$ mice fed a pro-atherogenic diet for eight weeks. They observed that the atherosclerotic lesion area was increased in orchidectomised mice compared to intact male mice and testosterone treatment in orchidectomised mice protected against this. The authors reported that administration of anastrazole, an aromatase inhibitor, to testes-intact males increased lesion formation to the same extent as that observed with orchidectomised animals. Furthermore, estradiol supplementation to orchidectomised males significantly decreased lesion area similar to that observed in testosterone-treated animals. They concluded that testosterone inhibits early atherogenesis by conversion to estradiol via aromatase. Whilst the anti-atherogenic effects of testosterone in the present study supports this finding, we did not observe any differences in the serum levels of estradiol. The present study did not

observe any differences in estradiol levels between groups. If in the present study, the effects of testosterone treatment were due to the conversion to estradiol, estradiol levels would be expected to be elevated in the sham-operated and the testosterone-treated mice compared to the placebo-treated orchidectomised mice. Bruck *et al.* (1997) demonstrated the sex-specific effects of testosterone and oestrogen in orchidectomised male and ovariectomised female rabbits fed a pro-atherogenic diet. Lesion area in the aortic arch was significantly reduced in testosterone-treated orchidectomised males and in oestrogen treated ovariectomised females. Testosterone treatment in ovariectomised females increased lesion area, whereas oestrogen treatment was insignificant in orchidectomised males. However, the greatest inhibition of lesion area in both sexes resulted from a combination of both testosterone and oestrogen treatment.

Increased lesion vulnerability that may result in lesion rupture is characterised by enhanced lesion content of macrophages, lipids and MMPs and reduced smooth muscle content, collagen content, fibrous cap thickness. The presence of monocytes/macrophages in atherosclerotic lesions of the ApoE$^{-/-}$ mice is well documented (Liang *et al.*, 2018, Zetterqvist *et al.*, 2013, Frodermann *et al.*, 2015), highlighting the role of inflammation. Kelly *et al.* (2012) demonstrated that monocytes/macrophages were present in both testosterone-treated and untreated Tfm mice and XY littermates. This suggests that monocytes and macrophages are present in the vessel wall at sites of atheroma regardless of the influence of androgens. However, this study did not quantify the positive staining for MOMA2 and therefore, could only report its presence in their animal groups but not the extent. In the present study, quantification of positive monocytes/macrophages staining was done, and we report a significant increase of positive staining in placebo-treated orchidectomised mice. Furthermore, testosterone treatment reduced this, following the same trend as lipid deposition. Kelly *et al.* (2012) suggested that as the positive staining for monocytes/macrophages was within the fatty streaks, and the size of the fatty streaks were smaller and fewer in the aortic root of testosterone-treated mice compared to controls, testosterone may not influence the proportion of monocytes/macrophages relative to lesion size, but may reduce leukocyte entry into

the vascular wall, resulting in inhibition of lesion formation. The present study supports this, reduced numbers of macrophages into the vessels wall would effectively reduce the resulting lipid uptake.

In advanced lesions, macrophage apoptosis promotes the development of the necrotic core, a key factor in rendering lesions vulnerable to disruption and rupture. Lesion complexity indicated by the necrotic core on Masson's trichrome staining revealed that small areas of necrotic core were present in all groups; however, there was no difference in total area between the groups. Shim *et al.* (2020) reported that the necrotic core area in the abdominal aorta, where the most advanced stages of disease were located was significantly increased orchidectomised Yucatan minipigs with hypercholesterolemia. Conversely, Bourghardt *et al.* (2010) observed that testosterone treatment in orchidectomised wild-type mice fed a pro-atherogenic diet reduced the frequency of a necrotic core by 68% compared to placebo-treated mice. However, they observed no significant difference in the necrotic core area following testosterone treatment in ARKO mice, indicating an AR-mediated effect. However, in the present study, we report no effect on the necrotic core area.

Collagen is synthesised by SMCs, and a reduction of lesion smooth muscle correlates with decreased lesion collagen content lending to lesion instability, characterised by reduced collagen content and a higher macrophage to smooth muscle ratio (Gojova *et al.*, 2003). The majority of the aforementioned animal studies did not investigate whether testosterone played a role in the stability of atherosclerotic lesion. The present study makes an advancing step in this investigation. We report that testosterone has no significant impact on lesion smooth muscle content, collagen content, fibrous cap thickness, or MMP expression in ApoE$^{-/-}$ mice. Conversely, Li *et al.* (2008) reported that physiological concentrations of testosterone enhanced lesion collagen content and fibrous cap thickness, thus increasing the stability of atheromatous lesion in the aortas of orchidectomised male rabbits fed a pro-atherogenic diet.

Our data does demonstrate, however, that the atherosclerotic lesions were rich in monocytes/macrophages and SMCs, both of which are potent producers of MMPs.

MMPs are responsible for both physiological, associated with production from SMCs, and pathophysiological, associated with production from macrophages, tissue remodelling and can cleave all structural elements of the ECM. MMP9 is one of the most widely investigated MMPs and regulates pathological remodelling in CVD. MMP9 deletion or inhibition has been shown to be beneficial in animal models of CVD (Halade *et al.*, 2013, Ducharme *et al.*, 2000, Gough *et al.*, 2006). As such, MMP9 expression is commonly measured in studies of atherosclerosis. In the present study, we did not observe any significant differences in intra-lesion MMP9 gene or protein expression in testosterone-treated mice. Furthermore, we did not observe any differences between the experimental groups for MMP10 expression, which has been implicated in reducing lesion stability. MMP10 deficiency has been shown to reduce intra-lesion inflammation, delay atherosclerosis progression and lesion calcification in $ApoE^{-/-}$ mice (Purroy *et al.*, 2018). Whereas, MMP13 inhibition has been shown to improve lesion stability by increasing collagen content of established mouse atheromas also in $ApoE^{-/-}$ mice (Quillard *et al.*, 2011). In the present study, we did observe a significant decrease in lesion MMP13 gene expression in placebo-treated orchidectomised mice compared to sham-operated and testosterone-treated mice, although this is not was expected, especially as this did not translate to lesion MMP protein expression analysed by immunohistochemistry. Data from the present study has shown that testosterone treatment is unlikely to influence lesion stability through the regulation of these MMPs. We have shown, however, that testosterone treatment may reduce monocyte/macrophage infiltration into the lesion and as macrophages are potent producers of MMPs, fewer intra-lesion macrophages are associated with a more stable lesion phenotype (Michel *et al.*, 2011). However, mouse lesions are not prone to rupture; therefore, factors that influence rupture such as MMPs are perhaps not the most suitable to be studied in such models as lesions may well remain relatively stable. Furthermore, lesion collagen content was high and smooth muscle, and fibrous cap thickness was stable in the present study regardless of treatment, again lending to an inability to rupture and an overall stable lesion. Therefore, testosterone may not influence MMP mediated plaque degradation an already stable lesion that is not prone to rupture. Still, in human studies, low testosterone is associated with an increased burden of aortic and carotid atheroma (Hak *et al.*, 2002, van den Beld *et al.*, 2003,

Malkin *et al.*, 2004b, Muller *et al.*, 2004, Svartberg *et al.*, 2006). Evidence from long‐term epidemiological studies supports a protective effect of TTh, evidenced by a reduction in major adverse CV events (MACEs) such as rupture and mortality in studies which have treated men with testosterone deficiency (Jones and Kelly, 2018). Additionally, hypogonadal men with a history of CVD treated with TTh have been reported to experience reduced frequency of cardiovascular events (Haider *et al.*, 2016).

The capacity of macrophages to accumulate or process lipids and to regulate proteases and cytokines in their local environment is a critical determinant of lesion development, lipid core formation and lesion stability (de Gaetano *et al.*, 2016). HDL particles facilitate reverse cholesterol transport by accepting cholesterol from lipid-laden macrophages in peripheral tissues and transporting it to the liver for excretion in bile. This prevents the deposition of cholesterol in the arterial wall and thereby protects against atherogenesis. *In vitro* findings suggest that testosterone upregulates scavenger receptor BI and as a functional consequence accelerates reverse cholesterol transport in macrophages (Langer *et al.*, 2002). Therefore reductions in HDL-cholesterol caused by TTh in clinical studies may actually reflect this accelerated process (Rubinow and Page, 2012). Testosterone has been shown to significantly increase the expression of genes encoding proteins involved in cholesterol efflux and metabolism by activating LXRα. Testosterone increased the rate of cholesterol efflux from macrophages, and this may be due to an increase in intracellular cholesterol transport (Kilby and Jones, 2013). Furthermore, macrophages exposed to testosterone *in vitro* stimulated the expression of LXRα and ApoE. The authors suggested that testosterone activates LXR acting through this nuclear receptor to control the expression of ApoE to aid cholesterol efflux. Supporting the role of testosterone as an anti-atherogenic factor (Bokhamada, 2014). Corcoran *et al.* (2011), however, reported that testosterone has no effect on cholesterol ester content and cholesterol efflux in macrophages. In the current study, we were unable to demonstrate direct effects of testosterone on lipid loaded macrophages *in vitro* with no androgen action on fatty acid uptake. Free fatty acids have been implicated in endothelial dysfunction and trigger inflammation and oxidative stress in the endothelium (Ghosh *et al.*, 2017). Fatty

acids also influence lipid deposition in the arterial wall by influencing levels of circulating lipoproteins, cholesterol efflux, and lipid metabolism (Sudheendran *et al.*, 2010). It may be apparent that oxLDL processing by macrophages in atherosclerotic lesion regions is more representative of atherogenesis and as the literature reports conflicting data regarding androgen action on isolated macrophage function, more detailed investigation is required (Barud *et al.*, 2002).

3.5.1 Summary

In summary, the current study demonstrates that testosterone protects against lipid deposition within the aortic root of ApoE$^{-/-}$ mice receiving both standard chow and pro-atherogenic diets. This suggests that testosterone may be cardioprotective and may explain some of the benefits of TTh shown clinically in men with low testosterone. Lesion-specific mechanisms of this testosterone action remain to be uncovered as the present study was unable to detect differences in lesion stability including SMC migration into the lesion, intra-lesion collagen content, necrotic core, fibrous cap thickness or MMP expression. This may, however, be due to differences in the pathogenesis of atherosclerosis between mice and humans. Lesion distribution differs between species with lesions occurring more frequently in humans in the coronary and carotid arteries compared to mice with lesions more commonly found in the aortic root and aortic arch (Lee et al., 2017). Previous clinical studies have shown that testosterone can stabilise aortic lesions through reduction of lipid uptake and anti-inflammatory mechanisms. In addition, *in vitro* testosterone treatment did not affect fatty acid uptake in THP-1 macrophages as a mechanism to represent lipid uptake. Therefore, while the present study reports no effect of testosterone on parameters of lesion stability or complexity, the beneficial effects of testosterone in reducing aortic atherosclerosis may be due to a modulation of the mechanisms controlling lipid uptake into the arterial wall as well as anti-inflammatory effects directly at the level of the macrophage and endothelium. Clinical studies demonstrate beneficial effects of testosterone on surrogate markers of atherosclerosis; however, there is currently not an adequate number of large scale randomised controlled trials to definitively address whether testosterone reduces the atherosclerotic burden and further studies are required for validation of these findings and to uncover lesion-

specific mechanisms of testosterone action. The beneficial effects of testosterone on atherosclerosis observed in clinical and animal studies may be due to improvements in systemic and localised inflammation.

4. The effects of testosterone on inflammation associated with atherosclerosis

4.1 Introduction

4.1.1 Inflammatory atherosclerotic events

Atherogenesis is considered to start with endothelial dysfunction or inflammation of the endothelium (Table 4.1), and accompanying infiltration of immune cells such as leukocytes and monocytes (Wang *et al.*, 2015). Following extravasation from the circulation and migration across the endothelial layer into the vascular intima, monocytes transition into macrophages that internalise modified lipoprotein particles present within the developing atheroma, resulting in the formation of foam cells (Ley *et al.*, 2011). Foam cells secrete pro-inflammatory cytokines that activate inflammatory signalling pathways that promote activation of the endothelium and further leukocyte recruitment and adhesion. The additional leukocytes similarly uptake modified lipoprotein particles within the vessel wall causing continuous damage and inflammation which in turn can also affect vascular permeability to lipids creating a continuous cycle promoting atherogenesis (Hansson and Libby, 2006).

Pro- and anti-inflammatory mediators regulate the inflammatory events that contribute towards atherosclerosis development and progression (Figure 4.1). Initially, these inflammatory events aim to provide vascular protection by clearing the vessel of lipids and repairing tissue damage caused to the endothelium. Many cytokines and immune cells have both pro- and anti-inflammatory properties that can influence cellular functions depending upon cues from the local environment.

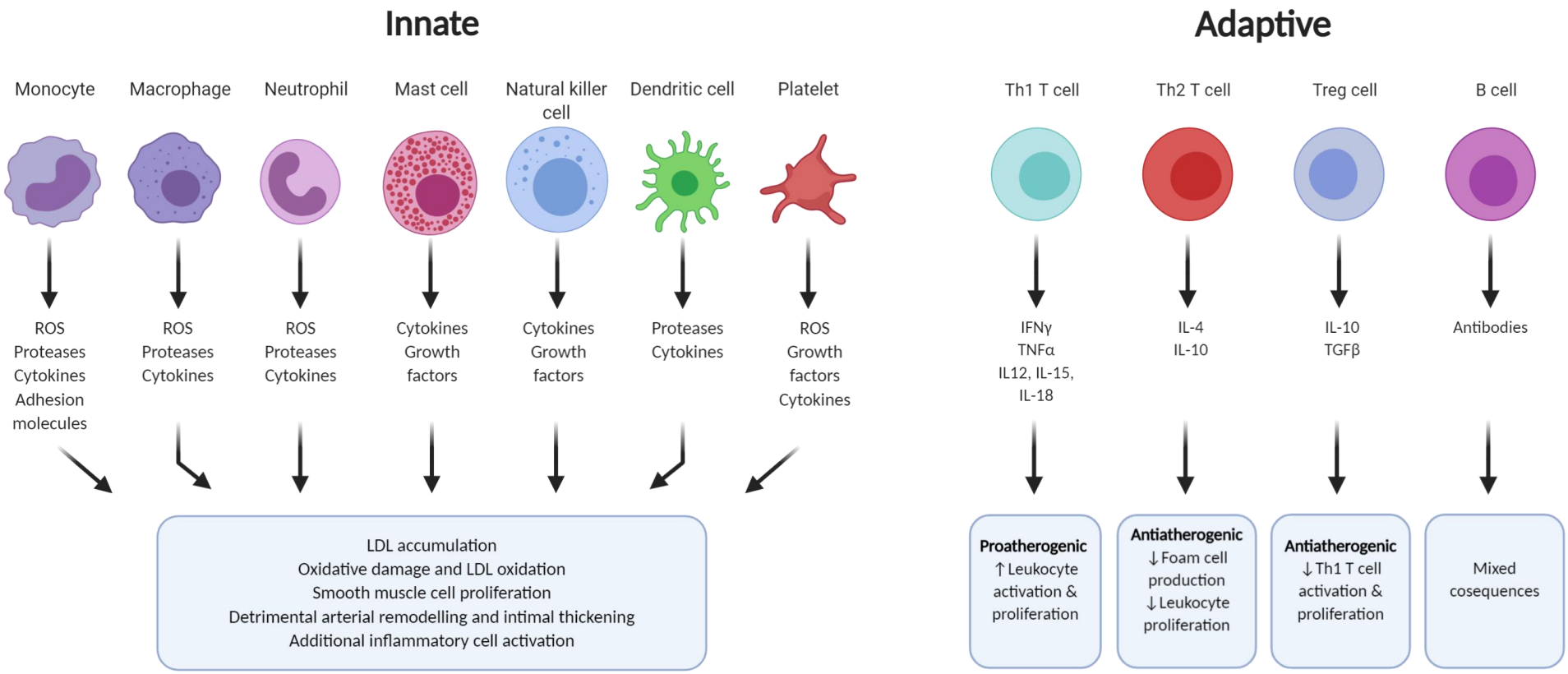


Figure 4.1 Summary of the immune cells involved in atherosclerosis. During atherosclerotic plaque development and progression, various types of innate immune cells are essential. The innate immune system responds to tissue injuries and can trigger inflammatory responses. This system is composed of diverse cellular components including monocytes, macrophages, neutrophils, mast cells, dendritic cells, and natural killer cells. Following an innate immune response, antigen-specific adaptive immune responses are triggered and mediated by T and B cells. The innate and adaptive immune response regulate the activation and function of other immune and vascular cells through secretion of inflammatory mediators such as cytokines, chemokines, growth factors, adhesion molecules and proteases. Additionally, immune cells are capable of altering oxidative status through generating reactive oxygen species (ROS). Adapted from Kelly (2010).

4.1.1.1 Cytokines

Cytokines are small pleiotropic signalling proteins that regulate inflammation and immune responses through autocrine, paracrine and juxtracrine activity. They can generally be divided into several classes: interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), colony-stimulating factors (CSF), transforming growth factors (TGF) and chemokines. There are both pro-inflammatory cytokines and anti-inflammatory cytokines, although many cytokines can act synergistically in their function and can be both pro- and anti-inflammatory. Inflammatory activation of immune cells in human plaques is regulated by inflammatory cytokines, including: IFN-γ, TNFα, IL-1β, IL-6, IL-8, IL-10 and IL-18 (Ait-Oufella *et al.*, 2011, Frostegård *et al.*, 1999).

Pro-inflammatory cytokines are secreted predominantly by activated macrophages and perpetuate the local inflammatory response. Specific pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα are involved in all stages of atherogenesis and can be produced by and act on all cell types involved in the pathology of atherosclerosis. They can also further induce their own production and the production of several other inflammatory mediators, to amplify the inflammatory response (Dinarello, 2009). Furthermore, cytokines can directly activate newly recruited immune cells such as monocytes and macrophages and regulate their phenotype in the lesions which consequently alters the inflammation state within the lesion (Ait-Oufella *et al.*, 2011).

4.1.1.2 Chemokines

Chemokines and their receptors are also considered key regulatory signalling peptides in atherogenesis. There are three major receptor-chemokine pairs considered to be involved in monocyte trafficking and transmigration into lesions: CCR2 - MCP-1/CCL2: CX_3C-chemokine receptor 1 (CX_3CR1) - CX_3C- chemokine ligand 1 (CX_3CL1); and CCR5-CCL5 (Rolin and Maghazachi, 2014).

Chemokines and their receptors are widely expressed and are prominently present on activated endothelial cells, SMCs, and leukocytes. Circulating leukocytes encounter inflammatory chemokines, released by activated endothelial cells, receptor-

ligand binding occurs, and the cells become activated. Activation occurs through actin rearrangement and thus a change in cell shape, allowing movement towards the site of inflammation via a chemoattractant gradient (Charo and Taubman, 2004, Apostolakis and Spandidos, 2013). Receptor activation also induces gene transcription that can lead to increased adhesion characteristics by modulation of integrins, selectins and other adhesion molecules in leukocytes and endothelial cells (Thelen, 2001).

4.1.1.3 Adhesion molecules

Adhesion molecules have a central role in the early processes of atherosclerosis. They are expressed on the vascular endothelium and circulating leukocytes in response to cytokines and chemokines, which are often co-expressed, aiding the immune cell recruitment (Springer, 1990). Under normal conditions, endothelial cells have a low adhesive capacity for leukocytes and platelets; however, under inflammatory conditions, adhesion molecule expression is increased in these cell types.

Adhesion receptors, E-selectin, P-selectin and L-selectin and their ligands (primarily P-selectin ligand) are involved in the rolling and tethering of leukocytes on the vascular wall and leukocyte adhesion molecules (ICAM-1 and VCAM-1), as well as some of the integrins, induce firm adhesion of inflammatory cells to the vascular surface (Davies *et al.*, 1993a, Johnson-Tidey *et al.*, 1994, O'Brien *et al.*, 1993, O'Brien *et al.*, 1996, DeGraba *et al.*, 1998). Monocyte rolling is predominantly mediated by E-selectin and P-selectin on endothelial cells and their ligand P-selectin glycoprotein ligand-1 (PSGL-1) on monocytes. E-selectin is rapidly up-regulated and presented on the surface of activated endothelial cells and is mediated by the transcription factor nuclear factor kappa B (NF-κB). TNFα, the principal atherogenic cytokine, mediates adhesion molecule activation via activation of the NF-κB (Hatakeyama *et al.*, 2002). Interferon regulatory factor 3 (IRF3), a proatherogenic mediator also directly modulates ICAM-1 and VCAM-1 (Okon *et al.*, 2017). This consequently initiates intracellular signalling of the leukocytes and endothelial cells, allowing leukocyte migration into the intima. CCL2 recruits monocytes to sites of inflammation and plays a vital role in the entry of monocytes and into the intima (Boring *et al.*, 1998). CCL2 is upregulated in mouse models of atherosclerosis (Veillard *et al.*, 2004) and CCL2

knockout mice display reduced plaque development and leukocyte infiltration (Gosling *et al.*, 1999, Gu *et al.*, 1998).

4.1.2 Monocytes

Monocytes and macrophages are the dominant cellular populations of atherosclerotic plaques (Zhang *et al.*, 2012, Napoli *et al.*, 1997). Monocytes infiltrate the vessel wall into the intima where they differentiate into macrophages promoted by local over-expression of macrophage-colony stimulating factor (M-CSF) (Libby *et al.*, 2002, Libby *et al.*, 2010).

Human monocytes are divided into three subsets based on their cell surface expression of cluster of differentiation (CD) 14 and 16 (Shi and Pamer, 2011, Zawada *et al.*, 2012). $CD14^{++}$ $CD16^{-}$ monocytes are termed classical monocytes are the most common subset in the peripheral blood (80-95%) and express high levels of CCR2; their function remains undefined. $CD14^{++}$ $CD16^{+}$ are intermediate monocytes constituting of around 2-11% of circulating monocytes and express high levels of CCR5 but low levels of CCR2 (Ancuta *et al.*, 2003). They secrete pro-inflammatory cytokines such as IL-1β and TNFα and significantly contribute to atherosclerosis progression (Cros *et al.*, 2010). $CD14^{+}$ $CD16^{++}$ monocytes are non-classical monocytes (2-8%) which express low levels of CCR2 and high levels of CX_3CR1 and fulfil patrolling and antiviral functions (Geissmann *et al.*, 2003, Woollard and Geissmann, 2010) (Table 4.2).

Mouse monocyte counterparts are also divided into three subsets based on their cell surface expression of chemokine receptor lymphocyte antigen 6 complex (Ly6C), a mouse specific marker of macrophage subsets (Wang *et al.*, 2015) termed the classical ($Ly6C^{high}$), intermediate ($Ly6C^{middle}$) and the non-classical ($Ly6C^{low}$) monocytes (Ziegler-Heitbrock *et al.*, 2010). $Ly\text{-}6C^{high}$ monocytes dominate hypercholesterolemia-associated monocytosis and are the primary subtype that will migrate toward atherosclerotic lesions and give rise to macrophages in atheroma (Swirski *et al.*, 2007).

Species	Subsets	Surface markers	% in MNCs	Chemokine receptors	Functions
Human	Classical	$CD14^{++}CD16^{-}$	80-95	$CCR2^{high}CX_3CR1^{low}$	Undefined
	Intermediate	$CD14^{++}CD16^{+}$	2-11	$CCR2^{low}CX_3CR1^{high}$	Pro-inflammatory
	Non-classical	$CD14^{+}CD16^{++}$	2-8	$CCR2^{low}CX_3CR1^{high}$	Patrolling
Mouse	$Ly6C^{high}$	$CD11b^{+}CD115^{+}Ly6C^{high}$	40-45	$CCR2^{high}CX_3CR1^{low}$	Pro-inflammatory
	$Ly6C^{middle}$	$CD11b^{+}CD115^{+}Ly6C^{middle}$	5-32	$CCR2^{high}CX_3CR1^{low}$	Pro-inflammatory
	$Ly6C^{low}$	$CD11b^{+}CD115^{+}Ly6C^{low}$	26-50	$CCR2^{low}CX_3CR1^{high}$	Patrolling - tissue repair

Table 4.1 Markers and functions of monocyte subsets in human and mouse. Human monocytes are divided into three distinct subsets based on cell surface expression CD14 and CD16, whereas mouse monocyte counterparts are divided into three subsets based on cell surface expression levels of the chemokine receptor Ly6C (Wang *et al.*, 2015).

4.1.3 Macrophages and atherosclerosis

The majority of monocytes differentiate into macrophages at the site of the atherosclerotic lesion (Moore et al., 2013, Moore and Tabas, 2011) and are involved in several aspects of disease progression.

Macrophages in atherosclerotic lesions actively participate in lipoprotein ingestion, and oxLDL is recognised by scavenger receptors, predominantly CD36 and CD68 on the macrophage cell surface (de Gaetano et al., 2016). Scavenger receptors mediate the uptake of oxLDL by macrophages and hydrolyzed into free cholesterol and fatty acids (Maxfield and Tabas, 2005, Pluddemann et al., 2007). Subsequently, free cholesterol will undergo re-esterification, forming cholesteryl esters that are stored in the cytoplasm as lipid droplets and are in dynamic equilibrium with free cholesterol transforming macrophages into foam cells (Lusis, 2000, Brown et al., 1980). The accumulation of foam cells is a key characteristic of early atherosclerotic plaques known as fatty streaks and are involved in lesion progression. Foam cells secrete pro-

inflammatory cytokines (IL-1β and TNFα) and chemokines (CX_3CL1, CCL2 and IL-8) that further propagate the recruitment of monocytes to the atherosclerotic lesion (Shirai et al., 2015, Tacke et al., 2007) and eventually undergo apoptosis, forming the necrotic core in the lesion (Seimon and Tabas, 2009).

4.1.3.1 Macrophage subsets and function

Macrophages can be classified into two major subsets and have been proposed to derive from different subsets of circulating monocytes in response to cytokine environments (Jablonski *et al.*, 2015). The "classically activated" (M1) pro-atherogenic macrophages are primed by exposure to T helper (Th)-1 cytokines such as IFN-γ and IL-1β. "Alternatively activated" (M2) anti-inflammatory macrophages are induced as a result of exposure to Th2 cytokines such as IL-4 and IL-13 (Shalhoub *et al.*, 2011, Chinetti-Gbaguidi *et al.*, 2011, Mantovani *et al.*, 2004). The M1 differentiation pathway is defined by responses to IFNγ and by the activation of toll-like receptors (TLRs), such as TLR4 (Martinez *et al.*, 2008). TLR4 macrophage expression is upregulated by oxLDL (Xu *et al.*, 2001) and TLR4 expressed by macrophages and have been implicated in the development of coronary artery disease through activation of NF-κB pathways (Cole *et al.*, 2010). M1 macrophages produce pro-inflammatory cytokines, such as TNFα and IL-6 (Zawada *et al.*, 2012, Shirai *et al.*, 2015), whereas M2 macrophages reduce this inflammatory response by producing anti-inflammatory factors (IL-10, TGFβ, IL-1 receptor antagonist (IL-1Ra)), consequently promoting angiogenesis and tissue repair (Gordon, 2003, Mantovani *et al.*, 2001, Shirai *et al.*, 2015). M2 macrophages aim to stabilise the plaque by phagocytosing cholesterol and become foam cells. However, macrophage cholesterol metabolism can become overwhelmed and dysregulated in the face of excessive cholesterol uptake (Moore *et al.*, 2013). This causes an increase in macrophage apoptosis resulting in secondary necrosis and the release of cellular components and lipids that form the necrotic core of the atherosclerotic plaque (Tabas, 2005).

Khallou-Laschet *et al.* (2010) investigated the phenotype of macrophages in $ApoE^{-/-}$ mice and found early atherosclerotic lesions contained mainly M2 macrophages, whilst M1 macrophages prevailed in more advanced lesions as inflammation increases, indicating that the macrophages are polarized according to

surrounding inflammation. Early lesions are dominated by plaque foam cells, plaque foam cell content decreases with atherosclerosis progression towards more advanced and fibrotic lesions. However, it is most likely that there would be multiple macrophage subsets at one in a plaque (Figure 4.1) (Willemsen and de Winther, 2020).

Stoger *et al.* (2012) investigated the presence and spatial distribution of polarized macrophage populations in human atherosclerosis and demonstrated a prominent and continued presence of both M1 and M2 macrophages during plaque development. In concordance with their pro-inflammatory characteristics, M1 macrophages dominated the rupture-prone shoulder regions of the plaque over M2 cells. However, adventitial (the outermost connective tissue covering the vessel) macrophages near atherosclerotic lesions are selectively polarized towards the M2 phenotype, and M2 macrophages were detectable in both complicated lesions and stable plaques.

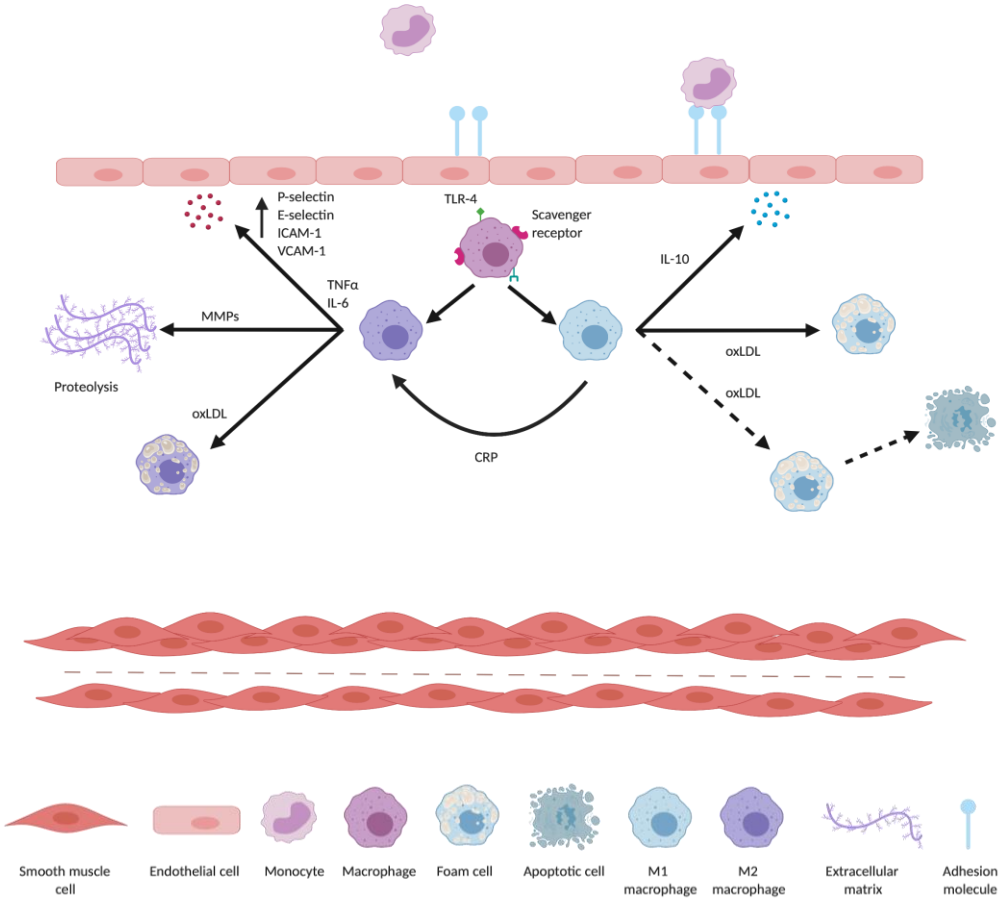


Figure 4.2 Macrophages in atherosclerosis. Monocytes are recruited to atherosclerotic lesions, where they undergo differentiation to either the M1 or M2 macrophage subset. M2 macrophages predominate in the early stages of atherosclerosis and are considered atheroprotective and can reduce plaque development. M2 macrophages are characterized by IL-10 secretion and accumulate smaller quantities of lipid. M2 macrophages undergo apoptotic death in the presence of oxLDL, and C-reactive protein (CRP) stimulation induces a phenotypical and functional change of M2 macrophages to M1 macrophages. M1 macrophages are found in advanced lesions where they accumulate a large quantity of lipids, which promotes their differentiation into foam cells. M1 macrophages secrete TNFα, IL-6 and metalloproteinases, which exacerbate and destabilise lesion development. In humans, M1 and M2 macrophages are also found in lesions. Adapted from Chávez-Sánchez *et al.* (2014).

4.1.4 Testosterone and inflammation

Clinical studies have shown that low testosterone levels are inversely associated with pro-inflammatory cytokines such as TNFα and IL-1β (Shores *et al.*, 2006, Laughlin *et al.*, 2008, Nettleship *et al.*, 2007b). Circulating pro-inflammatory cytokines are particularly evident in patients with low testosterone levels and obesity (Tivesten *et al.*, 2009). TTh has been reported to suppress inflammation in hypogonadal men. Nettleship *et al.* (2007b) reported a negative correlation between serum testosterone levels and IL-1β in testosterone-deficient men with stable CAD suggested that testosterone may regulate IL-1β activity in men with CAD. Malkin *et al.* (2004a) also reported that TNFα was significantly reduced, and IL-10 was significantly increased following one month of TTh in men with CHD. Similarly, hypogonadal men with ischaemic heart disease had a significant decrease in serum TNFα following a month of TTh (Malkin *et al.*, 2004b). In two RCTs of TTh in hypogonadal men, one in men with T2D and the other with MetS, TTh suppressed serum IL-1β and TNFα but did not cause significant changes in serum IL‑6 or IL-10 (Kalinchenko *et al.*, 2010, Dhindsa *et al.*, 2016). However, not all trials report a positive effect of TTh on inflammation (Pugh *et al.*, 2005, Kalinchenko *et al.*, 2010).

Low testosterone is also associated with T2D, which has been linked to elevated systemic inflammation. Patients with T2D have been shown to have low-grade inflammation, and inflammatory markers such as C-reactive protein (CRP) and cytokines are elevated in these patients (Duncan *et al.*, 2003, Schmidt *et al.*, 1999). Adipose tissue is suspected to be a large source of the inflammation, and increased adipose tissue is associated with both T2D and low serum testosterone levels. Giulietti *et al.* (2004), Giulietti *et al.* (2007) however, revealed that monocytes from T2D patients have a pro-inflammatory profile and are the key cells involved in the pathogenesis of atherosclerosis, the major vascular consequence of T2D and low testosterone.

A study investigating the effects of TTh in hypogonadal men with T2D did not observe any significant immuno-modulatory effect; however, baseline levels of IL-6 negatively correlated with total and bioavailable amounts of circulating testosterone, confirming that low testosterone is associated with inflammation (Kapoor *et al.*,

2007b). Similarly, Pugh *et al.* (2005) reported no change in serum TNFα levels in hypogonadal men with chronic heart failure following TTh. It is acknowledged that testosterone has immunomodulatory actions, but it is not clear how this relates directly to atherosclerosis *in vivo* as this has not been fully investigated (Kelly and Jones, 2013b). However, *in vitro* studies this could be due to direct anti-inflammatory actions on cells implicated in the pathogenesis of atherosclerosis (Corcoran *et al.*, 2010, Corrales *et al.*, 2006, Hatakeyama *et al.*, 2002, Mukherjee *et al.*, 2002).

4.1.5 Summary

Testosterone treatment is implicated as beneficial in reducing systemic inflammation in men with hypogonadism. *In vivo* and *in vitro* studies provides evidence that testosterone diminishes atherosclerosis possibly by anti-inflammatory mechanisms. However, very little is known regarding the effects of testosterone on localised inflammation at the atherosclerotic lesion and on specific cell types implicated in the pathogenesis of atherosclerosis.

4.2 Aims and objectives

The aim of the *in vivo* study was to use a mouse model of atherosclerosis to address the hypothesis that testosterone treatment reduces local and systemic inflammation, reducing atherosclerotic burden. Furthermore, the clinical study aimed to address the hypothesis that TTh would modulate inflammation in monocytes, additionally that testosterone treatment *in vitro* would modulate inflammation in monocytes and macrophages.

- To investigate the anti-inflammatory effects of TTh on monocytes isolated from hypogonadal men with T2D.
- To investigate the anti-inflammatory effects of testosterone treatment *in vitro* on monocyte-derived macrophages isolated from hypogonadal men with T2D.
- To investigate the effects of testosterone treatment *in vitro* using THP-1 macrophages differentiated into different macrophage subsets.
- To investigate the effects of testosterone treatment on systemic and localised using the $ApoE^{-/-}$ mouse model fed a pro-atherogenic diet.

4.3 Experimental design

Serum samples from the clinical trial: hypogonadal men with T2D diabetes following TTh (Section 2.3) and serum samples from ApoE$^{-/-}$ mice following testosterone treatment (Section 2.1) were analysed by cytometric bead array to assess systemic inflammation (Section 2.1.5.3). The inflammatory profile of monocytes isolated from patients in the clinical trial following TTh and THP-1 monocytes that were treated with testosterone *in vitro* was analysed by qRT-PCR (Section 2.3.1.3). Monocyte-derived macrophages isolated from clinical trial patients prior to TTh and THP-1 macrophages differentiated *in vitro* to macrophage subsets M1 and M2a were treated with testosterone *in vitro* and the inflammatory profile analysed by qRT-PCR (Section 2.2.2.1). Lesion inflammation within the aortic root lesions of the ApoE$^{-/-}$ mice was analysed by qRT-PCR following laser capture microdissection (Section 2.1.5.8) and immunohistochemistry (Section 2.1.5.7). Inflammation of the endothelial layer overlaying the aortic root lesions was also analysed by qRT-PCR following laser capture microdissection. The experimental design is summarised in Figure 4.3.

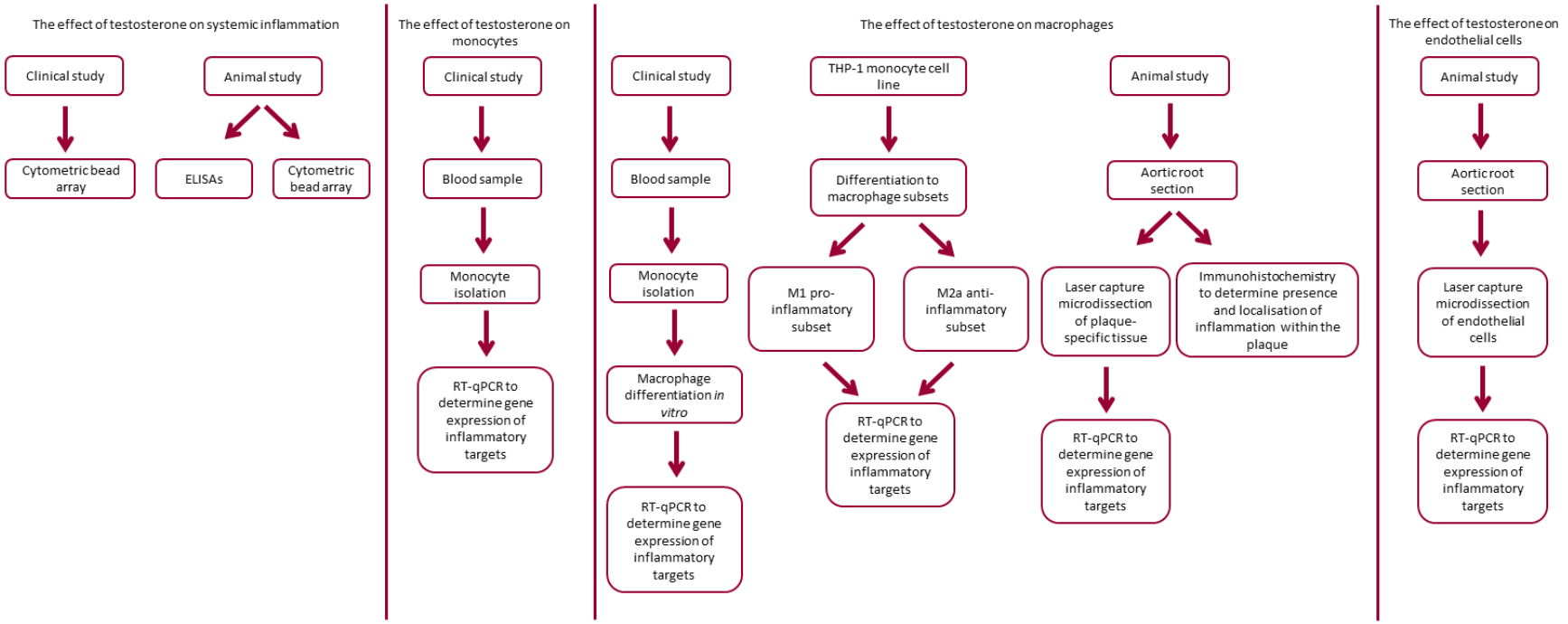


Figure 4.3 Experimental design to investigate the effect of testosterone on systemic and local inflammation in ApoE$^{-/-}$ mice, and in human monocytes in macrophages *ex vivo* and *in vitro*.

4.4 Results

4.4.1 The effect of testosterone on monocytes

4.4.1.1 Determination of the inflammatory profile of human monocytes isolated from male patients with hypogonadism and type 2 diabetes following testosterone replacement therapy

Gene expression of TNFα was significantly reduced following six months of TTh compared to baseline (0.568 vs 1.000, *P*=0.0014) and after three months of therapy (0.568 vs 1.027, *P*=0.0022). TNFα was also significantly downregulated after six months of treatment compared the patients receiving placebo (0.568 vs 1.361, *P*=0.0084). However, there we no differences in IL-1β, IL-6 and IL-10 gene expression following six months of TTh compared to the placebo group, (*P*=0.9673, 0.2586 and 0.8238 respectively) (Figure 4.4).

ICAM-1 gene expression was upregulated in the placebo and TTh groups after three months, however, after six months of treatment, the TTh group had lower expression compared to the placebo group, although this was not significant (*P*=0.0545) due to the low number of replicates. Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups. There were no differences observed in gene expression of CCL2 (*P*=0.4999), TLR2 (*P*=0.0683), TLR4 (*P*=0.202), SCARB1 (*P*=0.7065) and IRF3 (*P*=0.7065) (Figure 4.5). Power analysis indicates that there is unlikely to be significant differences between the two groups due to result variability.

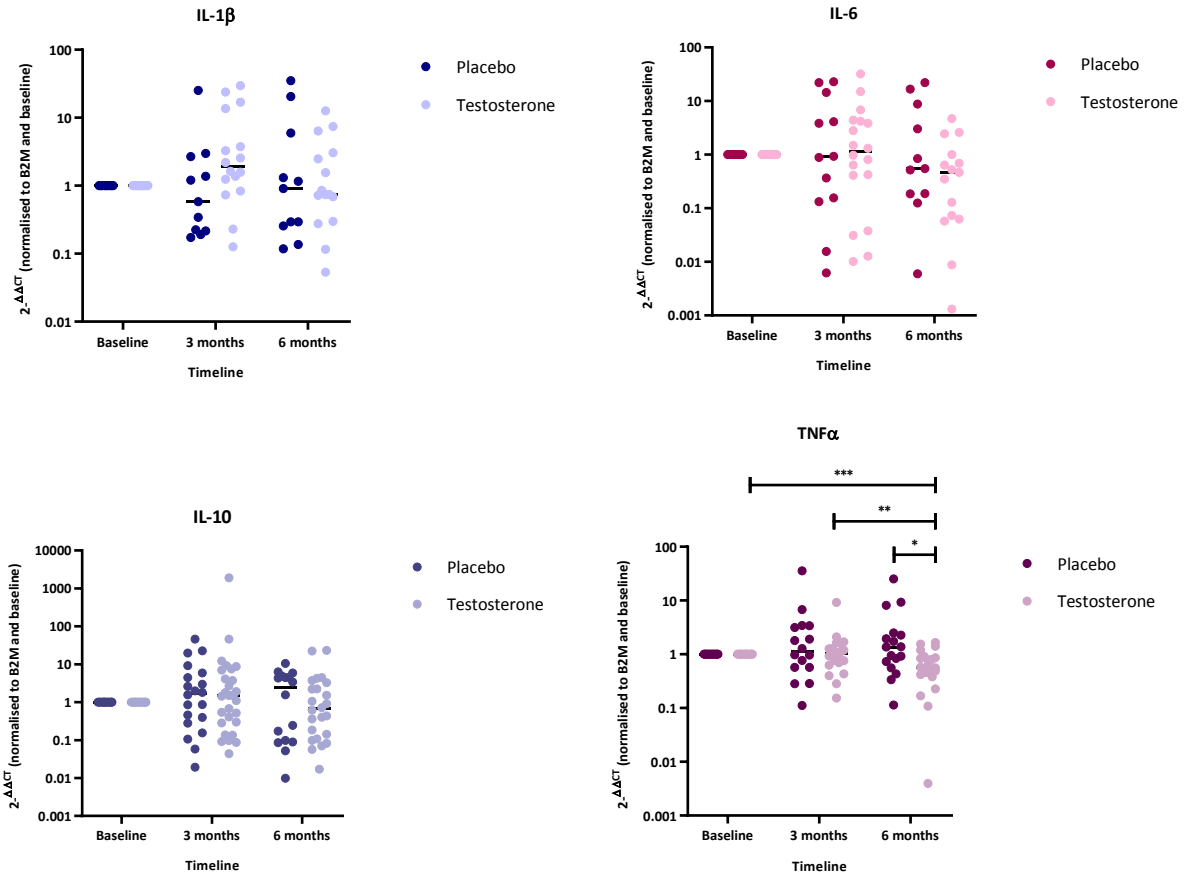


Figure 4.4 The effect of *in vitro* testosterone treatment on IL-1β, IL-6, IL-10 and TNFα gene expression in human monocytes. RT-qPCR to determine gene expression of IL-1β, IL-6, IL-10 and TNFα in monocytes isolated from hypogonadal men with T2D following three and six months of TTh (n=21) or placebo (n=31). Results were normalised to individual patient baselines. (**P*<0.05 ***P*<0.01 ****P*<0.001), Friedman test and Mann-Whitney U test.

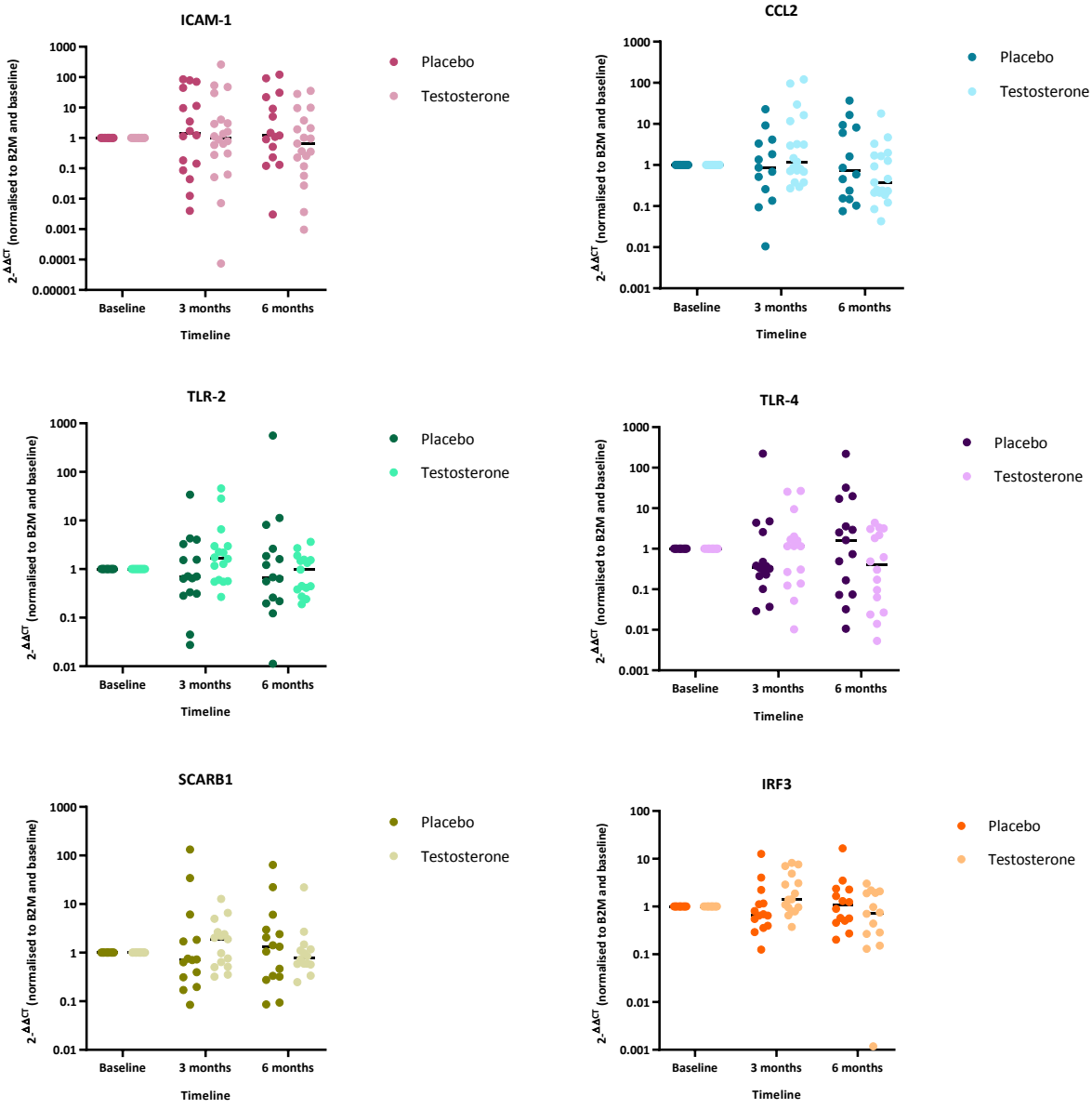


Figure 4.5 The effect of *in vitro* testosterone treatment on ICAM-1, CCL2, TLR2, TLR4, SCARB1 and IRF3 gene expression in human monocytes. RT-qPCR to determine ICAM-1, CCL2, TLR2, TLR4, SCARB1 and IRF3 gene expression in monocytes isolated from hypogonadal men with T2D following three and six months of TTh (n=16) or placebo (n=15). Results were normalised to individual patient baselines.

4.4.1.2 Determination of the inflammatory profile of THP-1 monocytes

There were no significant effects on the gene expression of IL-1β, IL-6, IL-10, TNFα, CCL2, TLR2, TLR4, SCARB1 or CX_3CL1 in THP-1 cells treated with 0-100nM of testosterone for 24 and 48H (Figure 4.6 and 4.7).

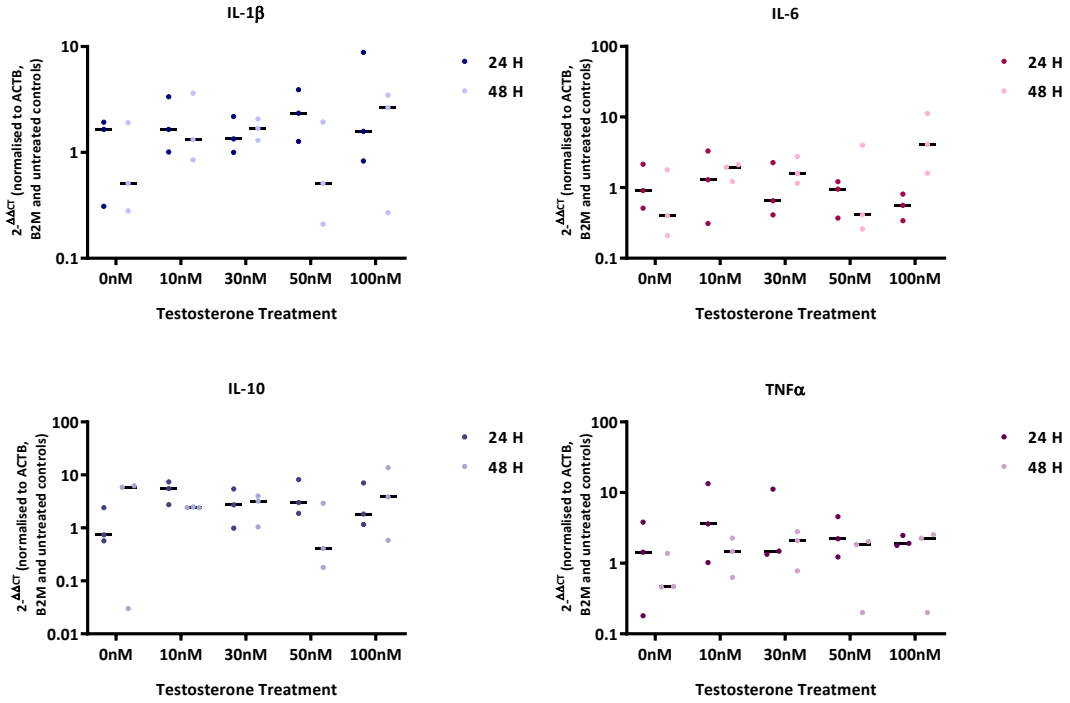


Figure 4.6 Gene expression of inflammatory cytokines in THP-1 cells following testosterone treatment. Monocytes were cultured alongside testosterone treatment. RT-qPCR analysis was performed to detect inflammatory cytokines.

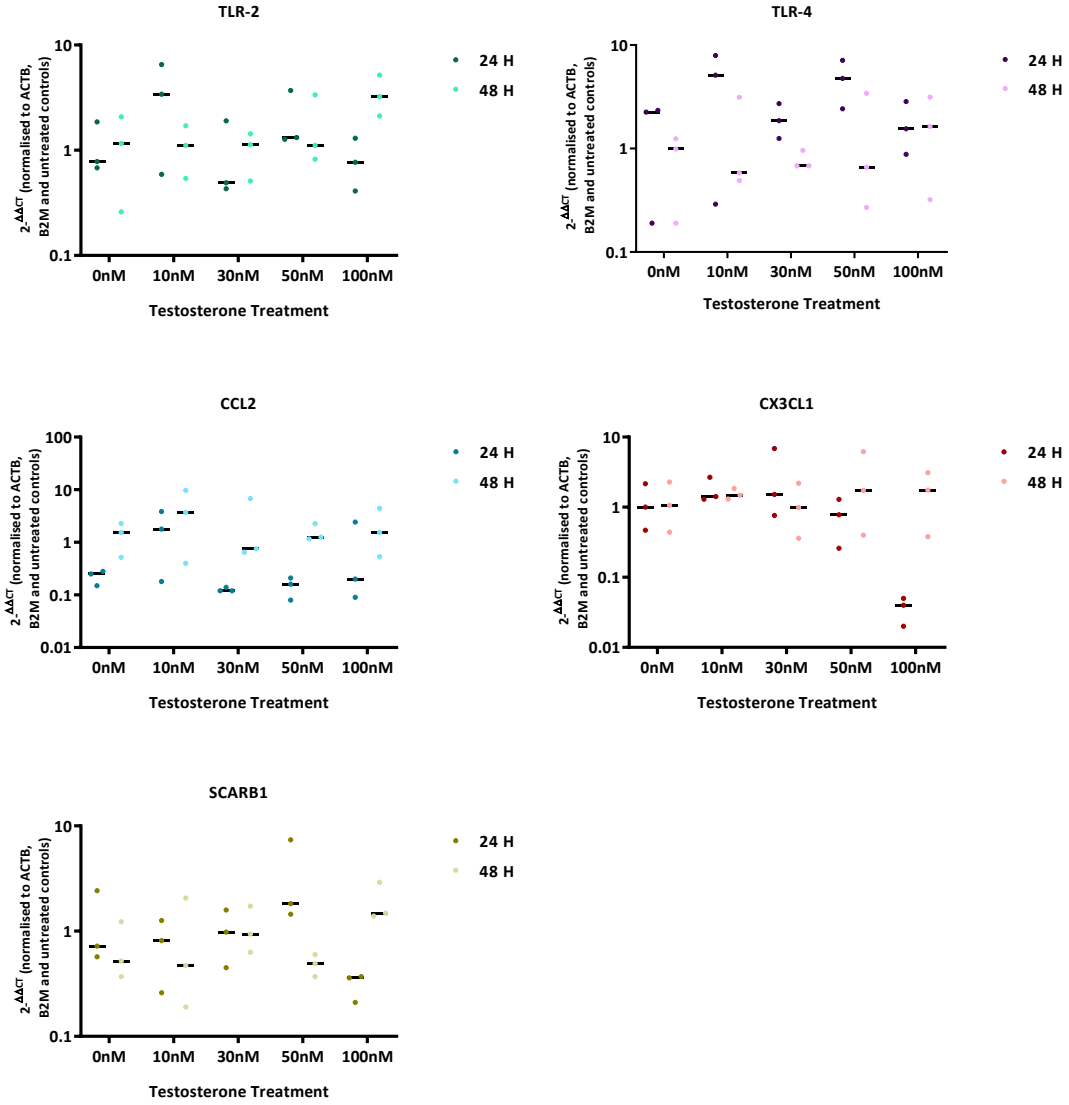


Figure 4.7 Gene expression of inflammatory targets in THP-1 cells following testosterone treatment. Monocytes were cultured alongside testosterone treatment. RT-qPCR analysis was performed to detect inflammatory targets

4.4.2 The effect of testosterone on macrophages

4.4.2.1 Determination of the inflammatory profile of human macrophages isolated from male patients with hypogonadism and uncontrolled type 2 diabetes following *in vitro* testosterone treatment

Testosterone treatment had no significant effects on the gene expression of IL-1β, IL-6, IL-10, TNFα, ICAM-1, CCL2, TLR2, TLR4, SCARB1 or IRF3 in *ex vivo* monocytes isolated from hypogonadal men with T2D and differentiated *in vitro* to macrophages (Figure 4.8 and 4.9). Power analysis indicates that there is unlikely to be significant differences between the three groups due to variability.

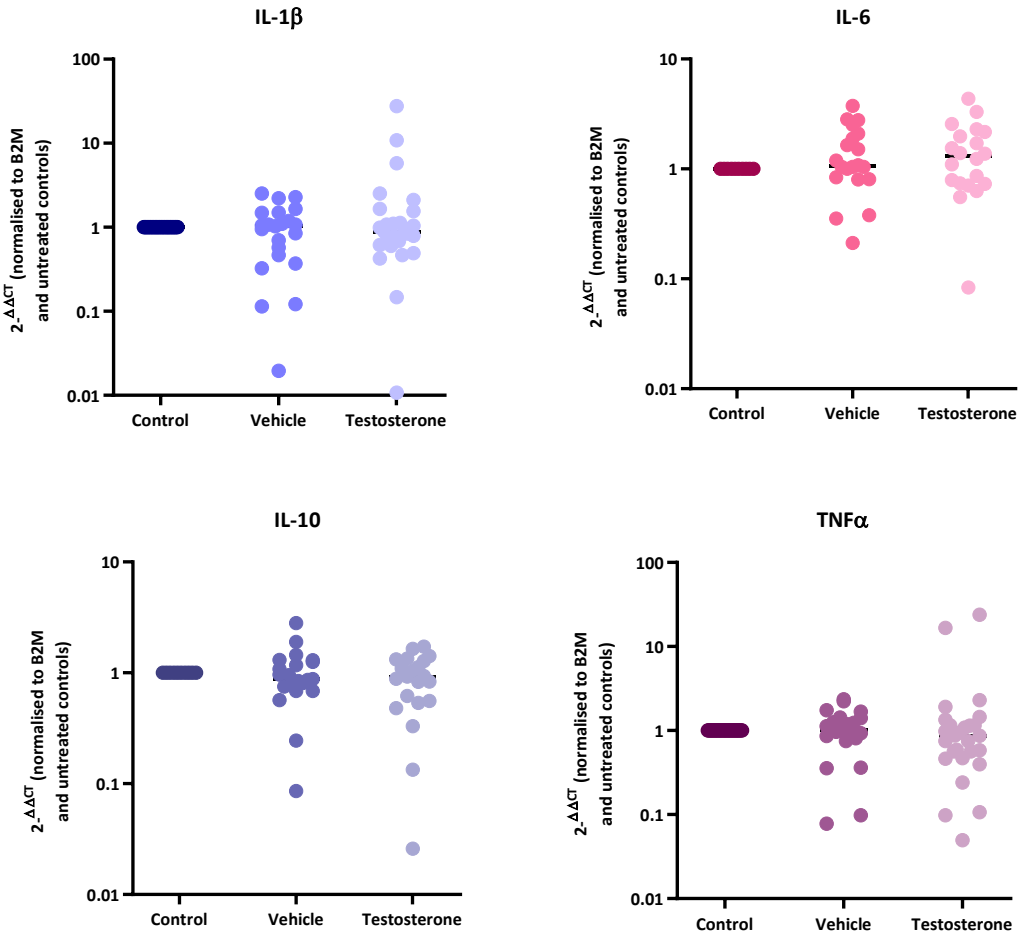


Figure 4.8 Gene expression of inflammatory cytokines IL-1β, IL-6, IL-10 and TNFα in human macrophages. Monocytes isolated from hypogonadal male patients with T2D were treated with testosterone *in vitro* following macrophage differentiation. Following 24 hours of testosterone treatment RT-qPCR analysis of IL-1β, IL-6, IL-10 and TNFα gene expression was determined.

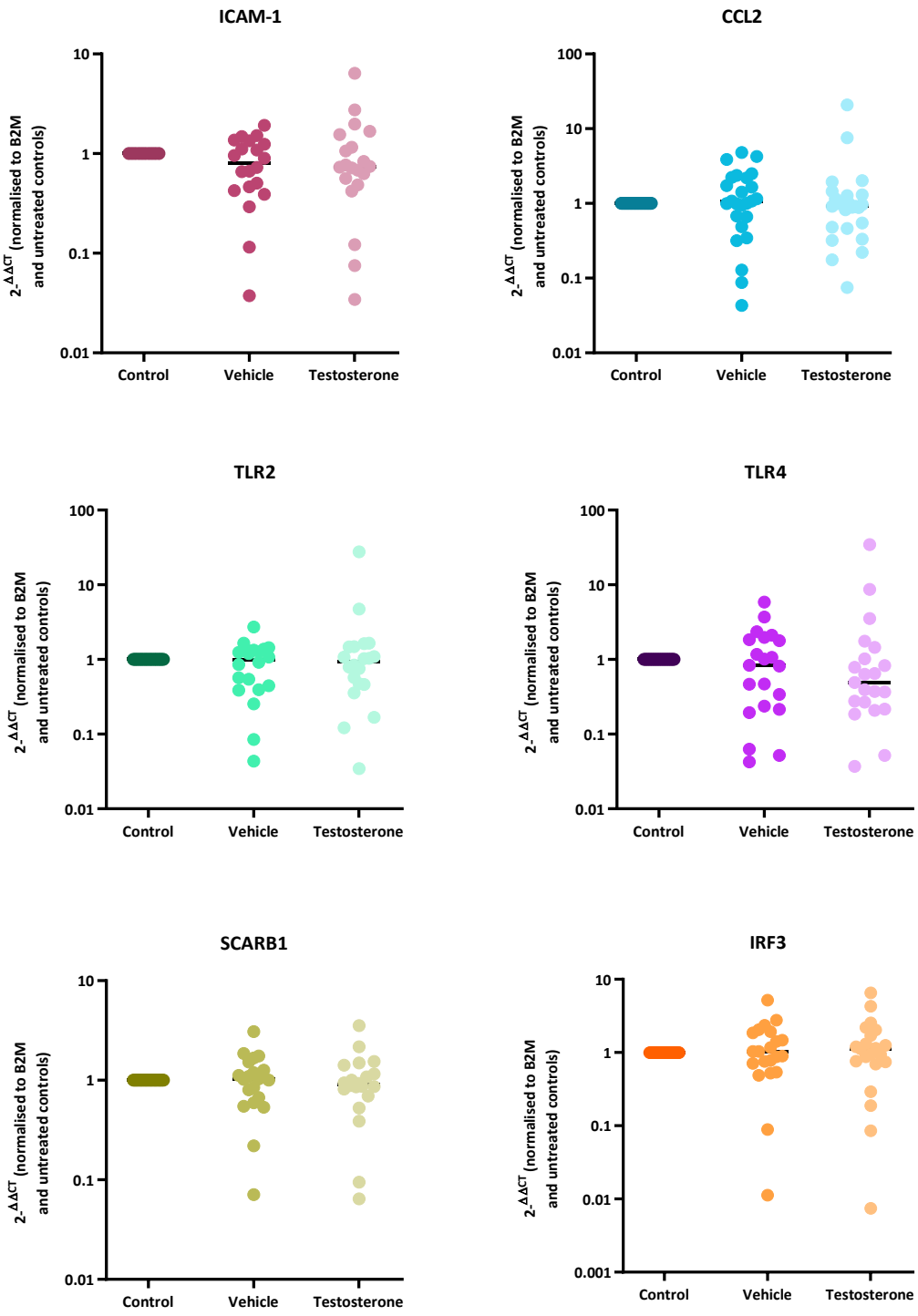


Figure 4.9 Gene expression of ICAM-1, CCL-2, TLR2, TLR4, SCARB1 and IRF3 in human macrophages. Monocytes isolated from hypogonadal male patients with T2D were treated with testosterone *in vitro* following macrophage differentiation. Following 24 hours of testosterone treatment RT-qPCR analysis for ICAM-1, CCL-2, TLR2, TLR4, SCARB1 and IRF3 gene expression was determined.

4.4.2.2 Determination of the inflammatory profile of the THP-1 macrophages

To evaluate whether testosterone has anti-inflammatory actions against specific macrophage subsets, THP-1 monocytes were differentiated *in vitro* to M1 and M2a macrophage subsets, treated with testosterone and gene expression analysed.

4.4.2.2.1 M1 macrophage subset

There was no significant change in gene expression of the inflammatory targets IL-β, IL-6, IL-10, TNFα, CCL2, CX_3CL1, TLR2, TLR4 and SCARB1 in the THP1 macrophage-like M1 subset following 24 and 48-hour treatment with testosterone (0-100nmol/L) (Figure 4.10 and 4.11).

4.4.2.2.2 M2a macrophage subset

In the THP1 differentiated M2a macrophage-like cell subset no significant change was observed in the expression of IL-β, IL-6, IL-10, TNFα, CCL2, CX_3CL1, TLR2, TLR4 and SCARB1 following 24 and 48-hour treatment with testosterone (Figure 4.12 and 4.13).

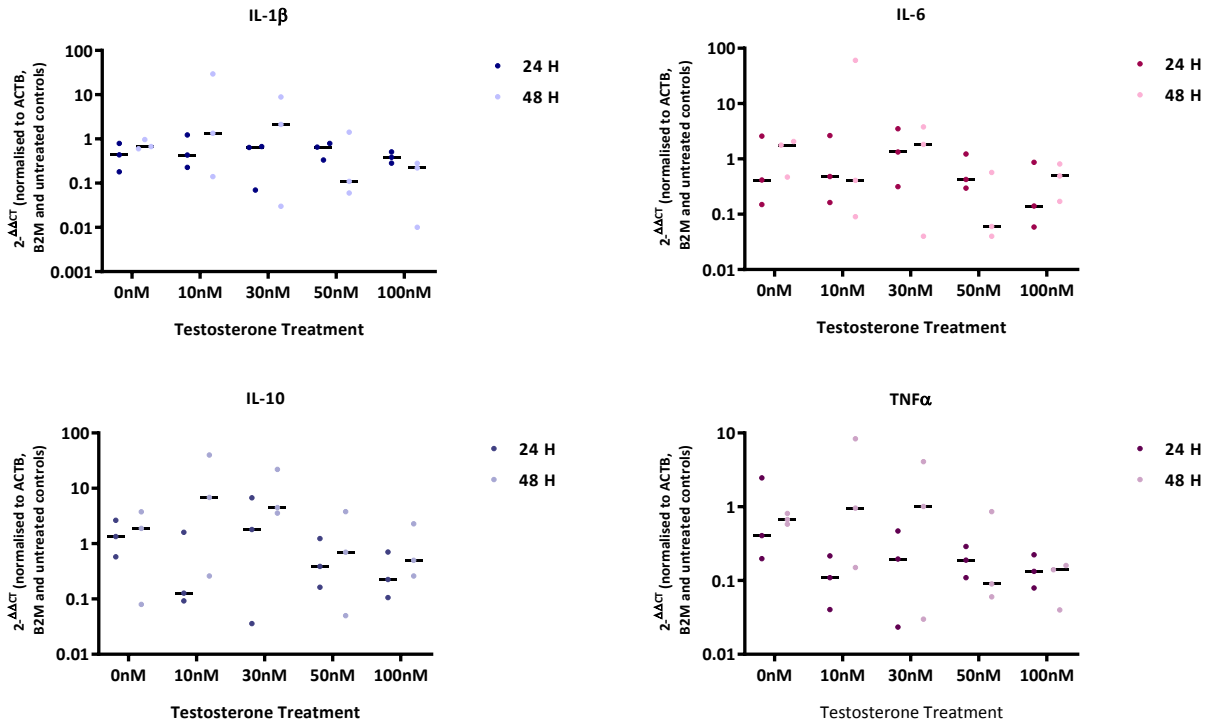


Figure 4.10 Gene expression of inflammatory cytokines in M1 macrophage-like cells following testosterone treatment. Cells were differentiated into macrophage subsets with cytokine/LPS treatment, followed by testosterone treatment. RT-qPCR analysis was performed to detect inflammatory targets.

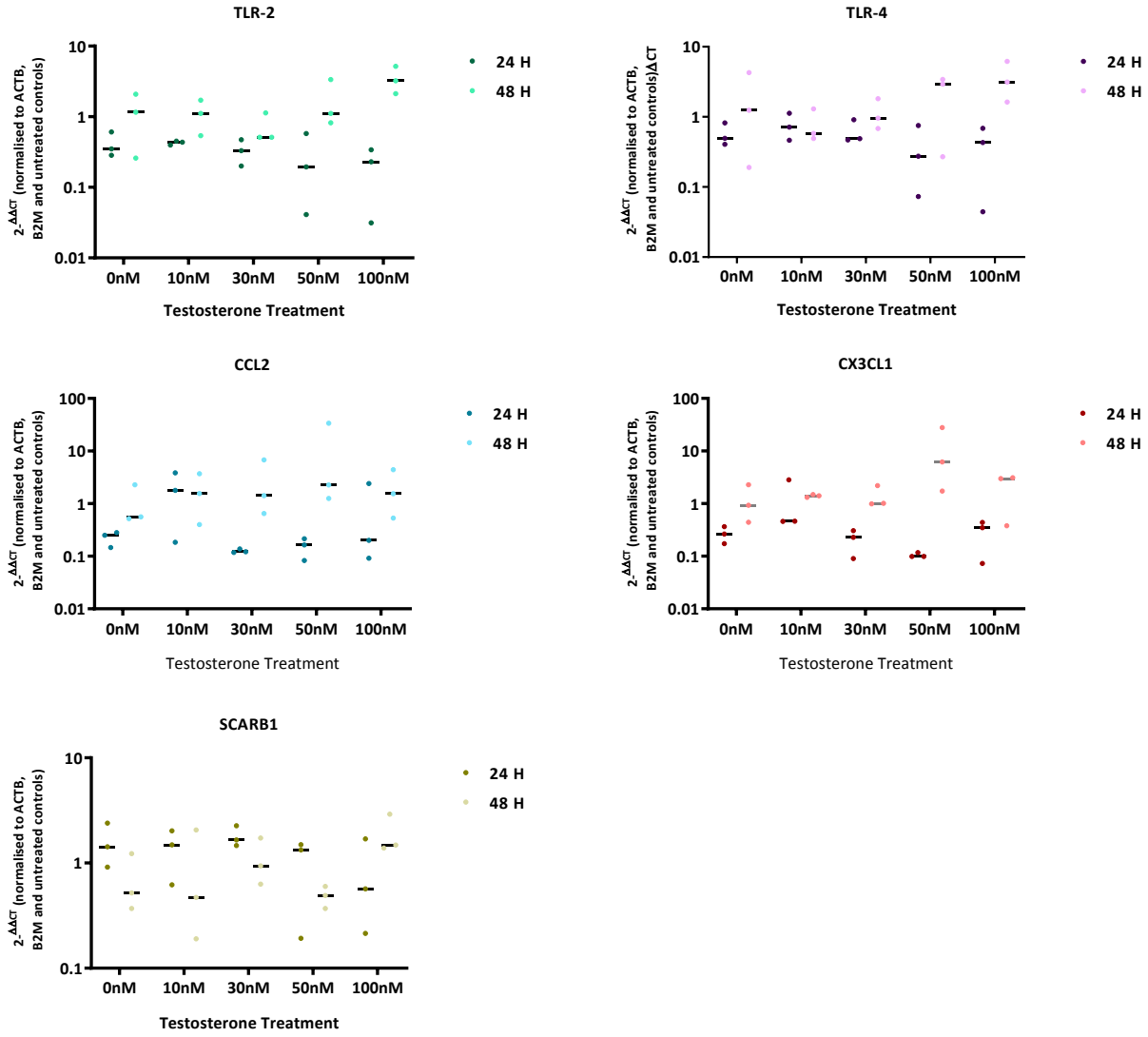


Figure 4.11 Gene expression of inflammatory targets in M1 macrophage-like cells following testosterone treatment. Cells were differentiated into macrophage subsets with cytokine/LPS treatment, followed by testosterone treatment. RT-qPCR analysis was performed to detect inflammatory targets.

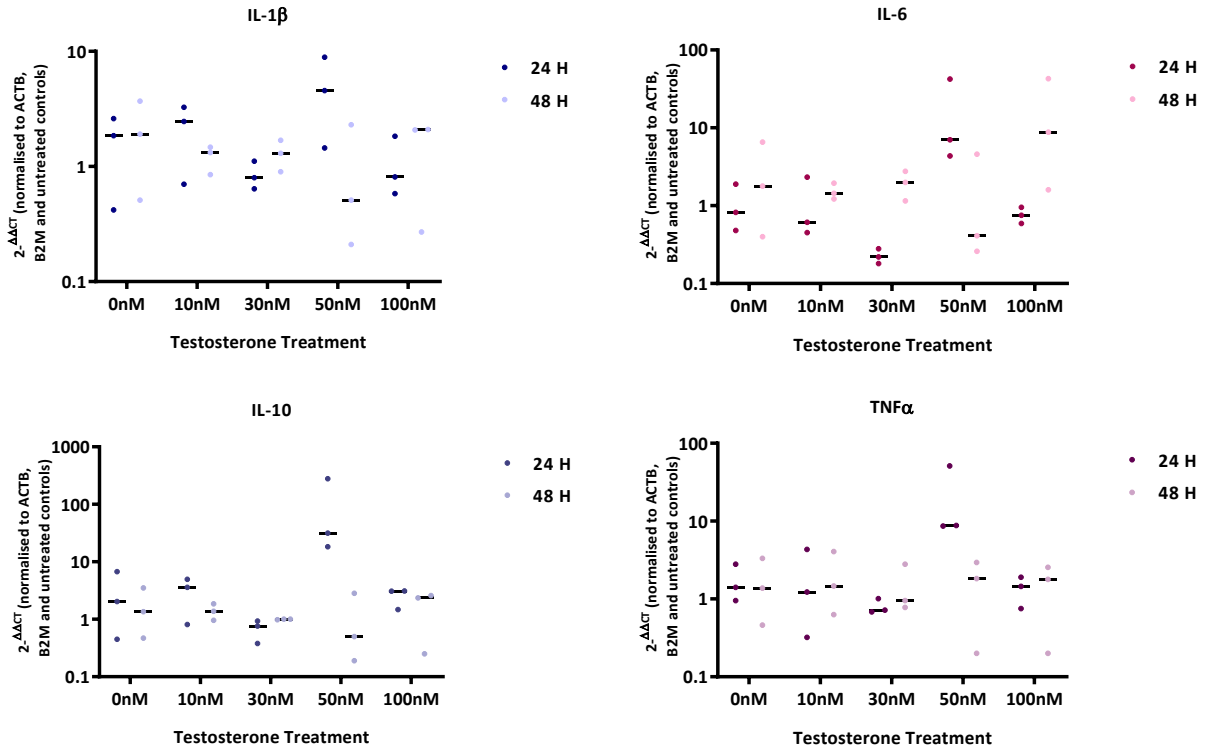


Figure 4.12 Gene expression of inflammatory targets in M2a macrophage-like cells following testosterone treatment. Cells were differentiated into macrophage subsets with cytokine stimulation, followed by testosterone treatment. RT-qPCR analysis was performed to detect inflammatory targets.

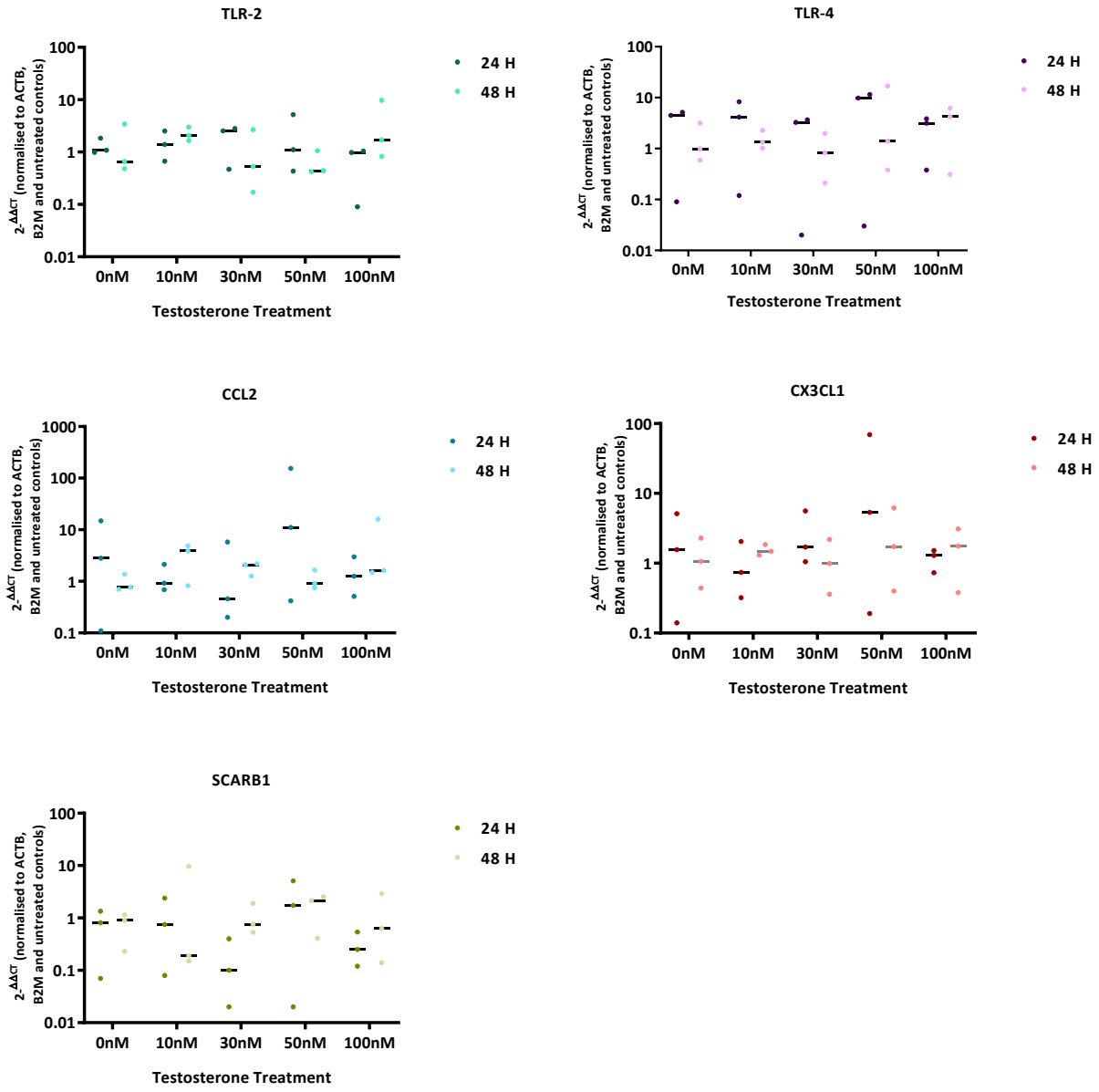


Figure 4.13 Gene expression of inflammatory targets in M2a macrophage-like cells following testosterone treatment. Cells were differentiated into macrophage subsets with cytokine stimulation, followed by testosterone treatment. RT-qPCR analysis was performed to detect inflammatory targets.

4.4.2.3 Analysis of lesion inflammation in ApoE$^{-/-}$ mice following testosterone treatment

To evaluate whether testosterone has an impact on the inflammatory status of the atherosclerotic lesion, lesion-specific tissue was isolated for gene analysis by LCM. Lesion specific tissue was isolated from regions that corresponded with positively stained areas for monocytes/macrophages (Figure 4.15). Lesion inflammation was also analysed by immunohistochemistry to identify specific cell types and adhesion molecules.

4.4.2.3.1 Gene analysis of lesion inflammation

Expression of pro-inflammatory cytokines IL-1β, IL-6, IL-10 and TNFα, CX3CL1 was not significantly modulated by testosterone status in the isolated lesion. Similarly, no differences in ICAM-1, CCL2 and CX3CL1 gene expression were observed in the isolated lesion (Figure 4.14). Power analysis indicates that there is unlikely to be significant differences between the groups due to result variability.

4.4.2.4 Lesion inflammation and composition analysis by immunohistochemistry

Aortic root sections adjacent to sections containing fatty streaks were selected for immunohistochemical analysis. ICAM-1 staining was consistently present in the regions of the aortic root that positively stained for monocytes/macrophages. There were no differences in ICAM-1 expression in orchidectomised mice receiving testosterone treatment compared to orchidectomised mice receiving placebo and sham-operated mice (37% vs 57% vs 45%, *P*=0.1345) (Figure 4.15). Power analysis suggested that the ICAM-1 analysis was underpowered and increasing the sample size would reveal any significant differences between the groups.

Similarly, VCAM-1 staining was present within the regions that positively stained for monocytes/macrophages. However, staining was more concentrated in the regions that corresponded with positive staining for vWF as well as smooth muscle cell regions, stained positively for αSMA, forming the fibrous cap and artery wall (Figure 3.15). VCAM-1 expression within the plaque area was not different between testosterone-treated orchidectomised mice compared to orchidectomised mice

receiving placebo and sham-operated mice (30% vs 45% vs 40%, *P*=0.1402) (Figure 4.15). Power analysis suggested that the VCAM-1 analysis was underpowered and increasing the sample size would reveal any significant differences between the groups.

E-selectin staining was present in the plaque and endothelial regions of all three groups. There were no significant differences between mice sham-operated and orchidectomised mice receiving placebo compared to testosterone-treated mice (53% vs 56% and 47%, *P*=0.4135). Power analysis suggested that the E-selectin analysis was underpowered and increasing the sample size would reveal any significant differences between the groups.

No staining was detected in negative controls, with the omission of the secondary antibodies, for both secondary antibodies, Alexa Fluor 488 and Alexa Fluor 594, indicating no autofluorescence. No staining was detected in the IgG controls, rat monoclonal 1gG2a or rabbit polyclonal IgG at optimised conditions indicating no non-specific binding (images not shown).

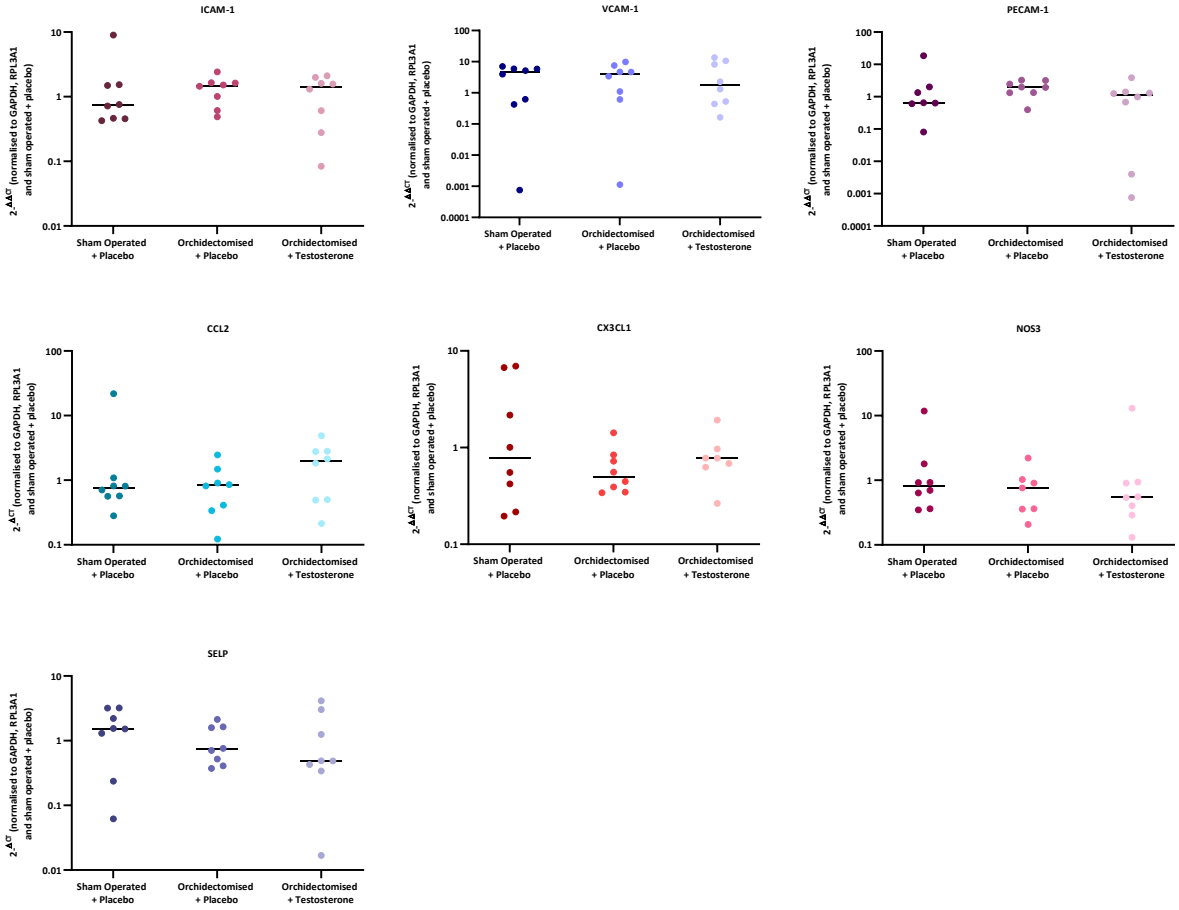


Figure 4.14 The effect of testosterone replacement in ApoE$^{-/-}$ mice fed a high-fat 'Western' diet on gene expression in lesion-specific tissue of inflammatory targets linked to the pathogenesis of atherosclerosis. Aortic root samples, identified as having atherosclerotic fatty streaks/lesions from positive ORO staining, were selected throughout the aortic root. Sham-operated mice (n=11) and orchidectomised receiving placebo (n=11) and orchidectomised receiving testosterone treatment (n=11) were compared for gene expression of inflammatory targets.

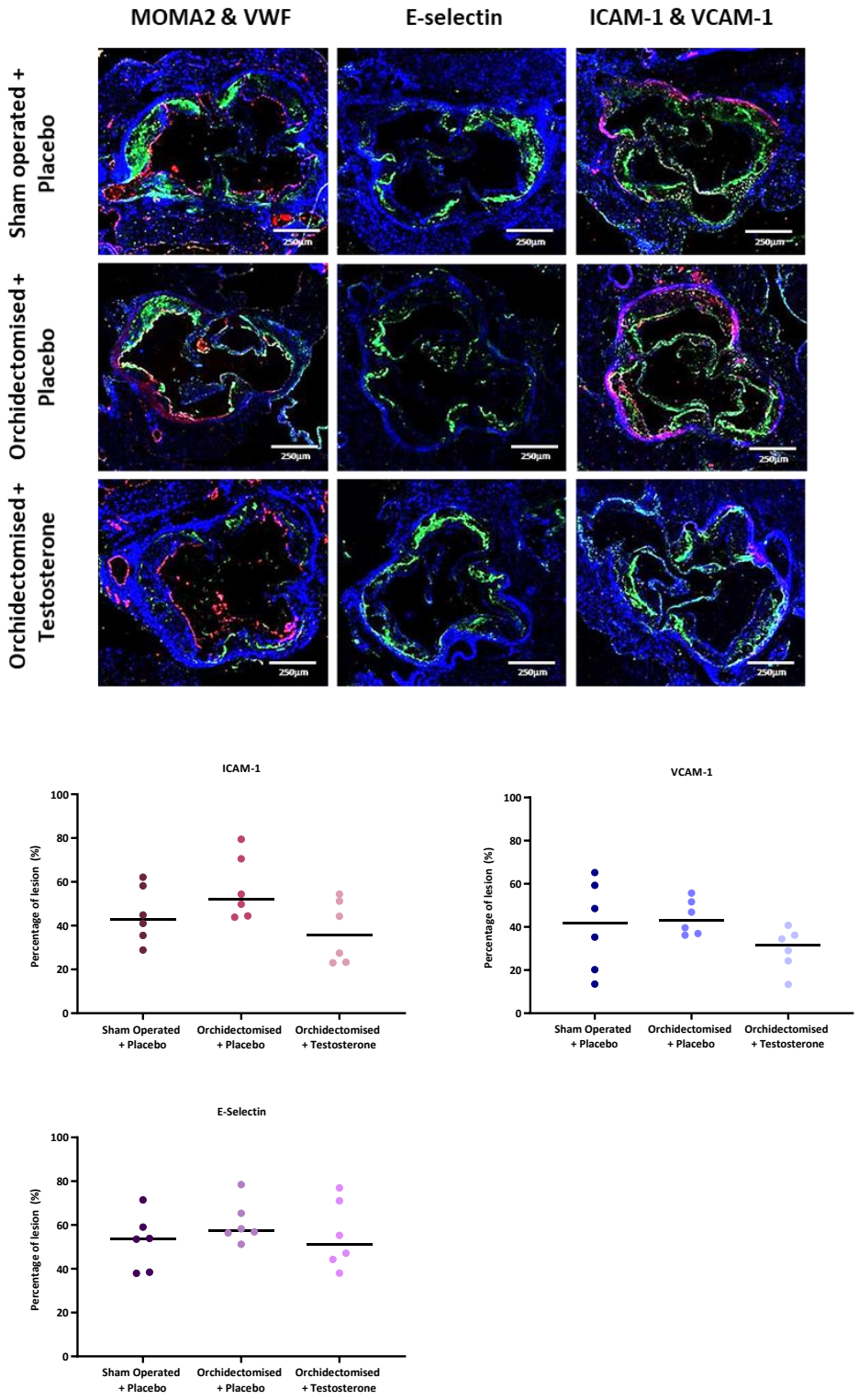


Figure 4.15 Immunohistochemical analysis of fatty streak composition in the aortic root of ApoE$^{-/-}$ mice fed a high-fat 'Western' diet following testosterone treatment. Aortic root samples were selected from the middle of the aortic root. The endothelial layer was marked by von Willebrand factor (VWF, red) and monocyte/macrophage infiltration was detected adjacent to fatty streaks (MOMA2, green). E-selectin (green) was detected in the lesion. ICAM-1 (green) and VCAM-1 (red) expression were also observed in the lesion. Immunopositivity was expressed as a percentage of the lesion area. Cell nuclei were stained with DAPI (blue). Scale bar 250µm. (*$P \leq 0.05$).

4.4.2.5 Analysis of endothelial inflammation in ApoE$^{-/-}$ mice following testosterone treatment

4.4.2.5.1 Gene analysis of endothelial inflammation

To evaluate whether testosterone has an impact on the inflammatory status of the endothelial cells lining the plaque, the endothelial layer, which corresponded with positively stained cells for vWF, was isolated for gene analysis by LCM.

Expression of adhesion molecules ICAM-1, VCAM-1, PECAM-1, SELP, CCL2, CX3CL1 and NOS3 were not significantly modulated by testosterone status in endothelial cells (Figure 4.16).

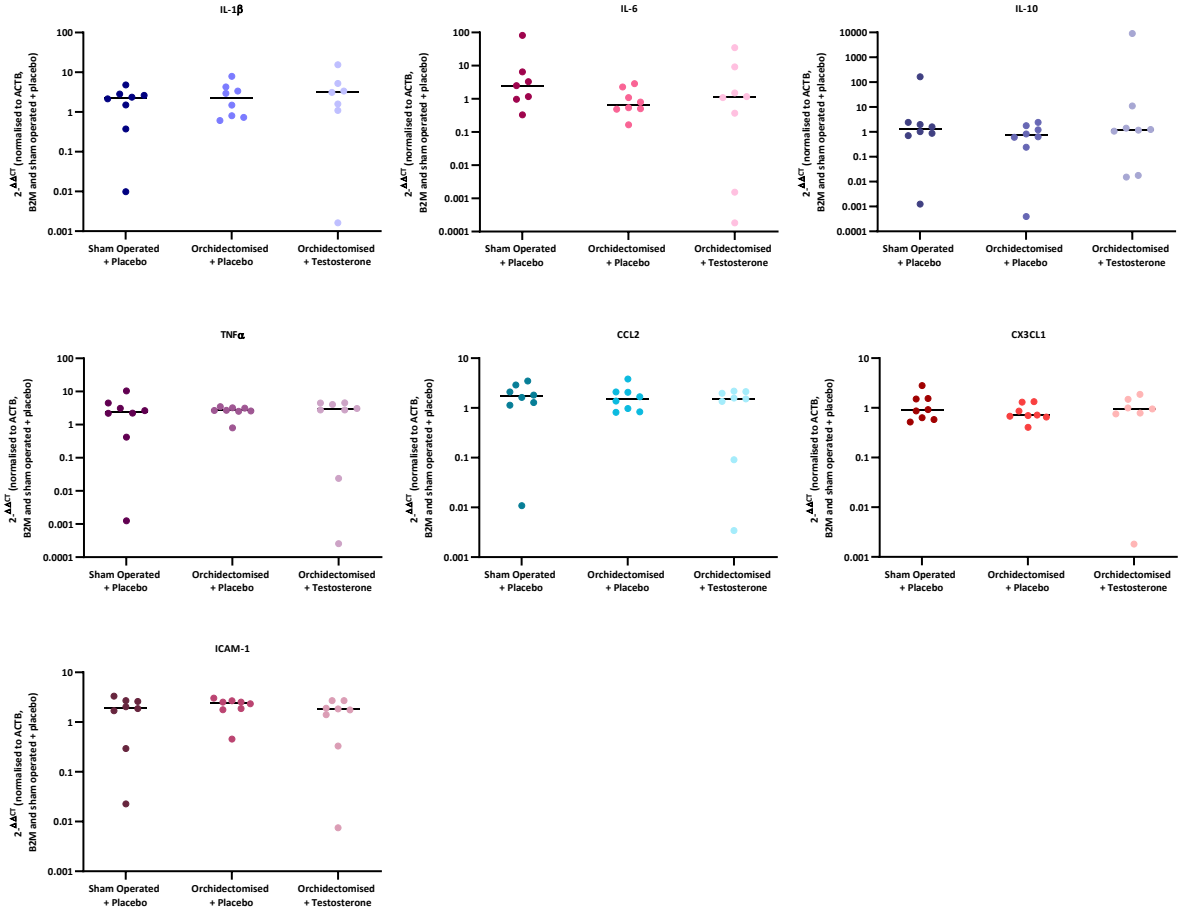


Figure 4.16 The effect of testosterone replacement in ApoE$^{-/-}$ mice fed a high-fat 'Western' diet on gene expression on endothelial cells lining the top of the fatty streak on inflammatory targets linked to the pathogenesis of atherosclerosis. Aortic root samples, identified as having atherosclerotic lesions/fatty streaks from positive ORO staining, were selected throughout the aortic root. Sham-operated mice (n=11) and orchidectomised receiving placebo (n=11) and orchidectomised receiving testosterone replacement (n=11) were compared for gene expression of inflammatory targets.

4.4.3 The effect of testosterone on systemic inflammation

4.4.3.1 Serum cytokine measurements in ApoE$^{-/-}$ mice

Serum concentrations of TNFα and IL-10 were unaffected by testosterone treatment in orchidectomised mice compared to placebo sham-operated and orchidectomised mice (*P*=0.9048 and *P*=0.388 respectively) (Figure 4.17). IL-1β, IL-6, IFNγ and MCP1 were also measured but fell below the lower limit of detection of the assay. Power analysis suggested that the cytokine analysis was sufficiently powered and is therefore unlikely there is significance in the data collected.

4.4.3.2 Serum soluble adhesion molecule measurements in ApoE$^{-/-}$ mice

Serum concentrations of ICAM-1 and VCAM-1 were unaffected by testosterone and there were no differences observed in placebo-treated orchidectomised mice compared to sham-operated mice and testosterone treated mice (*P*=0.1697 and *P*=0.4259). Similarly, there were no significant differences in concentrations of E-selectin (*P*=0.4427) and L-selectin (*P*=0.2202) between testosterone-treated and sham-operated mice compared to orchidectomised placebo-treated mice (Figure 4.18). Power analysis suggested that the adhesion molecule analysis was sufficiently powered and is therefore unlikely there is significance in the data collected.

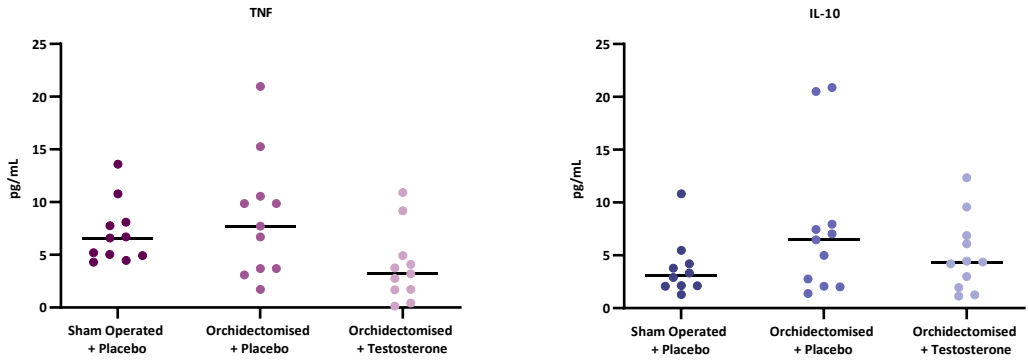


Figure 4.17 The effects of testosterone treatment on serum cytokines in ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. Sham-operated mice (n=11) and orchidectomised mice receiving placebo (n=11) and orchidectomised mice receiving testosterone replacement (n=11) were compared for serum cytokine concentrations.

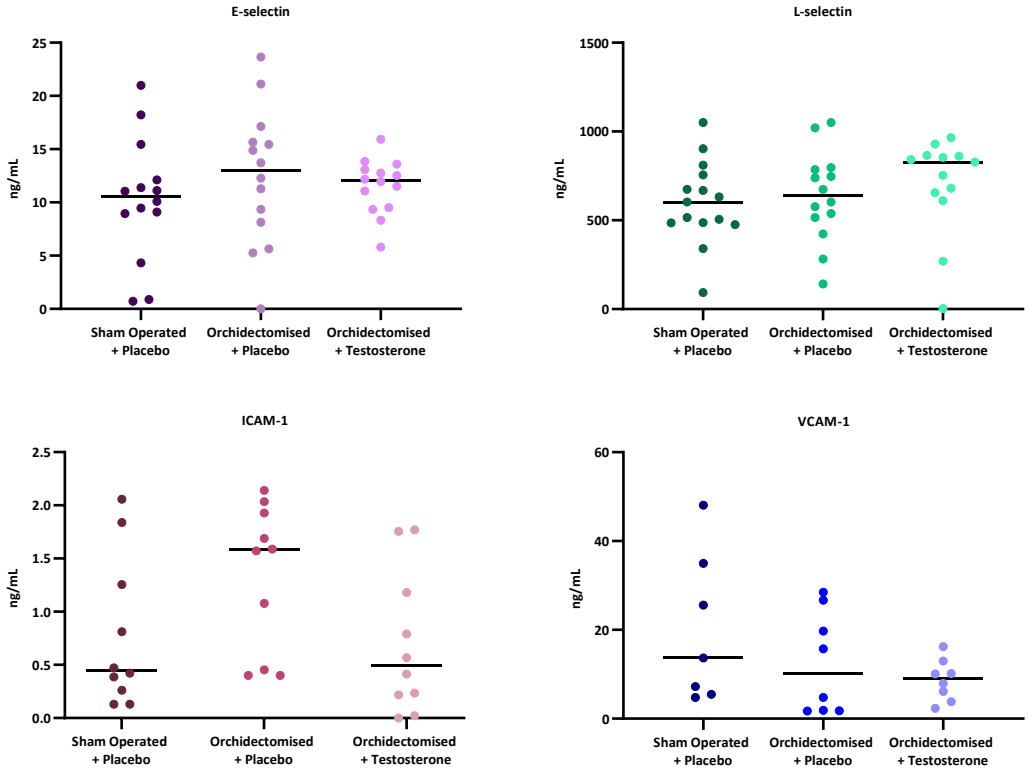


Figure 4.18 The effects of testosterone replacement on serum adhesion molecules in ApoE$^{-/-}$ mice fed a high-fat 'Western' diet. Sham-operated mice (n=11) and orchidectomised receiving placebo (n=11) and orchidectomised receiving testosterone replacement (n=11) were compared for serum adhesion molecule concentrations.

4.5 Discussion

These studies aimed to investigate the anti-inflammatory effects of testosterone through modulation of cytokines, chemokines and adhesion molecules. Systemic inflammation is also often present as a consequence of atherosclerosis risk factors such as MetS, T2D and testosterone deficiency (Jones, 2010b). TTh significantly reduced TNFα gene expression in monocytes isolated from male patients with hypogonadism and T2D but did not significantly modulate other factors implicated in the pathogenesis of atherosclerosis.

Atherosclerosis is driven by the inflammatory response of a complex sequence of interactions between circulating leukocytes and the vascular endothelium. It is established that this process involves a range of adhesion molecules on leukocytes and endothelial cells, as well as extensive intracellular signalling that drives adhesion and chemotaxis. Cells involved in the pathogenesis of atherosclerosis are activated by soluble factors, cytokines, chemokines and adhesion molecules that strongly influence the disease development. Pro-inflammatory cytokines accelerate atherosclerosis progression, while anti-inflammatory cytokines ameliorate the disease. Adhesion molecules and cytokines are critical participants in the vascular dysfunction, leukocyte recruitment and tissue injury associated with atherosclerosis; therefore, we sought to investigate whether testosterone influences these factors.

4.5.1 The effects of testosterone on monocytes

Monocytes are key cells in the atherogenic process (Spranger *et al.*, 2003). Circulating monocytes in patients with T2D are more prone to overexpression of pro-inflammatory cytokines. This inflammatory profile is not only important in the pathogenesis of T2D itself but may partially explain the higher incidence of macrovascular disease in this disease population (Giulietti *et al.*, 2007). Monocytes are essential cells in the atherosclerotic process, and their recruitment towards the plaque is a major pathogenic feature. The pro-inflammatory factors these cells have been shown to have increased expression of have been shown to contribute towards the inflammatory cascade leading to atherogenesis in humans and animal models (Whitman *et al.*, 2000, Frostegård *et al.*, 1999). Giulietti *et al.* (2007) reported high

levels of ICAM-1 and the co-stimulatory molecule B7-1 in monocytes isolated from T2D patients. Both have previously been implicated in atherosclerosis development (Jude *et al.*, 2002, Buono *et al.*, 2004).

Clinical studies highlight the benefit of testosterone on systemic inflammation (Malkin *et al.*, 2004a, Malkin *et al.*, 2004b, Zitzmann *et al.*, 2005, Kalinchenko *et al.*, 2010) but to date, there is very little information on the effect of testosterone on the specific cell types implicated in atherosclerosis. In the present study, we have shown that TTh significantly reduced TNFα gene expression in monocytes isolated from the patients after six months of therapy compared to their baseline and to the placebo group. It is currently unclear as to how TNFα expression in circulating monocytes contributes directly towards atherosclerosis *in vivo*. Autonomous TNFα is the mechanistic pathway by which monocytes have been shown to activate endothelial cells (Rainger *et al.*, 1996, Tsouknos *et al.*, 2003). TNFα promotes the inflammatory cascade within the arterial wall during lesion development, in part by promoting endothelial dysfunction (Zhang *et al.*, 2009, Zhang *et al.*, 2014). TNFα determines endothelial permeability to immune cells and small LDL (Steyers and Miller, 2014), promoting the first stage of atherosclerosis increasing the transport of LDL across the endothelium (Zhang *et al.*, 2014).

Furthermore, TNFα is released in response to oxLDL uptake in macrophages (Jovinge *et al.*, 1996) which transform into foam cells, directly contributing to atherosclerotic plaque progression (Ross, 1999). Experimental mouse studies consistently revealed diminished lesions in TNFα deficient animals and thereby clearly underline the crucial role of TNFα in atherogenesis (Boesten *et al.*, 2005, Brånén *et al.*, 2004, Ohta *et al.*, 2005). However, Oberoi *et al.* (2018) reported that anti-TNFα therapy diminished systemic inflammation and plaque burden. The authors also observed an increase in vascular inflammatory gene expression, although markers of plaque stability decreased in the $LDLr^{-/-}$ mouse model. The authors did conclude that this was due to the development of a pro-atherogenic plasma lipid profile rather than the anti-TNFα therapy.

TNF-α is a potent inducer of endothelial adhesion molecules such as VCAM-1 (Carlos *et al.*, 1990) and ICAM-1 (Pober *et al.*, 1987) with subsequently increased monocyte adhesion (Goerdt *et al.*, 1987). The expression of key adhesion molecules E-selectin, ICAM-1 and VCAM-1 were elevated in endothelial cells that had been co-cultured *in vitro* with monocytes and endothelial cells and reduced when TNFα was blocked in the monocytes (Chimen *et al.*, 2017). Whilst we observed no difference between groups for gene expression of ICAM-1 in monocytes, it is plausible that the downregulation of TNFα in the TTh group could reduce lesion expression of ICAM-1. The reduction in endothelium activation could, in turn, reduce monocyte infiltration and differentiation into macrophages and foam cells in the vessel wall, a critical process of atherosclerosis initiation and progression. It is unclear how relevant circulating monocytes isolated via venepuncture in the periphery are to monocytes that are recruited to and are localised to the site of atherosclerosis within the arteries. Still, other pro- and anti-inflammatory targets that are often elevated in men with hypogonadism and/or T2D and were also investigated in the present study, although, we report no change in gene expression of other inflammatory targets investigated following TTh.

Corrales *et al.* (2006) reported that *in vitro* testosterone treatment inhibited TNFα, IL-1β and IL-6 release from cultured peripheral blood monocytes isolated from patients of a similar cohort to the current study, androgen-deficient men with T2D. Gonadotropin treatment in patients with idiopathic hypogonadotropic hypogonadism, significantly decreased the production of IL-1β and TNFα by stimulated peripheral blood monocytes (Musabak *et al.*, 2003, Yesilova *et al.*, 2000). Another study reported a reduction of IL-6 production from isolated human monocytes in a small healthy male population following *in vitro* testosterone treatment (Kanda et al. 1996). Similarly, Li *et al.* (1993) also reported a reduction in IL-6 and IL-1β following *in vitro* testosterone treatment in human monocytes isolated from healthy male donors.

4.5.1.1 The effects of testosterone on localised inflammation

The role of adhesion molecules in the pathogenesis of human atherosclerosis has been well established (Davies *et al.*, 1993a, O'Brien *et al.*, 1993) and animal studies

have reported their presence in plaques of ApoE$^{-/-}$ mice (Smedlund *et al.*, 2015, Laschet *et al.*, 2006, Budatha *et al.*, 2018, Li *et al.*, 2019). ICAM-1, VCAM-1 and E-selectin staining were present in the plaques of all three animal groups of the current study, adjacent to ORO and MOMA2 staining. As dual staining was not carried out as part of this investigation, the localisation could not be specifically designated to cell type, although the majority of positive staining was within the region that was positively stained for monocytes/macrophages. Whilst we observed no difference in expression between the groups for E-selectin and VCAM-1, we did, however, observe that ICAM-1 expression was reduced in testosterone-treated mice.

Whilst testosterone treatment reduced localised protein expression of ICAM-1 in the aortic root; this was not the case for gene expression of isolated lesion-specific tissue. Furthermore, this investigation did not find any significant difference in gene expression of the inflammatory targets investigated.

In the current study, *in vitro* testosterone treatment of macrophages induced from isolated patient monocytes in the clinical study elicited no change in gene expression of key inflammatory targets linked to the pathogenesis of atherosclerosis. The anti-inflammatory effects of testosterone on macrophages have been previously demonstrated *in vitro*. Corcoran *et al.* (2010) reported reduced expression and secretion of TNFα and IL-1β following *in vitro* testosterone treatment in monocyte-derived macrophages obtained from men with CHD. Macrophage phenotype is dependent on environmental cues (Wang *et al.*, 2015) and the differentiation process *in vitro* may have caused the macrophages to take on a naïve or non-activated phenotype. Flow cytometry analysis for markers of a non-activated macrophage phenotype would have confirmed this. Testosterone may not influence non-activated macrophages in the absence of inflammation; therefore activation of macrophages into inflammatory subsets was investigated. Khallou-Laschet *et al.* (2010) investigated the phenotype of macrophages in ApoE$^{-/-}$ mice and found early atherosclerotic lesions contained mainly M2 macrophages, whilst M1 macrophages prevailed in more progressed lesions and lesions of aged ApoE$^{-/-}$ mice, indicating that the macrophages are polarized according to surrounding inflammation.

In the current study, we investigated the effects of testosterone on macrophage subsets M1 and M2a *in vitro* utilising the THP-1 cell line. Similarly to the macrophages isolated from patients and treated with testosterone *in vitro*, we found no change in gene expression of key inflammatory targets linked to the pathogenesis of atherosclerosis in either the M1 or M2 phenotype following treatment of varying concentrations of testosterone.

4.5.1.2 The effects of testosterone on endothelial cells

In the current study, we report no effect of testosterone treatment in ApoE$^{-/-}$ mice on gene expression of key inflammatory targets, including VCAM-1 in isolated endothelial cells lining the atherosclerotic lesion. However, unlike humans who have shown to have increased systemic inflammation that correlates with atherosclerosis progression, the ApoE$^{-/-}$ mice in the current study did not have systemic inflammation and therefore may not be representative of the *in vivo* situation in humans. Without systemic inflammation, it is plausible that testosterone would not have a downstream effect on the endothelium in this study. *In vitro* studies have shown that testosterone treatment in HAECs has been shown to attenuate TNFα-induced VCAM-1 expression via inhibiting activation of the transcriptional NF-κB, which is critical for the inducible expression of VCAM-1 as well as several other inflammatory gene targets (Hatakeyama *et al.*, 2002). This effect has also been demonstrated in HUVECs, and this attenuation of VCAM-1 by testosterone was abolished in the presence of an aromatase inhibitor, thus testosterones beneficial effects may be mediated by its conversion to estradiol via the enzyme aromatase present in the endothelial cells (Mukherjee *et al.*, 2002).

The promoter regions of the genes encoding ICAM-1, VCAM-1 and E-selectin all contain at least one κB site required for cytokine gene activation (Neish *et al.*, 1992, Kaszubska *et al.*, 1993, Hou *et al.*, 1994, Read *et al.*, 1994). Following stimulation with TNFα and LPS, Norata *et al.* (2006) also reported a reduction in VCAM-1 and ICAM-1 expression in HUVECs as well as decreased IL-6, MCP-1 and TNFα release following treatment with DHT, again mediated by NF-κB . Conversely, Zhang *et al.* (2002) observed that testosterone did not have an effect on TNFα receptor expression in TNFα-stimulated HUVECs and instead demonstrated that testosterone increased TNFα-

induced E-selectin and VCAM-1 upregulation. Similarly, McCrohon *et al.* (1999) and Death *et al.* (2004) reported that DHT treatment is associated with increased human monocyte adhesion to HUVECs and increased endothelial cell–surface expression of VCAM-1 via the activation of the transcriptional NF-κB pathway.

4.5.2 The effects of testosterone on systemic inflammation

In the present study, the majority of serum cytokines measured in the ApoE$^{-/-}$ mice fell below the lower limit of detection, and TNFα and IL-10 were only detectable at low levels (<25pg/mL) with limited differences between the experimental groups that were not significant, indicating that the mice did not have systemic inflammation. Other studies utilising the ApoE$^{-/-}$ mouse fed a pro-atherogenic diet have been able to detect the cytokines in high concentrations (>100pg/mL) that were undetectable in the present study (Wang *et al.*, 2017, Zhou *et al.*, 2018) however these studies used ELISAs. Levels of circulating cytokines have been shown to reflect the levels of pro-atherogenic cytokines in plaque tissue and may be used as surrogate markers for the identification of high-risk plaques (Edsfeldt *et al.*, 2015). Furthermore, soluble adhesion molecules have been shown to reflect plaque severity in the ApoE$^{-/-}$ mouse model whereby the concentration of soluble ICAM-1 (sICAM-1) increased over time in parallel with the progression of atherosclerosis (Kitagawa *et al.*, 2002). Similarly, Pradhan Aruna *et al.* (2002) reported that levels of sICAM-1 were higher in individuals who subsequently developed symptomatic arterial disease. In the current study, the levels soluble adhesion molecules ICAM-1, VCAM-1, E-selectin and L-selectin were investigated. There was a limited difference between the experimental groups, and testosterone did not appear to influence VCAM-1, E-selectin, and L-selectin. Orchidectomy did however increase circulating levels of sICAM-1, which is implicated in lesion progression. Moreover, testosterone treatment in the orchidectomised mice did reduce levels of sICAM-1, although not significantly.

Levels of soluble adhesion molecules were lower than expected for in the current study compared to previous studies that have reported much higher concentrations (Gustavsson *et al.*, 2010, Chan *et al.*, 2016, Tian *et al.*, 2005, Yuan *et al.*, 2008) with no differences between the placebo-treated sham-operated and

orchidectomised mice and the testosterone-treated orchidectomised mice. Controls and standards run alongside the samples were successful.

Kelly *et al.* (2012) reported that serum cytokines TNFα and IL-6 were significantly elevated in Tfm compared to XY littermates fed a standard chow diet for 28 weeks. Furthermore, the authors also reported a systemic increase in serum MCP-1 in Tfm and XY littermates fed a high cholesterol diet, suggesting that diet can generate a pro-inflammatory milieu. However, in the current study, all experimental mice were fed a high-fat diet and had undetectable serum MCP-1 levels. Following testosterone treatment, IL-6 was reduced by testosterone treatment in the Tfm mice fed the high cholesterol diet; however, there were limited differences in other serum cytokines (IL-1β, IL-10, TNFα, and MCP-1). Similarly, in the present study, there were no significant differences between the groups for IL-10 and TNFα with very low circulating levels. Bourghardt *et al.* (2010) also reported no effect of testosterone treatment on IL-6 or other cytokines in ARKO ApoE$^{-/-}$ mice.

Demirtaş Şahin *et al.* (2018) reported that 24-month-old Wistar rats displayed significantly increased serum TNFα, CRP, MCP-1, and sICAM-1 concentrations and decreased serum testosterone levels compared to controls. Chin and Ima-Nirwana (2017) reported that testosterone deficiency by surgical orchidectomy increased in serum IL-6 levels, but exogenous supraphysiological testosterone replacement did not suppress the inflammation. However, supraphysiological testosterone replacement in orchidectomised male rats has been shown to increase systemic inflammation, specifically IL-6 compared to physiological replacement which decreases inflammation (Freeman *et al.*, 2014). Inversely, Wang *et al.* (2005) observed that blocking the testosterone receptor with flutamide or castrating normal male rats decreased serum levels of TNFα, IL-1β, and IL-6 compared to controls.

Animal studies have highlighted that low endogenous testosterone levels are associated with systemic inflammation, and increased atherosclerosis and that testosterone treatment has beneficial effects in reducing inflammation and atherosclerotic burden. However, in the present study, we did not find elevated serum

cytokines in the mouse model and limited differences in soluble adhesion molecules between the experimental groups.

Clinical studies investigating the association between testosterone levels and inflammatory cytokines highlight the potential benefits of testosterone treatment. (Malkin *et al.*, 2004a, Malkin *et al.*, 2004b, Zitzmann *et al.*, 2005, Kalinchenko *et al.*, 2010). Nettleship *et al.* (2007b) reported a negative correlation between serum testosterone levels and IL-1β in testosterone-deficient men with stable coronary artery disease (CAD). The data implicated IL-1β and IL-10 in the pathogenesis of CAD and suggests that testosterone may regulate IL-1β activity in men with CAD. Another study investigated circulating pro- and anti-inflammatory cytokines in 27 hypogonadal men (20 of whom had CHD) and showed that levels of TNFα were significantly reduced, and IL-10 was significantly increased following one month of TTh (Malkin *et al.*, 2004a). Similarly, hypogonadal men with ischaemic heart disease had a significant decrease in serum TNFα following a month of TTh (Malkin *et al.*, 2004b).

In two RCTs of TTh in hypogonadal men, one in men with T2D and the other with MetS, TTh suppressed serum IL-1β and TNFα but did not cause significant changes in serum IL- 6 or IL-10 (Kalinchenko *et al.*, 2010, Dhindsa *et al.*, 2016). However, a study investigating the effects of TTh in hypogonadal men with T2D did not observe any significant immuno-modulatory effect; however, baseline levels of IL-6 but not TNFα were negatively correlated with total and bioavailable amounts of circulating testosterone, confirming that low testosterone is associated with inflammation (Kapoor *et al.*, 2007b). Similarly, Pugh *et al.* (2005) reported no change in serum TNFα concentration in hypogonadal men with chronic heart failure following TTh.

4.5.3 Summary

In summary, the current studies have revealed that testosterone treatment in ApoE$^{-/-}$ mice reduces ICAM-1, both circulating and localised in the aortic root lesions of ApoE$^{-/-}$ mice. However, testosterone treatment had little effect on other inflammatory targets. This effect on ICAM-1 was not observed in specific cell types monocytes, macrophages *in vitro* and endothelial cells isolated from ApoE$^{-/-}$ mouse lesions. Furthermore, TNFα gene expression was significantly reduced in freshly isolated

monocytes from patients with hypogonadism and T2D following six months of TTh. However, again this did not translate to *in vitro* studies utilising the THP-1 cell line. We report very little evidence to support the hypothesis that testosterone modulates plaque inflammation by acting directly on monocytes/macrophages. Whilst using *in vitro* studies is useful for determining the effect of testosterone on a cell type, the *in vitro* conditions used in present studies do not reflect the local *in vivo* environment in the artery wall. It is plausible that the anti-inflammatory effects of testosterone may act locally at the artery wall on a multitude of pathways and cell types, contributing to a reduction of lesion progression, rather than on a specific cell type *in vitro*. In addition, THP-1 cells are a cancerous cell line with altered metabolism and may not respond to hormones as primary cells would; consequently, they do not reflect typically normal human monocytes/macrophage cells. The beneficial effects of testosterone on atherosclerosis observed in clinical studies may be due to improvements in risk factors associated with atherosclerosis and testosterone deficiency, such as dyslipidaemia, obesity, insulin resistance and glycaemia control.

5. The effects of testosterone on risk factors for atherosclerosis

5.1 Introduction

Cardiovascular morbidity and mortality and the prevalence of atherosclerosis are increased in people with obesity, and central adiposity is an independent predictor of CVD mortality (Sucharda, 2010, Rocha and Folco, 2011). Increased adipose tissue is associated with T2D and components of MetS, including insulin resistance, hyperglycaemia, dyslipidaemia and hypertension, and obesity is also a component of MetS. These conditions lead to increased inflammation which may contribute directly and indirectly to local (adipose tissue) and distant (artery wall) inflammation (Glass and Witztum, 2001, Lusis, 2000). These consequences of obesity are the main reason for atherosclerosis, causing endothelial dysfunction (Rocha and Folco, 2011).

Epidemiological studies have demonstrated a negative correlation between measures of obesity with free testosterone, bioavailable testosterone and total testosterone levels (Allen *et al.*, 2002, Gapstur *et al.*, 2002, Jensen *et al.*, 2004, Svartberg *et al.*, 2004a, Svartberg *et al.*, 2004b), an association that is maintained throughout all age groups (Corona *et al.*, 2009). Low testosterone levels are associated with increased fat mass (particularly central adiposity) and reduced lean mass in males. Central obesity is also important in the incidence of atherosclerosis (Kershaw and Flier, 2004). Adipose tissue is an endocrine organ and adipocytes highly express aromatase that enzymatically converts testosterone to estradiol and thus lowers circulating testosterone (Cohen, 1999). Adipocytes also secrete pro-inflammatory adipokines, known as the hypogonadal–obesity–adipocytokine hypothesis, that influences the pathogenesis of obesity and negatively affect testosterone production to create a state of hypogonadism (Jones, 2007, Kelly and Jones, 2015). A bidirectional relationship between testosterone and obesity underpins this association indicated by the hypogonadal–obesity cycle, (the lowering of testosterone further contributing to adiposity creating a negative cycle) and evidence weight loss can lead to moderate increases in testosterone levels (Cohen, 1999, Kelly and Jones, 2015). TTh has been shown to have beneficial effects on measures of obesity that are partially explained by direct metabolic actions on adipose and muscle. Studies have reported that TTh in hypogonadal men improves body composition and consistently report an increase in lean body mass and a decrease in percentage fat mass. The majority of the studies also

found a reduction in waist circumference, which correlates positively with visceral adiposity (Haider *et al.*, 2016, Saad *et al.*, 2013, Saad *et al.*, 2016, Traish *et al.*, 2014, Traish *et al.*, 2017, Yassin *et al.*, 2016).

Low testosterone levels are highly prevalent in men with T2D and MetS and is associated with clinical characteristics associated with T2D and MetS, and total testosterone is negatively correlated to insulin levels and insulin resistance in men (Haffner *et al.*, 1994, Simon *et al.*, 1997). Insulin resistance is the major biochemical abnormality in men with MetS or T2D and is considered to be an intermediary cardiovascular risk factor that promotes hyperglycemia, dyslipidemia, hypertension, and endothelial dysfunction (Jones and Kelly, 2018). Hyperglycaemia induced endothelial dysfunction plays a fundamental role in the development of diabetic vascular complications and is thought to be due to hyperglycaemia induced oxidative stress (Funk *et al.*, 2012).

TTh has been shown to improve insulin sensitivity in as little time as three months and is maintained for at least up to 12 months (Kapoor *et al.*, 2006, Kalinchenko *et al.*, 2010, Aversa *et al.*, 2010, Jones *et al.*, 2011, Hackett *et al.*, 2014a, Dhindsa *et al.*, 2016).

Obesity and T2D frequently co-exist with dyslipidemia. Some studies have reported that low testosterone is associated with a pro-atherogenic lipid profile (Haidar *et al.*, 2007), characterised by elevated triglyceride and LDL-cholesterol (Haffner *et al.*, 1993, Barrett-Connor and Khaw, 1988, Simon *et al.*, 1997, Barud *et al.*, 2002, Wu and von Eckardstein, 2003, Dockery *et al.*, 2003, Nishiyama *et al.*, 2005, Braga-Basaria *et al.*, 2006, Yannucci *et al.*, 2006) and decreased HDL (Simon *et al.*, 1997, Van Pottelbergh *et al.*, 2003, Stanworth *et al.*, 2011). However, a few cross-sectional studies have found no association between serum lipid measurements and endogenous testosterone (Kiel *et al.*, 1989, Denti *et al.*, 2000).

5.1.1 Summary

Testosterone deficiency in men is associated with T2D and components of MetS, including insulin resistance, abdominal obesity, and dyslipidemia. In most but not all

studies, RCTs using TTh have shown improved insulin resistance, glycemic control, visceral adiposity, and cholesterol which correlates with reduced surrogate markers of atherosclerosis and consequentially improved CVD risk.

5.2 Aims and objectives

The aim of the *in vivo* studies was to use clinical trial data from hypogonadal men with T2D following TTh and a mouse model of atherosclerosis to address the hypothesis that testosterone treatment improves risk factors that contribute to the inflammatory pathogenesis of atherosclerosis. The aim of the *in vitro* study was to develop an *in vitro* model of diabetic monocytes to investigate the hypothesis that testosterone reduces inflammation in monocytes cultured in hyperglycaemic conditions indicative of T2D. Our specific objectives were to;

- Investigate the effects of low endogenous testosterone on risk factors (dyslipidaemia, obesity, hyperglycaemia and insulinaemia) for atherosclerosis using the $ApoE^{-/-}$ mouse model.
- Uncover the effects of testosterone treatment on risk factors for atherosclerosis using the $ApoE^{-/-}$ mouse model fed a pro-atherogenic diet.
- Develop a monocyte model of T2D and investigate the anti-inflammatory influence of testosterone.

5.3 Experimental design

Men participating in the clinical trial had serum hormones, HbA_{1c}, blood glucose, blood lipids and measurements of body composition taken at baseline, three and six months of treatment (Section 2.3). Initially, $ApoE^{-/-}$ mice were orchidectomised to reduce endogenous testosterone levels and fed a standard chow diet to investigate the effects of orchidectomy on serum hormones, body composition, serum lipids and blood glucose. Secondly, mice were fed a pro-atherogenic diet, and orchidectomised mice were treated with placebo or testosterone to investigate the effects of testosterone treatment on serum hormones, body composition, serum lipids and blood glucose (Section 2.1).

The THP-1 cell line was used as a model of high glucose-induced inflammation. THP-1 monocytes were treated *in vitro* with varying concentrations of glucose (5-25mmol/L). Inflammation was analysed with qRT-PCR (Section 2.2.3). The experimental design is summarised in Figure 5.1.

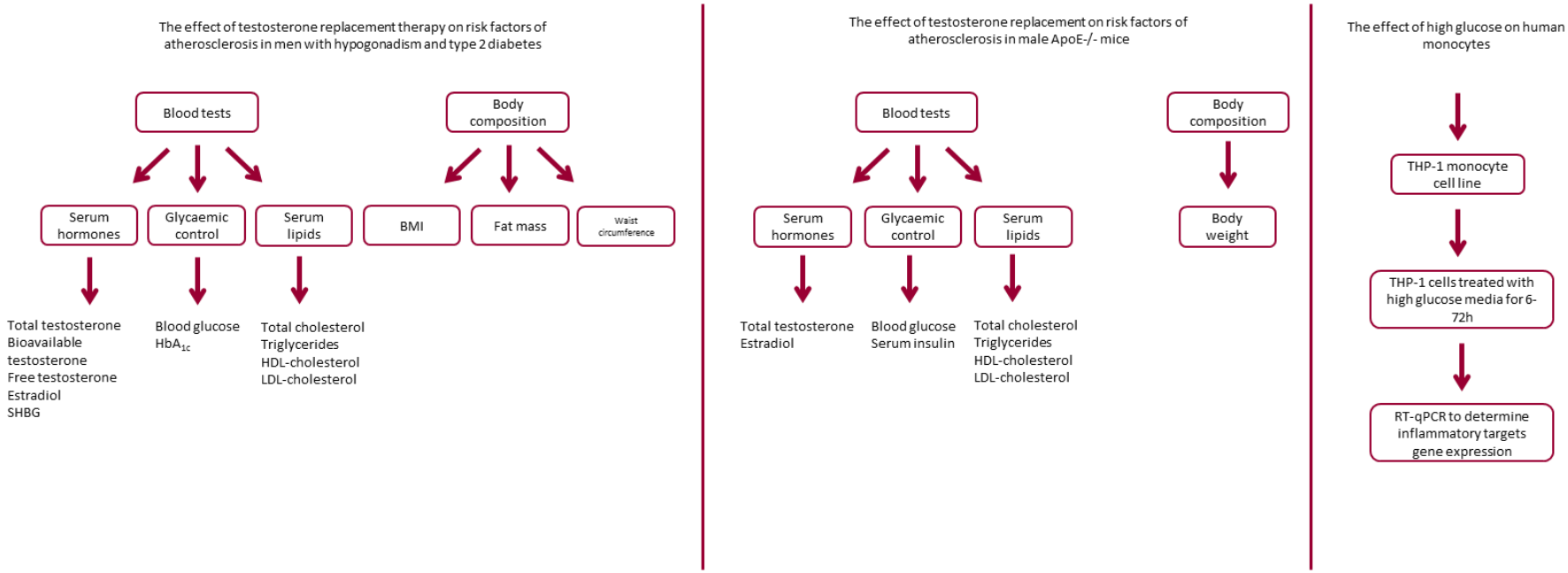


Figure 5.1 Experimental design to investigate the effect of testosterone on risk factors of atherosclerosis and an *in vitro* model investigating the effects of high glucose on human monocytes.

5.4 Results

5.4.1 Testosterone replacement in male patients with hypogonadism and type 2 diabetes

The study population of the men included in the clinical was a group of 33 men receiving TTh and 32 men receiving the placebo, all of which had hypogonadism and T2D. The baseline data of the treated group versus the placebo group were not statistically significant from one another (Table 5.1).

Parameter	Placebo (n=32) Median (lower & upper limit)	TTh (n=33) Median (lower & upper limit)	Standard range
Age (year)	60 (43-77)	59 (42-74)	-
HbA_{1C} (mmol/mol)	63.5 (55-80)	63 (53-80)	<42
Glucose (mmol/L)	9.6 (2.8-16.9)	9.2 (4.9-17.8)	4.0-7.8
Triglycerides (mmol/L)	1.65 (0.51-6.9)	2.03 (0.79-4.66)	<1.7
HDL-cholesterol (mmol/L)	0.96 (0.62-2.11)	1.02 (0.43-1.56)	>1.3
LDL-cholesterol (mmol/L)	1.90 (0.70-3.70)	1.80 (0.90-3.90)	<1.8
Total cholesterol (mmol/L)	3.65 (2.5-6.5)	3.90 (2.2-5.5)	<5.2
Weight (kg)	105.5 (74.5-186.9)	104.2 (71.2-141.0)	-
BMI	33.7 (24.3-51.8)	34.4 (26.0-47.0)	18.5-24.9
Fat mass (%)	34.6 (21.8-55.0)	35.7 (21.2-46.8)	14-25
Fat mass (kg)	35.9 (16.2-102.8)	35.4 (16.4-64.3)	-
Waist circumference	116 (89-160)	117 (91-146)	<89
Total testosterone (nmol/L)	9.35 (4.00-16.90)	9.00 (2.10-12.10)	10-30
Bioavailable testosterone (nmol/L)	2.93 (1.43-4.10)	2.95 (0.92-3.78)	3.87-14.7
Free testosterone (nmol/L)	0.19 (0.07-0.35)	0.19 (0.07-0.28)	0.26-0.71
SHBG (nmol/L)	31.95 (14.90-133.00)	30.65 (10.10-65.70)	10-57
Estradiol (pmol/L)	112.0 (65.0-198.0)	113.5 (45.0-182.0)	48-154
CRP (ng/mL)	1.90 (0.2-23.4)	3.15 (0.2-16.6)	<10

Table 5.1 Baseline characteristics median (lower and upper limit) of hypogonadal men with T2D assigned to either the placebo group or testosterone replacement therapy group. The baseline values of each parameter between the placebo and testosterone groups were not statistically different. HbA_{1c}: glycated haemoglobin, HDL: high-density lipoprotein, LDL: low-density lipoprotein, BMI: body mass index, SHBG; sex-hormone-binding globulin, CRP: C-reactive protein.

5.4.1.1 Serum testosterone concentrations

There were no significant differences in total, bioavailable and free serum testosterone at baseline between the two treatment groups (*P*>0.05) (Table 5.1). After three months of treatment, the patients receiving TTh had a higher total serum testosterone concentration compared to patients receiving the placebo (9.8 vs 9.1nmol/L), although this was not significant (*P*=0.0551). However, patients receiving TTh did have a significantly higher total serum testosterone concentration after six months compared to patients receiving the placebo (11.4 vs 8.6nmol/L, *P*=0.0047) (Figure 5.2). The normal total testosterone range for an adult male is between 10-30nmol/L; the patients receiving TTh are within the lower quartile of the normal range compared to placebo-treated males who remained in the hypogonadal range. After three months of TTh, the patients receiving TTh had significantly higher bioavailable testosterone levels compared to baseline (3.17 vs 2.95nmol/L, *P*=0.0004) (Figure 5.2). Similarly, after six months, the patients receiving TTh had significantly higher bioavailable testosterone levels compared to baseline (3.68 vs 2.76nmol/L, *P*<0.0001). This significance was also seen compared to placebo-treated patients after three months (3.17 vs 2.88nmol/L, *P*=0.0389) and six months (3.68 vs 2.76nmol/L, P=0.0005) (Figure 5.2). The normal bioavailable testosterone range for an adult male is between 3.8-14.7nmol/L; the patients receiving TTh were just below the normal range. After three months of TTh, the patients receiving TTh had significantly higher free testosterone levels compared to baseline (0.24 vs 0.19nmol/L, *P*=0.0133). Following six months of TTh, patients had significantly higher free testosterone levels compared to baseline (0.25 vs 0.19nmol/L, *P*=0.0006) (Friedman test). This significance was also seen compared to placebo-treated patients after three months (0.24 vs 0.19nmol/L, *P*=0.0376) and six months (0.25 vs 0.17nmol/L, *P*=0.0005) (Figure 5.2). The normal free testosterone range for an adult male is between 0.26-0.71nmol/L; the patients receiving TTh were at the lower end of the normal range.

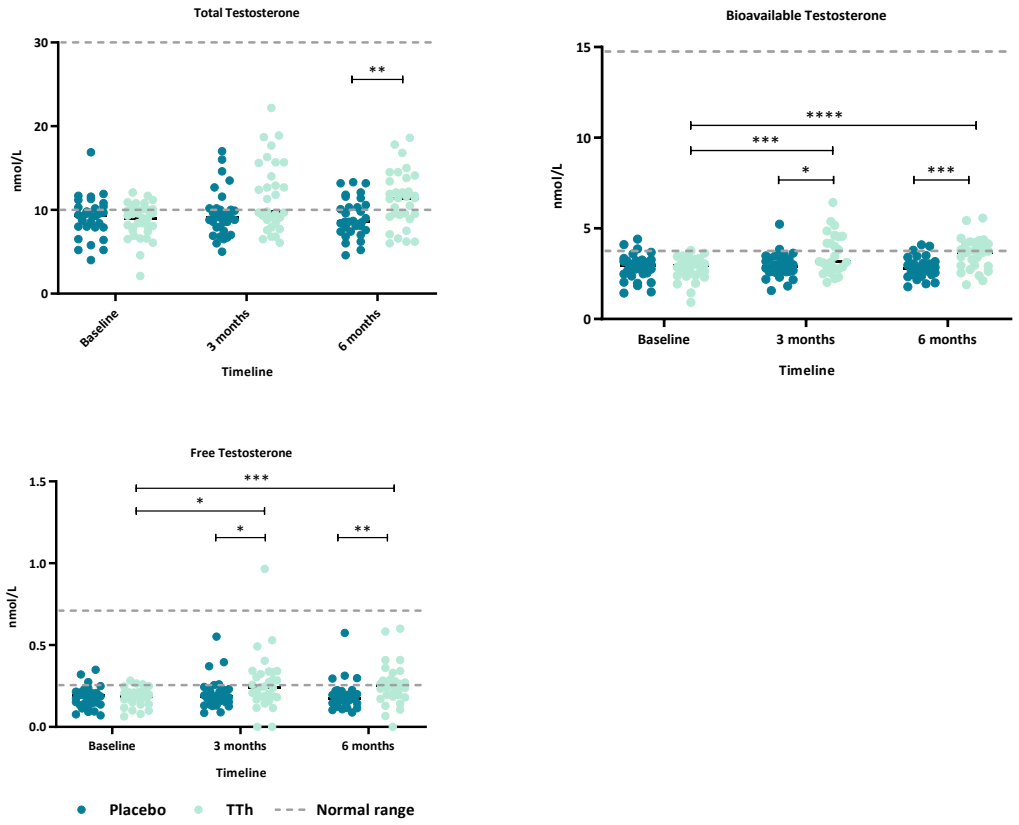


Figure 5.2 Comparative evaluation of serum testosterone levels at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. Total testosterone was measured, and free and bioavailable testosterone calculated for patients receiving either placebo (n=32) or TTh (n=33). (*P<0.05 **P<0.01 ***P<0.001 ****P<0.0001), Friedman test and Mann-Whitney U test.

5.4.1.2 Serum sex hormone-binding globulin concentrations

There were no differences observed between serum SHBG levels at baseline in the placebo group compared to the group receiving TTh (Table 5.1). SHBG levels did not change significantly and no differences were observed between the treatment groups throughout the treatment period.

5.4.1.3 Serum estradiol

There were no significant differences in serum estradiol levels at baseline placebo group compared to the group receiving TTh, with the vast majority falling within the normal range (P>0.05) (Table 5.1). After three months, the patients receiving TTh had significantly higher estradiol levels compared to baseline (149.00 vs 113.50pmol/L, P=0.0002). Similarly, after six months, the patients receiving TTh had significantly higher estradiol levels compared to baseline (169.00 vs 113.50pmol/L, P<0.0001). Serum estradiol levels were significantly increased in the group receiving TTh compared to the placebo group after three (149.00 vs 100.00pmol/L, P<0.0001.) and six months of treatment (169.00 vs 104.50pmol/L, P<0.0001) (Figure 5.4). However, this is likely a reflection of increased serum testosterone by conversion to estradiol via adipose aromatase.

5.4.1.4 Determination of blood glucose and HbA_{1c}

There were no significant differences in HbA_{1c} and fasting blood glucose at baseline for the placebo group compared to the group receiving TTh (P>0.05) (Table 5.1). TTh did not affect HbA_{1c} and fasting blood glucose after three and six months of treatment compared to placebo-treated patients and blood glucose. HbA_{1c} and blood glucose levels also did not change within the treatment groups over the study period compared to baseline measurements (Figure 5.5). The study was sufficiently powered to assess changes in HbA_{1c} and is therefore unlikely that TTh has a significant effect on HbA_{1c} in these men.

After three months of treatment, 61% of patients receiving TTh had an improved HbA_{1c} compared to 50% of patients receiving placebo. 26% of patients receiving TTh had a worse HbA_{1c} than their baseline measurement compared to 43% of

patients receiving placebo (Figure 5.5). However, after six months of treatment, 57% of patients receiving TTh had a worse HbA_{1c} than their baseline measurement compared to 59% of patients receiving placebo. 40% of patients receiving TTh had an improved HbA_{1c} after six months of treatment compared to 38% of patients receiving placebo (Figure 5.6).

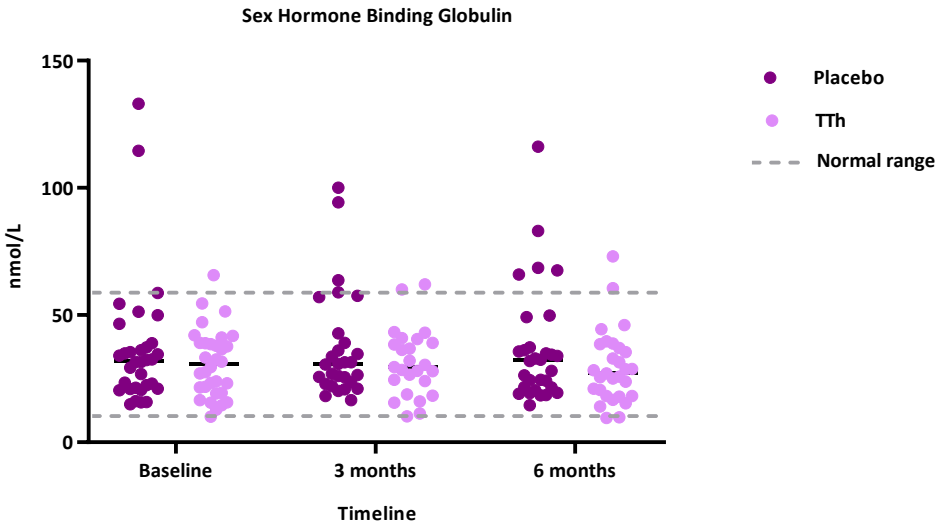


Figure 5.3 Comparative evaluation of serum SHBG at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. Serum SHBG was measured in patients receiving either placebo (n=32) or TTh (n=33).

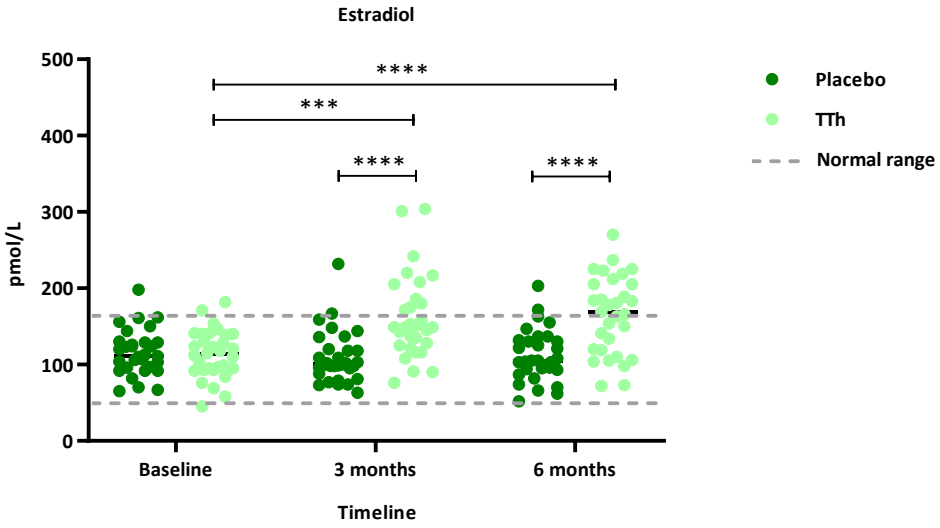


Figure 5.4 Comparative evaluation of serum estradiol at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. Serum estradiol was measured in patients receiving either placebo (n=32) or TTh (n=33). (***P<0.001 ****P<0.0001), Friedman test and Mann-Whitney U test.

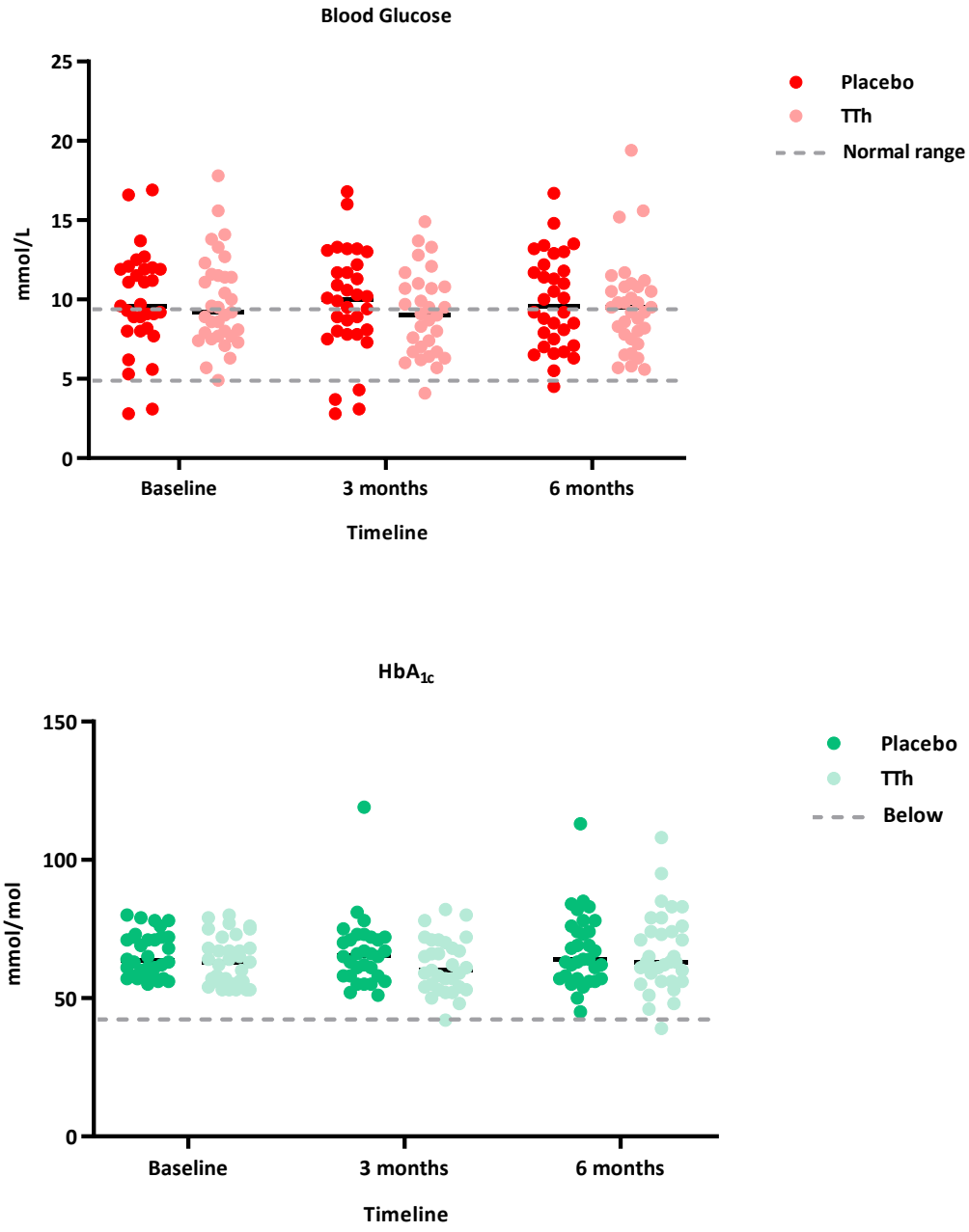


Figure 5.5 Comparative evaluation of fasting blood glucose and HbA_{1c} at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. Fasting blood glucose and HbA_{1c} was measured in patients receiving either placebo (n=32) or TTh (n=33).

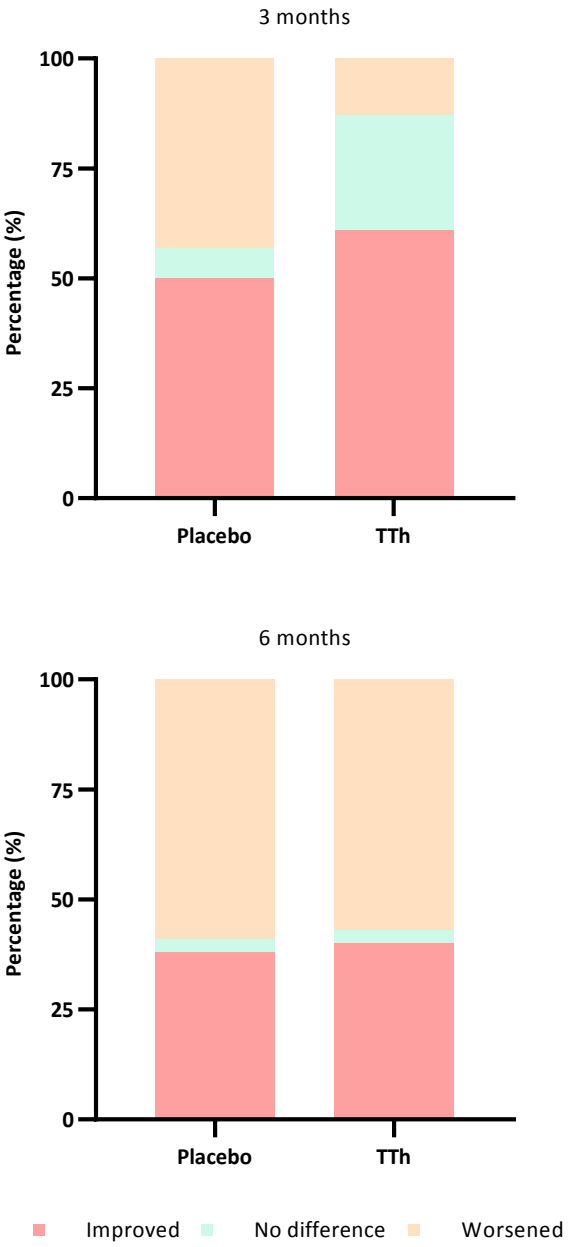


Figure 5.6 Comparative evaluation of HbA_{1c} following three and six months of treatment with TTh or placebo in male patients with hypogonadism and T2D. The percentage of patients in each group was compared for those whose HbA_{1c} had either improved, worsened, or no difference following three and six months of either TTh (n=33) or placebo (n=32).

5.4.1.5 Body mass composition

There were no significant differences in BMI at baseline for the placebo group compared to the group receiving TTh (P>0.05) (Table 5.1). BMI remained consistent between the placebo group and the group receiving TTh at three (33.6 vs 34.0) and six months (34.0 vs 33.9).

Fat mass in weight measured by DEXA scan and percentage body fat measured by Tanita body fat analyser also remained unchanged between the treatment groups and compared to baseline after three- and six-months treatment (Figure 5.7). BMI and percentage of fat mass in both treatment groups at baseline and throughout the study indicated obesity in patients. Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups.

5.4.1.6 Serum lipid profile

There were no significant differences in serum lipid fractions at baseline for the placebo group compared to the group receiving TTh (Table 5.1). Serum lipid concentrations remained consistent throughout the study compared to baseline measurements, and there were no significant differences between the patients receiving TTh and placebo following and six months of treatment (Figure 5.8). Total cholesterol in both treatment groups at baseline and throughout the study was within the healthy range (<5.2mmol/L). In contrast, triglycerides (<1.7mmol/L) and LDL-cholesterol (<1.8mmol/L) were slightly above the healthy range, and HDL-cholesterol was slightly below the healthy range (>1.3mmol/L). Power analysis indicates that the study was insufficiently powered to reveal differences between the treatment groups although due to the variability of the results it is highly unlikely that there are differences between the treatment groups.

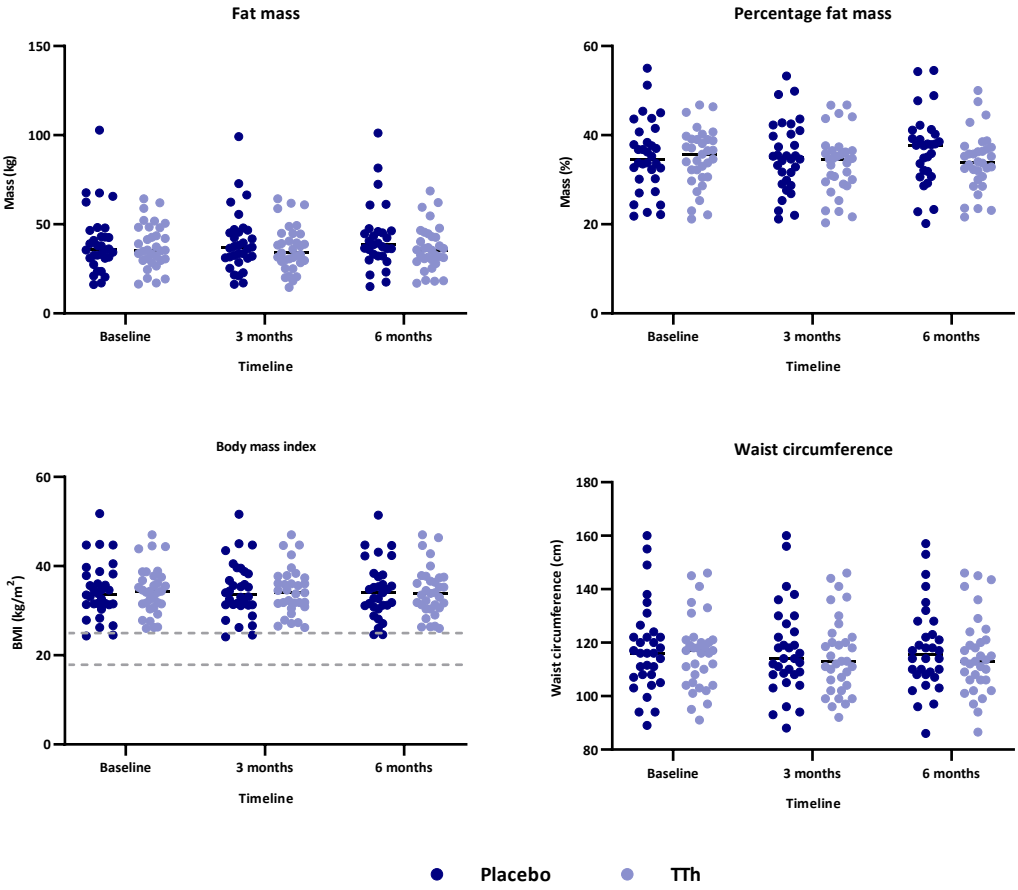


Figure 5.7 Comparative evaluation of fat mass, percentage fat mass, BMI and waist circumference at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. Fat mass, percentage fat mass and waist circumference were measured, and BMI was calculated for patients receiving either placebo (n=32) TTh (n=33).

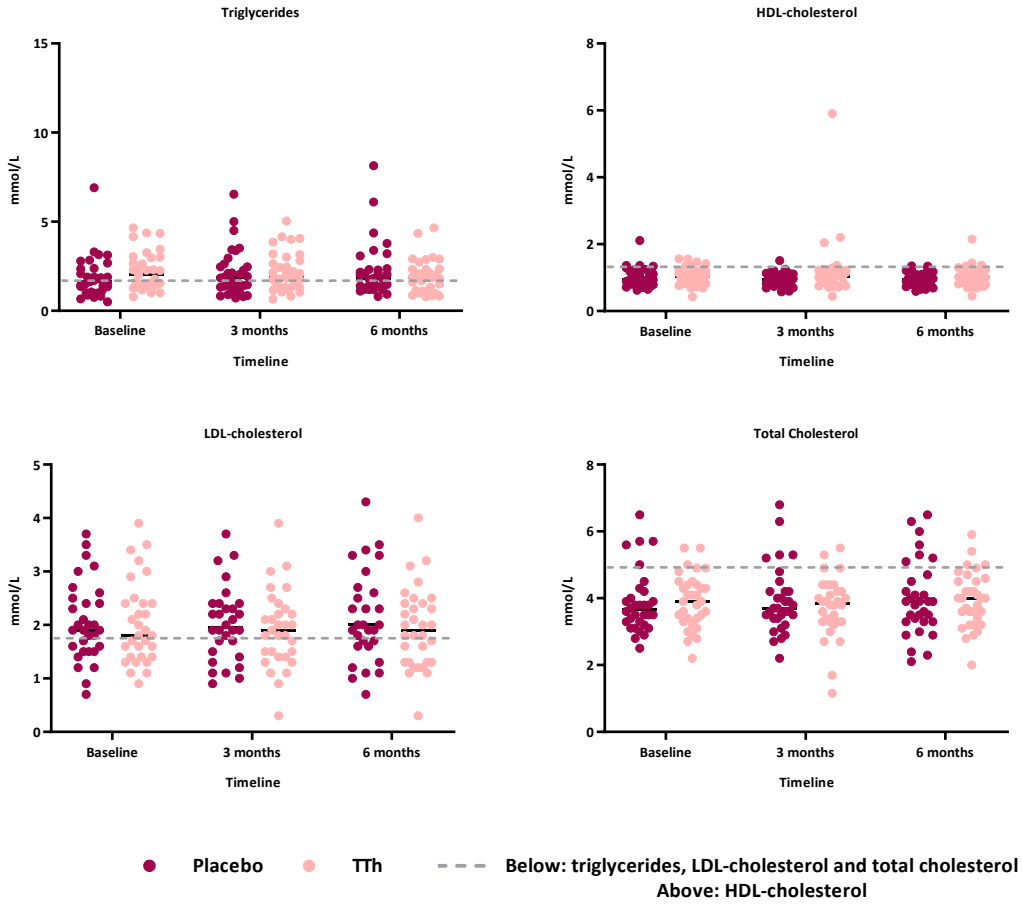


Figure 5.8 Comparative evaluation of the serum lipid profile at baseline, three and six months in male patients with hypogonadism and T2D receiving either placebo or TTh. For assessment of the lipid profile, triglycerides, HDL-cholesterol, and total cholesterol values were measured and LDL-cholesterol calculated in patients receiving either placebo (n=32) TTh (n=33).

5.4.2 Development of an *in vitro* model of diabetic THP-1 monocytes

THP-1 cells challenged with high glucose (15 and 25mmol/L) did not elicit any significant changes in mRNA expression of IL-1β, IL-6, TNFα or ICAM-1 after 48 hours of culture compared to normal glucose concentration (5.5mmol/L) (P>0.05) (Figure 5.9). 5.5mmol/L glucose and 9.5mmol/L mannitol were used as an osmolality control.

High glucose (15mmol/L) did not elicit any significant change in mRNA expression of IL-1β, IL-6, TNFα or ICAM-1 after 6, 12, 24, 48 or 72 hours of culture (P>0.05). mRNA expression was normalised to 15mmol/L after 6 hours of culture. Cells treated with TNFα was tested as a pro-inflammatory control; however this failed to elicit changes in mRNA expression of IL-1β, IL-6, TNFα (Figure 5.10). ICAM-1 was upregulated by TNFα treatment, although this was not significant (data not shown).

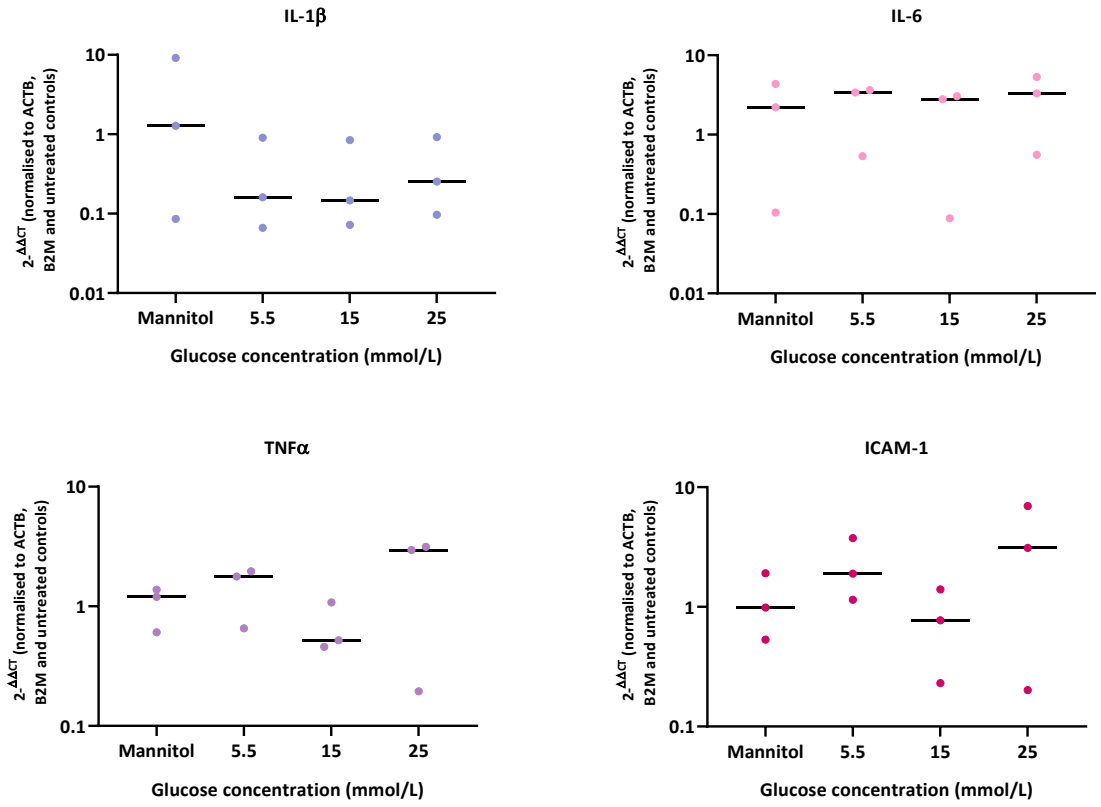


Figure 5.9 Dose-dependent effect of high glucose on IL-1β, IL-6, TNFα and ICAM-1 mRNA from THP-1 cells. Cells were challenged with increasing glucose concentration (15–25mmol/L) for 48 hours, and RT-qPCR analysis was performed to detect IL-1β, IL-6, TNFα and ICAM-1 mRNA. As a control, 9.5 mmol/l mannitol was added with 5mmol/L in simultaneous wells (n=3), normalised to 5.5mmol/L glucose.

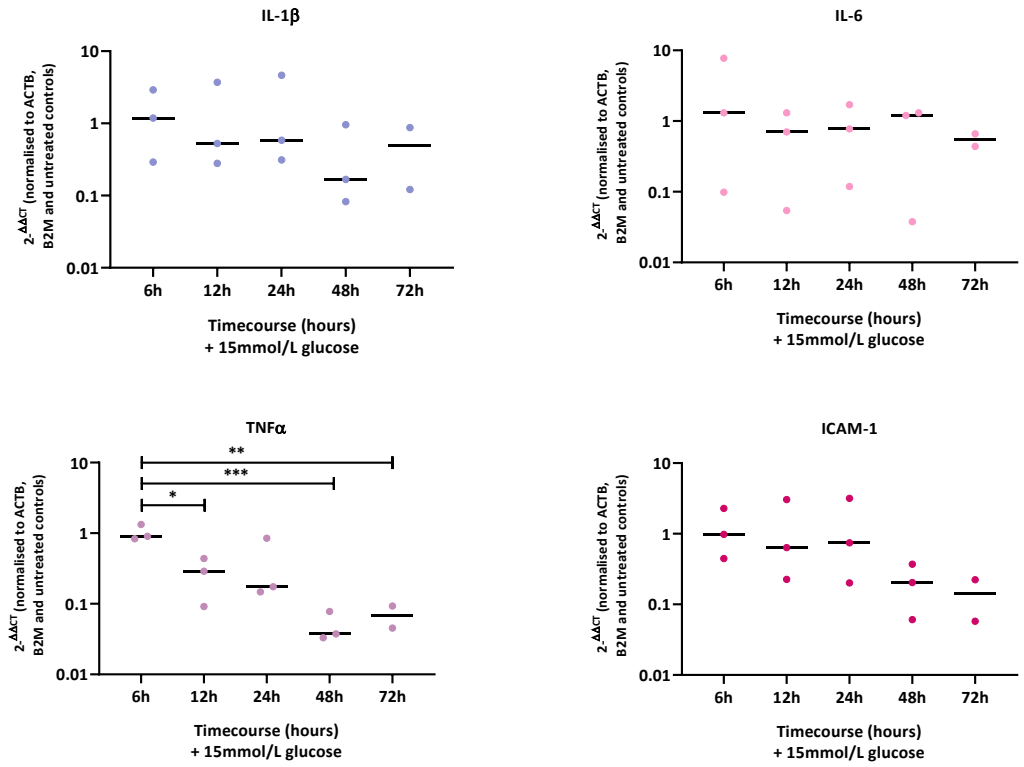


Figure 5.10 Time-dependent effect of high glucose on IL-1β, IL-6, TNFα and ICAM-1 mRNA from THP-1 cells. Cells were challenged with 15mmol/L glucose for 6–72 hours, and RT-qPCR analysis was performed to detect IL-1β, IL-6, TNFα and ICAM-1 mRNA. As a control, 9.5mmol/L mannitol was added with normal glucose in simultaneous wells (n=3). Normalised to 6 hours. (*$P \leq 0.05$, **$P<0.01$, ***$P<0.001$), Kruskal-Wallis test.

5.4.3 The effect of testosterone depletion on atherosclerotic parameters in ApoE$^{-/-}$ mice fed a standard chow diet

5.4.3.1 Serum estradiol measurements

Median serum estradiol concentration in sham-operated (11.19pmol/L) and orchidectomised mice (10.88pmol/L) were not statistically different from one another (*P*>0.9999) (Figure 5.11).

5.4.3.2 Blood glucose measurements

Fasting glucose was measured at the beginning, midpoint and end of the experimental period. Blood glucose measurements between the groups were elevated at the start of the study, approximately one and half times the concentration than that of wild-type male C57 mice at the same age (Amrani *et al.*, 1998, Andrikopoulos *et al.*, 2005). There were no significant differences in blood glucose concentration in the sham-operated mice after eight weeks (midpoint) and the end of the study compared to baseline (10.8 vs 9.1 vs 11.7mmol/L, *P*=0.8598). However, blood glucose concentration was significantly decreased in orchidectomised mice at the end of the study compared to baseline and eight weeks of experimental conditions (8.9 vs 10.5 vs 9.5mmol/L, *P*<0.014, 0.0009) (Figure 5.12).

5.4.3.3 Serum insulin measurements

Fasting serum insulin was measured at the end of the experimental period. There were no significant differences between serum insulin levels in sham-operated (1.90pmol/L) and orchidectomised mice (1.97pmol/L) (*P*=0.1) at the end of the experimental period (Figure 5.13). Insulin analysis was insufficiently powered to assess changes between the groups.

5.4.3.4 Animal body weights

Animal body weights were compared over the experimental period between 8 and 25 weeks old. Sham-operated mice (n=5) were significantly heavier (29.00g) orchidectomised mice (n=9) (24.65g) consistently up to the end of the experimental period (Figure 5.14A) (*P*<0.05-0.001).

The weight gain over the experimental period was also calculated, in relation to starting weight (aged eight weeks). Sham-operated mice demonstrated significantly greater increases in weight from aged eight weeks to 14 weeks when compared to orchidectomised mice (Figure 5.14B). The median weight gain over the experimental period for sham-operated mice was 4.8g and 3.1g for orchidectomised mice. However, weight gain when standardised to starting weight was only significant between aged nine to ten weeks. Orchidectomised mice remained heavier and gained slightly more weight throughout (Figure 5.14C).

5.4.3.5 Serum lipid measurements

There were no differences between sham-operated and orchidectomised mice for triglycerides (1.98 vs 1.52mmol/L, *P*=0.5854), total cholesterol (15.50 vs 14.26mmol/L, *P*=0.5303), HDL-cholesterol (2.25 vs 1.91mmol/L, *P*=0.1029) and LDL-cholesterol (11.60 vs 12.01mmol/L, *P*=0.7955) (Figure 5.15). Serum lipid analysis was insufficiently powered to assess changes between the groups.

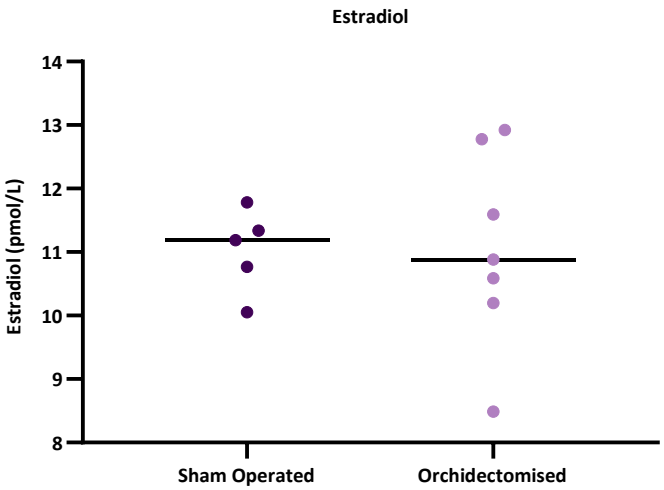


Figure 5.11 Serum estradiol concentration in ApoE$^{-/-}$ mice fed a standard chow diet. Sham-operated (n=4), and orchidectomised mice (n=9) on a standard chow diet were compared for serum estradiol concentration at the end of the 17-week experimental period.

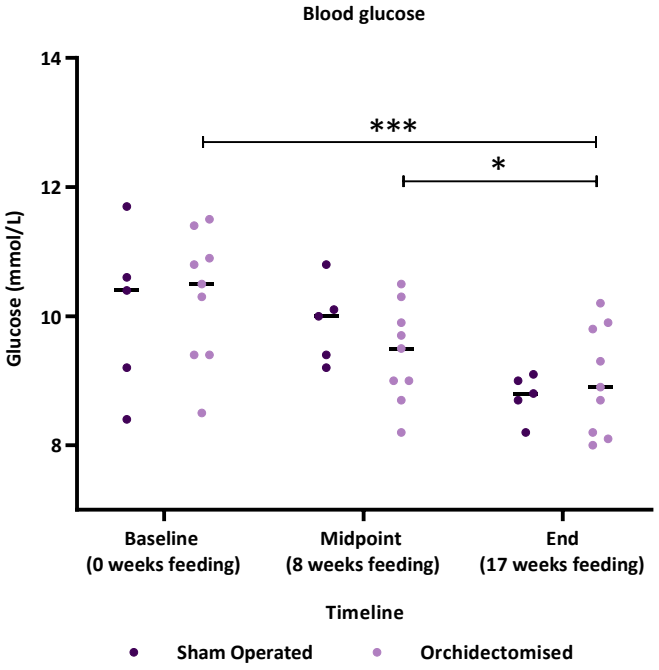


Figure 5.12 Blood glucose concentration of ApoE$^{-/-}$ mice fed a standard chow diet. Sham-operated (n=5), and orchidectomised mice (n=9) on a standard chow diet were compared for blood glucose levels at the beginning (week 0), midpoint (week 8) and end (week 17) of the 17-week experimental period. (*$P \leq 0.05$, ***$P < 0.001$ vs sham-operated), Friedman test.

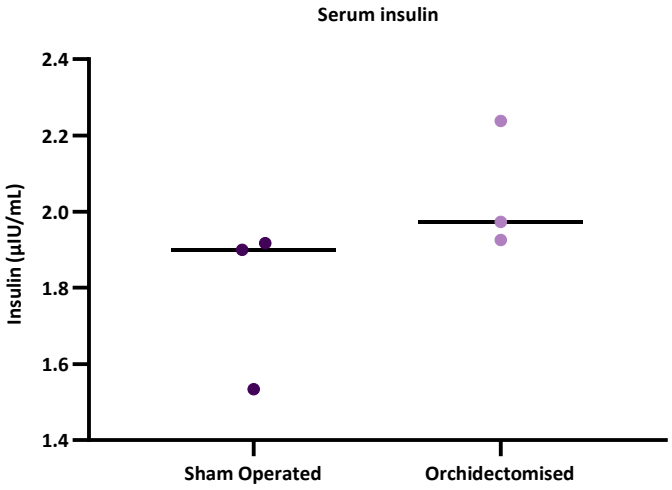


Figure 5.13 Serum insulin concentration of ApoE$^{-/-}$ mice fed a standard chow diet. Sham-operated (n=3), and orchidectomised mice (n=3) on a standard chow diet were compared for serum insulin concentration at the end of the 17-week experimental period.

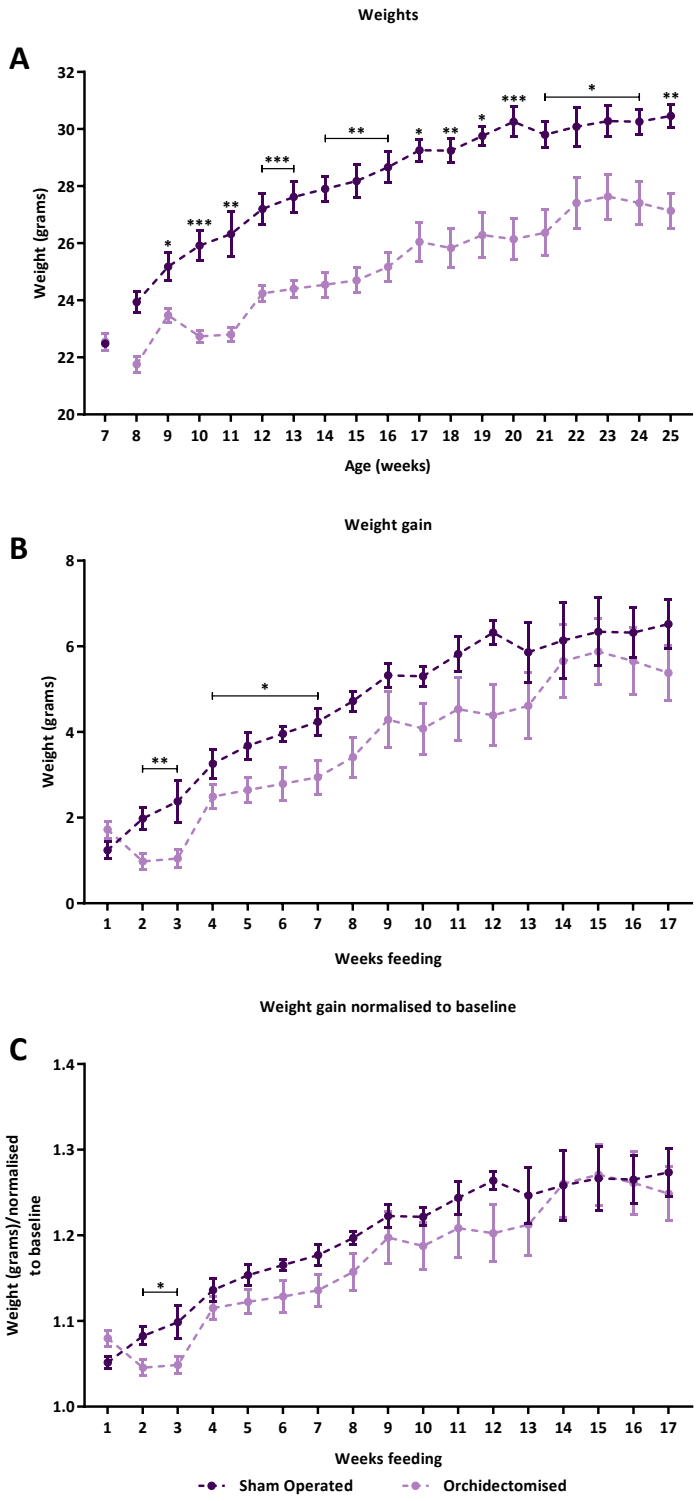


Figure 5.14 Total body weight and weight gain of sham-operated and orchidectomised male ApoE$^{-/-}$ mice fed a standard chow diet. Weekly weight measurements were compared between sham-operated (n=5) and orchidectomised mice (n=9) on a standard chow diet for the 17-week study period. (A) Mean weekly weights, (B) mean weight gains relative to week 1 (age eight weeks) start weights and (C) mean weight gains standardised to week 1 (age eight weeks) start weights (*P<0.05; **P<0.01; ***P<0.001 vs sham-operated), Mann-Whitney U test.

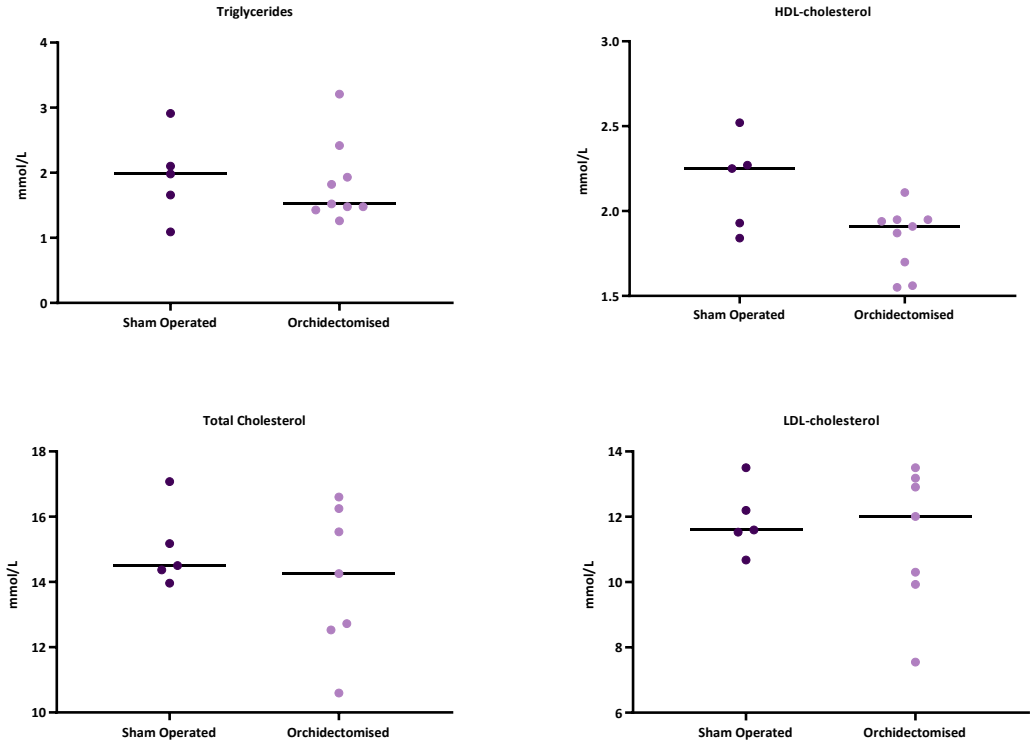


Figure 5.15 Serum lipid concentration of ApoE$^{-/-}$ mice fed a standard chow diet. Sham-operated (n=5), and orchidectomised mice (n=9) on a standard chow diet were compared for serum lipid levels at the end of the 17-week experimental period.

5.4.4 The effect of testosterone replacement on atherosclerotic parameters in ApoE$^{-/-}$ mice fed a high fat "Western-type" diet

5.4.4.1 Serum estradiol measurements

Median serum estradiol concentration in placebo-treated sham-operated (10.65pmol/L), orchidectomised (11.94pmol/L) and testosterone replaced orchidectomised mice (9.79pmol/L) were not statistically different from one another (*P*=0.4213) (Figure 5.16).

5.4.4.2 Blood glucose measurements

After eight weeks of treatment (midpoint), testosterone-treated mice had significantly lower blood glucose concentrations (9.3mmol/L) than placebo-treated sham-operated mice (10.9mmol/L) (*P*=0.0155) and orchidectomised mice (10.8mmol/L) (*P*=0.0083). However, this difference was lost at the end of the study, and there were no significant differences between the groups (*P*=0.2641) (Figure 5.17). Blood glucose concentration also decreased in the orchidectomised group receiving testosterone replacement after eight weeks compared to baseline readings (9.3 vs 11.4mmol/L) (*P*<0.01).

5.4.4.3 Serum insulin measurements

There were no significant differences between serum insulin levels in placebo-treated sham-operated (11.57pmol/L) and orchidectomised mice (11.76pmol/L) and orchidectomised mice receiving testosterone replacement (12.96mmol/L) (*P*=0.0592) at the end of the experimental period (Figure 5.18). Insulin analysis was adequately powered to assess changes between the groups.

5.4.4.4 Animal body weights

There were no significant differences in weight between the sham-operated receiving placebo (30.4g) (n=15), orchidectomised receiving placebo (29.6g) (n=15) or orchidectomised receiving testosterone replacement (29.52g) (n=15) (Figure 5.19A).

The weight gain over the experimental period was also calculated in relation to starting weight (Figure 5.19B and C). Orchidectomised mice receiving testosterone

replacement consistently gained more weight (7.7g) throughout the study compared to sham-operated receiving placebo (6.1g) and orchidectomised receiving placebo (7.0g); however, these differences only reached significance at limited time points ($P<0.05$-0.001). The study adequately powered to assess weight changes between the groups.

5.4.4.5 Serum lipid measurements

There were no significant differences between placebo-treated sham-operated and orchidectomised mice and orchidectomised mice receiving testosterone replacement for triglycerides (1.67 vs 1.56 vs 1.72mmol/L, P=0.5134), total cholesterol (26.50 vs 28.85 vs 30.58mmol/L, P=0.3058), HDL-cholesterol (2.27 vs 2.63 vs 2.54mmol/L, P=0.5286) and LDL-cholesterol (22.65 vs 25.48 vs 26.81mmol/L, P=0.4289) (Figure 5.20). Mice fed the high fat 'Western' diet had elevated serum lipids compared to ApoE$^{-/-}$ mice on a standard chow diet (Figure 5.20). The study was adequately powered to assess changes in serum lipids between the groups.

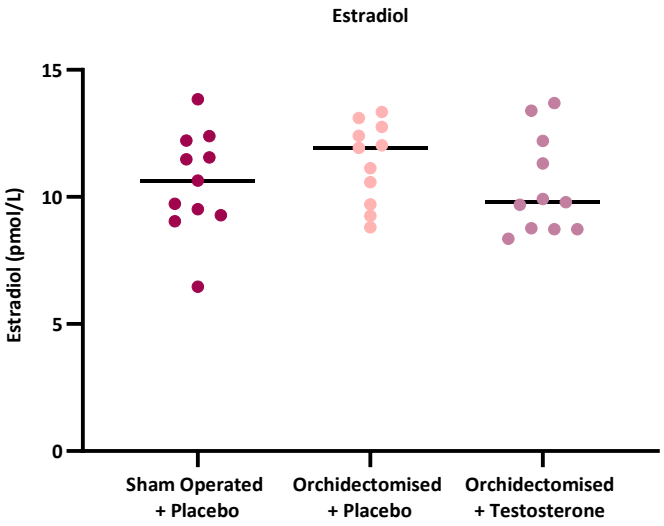


Figure 5.16 Serum estradiol concentration in ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Sham-operated (n=11) and orchidectomised mice (n=11) receiving placebo and orchidectomised mice receiving testosterone (n=11) were compared for serum estradiol concentration at the end of the 17-week experimental period.

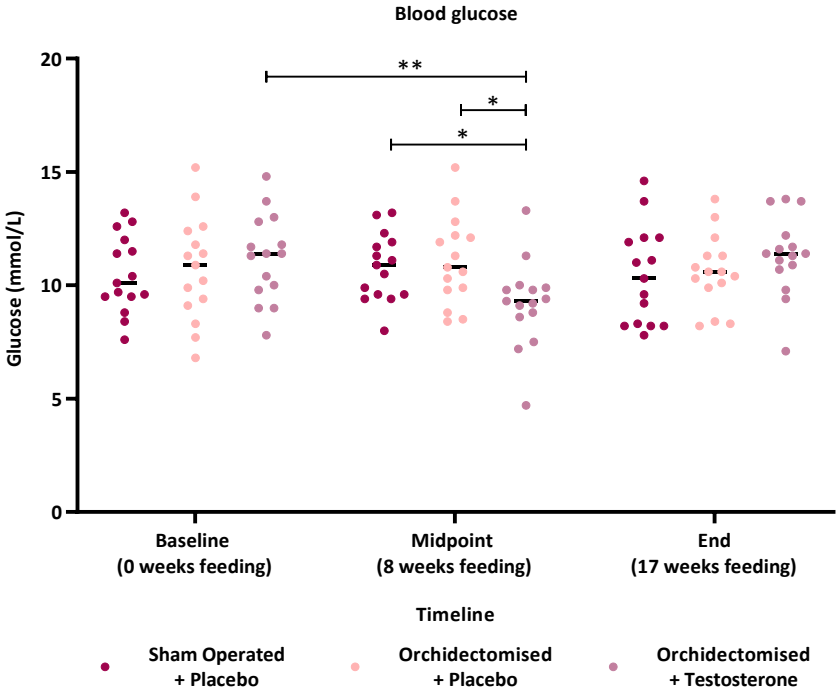


Figure 5.17 Blood glucose concentration of ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Sham-operated (n=15) and orchidectomised (n=15) receiving placebo and orchidectomised mice receiving testosterone (n=15) on a high fat 'Western' diet were compared for blood glucose levels at the beginning (week 0), midpoint (week 8) end (week 17) of the 17-week experimental period (*P≤0.05, **P<0.01), Kruskal-Wallis test and Friedman test.

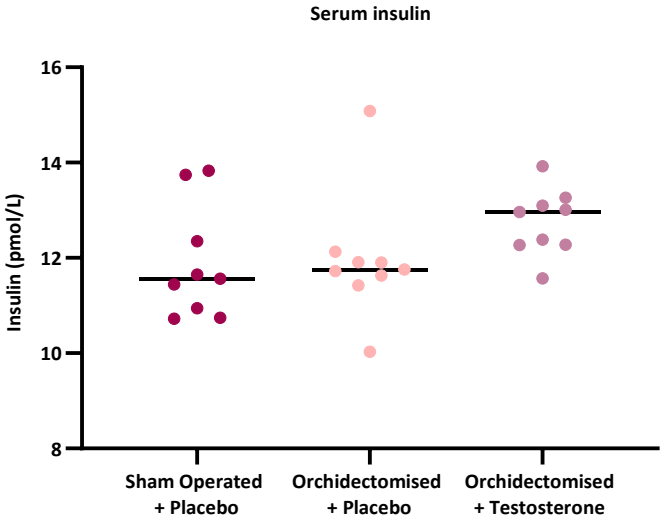


Figure 5.18 Serum insulin concentration of ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Sham-operated (n=9) and orchidectomised (n=9) receiving placebo and orchidectomised mice receiving testosterone (n=9) on a high fat 'Western' diet were compared for serum insulin levels at the end of the 17-week experimental period.

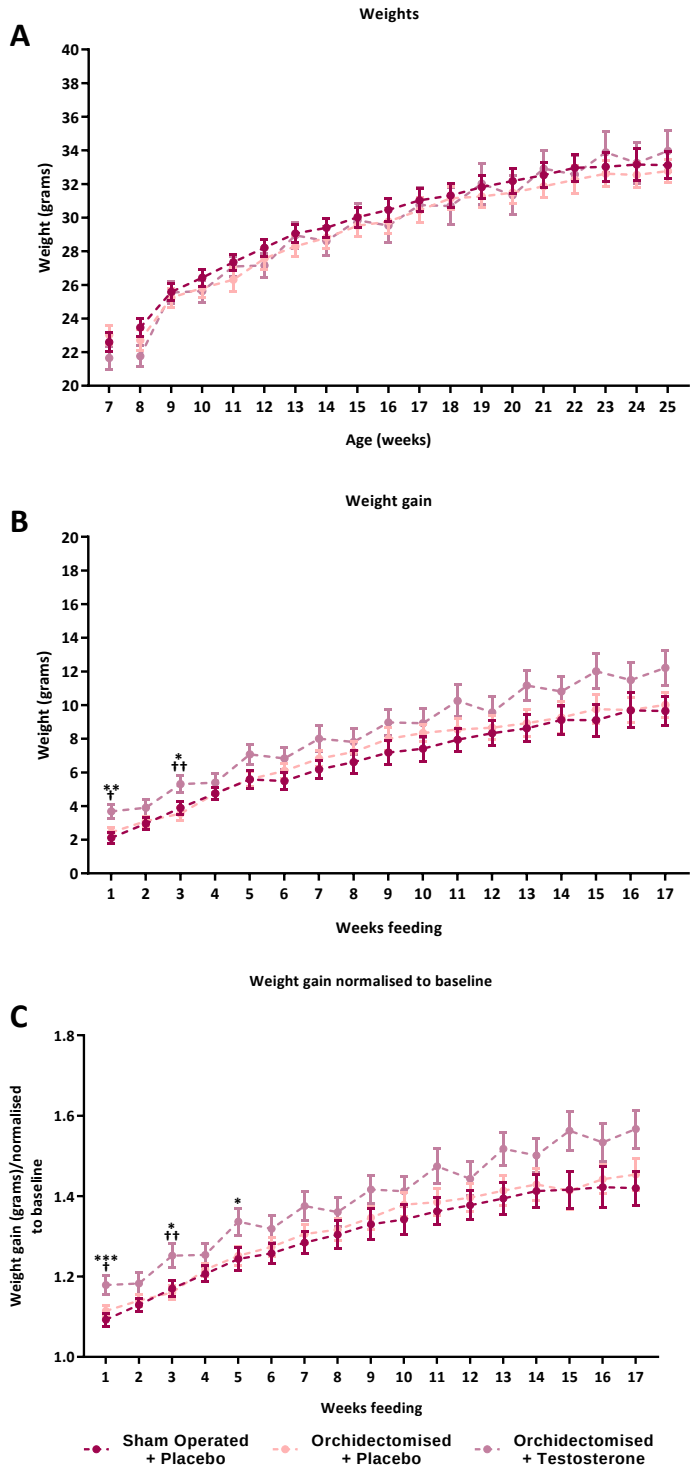


Figure 5.19 Total body weight and weight gain of saline-treated sham-operated, saline-treated orchidectomised and testosterone-treated orchidectomised male ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Weekly weight measurements were compared between sham-operated (n=15) and orchidectomised (n=15) receiving placebo and testosterone-treated orchidectomised (n=15) mice on a high fat 'Western' diet for the 17-week study period. (A) Mean weekly weights, (B) mean weight gains relative to week 1 (age eight weeks) start weights and (C) mean weight gains standardised to week 1 (age eight weeks) start weights. (*P≤0.05, **P<0.01, ***P<0.001 vs sham-operated (placebo), †P≤0.05, ††P<0.01 vs orchidectomised (placebo), Kruskal-Wallis test and Friedman test.

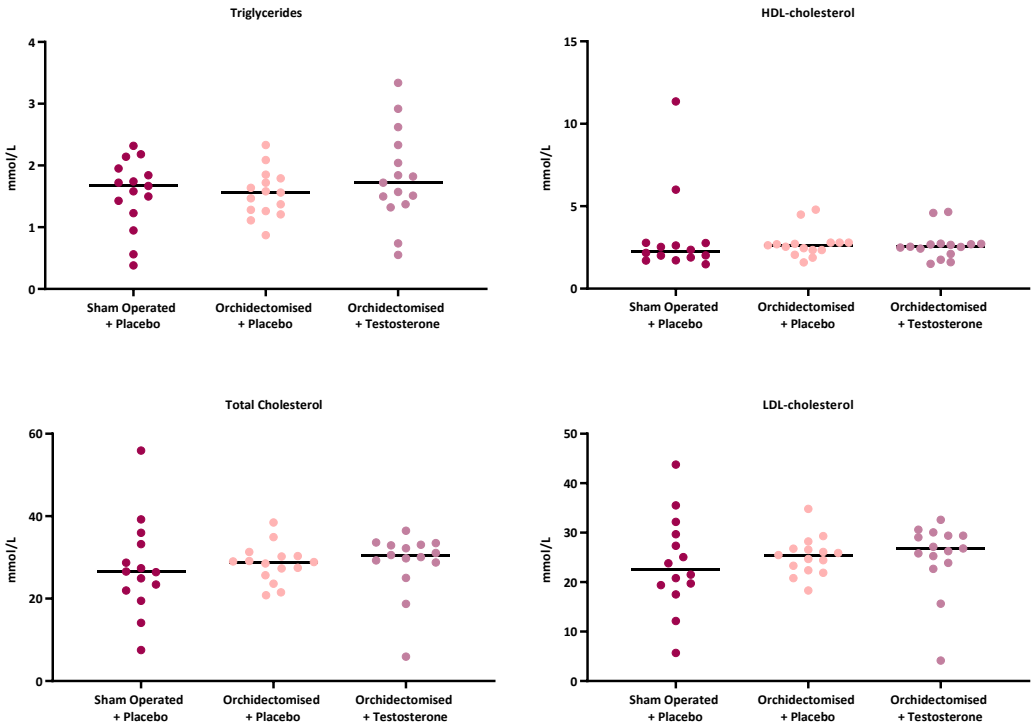


Figure 5.20 Serum lipid concentration of ApoE$^{-/-}$ mice fed a high fat 'Western' diet. Sham-operated (n=15) and orchidectomised (n=15) receiving placebo and testosterone-treated orchidectomised (n=15) mice on a high fat 'Western' diet were compared for serum lipid levels at the end of the 17-week experimental period.

5.5 Discussion

Epidemiological studies have consistently identified several risk factors for developing atherosclerosis including, but not limited to, elevated LDL-cholesterol and triglycerides, low HDL-cholesterol, poor diet and lack of exercise, abdominal obesity, insulin resistance and elevated HbA_{1c} irrespective of patient demographics (Lusis, 2000, Wang *et al.*, 2015). Low testosterone levels in men have also been indicated as an independent risk factor for the development of CVD, and TTh has shown improvements in cardiovascular mortality, CHD, and cardiovascular risk factors (Jones and Saad, 2009). The present study investigated the influence of testosterone on risk factors associated with the systemic cardiometabolic dysfunction known to contribute to the inflammatory pathogenesis of atherosclerosis in a specific population of hypogonadal men with T2D and an animal model of accelerated atherosclerosis. Furthermore, the development of an *in vitro* model to investigate the influence of diabetic conditions on macrophage-like cells was investigated.

5.5.1 The effect of testosterone on glycaemic control

Glycated haemoglobin (HbA_{1c}) measurement is universally used in patients with diabetes for the assessment of glycaemic control. Clinical studies have shown a high prevalence of testosterone deficiency in men with T2D and MetS (Saad *et al.*, 2011, Kapoor *et al.*, 2007a, Ding *et al.*, 2006), and that men with low serum testosterone concentrations have higher HbA_{1c} indicating poor glycaemic control (Derweesh *et al.*, 2007). Some interventional studies have shown significant improvement in HbA_{1c} after TTh compared to those receiving placebo; however, these are low in proportion to the number of studies that report no effect of TTh on HbA_{1c}. However, HbA_{1c} was not the primary outcome of these studies and many used a high proportion of participants that had good glycaemic control at the study outset (Kapoor *et al.*, 2006, Jones *et al.*, 2011, Heufelder *et al.*, 2009). TTh is unlikely to improve HbA_{1c} in patients with well-controlled T2D; therefore, the primary aim of the current clinical study was to investigate the effects of TTh as an 'add-on' treatment on HbA_{1c}, in hypogonadal men with poorly controlled T2D receiving diabetic medications.

The present study does not suggest that TTh may improve HbA_{1c} in hypogonadal men with T2D within six months. Conversely, Kapoor *et al.* (2007a) reported a significant reduction in HbA_{1c} following intramuscular TTh, and Boyanov *et al.* (2003) reported a reduction in HbA_{1c} after three months using oral testosterone treatment in men with poorly controlled T2D. However, the study conducted by Boyanov *et al.* (2003) was a non-blinded, non-placebo-controlled study and the changes observed in HbA_{1c} were greater than would be expected within three months using conventional diabetes medication. Two recent observational registry studies reported that long-term treatment with intramuscular testosterone undecanoate significantly decreased HbA_{1c} after the first year, and continued to decline over a period of eight years (Haider *et al.*, 2016). The patient cohort of these observation registry studies was similar to that of the current study; they consisted mostly of overweight men with T2D receiving standard treatment but with limited success according to their elevated baseline HbA_{1c}.

All patients in the current study were taking at least one standard medication to improve glycaemic control, but with limited success as baseline, HbA_{1c} was above the ideal range. Their standard diabetes treatment remained consistent for most patients throughout the study and is therefore unlikely to be a conflicting factor in HbA_{1c} results. The mechanism by which TTh improves glycaemic control is uncertain, studies have shown that insulin resistance is improved with TTh in men with T2D and MetS, although not significantly (Jones *et al.*, 2011, Kapoor *et al.*, 2006). Therefore, in this study, which is powered to assess glycaemic control, it would be valuable to determine the insulin resistance in patients to get a clearer picture of the effect of TTh on glycaemic control in hypogonadal men with T2D. There is considerable evidence linking abdominal obesity to insulin resistance and excess visceral fat results in the liver being exposed to higher amounts of free fatty acids leading to hepatic and eventually systemic insulin resistance (Kapoor *et al.*, 2005). An improvement in insulin resistance could be expected to result in better glycaemic control; however, no reduction in the primary endpoint, HbA_{1c} was demonstrated after six months of treatment. Furthermore, multivariant analysis did not reveal correlation between HbA_{1c} and any other parameters measured. Dhindsa *et al.* (2016) reported that TTh for

six months in hypogonadal men with T2D increases insulin sensitivity, increases lean mass, and decreases subcutaneous fat.

In the present study, ApoE$^{-/-}$ mice had considerably higher (up to 1.5 fold) baseline blood glucose levels than has been reported in wild-type XY C57 mice fed a standard chow diet (Amrani *et al.*, 1998, Andrikopoulos *et al.*, 2005). Although hyperglycaemia and T2D are acknowledged risk factors for atherosclerosis, it is unclear whether mice that are susceptible to atherosclerosis would have an increased tendency to develop T2D. Animal studies have indicated that ApoE$^{-/-}$ mice in a C57BL/6 strain, the atherosclerosis susceptible background strain used in the current study, have significant hyperglycaemia compared to atherosclerosis-resistant ApoE$^{-/-}$ in a BALB/c strain (Li *et al.*, 2011, Oppi *et al.*, 2019, Dansky Hayes *et al.*, 1999, Piedrahita *et al.*, 1992). The two ApoE$^{-/-}$ mouse strains have comparable serum lipids (Li *et al.*, 2011). This suggests that susceptibility to hyperglycaemia could increase susceptibility to atherosclerosis development, independent of serum lipid measurements. Li *et al.* (2011) induced hyperglycaemia in the ApoE$^{-/-}$ mice using a high fat 'Western' diet and observed an increase of fasting blood glucose of around two-fold compared to standard chow-fed mice. However, in the current study, there were no significant differences in blood glucose measurements at the same time points between the two diets. Furthermore, in contrast to the current investigation, (Li *et al.*, 2011) reported that a high fat 'Western' diet did not induce weight gain compared to the standard chow-fed group, indicating that hyperglycaemia in C57BL/6 ApoE$^{-/-}$ mice is diet-induced and not linked to increased weight. Whereas the data reported in this thesis suggest that hyperglycaemia was not diet-induced or linked to weight gain in this mouse model.

It was hypothesised that mice fed the high fat 'Western' diet would have higher blood glucose concentrations compared to age and surgery matched mice receiving the standard chow diet. It was also hypothesised that blood glucose levels would increase towards the end of the study with weight gain, which is associated with increased blood glucose and T2D, particularly abdominal obesity (Kelly and Jones, 2015). Although consistently hyperglycaemic throughout the experimental period, the blood glucose concentration did not increase with increased weight. Conversely, it decreased slightly at the end of the experimental period compared to baseline levels in

orchidectomised mice fed the standard chow diet. Towards the end of the experimental period, the orchidectomised mice stopped gaining and lost weight, perhaps causing the slight decrease in blood glucose concentration. Blood glucose concentrations often decrease in humans with T2D following weight loss (Haider *et al.*, 2016). Blood glucose at the end of the experimental period in mice fed the high fat 'Western' diet was mostly unchanged compared to baseline levels.

Animal studies have shown that mice with low testosterone have poorer glycaemic control than mice with normal testosterone levels (Dimakopoulou *et al.*, 2019, Fushimi *et al.*, 1989) and that testosterone replacement in mice can improve glycaemic control (Yabiku *et al.*, 2018, Pal *et al.*, 2019, Pal and Gupta, 2016). Pal and Gupta (2016) suggested that testosterone treatment improves glucose homeostasis despite increasing hepatic insulin resistance in male mice with T2D and further demonstrated that testosterone supplementation improves insulin responsiveness by potentiating insulin signalling in skeletal muscle in T2D male mice (Pal *et al.*, 2019). However, there are some methodological flaws with these studies; the animals used were wild-type C57BL/6 with diet-induced T2D. Whilst it has been shown that this background strain is susceptible to diet-induced T2D (Surwit *et al.*, 1988), the disease status was confirmed with a glucose tolerance test in comparison with standard-chow fed age-matched mice. However, whilst only at limited time points, the current study has shown that there was no difference between the blood glucose concentrations between standard diet and high-fat diet-fed mice suggesting that hyperglycaemia is not diet-induced in these mice. On the other hand, the animals in the current study did not have their glucose tolerance tested, so the difference between diets on glucose homeostasis is plausible. Perhaps the most serious methodological flaw in the aforementioned studies (Pal and Gupta, 2016, Pal *et al.*, 2019) is that testosterone supplementation was given to intact male mice and testosterone levels were not measured throughout or at the end of the experimental period. Supplementing intact male wild-type mice with testosterone could lead to supraphysiological serum concentrations, which in humans has been shown to have negative consequences such as causing sudden cardiovascular events including MI (Pirompol *et al.*, 2016). Thus, the positive effect the authors report on glucose homeostasis via insulin signalling in skeletal muscle could be a consequence of increased skeletal muscle mass associated

with supraphysiological testosterone supplementation rather than the effect of testosterone treatment on overall glycaemic control as suggested (Bhasin *et al.*, 1996).

Exogenous testosterone replacement in orchidectomised db/db mice partially restored disrupted standard glucose metabolism observed in orchidectomised mice and improved insulin resistance. Exacerbation of fatty liver was also alleviated in testosterone-treated mice compared to orchidectomised mice (Yabiku *et al.*, 2018). However, the effects of testosterone replacement on cardiovascular disease, specifically atherosclerosis in this model have not been investigated and would be an ideal model for future studies. The db/db mouse model is susceptible to obesity and insulin resistance leading to T2D due to leptin deficiency (Alpers and Hudkins, 2011). This model displays a more typical T2D profile and have hyperinsulinaemia at ten days of age, and significant hyperglycaemia is reported at eight weeks (Susztak *et al.*, 2004). The model is also on a C57BL/6 background strain, which is susceptible to atherosclerosis when fed a pro-atherogenic diet. To investigate the effects of testosterone replacement on glycaemic control, a mouse model of T2D would be a superior disease model.

Mice in the current study were hyperglycaemic and were hypothesised to be either insulin resistant or have disrupted insulin secretion. Li *et al.* (2011) demonstrated that ApoE$^{-/-}$ mice fed a pro-atherogenic diet are susceptible to diet-induced T2D and that this is due to defects in insulin secretion and not insulin resistance. Insulin secretion in response to glucose is usually biphasic, consisting of an initial transient peak of insulin release, followed by a prolonged second phase. Li *et al.* (2011) reported that the second phase was not observed, causing hyperglycaemia. This is supported by the findings of the current study, as serum insulin levels at the end of the experimental period were below the expected range indicating a possible dysfunction of insulin secretion in ApoE$^{-/-}$ mice (standard range 40-170pmol/L). Studies of isolated islet cells have observed that glucose metabolism and insulin release defects are associated with impairments in KATP channels and intracellular calcium flux in the C57BL/6J background mouse strain (Toye *et al.*, 2005). This aids in the explanation as to why mice in the current study had hyperglycaemia and hypoinsulinaemia irrespective of diet and body weight. Li *et al.* (2011) speculated that the pro-atherogenic diet-induced inflammation that exacerbated the insulin secretion

defect in mice in their study as a high-fat diet has been shown to induce an inflammatory state with infiltration of macrophages in local tissues in the db/db mouse model on the C57BL/6J background (Seitz *et al.*, 2010). Glycaemic control was not a primary or secondary outcome of the current study, and therefore, the study protocol did not incorporate experimental procedures to investigate glucose homeostasis. Given the conflicting reports on glucose homeostasis and insulin secretion in the ApoE$^{-/-}$ mouse model, it could have proved useful to investigate glucose homeostasis and measure beta cell function on isolated islets cells. Furthermore, histological analysis for macrophage infiltration on islets could also still be useful to investigate whether it was likely that the mice in the current study had islet inflammation that could enlighten as to whether it was likely they had islet β-cell functional failure. This could then potentially explain the hyperglycaemia in the current study and whether testosterone treatment, an acknowledged anti-inflammatory, has any effect. The effect of testosterone on body composition.

Most patients in the current study were classified as obese as defined by baseline BMI, body fat percentage and central adiposity, as defined by waist circumference. No significant changes in BMI, percentage body fat and waist circumference following six months of TTh were found in the current study. Studies have shown that free testosterone levels are low in obese men and negatively correlate with the degree of obesity (Kapoor *et al.*, 2005, Zumoff *et al.*, 1990). However, the current study found no correlation between total testosterone, free testosterone or bioavailable testosterone and percentage fat mass at baseline or following TTh.

Whilst many RCTs of TTh in hypogonadal men report a reduction in percentage fat mass, BMI and waist circumference, as well as an increase in lean body mass, independent of testosterone formulation used, this is normally only observed in longer-term studies (≥12 months) (Francomano *et al.*, 2014a, Hackett *et al.*, 2014b, Hackett *et al.*, 2014a, Saad *et al.*, 2013, Saad *et al.*, 2007). Longer-term studies of up to 60 months of TTh in hypogonadal men with T2D have shown a more positive effect on body composition with BMI, waist circumference and fat mass reduction and lean mass increase. Saad *et al.* (2013) reported a consistent reduction in BMI, body weight and waist circumference following TTh in hypogonadal men. These parameters were

statistically significant versus baseline and versus the previous year over a follow-up period of five years, indicating improvements were progressive over the full study period.

Shorter-term studies of a similar length to the current study often report no significant changes in body composition (Jones *et al.*, 2011, Kelly and Jones, 2015). Jones *et al.* (2011) observed no significant changes in the BMI, body fat percentage or waist circumference in hypogonadal males with T2D following six months of TTh but observed an improvement in fat mass and waist circumference after 12 months. Similarly, Heufelder *et al.* (2009) reported a reduction in waist circumference following 12 months of TTh but observed no changes in BMI and fat mass. However, Kapoor *et al.* (2007a) did observe a reduction in waist circumference and percentage body fat following only three months TTh in hypogonadal men with T2D but observed no changes in BMI. Differences in the results between the studies are unclear; however, the study by Kapoor *et al.* (2007a) had a much smaller population and broader age range as well as a different treatment course. The majority of other studies investigating TTh (six weeks – 70 months) in hypogonadal men also found a reduction in waist circumference, which correlates positively with visceral adiposity (Kelly and Jones, 2015). Although some studies have seen positive results in body composition following 3 -12 months of TTh, this may not be of sufficient length to detect significant changes of TTh as an add on therapy without lifestyle alterations of diet and exercise. Haider *et al.* (2016) observed a gradual, progressive, and sustained weight loss and reduction in waist circumference over the course of eight years of TTh, however, this was an observational registry study that did not have a control group to compare non-treatment. While all these studies report on body composition, they were not specifically designed to obtain evidence for evaluating the effect of TTh on body composition in hypogonadal men, at least as a primary endpoint. Therefore, information derived from randomised controlled trials designed for other purposes are fragmentary and often contradictory. The health benefits of TTh are time dependent and RWE studies provide valuable data about the true clinical significance of TTh, which cannot be derived from RCTs due to their short-term nature. The longest duration TTh RCTs are 3 years whereas there is RWE that provides evidence of the beneficial effects of TTh over the course of 12 years.

Many studies use BMI, body weight and waist circumference as predictors of obesity and related health risk. However, these parameters alone do not fully characterise obesity, and each has different predictive strength for associated chronic disease (Misra *et al.*, 2006, Eknoyan, 2008). Human body mass includes muscle mass and body water that can vary between populations; therefore, BMI and body weight does not accurately represent a measure of obesity. Waist circumference is commonly used as a proxy measure of abdominal fat that is often considered to correlate more with pathological consequences of obesity (Haslam and James, 2005) and is a strong predictor of CHD, T2D, metabolic syndrome, hypertension and dyslipidaemia (Lean *et al.*, 1995, Ross *et al.*, 2000, Janssen *et al.*, 2004, Vazquez *et al.*, 2007). The development of equipment to more accurately measure body composition is now more freely available. The current study utilised dual-energy X-ray absorptiometry (DEXA) for whole body composition and the Tanita body fat analyser to more accurately define individual phenotype according to body adipose tissue. However, despite the limitations of BMI and waist circumference, many studies still use these anthropometric measures as estimates of obesity, due to the more time consuming and expensive nature of the newer methods (Krachler *et al.*, 2013, Heymsfield *et al.*, 2009, Flegal *et al.*, 2008).

In the present study, sham-operated ApoE$^{-/-}$ mice were significantly and consistently heavier than orchidectomised mice fed a standard chow diet throughout the experimental period. This finding is surprising as low levels of endogenous testosterone have been linked to weight gain and obesity in men (Kelly and Jones, 2015). Towards the end of the experimental period, there were no significant differences between the two groups. Testosterone promotes myocyte and inhibits adipocyte development from pluripotent stem cells, thus increasing muscle mass, whereas testosterone deficiency enhances greater fat mass (Singh *et al.*, 2003). It may be proposed that the increased weight in sham-operated mice compared to orchidectomised mice could be due to increased muscle mass for which testosterone plays a pivotal role. Body fat and muscle was not measured in the present study but would be useful additional parameters to measure in future studies. Clinical trials have observed that TTh in hypogonadal men can cause a decrease in fat mass and an increase in lean body mass (Kelly and Jones, 2015).

In the current study, mice fed a high fat 'Western' diet receiving TTh gained significantly more weight for the first five weeks of the experimental period compared to placebo-treated sham-operated and orchidectomised mice, although they were not significantly heavier. Food intake and energy expenditure was not monitored in the present study but could be a useful additional parameter to monitor within future studies to evaluate dietary and exercise habits in relation to weight. This additional parameter, as well as metabolic cages, would be useful additional parameters for future investigations to investigate the effects of testosterone on not only body composition parameters but also measures of glucose metabolism, that could have an impact on atherosclerosis development.

5.5.2 The effect of testosterone on serum lipids

The cohort of hypogonadal men with T2D in the current study had total cholesterol that was within the healthy range, although LDL-cholesterol and triglycerides were above the healthy range and HDL-cholesterol below, which is indicative of atherogenic lipid profile (Haidar *et al.*, 2007, Wu and von Eckardstein, 2003). Studies have suggested that testosterone deficiency is associated with increased total cholesterol and LDL-cholesterol (Barrett-Connor, 1992, Barrett-Connor and Khaw, 1988, Haffner *et al.*, 1993, Simon *et al.*, 1997, Barud *et al.*, 2002), as well as decreased HDL-cholesterol (Simon *et al.*, 1997, Stanworth *et al.*, 2011, Van Pottelbergh *et al.*, 2003). However, a few cross-sectional studies have found no association between serum lipid measurements and endogenous testosterone (Kiel *et al.*, 1989, Denti *et al.*, 2000).

Studies have reported reductions in total cholesterol, triglycerides and LDL-cholesterol levels and increased HDL-cholesterol levels following TTh (Zitzmann *et al.*, 2005, Heufelder *et al.*, 2009, Saad *et al.*, 2007, Saad *et al.*, 2008, Tenover, 1992, Zgliczynski *et al.*, 1996, Tripathy *et al.*, 1998, Howell *et al.*, 2001, Ly *et al.*, 2001, Malkin *et al.*, 2004a, Malkin *et al.*, 2004b, Kapoor *et al.*, 2006, Jones *et al.*, 2011, Cornoldi *et al.*, 2010, Hackett *et al.*, 2014a, Mathur *et al.*, 2009). Although some studies report increased HDL-cholesterol following TTh, the majority report contraindicatory results with no difference (Zgliczynski *et al.*, 1996, Uyanik *et al.*, 1997, Boyanov *et al.*, 2003, Malkin *et al.*, 2004a, Kalinchenko *et al.*, 2010, Kapoor *et al.*, 2006, Kenny *et al.*, 2002,

Basaria *et al.*, 2015), or a decrease (Jones *et al.*, 2011, Thompson *et al.*, 1989). Differences in the results between the studies are unclear; it has been proposed that testosterone stimulation of reverse cholesterol transport may lead to increased consumption of HDL-C rather than testosterone having a negative effect on HDL-C (Wu and von Eckardstein, 2003).

The current study showed no significant differences in the lipid profiles of the TTh group compared to the placebo group following three and six months of treatment. A large proportion of men in both groups (78% of the placebo-treated group and 90% of the TTh group) were prescribed statins to control LDL-cholesterol and, therefore, TTh as an add on therapy may not have any influence of reducing LDL-cholesterol. Studies suggest that TTh reduces lipoprotein a, an LDL-like particle that possesses atherogenic and thrombotic properties and is an independent risk factor for atherosclerosis (Jones *et al.*, 2011, Zmunda *et al.*, 1996, Marcovina *et al.*, 1996, Ozata *et al.*, 1996). However, lipoprotein a was not measured in the current study.

Most data from studies investigating TTh in hypogonadal men show some improvements in lipid and lipoprotein profiles and meta-analysis of clinical trials in hypogonadal men report that significant reductions in total cholesterol and LDL-cholesterol are associated with intramuscular TTh (Isidori *et al.*, 2005, Jones and Saad, 2009, Jones and Kelly, 2018, Whitsel *et al.*, 2001). The discrepancies in many of these studies are attributed to differences in patient age, disease status, testosterone preparation and route of administration, dose and duration of treatment as well as many studies not considering the various changes in different lipoprotein sub-fractions (Jones and Kelly, 2018).

Studies have reported that low endogenous serum testosterone is associated with increased serum levels of total cholesterol and LDL-cholesterol in mice (Hatch *et al.*, 2012, Senmaru *et al.*, 2013, Kelly *et al.*, 2012) and that $ApoE^{-/-}$ mice demonstrate a pro-atherogenic serum lipid profile which is exasperated on a high-fat diet (Plump *et al.*, 1992).

$ApoE^{-/-}$ mice fed a standard chow diet had elevated serum lipid profiles compared to wild-type mice (comparative data not shown) although there were no

significant differences in total cholesterol, triglycerides and LDL-cholesterol between the sham-operated and orchidectomised. This contrasts with other studies that have shown this difference; however, these studies were not performed using $ApoE^{-/-}$ mice (Kelly *et al.*, 2012). There was, however, a significant decrease in HDL-cholesterol in the orchidectomised mice compared to the sham-operated mice. This concurs with studies that have reported that low endogenous testosterone is associated with decreased HDL-cholesterol in humans (Jones, 2010b).

Sham-operated and orchidectomised mice displayed elevated total cholesterol and LDL-cholesterol after a high fat 'Western' diet compared to the standard chow diet, and there were no significant differences in the lipid parameters between the two groups. Testosterone treatment in orchidectomised mice had no further modulatory effects on any of the lipid sub-fractions measured. The lack of effect on LDL-cholesterol and triglyceride fractions agrees with the study by Nettleship *et al.* (2007a). Nettleship *et al.* (2007a) reported that physiological testosterone treatment did not reduce non-HDL-cholesterol in the Tfm mouse; however, they did report an improvement in the serum concentrations of HDL-cholesterol in testosterone-treated Tfm mice compared to placebo-treated controls. The current study did not show any effect of testosterone treatment on the serum concentrations of HDL-cholesterol.

Although serum LDL-cholesterol levels are an important biomarker of CVD, subclasses of LDL-cholesterol may provide greater insight into cardiovascular risk. LDL-cholesterols are heterogeneous particles that consist of several subclasses that differ in size, density, physicochemical composition, metabolic and oxidative behaviour (Griffin *et al.*, 1990). Small dense LDL-cholesterol (sdLDL) is a distinct LDL-cholesterol subclass, which is associated with raised TG and decreased HDL-cholesterol levels in obese and T2D patients (Rizzo *et al.*, 2009, Sniderman *et al.*, 2018, Tsai *et al.*, 2018, Dekker *et al.*, 2005). sdLDL has a distinct metabolic role in atherosclerosis and is common in the serum of patients with atherosclerosis and is susceptible to oxidation, decreased receptor-mediated uptake and increased endothelial infiltration thus increasing their atherogenicity (Orekhov *et al.*, 1991, Steinberg *et al.*, 1989).

It is conceivable that testosterone treatment in the current study may reduce the atherogenicity of the lipids by altering the subclass, whilst not affecting the overall

concentration of serum LDL-cholesterol and total cholesterol. This is supported by testosterone treatment reducing plaque area and lipid deposition in the aortic root of orchidectomised ApoE$^{-/-}$ mice compared to orchidectomised mice receiving placebo, observed in the present study (Chapter 3). Furthermore, elevated sdLDL concentrations are associated with the presence of MetS and its components in men (Fan *et al.*, 2019), conditions that often co-exist with low testosterone levels and hypogonadism, further highlighting the possible benefit of testosterone on lipoprotein subclass.

5.5.3 The effect of high glucose on a human monocytic cell line

Although chronic inflammation in individuals with obesity and T2D occurs principally at metabolic sites such as liver, muscle and adipose tissue (Hotamisligil, 2003), mononuclear cells in a pro-inflammatory state have been found in obese and in severe hyperglycaemic T2D patients (Ghanim *et al.*, 2004, Cipolletta *et al.*, 2005).

Giulietti *et al.* (2007) reported that monocytes present in the peripheral blood of patients with T2D have a pro-inflammatory profile defined by elevated expression of pro-inflammatory cytokines and adhesion molecules compared to controls and patients with T1D. This pro-inflammatory profile confirms previous studies where patients with T2D presented higher plasma levels of pro-inflammatory cytokines (Spranger *et al.*, 2003, Moriwaki *et al.*, 2003, Pickup *et al.*, 1997, Zozuliñska *et al.*, 1999) and adhesion molecules (Ceriello *et al.*, 1996, Kado and Nagata, 1999). The pro-inflammatory status of circulating monocytes in patients with T2D could be a crucial factor of atherosclerotic plaque development when monocytes are recruited to the endothelial lesion.

The initial aim of the *in vitro* study performed here was to establish an inducible pro-inflammatory cell culture environment using high glucose as a model of diabetic human monocytes, whereby expression of pro- and anti-inflammatory targets could be reliably measured. However, the present study was unsuccessful in establishing a cell culture model of pro-inflammatory monocytes induced by high glucose. This study found that 15mmol/L did not induce an increase in IL-1β, IL-6 and TNFα and ICAM-1 mRNA expression after 48 hours. This is in contrast to previous

research which has demonstrated that glucose at this concentration does significantly increase mRNA expression of IL-1β after 48 hours in THP-1 cells (Dasu *et al.*, 2007). In fact, TNFα mRNA expression was significantly reduced after 12, 48 and 72 hours and ICAM-1 were reduced after 48 and 72 hours, although this was not significant. This study also found that even 25mmol/L glucose did not induce increased mRNA expression of IL-1β, IL-6 and TNFα and ICAM-1 compared to 5.5mmol/L glucose after 48 hours. Again, this is in contrast to Dasu *et al.* (2007) findings, where 25mmol/L glucose did significantly increase mRNA expression of IL-1β. This change was determined to be glucose specific as the mannitol osmolality control had no effect on IL-1β mRNA expression.

Although widely accepted as a model of human monocytes, it is important to highlight the limitations of the THP-1 cell line as a model for primary monocytes. THP-1 cells are an immortalised cancerous cell line and have been shown to not respond to stimuli in the same way as their physiological counterparts, i.e., human peripheral blood monocytes, which they are thought to represent (Bosshart and Heinzelmann, 2016). Cancer cells lines have altered metabolism, and THP-1 cells are more metabolically active than primary cells and require high glucose culture medium as standard (11mmol/L). Supplementing them with excess glucose may only stimulate the cells further and reducing glucose concentrations to those seen in humans (5.5mmol/L) may induce starvation effects (Ma *et al.*, 2019).

The initial aim of this *in vitro* study was to establish a model of diabetic human monocytes to investigate the effects of testosterone on the inflammatory profile *in vitro*. However, the THP-1 model was deemed inappropriate for this investigation, and future studies should use primary human peripheral blood monocytes obtained from healthy volunteers to determine whether using primary cells are more appropriate.

5.6 Conclusions

The present clinical study has supported previous findings that TTh does not improve HbA_{1c}, body composition or dyslipidaemia in the short-term. The majority of clinical studies have reported improvements in these parameters with long-term TTh. Randomised placebo-controlled clinical trials are the gold standard for assessing the

efficacy and safety of a given therapy, however, a long-term study of TTh (>eight years), ethical issues of not treating hypogonadal men who presented at the clinic would be raised. Therefore, research must utilise all available data irrespective of the nature of the study and primary endpoints to extract all relevant information on TTh to advance our knowledge of the benefits and risks of TTh while recognising methodological limitations. Furthermore, we did not observe any improvements in blood glucose, weight, and dyslipidaemia in the $ApoE^{-/-}$ mice receiving testosterone treatment compared to control mice. To gain a better understanding of the effect of testosterone treatment in these mice, body composition analysis would be useful to determine ratios of fat and muscle mass as anecdotally we did observe larger fat deposits viscerally, subcutaneously and hepatically in low testosterone animals. Similarly, more sophisticated glucose and insulin homeostasis tests would be greatly beneficial.

TTh has been shown to have beneficial effects on measures of obesity that are partially explained by both direct metabolic actions on adipose and muscle. The degree of these beneficial effects may be dependent on the treatment modality with longer-term administration often achieving greater improvements. Therefore, the relatively short-term nature of both the clinical and animal studies made preclude the detection of significant improvements in the parameters investigated, and long-term analysis is advocated.

6. General Discussion

6.1 General discussion

The main aim of this study was to investigate the potential anti-inflammatory actions of testosterone in atherosclerosis as a mechanism to explain the benefit of TTh on CVD shown clinically in men with low testosterone. The study design was three-pronged and used both *in vitro* and *in vivo* approaches. An *in vivo* mouse model of atherogenesis was used to investigate the effects of testosterone on atheroma composition, complexity, stability, and localised plaque inflammation. More specifically, this model was used to determine whether the known beneficial effects of testosterone on lesion formation in mice fed a pro-atherogenic diet were, at least in part, due to anti-inflammatory actions within the plaque. Secondly, a double-blinded randomised placebo-controlled trial of TTh in patients with poorly controlled type 2 diabetes and hypogonadism with the primarily outcome of glycaemic control (HbA_{1c}) was used to investigate atherosclerosis risk factors including systemic and isolated monocyte/macrophage inflammation as key players in the atherosclerotic process. Complimenting these investigations, *in vitro* cell culture models of macrophage inflammation were utilised, to specifically determine the effect of testosterone on key molecular regulators and factors produced by these prominent cells in the athero-inflammatory process.

Low serum levels of testosterone in elderly men are associated with aortic and carotid atherosclerosis (Hak *et al.*, 2002, van den Beld *et al.*, 2003). Testosterone levels have been shown in clinical studies to be inversely associated with the degree of atherosclerosis in the carotid artery (Demirbag *et al.*, 2005, Fukui *et al.*, 2003, Muller *et al.*, 2004, Svartberg *et al.*, 2006). Physiological testosterone levels in the higher quartile of the range are associated with an improved CIMT and lower occurrence of carotid lesions (Chan *et al.*, 2015). Furthermore, TTh has been reported to improve CIMT and lesion composition. However, these studies do not confer mechanisms by which testosterone improves markers of atherosclerosis. Animal studies have become invaluable in atherosclerosis research in investigating the effects of testosterone on lesion composition and stability may add mechanistic detail and clinical relevance to TTh in men. Testosterone treatment in animal models has shown cardio-protective effects by reducing aortic lipid accumulation and reduced atheroma formation as well as anti-inflammatory properties (Bruck *et al.*, 1997, Alexandersen *et al.*, 1999,

Nettleship *et al.*, 2007a, Kelly *et al.*, 2012, Larsen *et al.*, 1993, Qiu *et al.*, 2010, Bourghardt *et al.*, 2010). These animal studies show beneficial effects of testosterone supplementation on atherogenesis in male animals (Nettleship *et al.*, 2007a, Nathan *et al.*, 2001, Bruck *et al.*, 1997, Alexandersen *et al.*, 1999).

The findings reported in this thesis further support the hypothesis that testosterone modulates lipid accumulation within the aortic root. As there was no effect of testosterone on circulating lipids, that this may be mediated locally with testosterone protecting against the detrimental effects of pro-atherogenic lipids on lesion-associated immune cells. Indeed, the present study shows for the first time, that aortic root lesion monocyte/macrophage infiltration was reduced in testosterone-treated $ApoE^{-/-}$ mice. Lesion macrophages ingest lipoproteins that accumulate giving rise to foam cells. Accumulation of foam cells contributes to lipid storage lesion progression. Therefore, testosterone may protect again atherosclerosis by reducing monocyte/macrophage infiltration, thus reducing lesion lipid accumulation. However, macrophage numbers within the lesion can result from infiltration or proliferation. Therefore, without measurement of circulating leukocyte populations by flow cytometry, or markers of proliferation then it is not possible to delineate the two for certain and changes in infiltration from testosterone treatment should not be assumed. Furthermore, lesion macrophages have a decreased ability to migrate, which leads to failure of inflammation resolution and to further progression of the lesion into a complicated and vulnerable atherosclerotic lesion that may be prone to rupture causing a MACE.

In vitro, testosterone treatment has been shown to reduce foam cell formation and enhance reverse cholesterol transport, often considered a protective mechanism in early atherogenesis, in cultured monocyte-derived macrophages (Langer *et al.*, 2002, Qiu *et al.*, 2010, Kilby and Jones, 2013). However, the data presented in this thesis did not observe a testosterone effect on fatty acid uptake in monocyte-derived macrophages *in vitro*. Testosterone may modulate lipid accumulation within the developing atheroma by enhancing reverse cholesterol transport in macrophages.

Vulnerable lesions that may result in rupture and MACE are characterised by an enhanced content of macrophages, lipids and MMPs. Reduced collagen, smooth

muscle cell content and fibrous cap thickness are also characteristics of an unstable lesion. Whilst the findings of this thesis demonstrate that physiologic concentrations of testosterone impede lipid and macrophage content within the lesion, it had no effect on lesion stability as collagen and smooth muscle content were unaffected, nor did treatment enhance fibrous cap thickness. Furthermore, MMP expression within the lesion was not affected by testosterone treatment, with the exception of MMP13 gene expression, although this did not translate to protein expression. This suggests that that testosterone may not affect lesion stability by modulating collagen, smooth muscle or MMP content in ApoE$^{-/-}$ mice but rather reduce local lipid content and immune cell infiltration in the developing atheroma. Analysis of plaque components in humans found that TTh significantly increased the fibrous plaque volume compared to placebo indicating a stable lesion. Post-mortem studies have revealed that atherosclerotic lesions associated with coronary thrombosis, sudden death, and rupture typically have less fibrous tissue (Davies *et al.*, 1993b, Davies and Thomas, 1984, Burke *et al.*, 1997, Cheruvu *et al.*, 2007). Lesion and necrotic core size were also unaffected by testosterone treatment in the present study which is in contrast to Bourghardt *et al.* (2010) who did report significant improvements in lesion area and necrotic core area following testosterone treatment in ApoE$^{-/-}$ mice. The current study was powered to assess significant changes in aortic root lipid deposition. It is plausible that increased numbers in these parameters would reveal significant differences between the groups.

The reduction in monocyte/macrophage lesion infiltration could be in part due to the reduction in adhesion molecule expression, particularly ICAM-1. Circulating levels of ICAM-1 have been shown to be associated with the severity of atherosclerosis in ApoE$^{-/-}$ mice, and also that inhibition of ICAM-1 may delay the progression of atherosclerosis (Nakashima *et al.*, 1998, Kitagawa *et al.*, 2002). Whilst not significant, the present study suggests for the first time that circulating levels and local expression within the atheromatous lesion of ICAM-1 were positively modulated by testosterone in ApoE$^{-/-}$ mice. Increased levels of circulating ICAM-1 are implicated in lesion progression and its modulation by testosterone both systemic and locally may be a factor contributing to reduced monocyte/macrophage infiltration and therefore lipid content in the atheromatous lesion. Moreover, ICAM-1 and adhesion molecules were

not modulated by testosterone in monocytes isolated from hypogonadal men with T2D, cultured monocytes or monocyte-derived macrophages. This suggests that the beneficial effects of testosterone may not be seen in isolated circulating cells *ex vivo* or cultured cells *in vitro*. However, the novel molecular analysis performed in this thesis of isolated lesion-specific cells and endothelial cells from the aortic roots showed no significant changes in cytokine, chemokine or adhesion molecule gene expression following testosterone treatment.

Low serum testosterone levels are associated with several cardiovascular risk factors such as visceral obesity, hyperglycaemia, insulin resistance, dyslipidaemia and hypertension (Jones, 2010b). Although the relationship between testosterone and CVD has shown conflicting results, the majority of studies investigating TTh in patients with CVD risk factors suggest that testosterone is beneficial. These studies suggest that TTh may positively influence several of the risk factors that shape the pathophysiology of atherosclerosis (Malkin *et al.*, 2004b, Pugh *et al.*, 2004, Jones *et al.*, 2011, Malkin *et al.*, 2006, Zitzmann, 2008, Webb *et al.*, 1999, English *et al.*, 2000b). It is well established that low testosterone levels are associated with risk factors of atherosclerosis including dyslipidaemia, abdominal obesity and insulin resistance, with insulin resistance the key central biochemical defect in development of T2D and atherosclerosis (Jones, 2010a). It is plausible that testosterone may have positive effects on atherosclerosis by improving risk factors of atherosclerosis. However, both previous animal and clinical studies have reported mixed results of the effects of testosterone on circulating lipids, body composition and glycaemic control. The present six-month clinical study found that TTh had no effect on body composition (BMI, WC and fat mass), and this pattern was also observed in ApoE$^{-/-}$ mice receiving testosterone treatment. However, body composition as such was not measured in the animal study, and this pattern is based purely on weight and weight gain, not the percentage of body fat and lean muscle. This is supported by previous data from hypogonadal men with and without T2D where no change was observed in body composition following short-term TTh (Bokhamada, 2014, Jones *et al.*, 2011, Lee *et al.*, 2005). Longer-term studies of TTh, however, have reported positive effects of TTh on serum lipids, body composition and glycaemic control of up to 12 years. The key difference in these studies is the duration of treatment and follow-up. Observational

studies have the capability of following men on TTh for a more extended period compared to RCTs which tend to be considerably shorter (<two years) (Haider *et al.*, 2016, Saad *et al.*, 2013, Saad *et al.*, 2016, Pietri *et al.*, 2015, Nedogoda *et al.*, 2015, Francomano *et al.*, 2014b, Aversa *et al.*, 2012). Observational studies may be more appropriate for assessing the effectiveness of long term TTh than an RCT, as ethical issues of not treating hypogonadal men be raised. TTh has been shown to reduce body fat mass and increase lean mass in men treated for hypogonadism significantly and progressively over a treatment period of at least two years (Haider *et al.*, 2016, Saad *et al.*, 2013, Saad *et al.*, 2016, Traish *et al.*, 2014, Traish *et al.*, 2017). In the present study, six months of TTh was determined to be of insufficient treatment length to observe consequential metabolic improvements.

TTh has been shown to improve insulin resistance in hypogonadal men with and without T2D (Kapoor *et al.*, 2007a, Kapoor *et al.*, 2006, Jones *et al.*, 2011, Heufelder *et al.*, 2009, Dhindsa *et al.*, 2016). Additionally, glycaemic control (HbA_{1c} and fasting glucose) in hypogonadal men with T2D improves significantly with TTh (Kapoor *et al.*, 2007a, Kapoor *et al.*, 2006, Jones *et al.*, 2011, Heufelder *et al.*, 2009). The present study was powered to assess changes in HbA_{1c} as its primary endpoint in hypogonadal men with poorly controlled T2D, however, no changes were observed, and glycaemic control was not improved with TTh. Similarly, blood glucose and insulin levels were not improved following testosterone treatment in the animal study. Again, in humans, this is likely a result of the short treatment duration. Improvements in glycaemic control in previous studies is often associated with improvements of other cardiovascular risk factors such as weight loss and insulin resistance and is progressive and continuous over several years. Studies that have observed this are observational studies that do not have a control group but have been able to follow men over time up to 12 years (Haider *et al.*, 2016, Saad *et al.*, 2016, Traish *et al.*, 2014, Traish *et al.*, 2017). Although no changes were observed in the present study, these are clearly important findings as long-term TTh has been shown to be beneficial. The present study suggests that six months is not a long enough therapeutic window to observe improvements in HbA_{1c} and indeed glycaemic control in hypogonadal men with poorly controlled T2D. Indeed, a recent prospective observational clinical study (first year double-blind, randomised, placebo-controlled trial; second-year open-label follow-up)

observed improvements in glycaemic control, body composition and serum lipids in hypogonadal obese men with T2D following two years TTh (Groti Antonič *et al.*, 2020). The authors concluded that TTh for at least two years is needed to achieve significant improvements in body composition, glycaemic control and serum lipids and at least in part explains why a lack of change was observed in the current study and the benefit of TTh in the current study may be observed with continuous treatment and follow up. Studies have shown that improvement in metabolic parameters is associated with reduced cardiovascular events and surrogate markers of atherosclerosis (Haider *et al.*, 2016, Heufelder *et al.*, 2009, Kalinchenko *et al.*, 2010, Saad *et al.*, 2016, Traish *et al.*, 2017, Yassin *et al.*, 2016).

Together, the data presented in this thesis have contributed novel findings and increased knowledge in the field of the effects TTh of atherosclerosis and cardiovascular risk factors. Results have highlighted that testosterone treatment in mice reduces monocyte/macrophage infiltration into the vessel wall, thus reducing lipid deposition. This may be due to the reduction in systemic and localised expression of ICAM-1, which is associated with the progression and extent of atherosclerosis. Novel analysis of lesion-specific tissue and the endothelial layer did not reveal any significant differences between treatment groups, nor did analysis of lesion stability. This could be due to insufficient numbers and further analysis may highlight the possible benefits of testosterone treatment on lesion stability. However, it must be noted that the process of atherosclerosis in $ApoE^{-/-}$ is genetically altered to be sped up on a pro-atherogenic diet, therefore the process differs significantly than that in humans that develops over decades. The morphology of atherosclerotic lesions observed in mice is different from that of humans, most notably in that the thick fibrous cap is absent (Rosenfeld *et al.*, 2008). The predilection sites for atherosclerosis development in mouse models are the aortic sinus and innominate artery, whereas the coronary arteries are commonly affected in humans. Unstable lesions are also rarely observed in mouse models, and it is therefore difficult to examine lesion rupture and thrombosis, which is the direct cause of acute ischemic events responsible for cardiovascular deaths, using these systems (Williams *et al.*, 2002, Johnson *et al.*, 2005, Zhou *et al.*, 2001).

Furthermore, the clinical study revealed that monocyte expression of TNFα was significantly reduced over time in monocytes from patients treated with testosterone and significantly reduced compared to placebo-treated patient monocytes after six months. However, other targets were not significantly altered over time or between treatment groups. These findings importantly indicate for the first time that testosterone influences monocyte inflammatory activation in men with T2D by altering expression a potent pro-inflammatory cytokine TNFα, as a potential mechanism to protect against atherosclerotic plaque development in hypogonadism. Additionally, this PhD project has raised new avenues of research for future study, which have the potential to unravel the role of testosterone treatment in the fundamental cellular processes of atherosclerosis.

6.2 Future directions

Conflicting reports regarding testosterone as anti-inflammatory still remain, although accumulating evidence strongly supports a role for testosterone as a cardioprotective hormone in men and warrants further scientific and clinical research. It is important that future research focuses on defining the physiological, biochemical and molecular mechanisms underlying the atheroprotective role of testosterone.

6.2.1 Clinical studies

The present study would benefit from having the serum cytokines, chemokines and adhesion molecules, as well as CIMT, analysed to see if there is a correlation between these parameters and testosterone levels as suggested in the literature. These analyses were originally designed into the study before COVID-19 restrictions prevented them being undertaken. It is unlikely however, given the treatment duration, that TTh would have affected these parameters as the literature indicates that longer treatment duration is required to fully elucidate the clinical benefits of TTh. The extent of atherosclerotic disease has been shown to correlates with systemic inflammation and it would be interesting to investigate whether this is the case for patients included in this study and if this correlates with testosterone levels. TTh has shown cardioprotective effects in RCTs that have had a more extended follow-up period than six months. A longer study duration powered to assess changes in

measures of atherosclerosis and risk factors is required. MRI would allow for the characterisation of plaque composition, i.e., the discrimination of lipid core, fibrosis, calcification, and intraplaque haemorrhage deposits. Furthermore, advancing MRI techniques capable of imaging biological processes, including inflammation, neovascularisation, and mechanical forces, that may aid in advancing the understanding of the disease and allowing accurate non-invasive assessment of atherosclerosis. Thus, MRI opens new strategies ranging from the screening of high-risk patients for early detection and treatment as well as monitoring of the target lesions for pharmacological intervention. Identification of subclinical atherosclerosis and early treatment initiation has the potential to surpass conventional risk factor assessment and management in terms of the overall impact on cardiovascular morbidity and mortality.

6.2.2 Animal Studies

The current study was powered to assess significant changes in aortic root lipid deposition, and therefore the data collected in this thesis could be used to calculate numbers required to assess significant differences between groups in localised inflammation and parameters of atherosclerosis such as lesion stability that did not reveal significant differences. Tissue staining for markers of apoptosis in the necrotic core to determine if this is apoptosis or cell necrosis would give a clearer indication of what cellular process are occurring in the lesion and how this may contribute to lesion vulnerability or plaque progression/regression. There are still however, fundamental flaws with using animal models to investigate treatments for human atherosclerosis. Whilst studies in mouse models particularly have without doubt contributed significantly to our understanding of the mechanisms of atherogenesis, the extent to which the mouse serves as an accurate model of the human disease remains open to discussion. Mouse models are not typically prone to lesion rupture and do not demonstrate the unstable atherosclerotic lesion with overlying thrombosis, that is associated with clinically significant acute cardiovascular episodes. Murine lesions also do not characteristically develop the thick fibrous cap observed in human atherosclerosis from the chronic progression over decades, compared to accelerated development from genetic manipulation and pro atherogenic diets in mouse models. Locations of lesion development also differ between species. Unlike humans, mice

rarely develop atherosclerosis in the coronary arteries, but instead develop atherosclerosis in the aortic root. The much more rapid heart rate of the mouse and hence disturbed blood flow likely accounts for the atherosclerosis development at this site. With this in mind, when investigating the effect of testosterone one atherosclerosis the features that humans and mice share such as leukocyte infiltration and macrophage behaviour within the lesion may still give insight into how this therapy may benefit human atherosclerosis. Macrophage numbers within the lesion can result from infiltration or proliferation, and measurements of proliferation markers such as Ki67 would reveal whether the lesion macrophages are the result of infiltrated leukocytes or are actively proliferating within the lesion. For future murine studies, flow cytometric investigation of circulating leukocyte numbers would also aid in discriminating between proliferating and infiltrating macrophages within the atherosclerotic lesions.

6.2.3 *In vitro* studies

The *in vitro* cell culture studies in this thesis were designed to simulate the macrophage inflammation that would be associated with atherosclerosis via cytokine stimulation of cells, with the subsequent investigation of the potential influences of testosterone on this. The effect of testosterone on monocytes and macrophages lesion infiltration and inflammation may be clearer if the cells are co-cultured in conditions more representative of the *in vivo* environment such as in a collagen matrix with relevant cell types. Indeed, this thesis does report a role for testosterone in the reduction of aortic root lipid deposition and monocyte/macrophage infiltration in vivo. Thus, the in vitro actions of testosterone on macrophage lipid handling are of interest. Studying the effects of native LDL and oxLDL on inflammation in primary macrophage cells, and the potential protective effects of testosterone would offer a more representative model of atherogenesis. Inducing conditions *in vivo*, including the formation of foam cells and secretion of pro-inflammatory cytokines, may highlight a link between the beneficial effects of testosterone on lipids and inflammation within the local aortic environment preceding, during and late stages of the pathogenesis of atherosclerosis. Furthermore, co-culture studies in a collagen matrix could investigate in detail the effect on vulnerability measured by MMP expression and matrix and collagen degradation/synthesis. However, possibly the most critical factor for future *in*

vitro studies is the origin of the cells utilised. The usefulness of the cancerous THP-1 cell line for investigating macrophage mechanisms remains open to discussion. THP-1 cells have altered mechanisms giving them characteristics not consistent with primary monocytes and macrophages and therefore could hide any important findings that would be revealed using primary cells. Future work would aim to build and expand on the work presented in this thesis therefore improving the validity of the findings and therefore should utilise primary monocytes, and macrophages or differentiated hematopoietic stem cells to investigate the effects of testosterone on atherosclerotic mechanisms.

6.3 Conclusion

Although complicated by many conflicting reports, the majority of the evidence investigating hormone intervention in men with hypogonadism suggests that long-term TTh may be beneficial on cardiovascular outcomes, exerting positive effects on several of the contributory factors that shape the pathophysiology of atherosclerosis. This thesis has investigated potential cardiovascular benefits of testosterone with a focus on anti-inflammatory mechanisms within the atherosclerotic plaque. Evidence presented in this thesis demonstrates that physiological testosterone treatment in $ApoE^{-/-}$ mice reduces lipid deposition and monocyte/macrophage infiltration into the vessel wall and that this may be, in part, due to anti-inflammatory actions on key mediators of atherosclerosis; TNFα and ICAM-1 both locally and systemically. Whilst no significant changes in lesion stability were observed, there are some indications that testosterone may reduce lesion smooth muscle content but increase fibrous cap thickness that indicates that testosterone may improve the overall vulnerability of the lesion and reduce MACE events in men. There were no significant findings from the clinical trial; however, it has confirmed, along with other recent trials, that a longer treatment period and follow-up may be required to observe beneficial effects of testosterone on cardiovascular risk factors particularly in men with long standing comorbidities such as T2D and obesity. Due to the complexity of the pathogenesis of atherosclerosis involving multiple cells and intrinsic and extrinsic factors, more studies needed to uncover the anti-inflammatory effects of testosterone in the atherosclerotic process.

7. References

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