The effect of testosterone on factors associated with diabetes, atherosclerosis and obesity

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Dedication

This thesis is dedicated the soul of my baby

Yusef

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Abstract

Obesity has recently become a major global health problem. Epidemiologic studies indicate that obesity is an important risk factor for type 2 diabetes (T2DM), atherosclerosis and low testosterone in men. Importantly, testosterone replacement treatment (TRT) can improve the condition of these diseases. According to human and animal studies testosterone can act as an anti-inflammatory and anti- atherogenic factor leading to inhibition of the risk factors and consequence of T2DM and atherosclerosis. Consequences of these diseases include dysregulation in atherogenic factors such as apolipoproteins or pro-inflammatory mediators such as cytokines and chemokines and their receptors. The effect of testosterone on these factors remains unclear.

The objective of the present study was to demonstrate whether testosterone has anti-inflammatory and anti-atherogenic action and by which mechanisms. This was achieved by using in vivo human and mice studies as well as in vitro models. The in vivo human study was conducted on short and long term studies, to determine the effect of TRT on anti and pro-inflammatory cytokines, HDL fractions and apolipoproteins in diabetic hypogonadal patients. Samples of liver tissue from testicular feminization mice (Tfm) were studied to investigate the effect of testosterone therapy on mRNA expression of adiponectin, PPARβ/δ, PAI-1, apolipoproteins and pro-inflammatory chemokines with their receptors. Additionally, models of cell culture were studied including human macrophage THP-1 cells and mouse 3T3L1 cells to study the effect of testosterone with or without blocked androgen receptor (AR) on CX3CR1 and CCR2 and pro-inflammatory cytokines in macrophage cells and on adiponectin, PPARβ/δ, PAI-1, leptin and chemokines in adipocyte cells, respectively.

In the clinical studies, a reduction in adiponectin levels after 3months was seen in the short-term study and an increase in HDL2 and HDL2/HDL3 ratio in the long-term study. No significant effect of testosterone was observed on body composition and atherogenic factors in either the short or long-term studies. In the animal study, testosterone increased hepatic expression of mRNA adiponectin, PPARβ/δ and PAI-1 mRNA expression in Tfm. In the cell culture studies, testosterone treatment increased CCR2 mRNA expression and decreased secretion of IL-8 and TNF level in the supernatant of THP-1 macrophages. Testosterone decreased secretion of CCL2 and CX3CL1 from 3T3L1 adipocytes while increasing PAI-1 mRNA expression in these cells. The action of testosterone was based on the type of cells and time, route and dose size of treatment.

In conclusion, although testosterone therapy showed a positive effect on some risk factors of obesity and its associated conditions, negative effects were also seen. However the exact mechanism of action of testosterone that influences risk factors of obesity and its associated conditions in men with low testosterone remain unclear, therefore further studies are needed to fully elucidate the above finding.

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Abbreviation

ABPI	Ankle brachial pressure index
ABCA1	ATP-binding cassette transporter
A-COA	Acetyl-CoA
ADP	Androgen deprivation therapy
AdipoQ	Adiponectin Q
AML	Acute myeloid leukemia
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activating Protein-1
APCs	Antigen presenting cell
APM1	Adiponectin gene with parameters of metabolic syndrome
Apo	Apolipoprotein
AR	Androgen Receptor
ATPase	Adenosine triphosphates
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BT	Bioavailable testosterone
cAIS	Complete androgen insensitivity syndrome
cAMP	Cyclic adenosine monophosphate
CBA	Cytometric bead assay
cDNA	Complimentary DNA
CHD	Coronary heart disease
CM	Centimetres
CRP	C-selective reactive protein
Ct	Cycle threshold
CVD	Coronary vascular disease
DEX	Dexamethasone
DHEAS	Dehydroepiandrosterone
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTH	Dihydrotestosterone
E	Estradiol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estradiol receptor
F	Flutamide
FBS	Foetal bovine serum
FBS	Fasting Blood Sugar
FFA	Free fatty acid
FSH	Follicle-stimulating hormone
FT	Free testosterone
G-6-PH	Glucose-6- phosphatase
GBP28	Gelatine-binding protein of 28
GnRH	Gonadotropin releasing hormone
GLM	Generalized linear model
GLUT4	Glucose transporter type 4
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HMDMS	Human monocyte- derived macrophage
HSP	Heat shock protein
HTGI	Hepatic triglyceride lipase
IBMX	Isobutylmethylxanthine
ICAM	Intercellular adhesion molecule
IDL	Intermediate-density lipoproteins
IHD	Ischemic heart disease
IKK	Inhibitor of nuclear factor kappa- kinase subunit
IL-	Interleukin
IL-IRN	Interleukin-1 receptor nuclear
IR	Insulin resistance
IRS-1	Insulin receptor substrate -1
JAK/STAT	Janus kinase/ Signal Transducer and Activator of Transcription

30KDa	30Kilodalton
JNK	Jun N-terminal kinase
Kg/m^2	Kilogram Square Metre
LCAT	Lecithin—cholesterol acyltransferase
LDL	low density lipoprotein
LH	Luteinizing hormone
LP (a)	lipoprotein (a)
LPL	lipoprotein lipase
LPS	lipopolysaccharides
LXR	Liver X receptor
M1	Macrophage type 1
M2	Macrophage type 2
Macl2	Magnesium Chloride
MCP-1	Monocyte chemoattract protein
M-CSF	Macrophage colony-stimulating factor
MetS	Metabolism syndrome
MFI	Mean fluorescence index
MI	Myocardial infarction
MIF	Macrophage migration inhibitory factor
Mmol/l	Millimoles/litre
MRI	magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NA/K	Sodium-potassium adenosine triphosphates
ATPase	Adenosine triphosphatase
NF-κB	Factor nuclear factor kappa B
NO	Nitric oxide
NRC	Neo Red Cells
iONS	inducible NO synthase
oxLDL	Oxidised low density lipoprotein
Pal-1	plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PE	Phycoerytherin
PPARS	Peroxisome proliferator activated receptors

PMA	Phorbol myristate acetate
PPRE	Peroxisome proliferator response elements
PSA	Prostate-Specific Antigen
PUFA	Polyunsaturated fats
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RPM	Revolutions per minute
RMR	Resting Metabolic Rate
rRNA	Ribosomal ribonucleic acid
RT	Random primer
RXRα	Retinoid-X receptor alpha
SAT	Subconscious adipose tissue
SEM	Standard error of the mean
SHBG	Sex hormone-binding globulin
SMC	Smooth muscle cell
SNPs	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signalling proteins
S-HRP	Streptavidin, Horseradish Peroxidase
SR-BI	Scavenger receptor class B member 1
TACF	TNF-alpha converting enzyme
T2DM	Type 2 Diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
TH1	T – Helper 1
Tfm	Testicular feminised mouse
TLRs	Toll- like receptors
TNF-α	Tumour necrosis factor –alpha
TRT	Testosterone replacement treatment
TT	Total Testosterone
VAT	Visceral adipose tissue
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscular cell
WC	Waist circumference
WHR	Waist to hip ratio

Conference presentations

Abstracts presented

H.K. Bokhamada, M.N. Woodroofe, T.H. Jones, S.H. Small, W. Kay, C.F. Dalton. (2013). Investigation of the effect of testosterone treatment on the expression of chemokine receptors in monocyte/macrophage THP-1 cell line. Abstract. Atherosclerosis, Vol. 231, Issue 2, e6.

Posters presentations

H.K. Bokhamada, M.N. Woodroofe, T.H. Jones, S.H. Small, W. Kay, C.F. Dalton(2013). Investigation of the effect of testosterone treatment on the expression of chemokine receptors in monocyte/macrophage THP-1 cell line. Poster presented at Heart UK 27TH Annual conference, Familial hyperchoesterolaemia, Bristol, UK.

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1.1 Introduction

Obesity leads to a wide range of serious diseases such as metabolic syndrome, type two diabetes (T2DM), and atherosclerosis as well as in men, testosterone deficiency, which has resultant complications e.g. increased risk of cardiovascular disease (CVD). Obesity is a substantial public health problem that increases morbidity, mortality and has substantial long term economic and social costs. This raises public-health concerns (Saad *et al.,* 2012; van Stijn *et al.,* 2014).

1.2 Obesity

The prevalence of obesity has increased substantially over past decades in most industrialised countries. It is a disease that is present among all age groups in both genders and is rapidly reaching pandemic proportions (Ogden *et al.,* 2014). Prospective causes of this epidemic are attributed to a combination of factors in an individual, which lead to an imbalance between food intake and energy expenditure. The main factors are endocrine disorders, environmental and genetic contributors with inappropriate life style such as changes in dietary patterns and a lack of regular physical activity (Pradhan, 2007).

1.2.1 Measurement of obesity

A clinical definition of obesity is known as a body mass index (BMI) or Quetelet Index of $\geq$ 30 % Kg/m^2 (Poirier *et al.,* 2006). Therefore, BMI is considered the main indictor of obesity (Table 1.1). Waist to hip ratio (WHR) is a second measure used for estimating the degree of obesity. Some studies established that the waist circumference, not WHR, is a better indicator of abdominal fat and a predictor of risk of cardiovascular disease and hypertension in diabetic patients (Balkau *et al.,* 2007; Alberti *et al., 2009*). In a number of studies, WHR or waist circumference was considered more significant than BMI in measuring the body fat distribution (Table 1.2) (Seidell *et al.,* 1997, Yusuf 2005, Van Gaal *et al.,* 2006).

Classification	BMI (kg/m²)	Risk of co-morbidities
Underweight	< 18.5	Low (but risk of other clinical problems increased)
Normal Range	18.5 – 24.9	Average
Overweight (pre-obese)	25.0 – 29.9	Mildly increased
Obese	≥ 30.0	
Class 1	30.0 – 34.9	Moderate
Class 11	35.0 – 39.9	Severe
Class 111	≥ 40.0	Very severe

Table 1.1: Classification of overweight and obesity in adults according to BMI and co-morbidities. (Adapted from Cheah and Kam, 2005).

Classification	Males	Females
Not overweight	<94.0	<80.0
Pre-obese	94.0 – 101.9	80.0 – 87.9
Obese	≥102.0	≥88.0

Table 1.2: Classification of abdominal obesity by waist circumference (cm). (Adapted from Cheah and Kam, 2005).

It also indicates abdominal (central, visceral, android) obesity, which is associated with an increased risk of morbidity, compared to gluteofemoral (peripheral, gynoid) obesity (Wang *et al.,* 2005; Yusuf *et al.,* 2005). The body fat percentage has been considered an additional approach to obesity measurement, which is calculated by taking height, age, gender, weight and waist measurement into account. This calculation gives the exact body fat and the measure can be used to determine the amount of body fat that should be lost to reduce risk of obesity associated conditions. A number of other measurement tools have been used to assess body fat composition, including the measurement of subcutaneous skin fold by means of a caliper or ultrasound, bioelectrical impedance analysis (BIA), densitometry, or imaging procedures; nevertheless, most of these measurements are not readily used in clinical practices, and do not add considerable information involving risks compared to BMI and waist circumference measurements (Jagannathachary and Kamaraj, 2010; Pischon *et al.,* 2007).

Functional differences between peripheral and visceral adipose tissue (VAT) have been reported (Gesta *et al.,* 2007). Visceral adipose tissue has higher metabolic activity than peripheral adipose tissue (Abate and Nicola, 2013). Furthermore, the subcutaneous fat accumulation in the abdomen is more contributory to diabetes, coronary vascular diseases (Hajer 2008; Bastard, 2006) and hypogonadism (Abate *et al.,* 2002) than peripheral adiposity. This may explain why these diseases, in particular heart disease, are more widespread among men than women, because men have more upper fat body than women, who have more lower body fat in general. Also these conditions are more common among women with central obesity compared to women with peripheral obesity (Blouin *et al.,* 2008).

1.2.2 Structure of adipose tissue

Mammalian adipose tissue is histologically identified as a loose association of lipid-filled cells, called adipocytes, which are held together by a framework of collagen fibres. Adipose tissue also contains fibroblastic connective tissue, leukocytes, macrophages, and preadipocytes, in various stages of development. It can be classified into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). The presence, amount, and distribution of both types depend on the species, but the WAT is the main type of adipose tissue and is located under the skin (Witkowska-Zimny and

Walenko, 2011). It usually surrounds the internal organs and prevents them from jarring. WAT is also found in the lymph nodes and the skeletal muscle. The WAT plays a significant role in insulation against heat loss and mechanical and/or structural support and most prominently, is a storage site for energy in the form of long chain fats Vázquez-Vela *et al.,* 2008). Approximately 60 to 85% of the weight of WAT is lipid, with 90-99% being triglycerides. Small amounts of free fatty acids, diglyceride, cholesterol, phospholipid and minute quantities of cholesterol ester and monoglyceride are also present (Satterfield and Wu, 2011). BAT derives its name from the rich blood vessels and densely packed mitochondria in it. It is located near or around some organs including: brain, spinal cord, heart, lungs, and kidneys during cold stress. In infants, BAT comprises 5% of the total body mass and it decreases in humans as they age; it almost disappears by adulthood (Symond *et al.,* 2013; Enerbäck 2010).

1.2.3 Development of adipose tissue

The differentiation of these cells starts at the adipoblast to pre-adipocyte stage and then to an immature adipose cell until it becomes a mature adipose cell, typically referred to as an adipocyte (Tang *et al.,* 2008). Concerning the level of adipocytes, different cell populations at different stages of development are known to be present in adipose tissue during foetal life and early infancy. Some of these cells contain small amounts of lipid and represent the adipose cell mass at an early age. Then the level of the body fat increases after birth by increasing the fat cell size (Lonn *et al.,* 2010). These processes are influenced by several factors including catecholamines, β-adrenoreceptor-mediated agents, thyroid hormones, insulin, growth hormone, glucocorticoids and sex steroids, which have been studied *in vitro* by using pre-adipocyte clonal cell lines, principally of mouse and rat origin (Zancanaro *et al.,* 1995, Yang 2005). Both adipocyte number and size are responsible for increasing the size of adipose tissue mass (Tang *et al.,* 2008). Hyperplastic growth is a means of enhancing the number of adipocytes by mitotic activity in the precursor cells. In contrast, hypertrophic growth is a means of increasing the size of adipocytes as a result of augmenting lipid accumulation within the cells (Schling and Löffler, 2002).

1.2.4 Functions of adipose tissue

The fat storage capability of adipose tissue is associated with its traditional function as an energy storage tissue, but its role as an active immune-modulator and endocrine organ is a relatively new concept (Trayhun, 2005; Rosen *et al.,* 2014). This concept has been an area of intense investigation and has been examined by highly varied experimental approaches to study the main secreted molecules from adipose tissue. Adipose tissue produces various substances with autocrine, paracrine and neuroendocrine actions (Figure 1.1). These substances have a role in glucose metabolism, insulin sensitivity, lipid oxidation, immune and vascular function and hormonal status (Lago *et al.,* 2007; La Merrill *et al.,* 2013).

1.2.5 Adipocyte endocrinology

At the beginning of the 1990s, a big advance in the recognition of the endocrine and secretory role of adipose tissue occurred (Rasouli and Kern, 2008). Adipocytokines or Adipokines is a general term that refers to a bioactive produced by the adipose tissue. Hotamisligil *et al.* (1993) reported that tumour necrosis factor alpha (TNFα) expression increases in obesity and has a significant responsibility in insulin resistance mechanisms (Hajer *et al.,* 2008) and adipose tissue metabolism such as the stimulation of lipolysis and apoptosis (Dunger *et al.,* 1996; Rabinovitch *et al.,* 1999). However, a radical change in viewpoint occurred following the discovery of leptin in 1994 by Friedman and colleagues (Zhang *et al.,* 1994*)*. This vital hormone is made predominantly by white fat, assigning to the tissue an endocrine function. It plays a role in the regulation of food intake and energy expenditure by stimulating the hypothalamus, the centre of appetite and energy balance (Lago *et al.,* 2007). Therefore, the discovery of leptin helped scientists understand the secretory role of WAT and its role in the regulation of metabolic and physiological homeostasis. It is now well established that adiponectin, leptin, adipsin, resistin, visfatin, complement factor 3, interleukin-6 (IL-6), IL-1β, IL-8 IL-10, TNF-α, angiotensinogen and plasminogen activation inhibitor-1 (PAI-1) are also all produced and released by adipose tissue (Trayhurn and Wood, 2005; Bullo′ *et al.,* 2013).

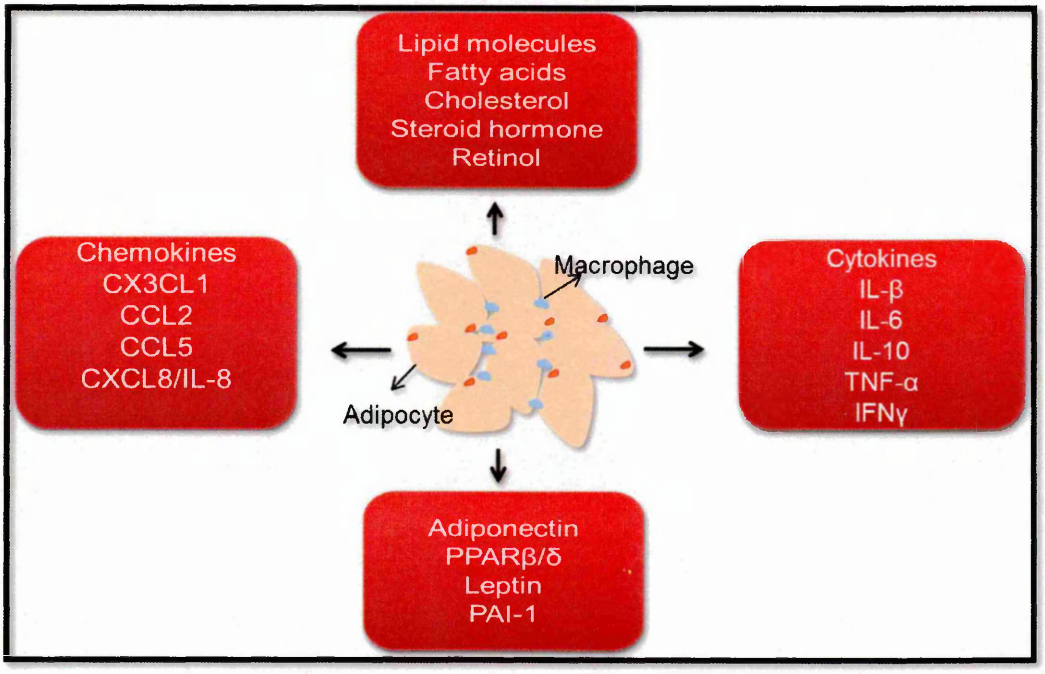

Figure 1.1: The multiple functions of white adipose tissue. The function of this tissue includes: synthesis, secretion and storage of lipids and production of cytokines, chemokines and other adipokines such as adiponectin, leptin, PPARβ/δ and PAI. CCL: CC-chemokine ligand (CCL); CXCL: CXC-chemokine ligand (CXCL); IL: interleukin, PAI-1: plasminogen activator inhibitor 1, PPARβ/δ: peroxisome proliferator-activated receptor, TNF-α tumour necrosis factor, IFNγ: Interferon gamma (Adapted and modified from Lago *et al.,* 2007).

Furthermore, adipose tissue is a source of chemokines including CCL2 (MCP-1) and fractalkine (CX3CL1) (Suganami and Ogawa 2010; Shah *et al.,* 2011). In addition, overwhelming evidence has established that the residence of macrophage cells in WAT assists strongly in the secretory function of adipose tissue (Weisberg *et al,* 2003) (Figure 1.2).

It has been suggested that the main source of pro-inflammatory adipokines is macrophages, although other cells in adipose tissue such as adipocytes, preadipocytes and vascular cells contribute to their secretion (Fain, 2006, Sell and Eckel, 2009). Two types of macrophage (M1 and M2) are located in adipose tissue. M1 macrophages are predominantly found in adipose tissue of fatter people and they produce TNF-α and IL-6; thereby increasing inflammation, whereas M2 macrophages secrete IL-10 which is an anti-inflammatory cytokine. Both types of macrophage, together with fat cells have the ability to accumulate lipids and release cytokines (Fuentes *et al.,* 2010).

Adipose tissue is an active endocrine organ, and its relationship to inflammation and the immune system has become the focus of much research aimed at understanding this relationship, its causes and consequences, in the hope that will offer new targets for drug development for controlling obesity and reducing its role in the occurrence of cardiovascular disease (CVD), T2 DM and testosterone deficiency.

1.3 Inflammation and key molecules

The key markers of inflammation include: immune cells, biological molecules such as cytokines, chemokines and innate effector mechanisms (Romagnani, 2002). Chronic systemic inflammation is associated with a reduced human life span (Finch and Crimmins, 2004). Although there is limited information on markers of systemic inflammation and their mechanism of action, there is an association between chronic inflammation and several diseases found in the developed world, including obesity (De Heredi *et al.,* 2012), diabetes (Wang *et al.,* 2013), atherosclerosis (Libby *et al.,* 2009) as well as low testosterone, which leads to increased risk of other disease (reviewed from Saad *et al.,* 2012). Therefore, a key to preventing and treating these conditions is reducing inflammation.

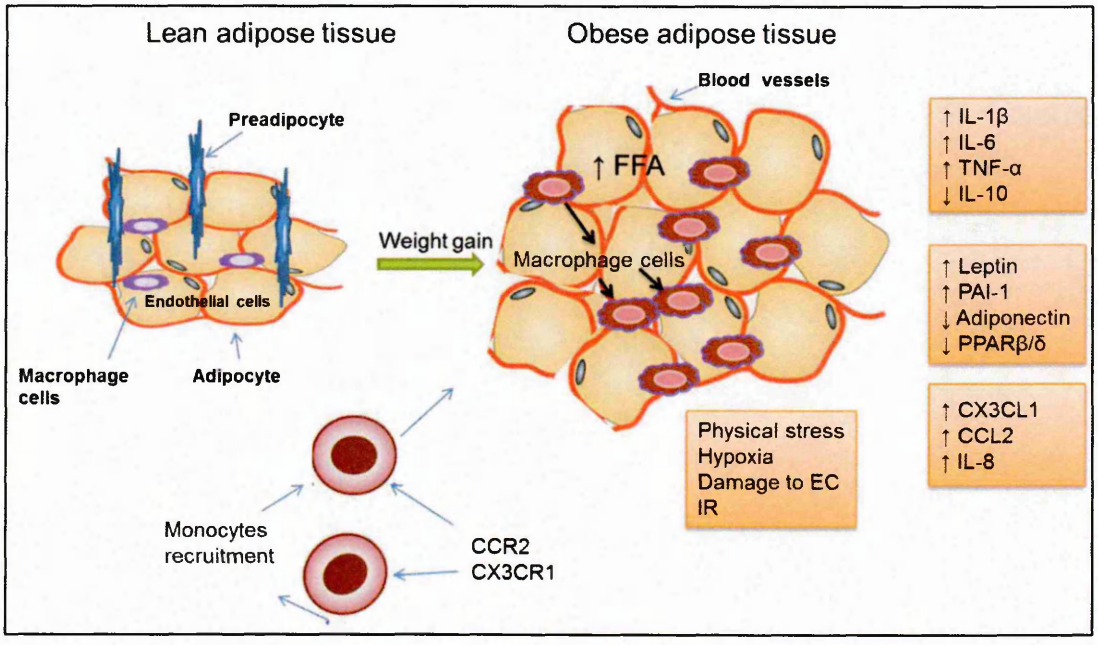

Figure 1.2: Adipose tissue cellular components and molecules synthesized in lean and obese adipose tissue. Changes in adipocyte and fat size lead to physical changes in the surrounding area and modifications of the paracrine function of the adipocyte. Increased migration of monocytes to adipose tissue in response to CX3CL1and CCL2 via their receptors CX3CR1and CCR2 leads to activation of macrophages from M2 to M1phenotyp. This leads to reduction in anti-inflammatory factors including: adiponectin, IL-10 and PPARβ/δ and increased secretion of free fatty acid (FFA) and pro-inflammatory factors including: IL-1β, IL-6, and leptin, PAI-1, CCL2, CX3CL1 and IL-8. The result of all these changes is damage to endothelial cells (EC) and development of insulin resistance (IR). (↑) increase, (↓) decrese. (Drawn using information from Ouchi *et al.,* 2011, Shah *et al.,* 2011; Yao *et al.,* 2014).

1.3.1 Factors linking obesity with inflammation

There is a causal link between obesity and the activation of the innate immune system (Bastard *et al.,* 2006) leading to a low-grade inflammatory state, which has been observed in obese individuals and this could play a central role in obesity, T2DM-related IR and cardiovascular complications (De Heredi *et al.,* 2012).

However, the occurrence of low-grade inflammation in obesity is not clearly understood, although hypotheses to explain this have been proposed. It could be a result of insufficient blood supply in growing adipose tissue, which leads to decreased oxygenation (Pasarica *et al.,* 2009). Further evidence implicating adipocytes in innate immunity, is that fat cells have the ability to synthesize and release pro-inflammatory cytokines and chemokines (see section 1.2.5) (Monteiro and Azevedo 2010, Skurk *et al.,* 2005, Wood *et al.,* 2009).

These factors can activate inflammatory signalling pathways, resulting in the induction of further biological markers of inflammation. Although the expression, production and secretion of inflammation-related adipokines are augmented in adipose tissue with obesity, the major exception to this example of increased inflammation is adiponectin, the expression and concentration of which falls in obese individuals, given the anti-inflammatory action of the hormone. Adiponectin controls the level to which adipose tissue is in a state of 'inflammation' in the obese (Von Eynatten *et al.*, 2006; Hoffstedt *et al.*, 2004; Kumada *et al.*, 2003).

In addition, in rodent genetic models of obesity, the expression of genes coding for these factors in WAT result from the infiltration of macrophages of obese mice (Bastard *et al.,* 2006). This is a further biological clue demonstrating that immune cells are implicated in obesity. There is substantial evidence supporting the significance of body weight loss in reducing gene expression of pro-inflammatory factors and the infiltration of macrophages in WAT (Clement *et al.,* 2004). Furthermore, there have been several suggestions that explain and clarify the process that led to such macrophage cells residing specifically in WAT. One of the explanations was that macrophage cell accumulation in the adipose tissue was a low-grade inflammation state in obesity (Wellen 2005; Ford 2003).

Other research suggests that macrophages may derive from preadipocytes (Cousin *et al.,* 2003) while some studies have indicated that macrophages originate from bone marrow precursors (Weisberg *et al.,* 2003). In addition, leptin may have a stimulatory role in macrophage infiltration from the blood circulation to the WAT (Curat, 2004). Moreover; adipocytes can produce and release chemokines including CCL2 and CX3CL1 which are chemoattractant recruiting factors for circulating monocytes. Both chemokines are now well acknowledged for over-production in obesity (Martinovic *et al.,* 2005; Shah *et al.,* 2011). Moreover, some factors can link obesity to the immune system via toll-like receptors (TLR). These receptors are a family of pattern-recognition receptors that play a significant role in the innate immune system. They can be stimulated by saturated fat to enhance the expression of pro-inflammatory IL-6 and TNF-α (Huang *et al.,* 2006). There is a signalling cascade that promotes the NF-κB pathway of both TLR2 and TLR4 in fat and macrophage cells, which then activates the transcription of many pro-inflammatory genes that encode pro-inflammatory molecules including cytokines, chemokines and other effectors of the innate immune response (Brown *et al.,* 2011).

1.3.2 Adipokines and their effects

Adipocytokines are biologically active factors which work locally and systemically to influence glucose and fatty acid metabolism, insulin sensitivity, adipocyte differentiation, inflammation and the immune response. These biomarkers are classified according to their action as pro or anti-inflammatory factors.

1.3.3 Pro-inflammatory Adipokines

1.3.3.1 Interleukin-1β

IL-1β is a pro-inflammatory cytokine produced by a variety of cells including macrophages, monocytes and adipocytes. The IL-1 family contains three polypeptides IL1-α, IL-1β and an interleukin-1 receptor antagonist (IL-Ira) (Kim *et al.,* 2008). They play a potent role in the regulation of immune cells and inflammation. A positive correlation between IL-1β and BMI has been reported in humans. In both human and rodent obesity, adipose tissue expression of IL-1β is highly up-regulated in visceral

compared with subcutaneous fat (Moschen *et al.,* 2011; Shaul *et al.,* 2010). Nov *et al.* (2013) suggesting that IL-1β is a regulator of adipose inflammation by promoting leukocyte and macrophage recruitment and enhancing macrophage lipid accumulation. IL-1β is also positively associated with IR; the mechanism by which IL-1β causes IR and subsequently affects the development of diabetes is under investigation. It has been suggested that it could impair insulin secretion and its signalling by stimulating insulin receptor substrate-1 (IRS1) serine phosphorylation (Zeyda and Sultnig, 2007). In addition, it may induce pancreatic beta cell apoptosis in diabetic patients resulting in Type 1 diabetes (Zeyda and Sultnig, 2007). The available data certainly emphasise that the adipocytokines, IL-1β with IL-6, have been closely linked to endothelial cell dysfunction (Caballero, 2003). Both IL-1β and TNF-α play a role in the induction of CX3CL1 by primary endothelial cells where CX3CL1 can contribute to the pathogenesis of atherosclerosis (Jones *et al.,* 2010). Furthermore, IL-1β similarly to IL-6 and TNF-α, can also induce the liver to produce C-reactive protein (CRP) a marker of inflammation (Malkin *et al.,* 2004a).

1.3.3.2 Interleukin 6

IL-6 is produced by a number of cell types including macrophages, T helper lymphocytes and adipocytes (Papanicolaou and Vgontzas, 2000). A positive correlation between IL-6 and obesity has been reported (Rocha *et al.,* 2011). In particular, circulating IL-6 levels increase with an increase in BMI. Abdominal subcutaneous and visceral depots are both considered as the main source of this cytokine as well as white blood cells. Other studies confirmed that IL-6 mRNA expression is higher in VAT than in subcutaneous adipose tissue (SAT) (Mohamed *et al.,* 1998, Rocha *et al.,* 2011).

IL-6 plays an important role in the development of inflammatory conditions such as T2DM and CVD. According to animal model and human studies, IL-6 is involved directly in progressing T2DM by causing IR in some distant tissues such as skeletal muscle, liver and vascular endothelial cells (Hu *et al.,* 2004, Wallenius *et al.,* 2002; Klover *et al.,* 2003). The possible mechanism of action of IL-6 is by impairing the action of insulin (Bastard *et al.,* 2006). IL-6 binds to its receptor (IL6R) which pathway leads to phosphorylation signals via the Janus-kinase/signal transducers and activators of transcription (JAK/STAT). JAK/STAT pathway is employed in the signalling of many cytokines. The phosphorylated sites on the receptor and JAKS serve as docking sites for the SH2 (Src Homology 2) containing STATS. Phosphorylated STATS

dimerizes and translocates into the nucleus to regulate target gene transcription. However, the stimulation of the JAK/STAT receptor system via IL-6 could dysregulate insulin action by inhibiting serine/threonine phosphorylation of Insulin Receptor Substrate (IRS), which is an important ligand in the insulin response of human cells, or by increasing suppressors of cytokine signalling (SOCS) (Bastard *et al.,* 2006; de Luca and Olefsky, 2008).

IL-6, in addition to its role in the reduction of insulin action, also has a role in causing CVD that is linked to inflammation by inducing hepatic CRP secretion (Rocha and Folco, 2011, Lee *et al.,* 2012). It is established that the IL-6 content of adipose tissue is strongly correlated with circulating levels of both IL-6 and CRP, particularly, IL-6 is produced mainly via VAT, which might demonstrate the causal relationship between central depots and CVD (Hu *et al.,* 2004). IL-6 secreted from the adipose tissue directly passes to the liver via the blood, where it augments hepatic triglyceride and very low density lipoprotein (VLDL) production, reduces lipoprotein lipase (LPL) activity and enhances lipolysis. This leads to dyslipidemia development which is considered as a cardiovascular risk complication in humans. Thus, IL-6 seems to play a role in the development of coronary disease and diabetes linking both diseases with obesity and inflammation (Bastard *et al.,* 2006). Besides these diseases, IL-6 also has a role in reduction of testosterone levels by inhibiting hypothalamic production of GnRH and subsequent release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. This causes a state of hypogonadotrophic hypogonadism (Kelly and Jones, 2013).

1.3.3.3 Tumour necrosis factor

TNF-α is a multifunctional cytokine secreted from a number of different cell types, principally macrophages and lymphocytes. It can also be released from muscle cells and adipose tissues (Sewter *et al.,* 1999). It was initially identified as a suppressor of tumours; it is now involved as a significant factor in the progression of IR and heart disease (Pischon, 2007). TNFα was the first cytokine to be implicated in the pathogenesis of obesity and IR (Hotamisligil *et al.*, 1993). TNF-α mRNA expression is similar in both VAT and SAT, where VAT is strongly associated with IR and hyperinsulinaemia (Dusserre *et al.,* 2000).

Other factors, such as leptin, have been proposed to induce TNF-α by other cell types including macrophages. TNF-α mRNA is over-expressed in adipose tissue in rodent models of obesity, however, the actual role of TNF-α in obese humans remains to be elucidated (Sakurai *et al.,* 2013). Weight loss in fatter people is associated with a significant reduction in TNF-α mRNA expression in adipose tissue and improved insulin sensitivity (Manco *et al.,* 2007). Therefore, it is thought that TNF-α expression in obese adipose tissue could originally derive from macrophage infiltration, under the control of leptin, rather than from the adipocytes themselves (Bastard *et al.,* 2006). In addition, there is also a debate related to the physiological role of TNF-α in the development of IR and CVD. One possible link between IR and diabetes is that TNF-α can cause the phosphorylation of the insulin receptor substrate-I (IRS-I) protein on serine residues. This leads to impeding the interaction of insulin with its receptor beta subunit and its signalling pathway. Another mechanism suggested is that TNF-α may down-regulate glucose transporter type 4 (GLUT 4) an insulin-responsive glucose transport protein (Hotamisligil *et al.,* 1996; Shi *et al.,* 2004). TNF-α also reduced peroxisome proliferator-activated receptor (PPAR) expression a transcription factor that is abundant in adipose tissue, which may also play a role in the sensitivity of cells to insulin (Zehda and Stulnig, 2007). As for the role of TNF-α in the incidence of micro-vascular or macro-vascular complications in diabetes, TNF-α can also directly cause endothelial dysfunction by inducing endothelial cells, hepatocytes and adipose tissue to express plasminogen activator inhibitor (PAI-1) that leads to a pro-coagulant state (Laaksonen *et al.,* 2005). TNF-α also sustains the development of CVD by promoting hepatic CRP synthesis that is the main predictor of CVD, (Figaro *et al.,* 2006, Hu *et al.,* 2004, Malikan *et al.,* 2004a). In addition, TNF-α controls IL-6 synthesis and both are pro-inflammatory factors associated with obesity, inflammation with diabetes and CVD (Hoffstedt *et al.,* 2004). Additionally, TNF-α can, like IL-6, be associated with low testosterone and a state of hypogonadotrophic hypogonadism (Kelly and Jones, 2013).

1.3.3.4 C-reactive protein

CRP is the one of the acute phase proteins secreted by the liver. It is increased considerably during systemic inflammation/infection and is found to be a sensitive marker of low grade systemic inflammation. Several studies have observed that the level of CRP increses with increasing BMI (Ford, 2003), serum lipid and fasting blood

glucose (Wexler *et al.,* 2005, Thorand *et al.,* 2003, Santos *et al.,* 2005). In addition, following the Women's Healthy Lifestyle study, losing body weight contributed to a reduction in CRP levels, indicating the important role of inflammation in body fat (Isidoris *et al,* 2000).

The nature of the association of CRP with IR and T2DM is not fully understood. Previous studies have confirmed that CRP concentration in plasma increased two fold in diabetic patients compared with non-diabetic subjects with acute coronary syndrome before and after coronary stenting (Aggarwal *et al.,* 2003; Huoya *et al.,* 2009). Furthermore, Barzilay *et al.,* (2001) found at least a two-fold higher risk of T2DM within 3-4 years in obese individuals with higher CRP levels. Moreover, it is expected that a decrease in insulin sensitivity could lead to increased CRP expression by counteracting the action of insulin on hepatic acute-phase protein synthesis. The circulating level of CRP is a useful marker of CVD and stroke in men and women (Lowe *et al.,* 2014, Kapoor *et al.,* 2007). Evidence suggests that the production of CRP is induced by IL-1, IL-6, and TNF-α leading to the correlation of CRP with heart disease and inflammation (Klover *et al.,* 2003, Laaksonen *et al.,* 2005). Thus, CRP is considered as a marker of both inflammation and cardiovascular events; including myocardial infarction, stroke, peripheral arterial disease and sudden cardiac death (Spagnol *et al.,* 2007).

1.3.3.5 Chemokines

Chemokines constitute a family of over 40 different cell signalling molecules. The structure of these chemokines is small disulphide-linked polypeptides of typically 60 - 70 amino acids (Linton and Fazio, 2003). They have been classified into different subfamilies on the basis of these conserved structural features. Most chemokines contain four conserved cysteines which form two essential disulphide bonds, Cys1–Cys3 and Cys2–Cys4. The backbone of the chemokine molecule consists of beta strands, while the N and C terminus of the protein appear to have a less ordered structure (Table 1.3). Chemokines are also associated with chronic inflammation related to obesity such as IR, T2DM and CVD (Ota *et al.,* 2013). Adipose tissue chemokines increase in obesity and have an important role in leukocyte recruitment into inflammatory adipose tissues. It has been proposed that fractalkine (CX3CL1) and monocyte chemotactic protein-1 (MCP-1/ CCL2) are vital factors for adipose tissue macrophage recruitment and IR in obesity (Ferrante *et al.,* 2007; Dahlman *et al.,* 2005).

Family	Other Nomenclature	Common Synonyms	Receptors
C chemokines	XCL1	Lymphotactinα/SCMα	XCR1
	XCL2	Lymphotactinβ/SCMβ	XCR1
CC chemokines	CCL1	I-309, TCA3, P500	CCR8
	CCL2	MCP-1, MCAF, (mouse; JE)	CCR2
	CCL3	LD78a, LD78b, MIP-1a	CCR1, CCR5
	CCL4	Act-2, HC21or 400, MIP-1b,	CCR5
	CCL5	RANTES	CCR6
	CCL7	MCP-3	CCR1,CCR2, CCR5
	CCL8	MCP-2, HC14	CCR3
	CCL11	eotaxin	CCR3
	CCL13	CCL12 NA I (mouse) MCP	CCR2, CCR3
	CCL14	MCP-4, NCC-1, CKb10	CCR1
	CCL15	HCC-1, HCC-3, NCC-2, MCIF	CCR1, CCR4
	CCL16	NCC-4, LEC, HCC-4, LMC,	CCR1
	CCL17	TARC	CCR4
	CCL18	DC-CK1, PARC, MIP-4, CKb7	Unknown
	CCL19	ELC, MIP-3b, exodus-3, CKb11	CCR7
	CCL20	MIP-3a, LARC, exodus-1, CKb4	CCR6
	CCL21	SLC, 6Ckine, exodus-2, CKb9	CCR7
	CCL22	MDC, STCP-1, DC/B-CK	CCR4
	CCL23	MIP-3, MPIF-1, CKb8	CCR1
	CCL24	MIP-3, MPIF-1, CKb8	CCR3
	CCL25	TECK, Ckb15	CCR9
	CCL26	eotaxin-3, IMAC, MIP-4a	CCR3
	CCL27	ALP, ILC, ESkine, CTAK	CCR10
	CCL28	MEC, CCK1	CCR10
CXC chemokines	CXCL1	GROa, MGSA-a, NAP-3	CXCR1, CXCR2
	CXCL2	GROb, MIP-2a, MGSA-b	CXCR2
	CXCL3	GROg, MIP-2b, CINC-2b	CXCR2
	CXCL4	PF4	CXCR3B
	CXCL5	ENA-78	CXCR2
	CXCL6	GCP-2	CXCR1, CXCR2
	CXCL7	NAP-2, LA-PF4, MDGF, LDGF	CXCR2
	CXCL8	IL-8, NAP-1	CXCR1, CXCR2
	CXCL9	mig	CXCR3
	CXCL10	IP-10	CXCR3
	CXCL11	I-TAC	CXCR3
	CXCL12	SDF-1a, SDF-1b, PBSF	CXCR4
	CXCL13	BLC, BCA-1	CXCR5
	CXCL14	BRAK, MIP-2g, BMAC, KS1	Unknown
	CXCL16	-	CXCR6
CX3C chemokines	CX3CL1	Fractalkine	CX3CR1

Table 1.3: Classification of chemokines and their receptors. To date, over 40 human chemokines and 20 receptors have been discovered in humans. These chemokines are classified into four families (C, CC, CXC, and CX3C) dependent on the position of the first two cysteine motifs while chemokine receptors are classified on the basis of their ligand (Adapted from Mantovani *et al.*, 2006).

On the basis of their molecular structure, both chemokines are derived from the two subgroups: CC chemokine ligands (CCL) and CX3C chemokine ligand (CX3CL1) (see table1.3), which bind to CC chemokine receptor (CCR), (Bruserud *et al.,* 2010) or CX3C chemokine receptor (CX3CR) (Shah *et al.,* 2009), respectively.

There is evidence demonstrating that CCL2, with its receptor CCR2, plays a key role in the recruitment of monocytes and infiltration of macrophage cells from the blood into adipose tissue. This comes from the study of CCR2-knock-out mice, where IR, hepatic steatosis, and macrophage accumulation in adipose tissue induced by a high-fat diet were reduced (Kanda *et al.,* 2006). Moreover, there are high levels of CCL2 in both white adipose tissue and plasma in obese mice (Sartipy *et al.,* 2003, Weisberg *et al.,* 2003, Xu *et al.,* 2003) and it is secreted by the mouse 3T3L1 adipocyte cell line (Kanda *et al.,* 2006). Additionally, CCL2- or CCR2-deficient mice showed a relative reduction in adverse metabolic outputs (Inouye *et al.,* 2007).
In contrast, other studies found that lack of CCL2 neither attenuates obesity-associated macrophage recruitment to adipose tissue nor improves metabolic function, which indicates that CCL2 is not critical for obesity-prompted macrophage migration and systemic IR (Inouye *et al.,* 2007 and Kirk *et al.,* 2008). A further study reported that although CCR2/CCL2 is important for adipose tissue macrophage accumulation, deletion of these molecules only decreases macrophage recruitment to adipose tissue by ~40% indicating that there are further chemotactic signals that are key to this process (Oh *et al.,* 2012).

One of these signals is CX3CL1, which is a chemokine that has a dual structure and function as both an adhesion molecule and chemoattractant. Soluble CX3CL1 released from the cell membrane, strongly chemoattracts leukocytes (T cells and monocyte cells), while the membrane-bound chemokine induces strong adhesion of cells expressing the G protein–coupled receptor CX3CR1, through which it exerts its effect (Maegdefessel *et al.,* 2009; White *et al.,* 2010). It is thought that CX3CL1, with its receptor, is involved in recruitment of inflammatory monocytes and macrophages to adipose tissue as well. Shah and co-workers (2011) reported that CX3CL1 is elevated in the serum of obese patients, suggesting that CX3CL1/CX3CR1 may also be implicated in adipose tissue inflammation (Umehara *et al.,* 2004; Morris *et al.,* 2012).

Recent data has reported that CX3CL1 is expressed in adipocytes and that CX3CR1 signalling in macrophages is down-regulated by peroxisome proliferator–activated receptor (PPAR-γ) agonists (Digby *et al.,* 2010). It has been established that CX3CL1 is significantly increased in obesity but it can also be induced by induction of inflammation *in vivo* (Shah *et al.,* 2011). In this study it was found that both the stromal vascular fraction and specific adipocytes were responsible for production of CX3CL1, promoting monocyte adhesion to human adipocytes. In addition, visceral adipose tissue is known to have greater inflammatory leukocyte infiltration (Baranova *et al.,* 2005) and has higher CX3CL1 levels than subcutaneous fat. Adipose tissue macrophages in obese individuals express high levels of CX3CR1 (Zeyda *et al.,* 2010). Moreover, humans with two common non-synonymous coding SNPs (single-nucleotide polymorphism) in the CX3CR1 gene and the variants causing greater expression of CX3CR1, tended to be associated with greater waist circumference, were more IR, and had higher leptin levels, but lower adiponectin levels in serum. Furthermore, these alleles tended to be associated with the presence of metabolic syndrome and T2DM. The association of SNPs in CX3CR1 with atherosclerosis and obesity (Sirois-Gagnon *et al.,* 2011) was also reported (Kasama *et al.,* 2010).

This suggests that CCL2 and CX3CL1 play important roles in the pathogenesis of metabolic syndrome, T2DM and CVD, therefore, inhibition of the interaction of these chemokines with their receptors might provide the basis for development of new therapies for these conditions.

1.3.3.6 plasminogen activator inhibitor

PAI-1, which is encoded by the SERPINE1 gene, is synthesised by WAT and is a key inhibitor of tissue plasminogen activator (tPA) (Kathiresan *et al.,* 2005). Additionally, PAI-1 is produced by liver and endothelial cells but is synthesised largely by visceral adipocytes (Skurk and Hauner, 2004; Bastelica *et al.,* 2002). It is noticeably elevated in serum in obese people and patients with IR, T2DM and CVD (Chen *et al.*, 2006). An increased in plasma PAI-1 level in obesity is well recognized in animals and humans (Bastard and Pieroni, 1999, Morange *et al.,* 2000; Skurk and Hauner, 2004). More recently PAI-1 levels have been considered as one of the biomarkers used to predict

obesity-associated diseases; weight loss leads to normalized or decreased levels of PAI-1 (Gonzalez *et al.*, 2012).

There is increased expression of PAI-1 mRNA in adipose tissue through adipose differentiation processes *in vitro* (Bastelica *et al.,* 2002). PAI-1 as an acute-phase protein, increases in response to inflammation or injury in the circulation. Inflammatory mediators, such as TNF-α, IL-1, IL-6 and transforming growth factor β (TGFβ), have been shown to stimulate PAI-1 production (Hermans and Hazelzet, 2005). There are also hormones that regulate the expression of PAI-1 mRNA including insulin, glucocorticoids and sex hormones (Skurk and Hauner, 2004). Local hypoxia and the associated cellular stress stimulate the expression of angiogenic factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor and PAI-1. These induce the inhibition of adiponectin gene transcription leading to reduction in adiponectin mRNA stability and in adiponectin expression in obese mice (Hosogai *et al.,* 2007; Bell *et al.,* 2008). The induction of leptin and PAI-1 gene transcription simultaneously in adipose tissue suggests that the dysregulation of adipokine secretion is part of the cellular mechanism in response to hypoxia and associated cellular stress conditions (Chen *et al.,* 2006). Clinical studies have shown that PAI-1 correlates obesity with T2DMM, CVD and low testosterone through indirect effects on insulin signalling, by influencing adipocyte differentiation or by regulating recruitment of inflammatory cells within adipose tissue, which ultimately leads to IR, characterized by visceral fat accumulation (Pergola *et al.,* 2000; Alessi *et al.,* 2007). Moreover, over-production of PAI-1 in excess visceral fat inhibits the fibrinolytic system and consequently may lead to thrombotic vascular disorders (Ryo *et al.,* 2004). This is due to a shift in the balance between fibrinolysis and thrombosis, towards thrombosis facilitating the formation of micro-thrombi and by the ability of PAI-1 to inhibit plasminogen-induced migration of vascular smooth muscle cells (VSMCs) resulting in plaques prone to rupture with thin fibrous caps, necrotic cores and rich in macrophages (Chu *et al.,* 2001).

1.3.3.7 Leptin

Leptin is a 16-kDa protein and was discovered by Friedman and colleagues (Zhang *et al.,* 1994). It is one of the most important adipose-derived hormones and is encoded by the leptin gene (*Lep (ob)*) located on chromosome 7 in humans. It is a hormone that is almost exclusively expressed and produced by WAT and more particularly by

differentiated mature adipocytes, which binds to its receptor in the hypothalamus and other tissues (Ahima *et al.,* 2000). This is evidenced from several human and animal studies (Trayhurn and Beattie, 2001).

Leptin is a key appetite-regulating hormone, with effects on energy expenditure. There is a significant association between leptin and both BMI and fat mass. Circulating levels of leptin (Considine *et al.,* 1996) and adipose tissue leptin mRNA expression are increased proportional to body fat mass, in obesity (Vidal *et al.,* 1996). However in obese people, receptors of leptin are insensitive to the effects of leptin leading to leptin resistance (Myers *et al.,* 2010). The pathway of leptin control in obese people is defective to some extent, thus the body does not sufficiently receive the satiety effect, subsequent to eating. Furthermore, although leptin functions predominantly at the level of the central nervous system to control food intake and energy expenditure, there is a relationship between leptin and the low-grade inflammatory state in obesity, suggesting that leptin could display peripheral biological activities as a function of its cytokine-like structure (Ahima *et al.,* 2000). The role of leptin in the inflammatory response by association with the presence of hyperleptinemia, without obesity (Loffreda *et al.,* 1998, van Dielen *et al.*, 2001; Bastard *et al.*, 2006) and in controlling TNF-α production by macrophages, although, the exact mechanisms have not been identified (Bastard *et al.*, 2006). Moreover, the leptin receptor has been recently detected in peripheral tissues, suggesting further roles for leptin and a much broader range of actions than initially supposed. One of these functions is regulation of glucose metabolism, where considerable evidence suggests that insulin and leptin act in the brain as adiposity negative feedback signals (Morton and Schwartz, 2011). Certainly, recent study revealed that leptin has an effect on modulation of insulin action in the liver, normalizes hyperglycemia and hyperinsulinemia and increases insulin sensitivity (Amitani *et al.,* 2013).

An additional role for leptin in vascular diseases has been reported, with elevated plasma leptin leading to adipocyte dysfunction, which is associated with the presence of risk factors for vascular disease such as increased BMI, CRP, low density lipoprotein (LDL) and triglycerides (TG) (Wolk *et al.,* 2004, Brennan *et al.,* 2007; Iribarren *et al.,* 2007). Finally, leptin has an inhibitive role on the hypothalamic -pituitary -testicular axis causing a state of hypogonadotrophic hypogonadism and low testosterone levels (Jones, 2010). Therapeutic potential of leptin in obesity, T2DM, testosterone deficiency and CVD has been proposed (Amitani *et al.,* 2013).

1.3.4 Anti-inflammatory adipokines

1.3.4.1 Adiponectin

Adiponectin is highly expressed in adipose tissues and has a varied nomenclature: ACRP30 (adipocyte complement-related protein of 30kDa), adipoQ in mouse and GBP28 (gelatin-binding protein 28) and/or APM1 (adipose most abundant gene transcript 1) (Kadowaki and Yamauchi 2005). Recent genetic studies have reported that the expression of adiponectin mRNA is dependent on the adipose tissue localization, with higher expression in SAT than in VAT (Bastard *et al.,* 2006). Adiponectin has two receptors, adipoR1 and adipoR2, adipoR1 is expressed in skeletal muscle whereas adipoR2 is in the liver (Rocha and Folco, 2011; Yamauchi *et al.,* 2003). In addition, as opposed to other adipose derived proteins, the level of adiponectin decreased with increased body fat and increased with weight loss. Therefore, low levels of adiponectin are correlated with T2DM, CVD and dyslipidemia (Lihn *et al.,* 2004). Further investigation is required to understand how low levels of adiponectin lead to IR and its components. Studies in mice indicate injection of adiponectin stimulate non-esterified fatty acid oxidation and at the same time, reduce plasma levels of glucose (Fruebis *et al.,* 2001). After treating these animals with adiponectin for the long term, there is a decrease in the triglyceride storage in liver and muscle, an improvement in insulin sensitivity by reducing hepatic glucose, a decline in triglyceride and free fatty acid levels and a rise in HDL concentration (Yamauchi *et al.,* 2001; Hoffstedt *et al.,* 2004). This was confirmed by clinical studies suggesting that circulating adiponectin was positively associated with enzymes that control lipid metabolism and also explained the negative correlation between adiponectin and CVD (Siasos *et al.,* 2012). Furthermore, adiponectin is also considered as a potential anti-atherogenic factor, but it remains unclear which pathways mediate the inverse relationship between adiponectin and CVD and diabetes (Ouchi *et al.,* 2001, Diez and Iglesias *et al.,* 2003; Siasos et al., 2012). This relationship may relate to the role of adiponectin in the inhibition of TNF-α induced activation of NF-κB suggesting a role as an anti-atherosclerotic factor. TNF-α and IL-6 both decrease human adipocyte mRNA expression of adiponectin, which is an additional means by which these two adipokines induce IR (Ouchi *et al.,* 2000, Bruun *et al.,* 2003). Additionally, adiponectin can stimulate adenosine monophosphate-activated protein kinase (AMPK), which it is known to mediate the cellular malonyl CoA concentration by suppressing acetyl CoA and this demonstrates additional mechanisms

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of action for adiponectin in improved insulin sensitivity (Bastard *et al.,* 2006). Adiponectin also plays a role in regulating liver glucose production by reducing mRNA expression of phosphoenol pyruvate carboxykinase and glucose-6-phosphatase, two key enzymes in neoglucogenesis (Kadowaki and Yamauchi, 2005). Furthermore, adiponectin might protect the vascular wall by controlling the atherogenesis process: 1) by alteration of endothelial cell adhesion molecules, 2) conversion of macrophages into foam cells and 3) modulating vascular smooth muscle cell proliferation. Moreover, an inverse relationship was seen between adiponectin and the level of testosterone in diabetic men (Kapoor *et al.,* 2007; Bai *et al.,* 2011). In addition, testosterone administration is associated with a decline in serum adiponectin in animals and by reduced protein secretion in cultured adipocytes (Nishizawa *et al.,* 2002). However, the mechanism of the reduction in adiponectin levels during testosterone treatment is not understood.

1.3.4.2 Interleukin -10

Interleukin-10 is an anti-inflammatory cytokine, which is a major inhibitor of pro-inflammatory cytokine and chemokine production (Hong *et al.,* 2009). It is secreted by lymphocytes, macrophages and monocytes. Juge-Aubry et al. (2008) have found that IL-10 is also produced by human WAT explants following lipopolysaccharide (LPS) and TNF-α treatment *in vitro*. In addition, Esposito et al. (2003) conducted the first study which showed a significant relationship between lower levels of IL-10 and metabolic syndrome in women, independent of age and body weight. However, the main actions of anti-inflammatory cytokines in human obesity are unclear. Studies have reported that anti-inflammatory cytokines are suitable for the treatment of T2DM as IL-10 was shown to promote pancreatic β-cell function in response to glucose *in vitro* (Pennline *et al.,* 1994). In this regard, plasma levels of IL-10 are strongly associated with insulin sensitivity in healthy subjects and decrease in obese and diabetic patients (Scarpelli *et al.,* 2006).

Animal studies have demonstrated the protective effect of IL-10 against some inflammatory conditions including atherosclerotic lesion formation and stability (Esposito *et al.,* 2003). All these anti-inflammatory effects for IL-10 may relate to its role in the inhibition of TNF-α effect, lowering CRP (Manigrasso *et al.,* 2005) and IL-6 (Ouyang *et al.,* 2011). Furthermore, a positive relationship has been noted between IL-

10 and adiponectin, where adiponectin is able to increase the level of IL-10 in human macrophages (Kumada *et al.,* 2003).

1.4 Testosterone deficiency

Low testosterone is associated with a number of health problems in men including obesity, metabolic syndrome, diabetes and CVD, but the exact mechanisms involved are unclear. There is an association between testosterone levels and mortality in men, but causality has not been proven (Ma and Tong, 2010). Testosterone replacement therapy leads to improvement the signs and symptoms of low testosterone in men (Jones and Saad, 2010). Testosterone is an androgen male hormone, synthesised in Leydig cells of the testis and to a lesser extent in the adrenal glands and female ovaries. The core of its structure is cholesterol, which is the initial substrate in steroidogenesis (Figure 1.4). The steroidogenesis of testosterone is usually under luteinizing hormone (LH) control, which is produced by pituitary gland. The secretion of LH hormone is regulated by GnRH (Veldhuis *et al.,* 2009).

The binding of testosterone to specific proteins is required for transportation through the blood stream. 60-80% is bound to the sex hormone binding globulin (SHBG) and 20-40% is bound to albumin, whereas approximately 2% is transported as free testosterone (FT) (Kapoor *et al.,* 2005; Schwarcz and Frishman, 2010) (Figure 1.3). The physiological action of the androgen depends on diverse factors such as the number of androgen molecules, distribution of the androgen and its metabolites inside the cells and interaction with its receptors. The key functions of testosterone are maintenance of male reproductive organs and spermatogenesis, the induction of primary and secondary sexual characteristics in males and in the regulation of lipid metabolism (Nieschlag *et al.,* 2012). Testosterone acts following conversion to dihydrotestosterone by 5α-reductase, which is the bioactive form, in order to bind to its receptors (AR). It is converted to estradiol by aromatase and then stimulates the estrogen receptor (ER) (Corbould, 2007) (Figure 1.4). Testosterone action is regulated by both genomic and non-genomic mechanisms. For genomic mechanisms, the AR plays a role in the mediation of androgen action via activating transcription and altering gene expression. Testosterone can directly bind to inactive ARs in the cytoplasm, which forms a complex with its ligand (Pratt and Toft 1997, Defranco 2000; Wilson et al., 2011).

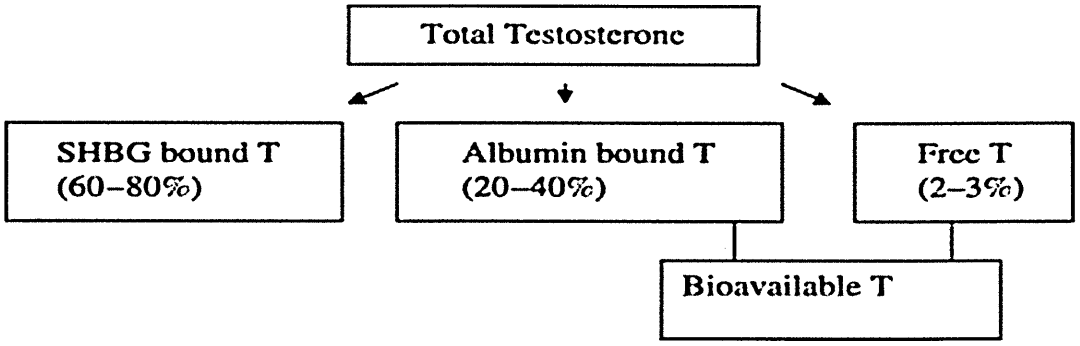


Figure 1.3: Fractions of circulating total testosterone in men. Non-SHBG-bound testosterone is called bioavailable testosterone and comprises both albumin-bound testosterone and free testosterone. Bioavailable testosterone is readily available to the tissues. T: testosterone. SHBG: Sex hormone binding Glubin (Adapted from Kapoor, 2005).

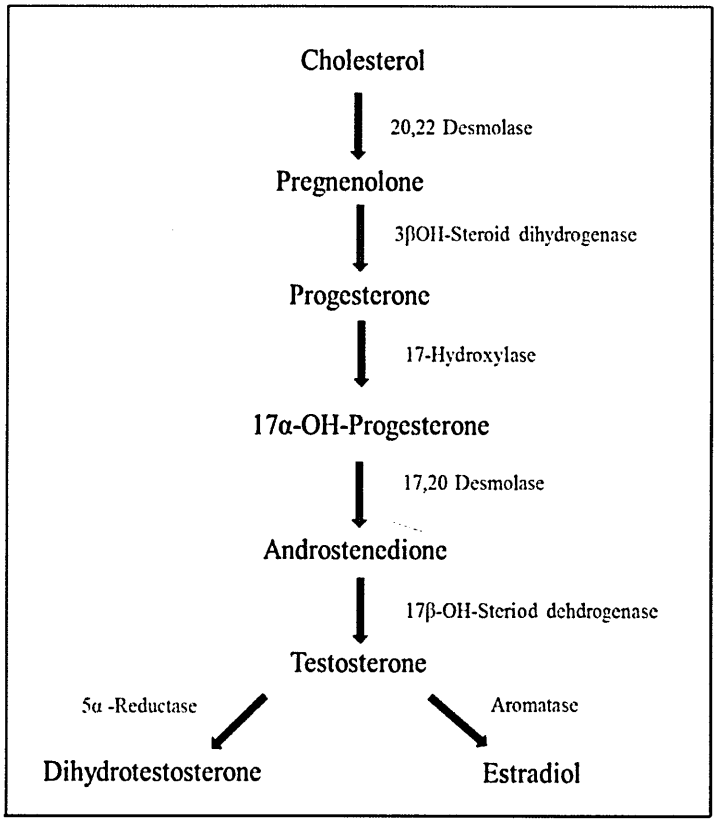


Figure 1.4: The Synthesis of testosterone pathway from cholesterol in human testis. Blue: Enzyme (Redrawn using information from Nieschlag *et al.*, 2012).

Upon ligand binding, the nuclear receptors translocate from the cytosol to the nucleus, where they dimerize and bind to regulatory DNA sequences on target genes, activate transcription leading to protein synthesis (Simental *et al.,* 1992, Isaacs, 2004; Wilson *et al.,* 2011). In addition, testosterone can act in a genomic mode, when it is converted to DHT in the cytoplasm by 5α-reductase by the same process (Rommerts, 2004). However, it should be noted that DHT has a much higher affinity for the AR than its precursor testosterone and has a slower detachment rate from the receptor complex, amplifying the effects of testosterone more than two-fold, therefore it is considered a more potent androgen. The other form of androgen activation is transformation of testosterone to estradiol by the enzyme aromatase, which diversifies androgen action by facilitating effects mediated via ERs. Consequently, while DHT may be considered a potent androgen because of its ability to bind to the AR, testosterone has a wider spectrum of action which includes diversification by aromatisation and hence ER mediated effects (Rommerts, 2004). In addition to this genomic effect for testosterone and DTH, it has been reported that androgens, as well as progesterone and estrogen, can exert quick, non-genomic actions (Falkenstein *et al.,* 2000).

Non-genomic androgen activity implicates the rapid induction of conventional second messenger signal transduction cascades, compared to significant delay between ligand binding and the effect on the genomic androgen receptor pathway. Furthermore, second messenger induction by non-genomic steroid action is insensitive to inhibitors of transcription and translation. Testosterone may display these non-genomic effects by binding to a G-protein coupled receptor specific for the SHBG-testosterone complex, which, in turn, initiates a cAMP-mediated non-transcriptional pathway, rapidly affecting intracellular calcium concentrations (Heinlein *et al.,* 2002). Calcium fluctuations are subsequently involved in the activation of intracellular signalling; ultimately influencing specific target proteins and cellular responses (Heinlein *et al.,* 2002) (Figure 1.5). As testosterone acts in genomic and non-genomic modes, it can have multiple functions in several tissues (Diano *et al.,* 1999; Schwarcz and Frishman, 2010). Several types of androgen receptor blockers such as flutamide, nilutamide and bicalutamide are used for determining the mechanism by which these androgens act. Inhibition of the main enzymes involved in metabolism of androgen, such as aromatase or 5α-reductase, is a further approach for understanding the action of this androgen.

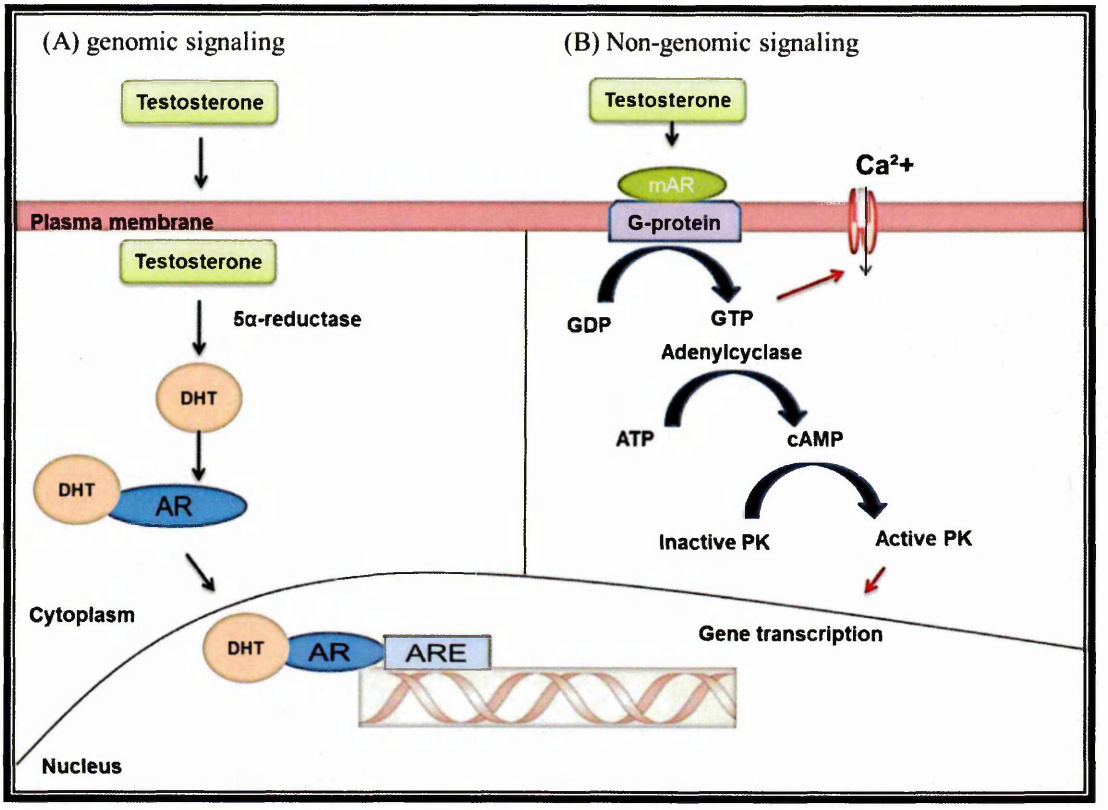


Figure 1.5: Summary of genomic and non-genomic of androgen action. The genomic signalling of androgen (A) converts testosterone to dihydrotestosterone by 5α reductase which binds to the AR. The binding of dihydrotestosterone to AR results in translocation to the nucleus. The androgen receptor dimer binds to a specific sequence of DNA known as a hormone response element. In the non-genomic of androgen action (B), testosterone mediates rapid effects through G-protein coupled receptors leading to effects including fast intracellular Ca^{2+} increases, activation of Ca^{2+}-dependent enzymes and second messenger cascades including cAMP, which activates protein kinase. AR: androgen receptor; DHT: dihydrotestosterone, mAR: putative membrane AR, ARE: androgen response element, GDP: Guanosine diphosphate, GTP: Guanosine-5'-triphosphate, ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate, PK: protein kinase. (Drawn using information from Lattouf *et al.,* 2006; McGrat *et al.,* 2008).

Advancing age and conditions such as diabetes and obesity are common causes of low testosterone (Gooren, 2003). According to the Endocrine Society's Clinical Practice Guideline, low testosterone is defined as a total testosterone (TT) concentration of less than 300 ng/dl or less than 10.4 nmol/l for total testosterone and less than 5 ng/dl or less than 0.17 nmol/l for free testosterone (Hall *et al.,* 2008).

Diagnosis of testosterone deficiency is dependent on both biochemical and clinical data. The period of the secretion of testosterone, which is usually in the early morning between 8:00 am and 11:00 am, should be taken into consideration when measuring TT in serum. Variations in laboratory methodology are another factor to be considered when reviewing these data (Crawford, 2007, Kapoor *et al.,* 2007; Schwarcz and Frishman, 2010).

1.4.1 Low testosterone and the link to obesity

The incidence of testosterone deficiency has increased along with the increase in obesity in aging men (Surampudi *et al.,* 2012). In recent studies, where body fat has been determined in relation to sex hormone concentrations in men, testosterone (or free testosterone) concentrations associate negatively with measures of central fat accumulation, rather than other peripheral adipose tissues (Alexandersen and Christiansen, 2004). Several epidemiological studies indicate an inverse relationship between serum testosterone and obesity. Low serum testosterone predicts the development of central obesity and accumulation of intra-abdominal fat. Also, low total and free testosterone and SHBG concentration are related to an increased risk of developing MetS, independent of age and obesity (Allan and McLachlan, 2010, MacDonald *et al.,* 2010; Brand *et al.,* 2011). Lowering serum testosterone levels in older men with prostate cancer, treated with androgen deprivation therapy (ADT), increases body fat mass (Faris and Smith, 2010). In the same way, high BMI and central adiposity are accompanied by and predict low serum total, and to a lesser extent free, testosterone and SHBG concentrations (Laaksonen *et al.,* 2005; Wang *et al.,* 2011). Low testosterone influences the increase in the degree of obesity, particularly in central positions, which indicates the inverse relationship between both total and free testosterone and SHBG and WHR (Van Aders *et al.,* 2005, Chandel *et al.,* 2008 and Mohasseb *et al.,* 2013). This may explain the development of IR as a result of obesity among low testosterone patients (Pradhan, 2007). It also explains the significant

relationship between the spread of T2DM and CVD among hypogonadal patients (Fu et al., 2008). Furthermore, in ADT of prostate cancer patients, both visceral and subcutaneous abdominal fat increased in a 12-month prospective observational study (Hamilton *et al.,* 2011). Generally, androgens can regulate different phases of adipocyte functions such as lipid metabolism and differentiation by their effect on ARs, which regulate its functions by activating the downstream genes in human adipose tissues. This seems to be related to the function of testosterone as a suppressor of lipoprotein lipase in adipose tissue, whereby this enzyme plays a significant role in the regulation of triglyceride uptake and lipid mobilization; it ultimately leads to reduction in visceral fat mass (Cohen, 1999; Traish *et al.,* 2009). This action is usually absent in the low testosterone individual and it consequently lead to augmentation of the level of abdominal fat body in a cycle of low testosterone levels and obesity (the hypogonadal–obesity cycle hypothesis) (Figure 1.6).

Other possible mechanisms of testosterone actions are related to its structure as a steroid hormone, which can be aromatized by aromatase in the adipose tissue to estradiol, causing a reduction in the level of testosterone. The aromatase level is often high in adipose tissue of obese individuals and is associated with testosterone conversation to 17b estradiol (E2). E2 has inhibitive action on hypothalamic production of GnRH and subsequent release of LH and FSH from the pituitary. This, in sequence, decreases gonadal stimulation and inhibits testosterone release, thus causing a state of hypogonadotrophism and the increase in obesity (Kelly and Jones, 2013). As a result of the increase in obesity, there is an increase in production of IL-6, TNF-α and leptin from visceral adipocytes. These mediators have the ability to inhibit LH and FSH hormone secretion by binding to their receptors in the pituitary gland. Therefore, the role of both hormones in the stimulation of released testosterone from the testes is absent. This leads to exacerbation of the testosterone deficiency state in obese people (Ding *et al.,* 2006, Jones, 2010). In addition, testosterone therapy led to a decrease in obesity in hypogonadal and eugonadal men (Dandona and Rosenberg, 2011). Moreover, testosterone replacement reduced IR and increased insulin sensitivity in obese people, which leads to decreasing symptoms of T2DM and CVD (Jones *et al.,* 2011). However, clinical findings are not as clear, for example, there was no change in visceral fat mass in aged men with low testosterone levels following 6 months of transdermal TRT, yet subcutaneous fat mass was significantly reduced in both the thigh and the abdominal areas when analysed by magnetic resonance imaging (MRI) (Frederiksen *et al.*, 2012b).

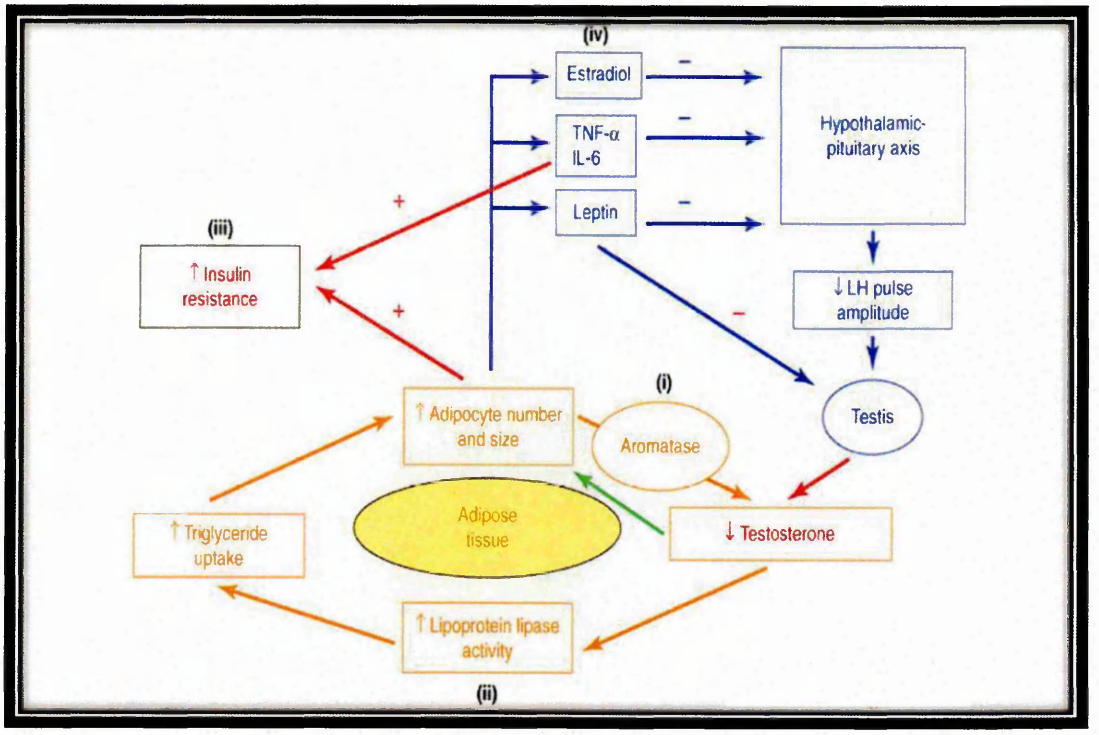


Figure 1.6: The Hypogonadal–Obesity–Adipocytokine hypothesis. (i) An increase in aromatase activity in adipocytes converts testosterone to estradiol. This decreases the suppressive testosterone action on lipoprotein lipase. (ii) Activation of lipoprotein lipase increases triglyceride up-take by adipose tissue. (iii) Increased adipocyte mass is associated with greater insulin resistance. (iv) Estradiol and adipocytokines TNF-α, IL-6 and leptin (as a result of leptin resistance in human obesity) impairs the hypothalamic–pituitary–testicular axis response to declining androgen levels (blue arrows). The orange arrows represent the hypogonadal–obesity cycle. Green arrow, low testosterone stimulates the formation of adipocytes from pluripotent stem cells. LH: luteinizing hormone +, positive effect; − negative effect. (Adapted from Jones, 2010).

In addition, testosterone treatment in the short-term decreased WC in diabetic patients (Jones 2010), whereas a placebo-controlled clinical study in middle-aged obese men found a neutral effect of testosterone on lean tissue mass after eight months of treatment and effects on fat tissue mass depended on the region measured (visceral fat tissue mass decreased, whereas subcutaneous fat tissue mass remained unchanged) (reviewed from Saad *et al.*, 2012).

1.5 Type two diabetes

Type two diabetes mellitus (T2DM) is a set of related diseases in which the body cannot regulate the amount of glucose in the blood. This is a result of IR, which is identified as the failure of target organs to respond normally to the action of insulin. IR causes the incomplete suppression of hepatic glucose output and impaired insulin-mediated glucose uptake in the periphery (skeletal muscle and adipose), leading to an increase in insulin requirements (Adilson *et al.,* 2008). The outcomes of IR include impaired insulin action which is known as the first stage of T2DM, glucose intolerance, hyperglycemia, hyperinsulinemia and dyslipidemia, which are collectively referred to as metabolic syndrome (MetS) (Tony and Sudhesh, 2005; Van Gaal *et al.*, 2010). Progressive development of IR is a prediabetic state which is today a common metabolic abnormality of people living in developed societies. According to the American Heart Association and the National Heart, Lung and Blood Institute (Tony and Sudhesh, 2005) clinical criteria for diagnosis of IR or MetS is dependent on the following features:

- Waist circumference >102cm in men and in women more than 88cm.
- Fasting triglyceride (TG) concentration >150mg/dl (1.7 mmol/l).
- HDL cholesterol <40 mg/dl
- Blood pressure ≥130/85mmHg
- Fasting glucose level >110mg/dl (>6.1 mmol/l).

1.5.1 The role of insulin in glucose and lipid metabolism

Insulin assists the transport of glucose from the blood by stimulating the translocation of GLUT4 to the plasma membrane (de Luca and Olefsky, 2008). It promotes storage of glucose in the form of glycogen in the liver by stimulating enzymes that are directly involved in glycogen synthesis such as phosphofructokinase and glycogen synthesase. When the liver is saturated with glycogen, any further glucose is converted to fatty acid synthesis (De Luca and Olefsky, 2008; Karpe *et al.,* 2011). These fatty acids are exported from the liver to other tissues by lipoproteins in the blood circulation. Lipoproteins act as transporters of fatty acids to other tissue such as adipocytes, which use them to synthesise triglycerides. Insulin also suppresses the degradation of fat in adipose tissues by inhibiting the lipoprotein lipase that hydrolyses triglycerides to release fatty acids (Frayn *et al.*, 2006). By these mechanisms, insulin is associated with further accumulation of triglyceride in fat cells leading to an increase in fat accumulation in adipose tissue (Nussey and Whitehead, 2013).

1.5.2. The effect of absence of insulin action on glucose and lipid metabolism

The absence of insulin action is usually as a result of a defect in insulin secretion or IR (Bailey, 2007). In cases of IR, there is a reduction in tissue capacity to respond to insulin even though there is hyperinsulinemia. Because of this, the body acts as if it is fasting in an insulin absence state. Thus, all the aforementioned effects of insulin are reversed. Firstly, an increase in the glucose concentration in blood leading to hyperglycemia is seen. Secondly, glycogenolysis and hepatic gluconeogenesis are increased (Silvio *et al.,* 2012). Finally, it provokes lipolysis in fat tissue as a consequence of the stimulation of hormone sensitive lipase and the inhibition of lipogenesis. This causes hydrolysis of the stored triglyceride, releasing large quantities of fatty acids into the blood (Figure 1.7). Consequently, the plasma FFAs then become the main energy substrate used by essentially all tissues of the body besides the brain. The excess of fatty acids in plasma also promotes liver conversion of some of the fatty acids into phospholipids and cholesterol, two of the major products of fat metabolism (Blaschke *et al.,* 2006). These two substances, along with excess triglycerides formed at the same time in the liver, are then discharged into the blood as lipoproteins with their co-receptors such as, Apo A1 and Apo B, Apo E.

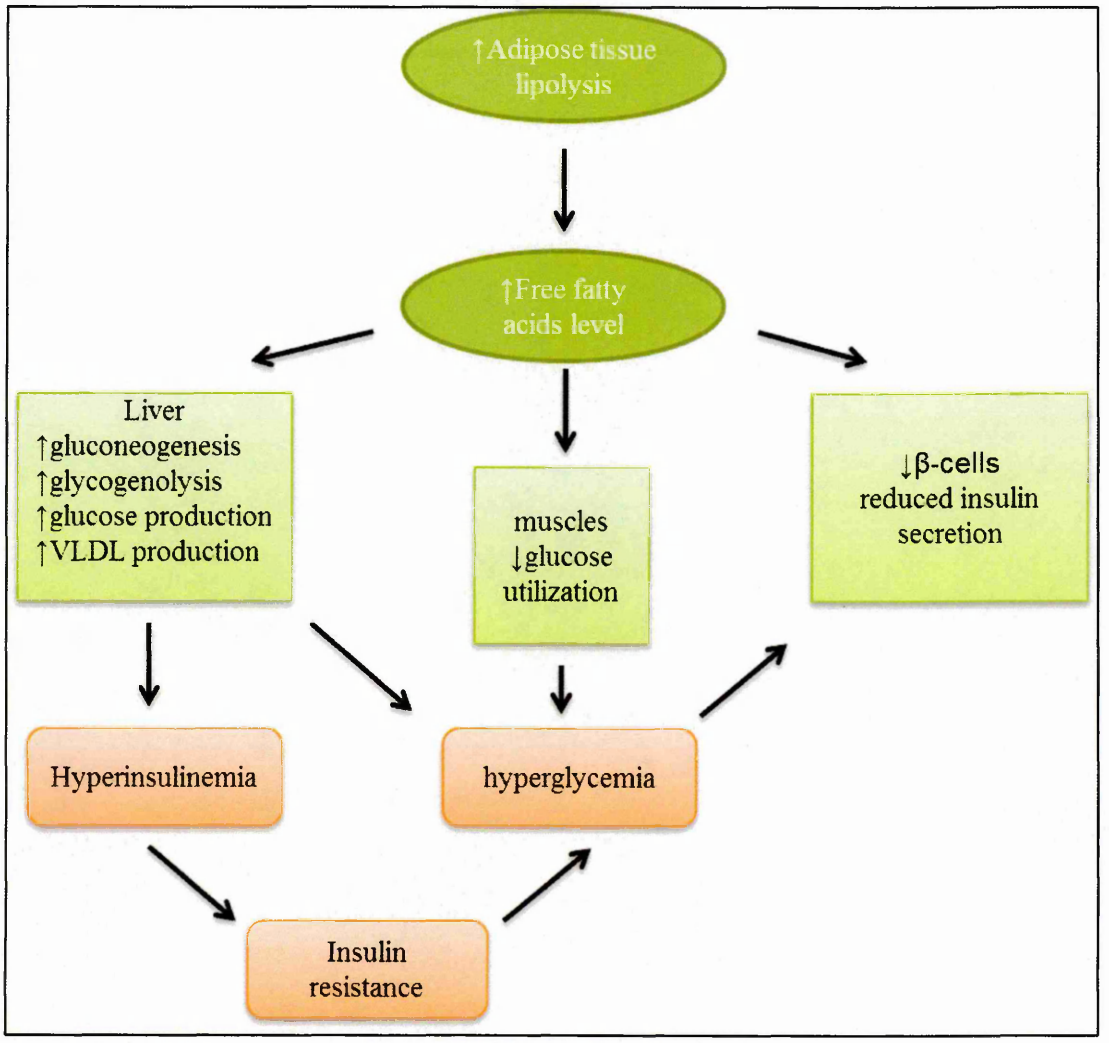


Figure 1.7: Association of lipolysis in adipose tissue with insulin resistance development. The exposure of the liver to elevated free fatty acid concentration results in increased (↑) very low density lipoprotein (VLDL) and glucose production resulting in peripheral hyperinsulinaemia which enhances insulin resistance and hyperglycemia. (Adapted and redrawn from Tony and Sudhesh, 2005).

Occasionally, plasma lipoproteins increase as much as three fold in the absence of insulin leading to a dyslipidemia state leading to CVD in T2DM patients (Tony and Sudhesh, 2005; Mooradia 2009).

1.5.3 Obesity and diabetes

The development of T2DM relates to several risk factors, but the most significant ones are defined by an increase in the prevalence of sedentary lifestyles and the incidence of obesity. Obesity is a powerful risk factor involved in the development of T2DM with more than two-thirds of patients with T2DM being obese (Hossain *et al.,* 2007). The risk of developing diabetes increased five-fold with BMI of 25kg/m^2 compared with those with BMI of 22 kg/m^2. The risk becomes higher, reaching 28-fold with BMI of 30 kg/m^2 and 93-fold with a BMI > 35 kg/m^2. IR is a common association with obesity, where obese people cannot use insulin efficiently and this leads to the progression to T2DM and its complications including, hyperglycaemia, dyslipidemia, hypertension and CVD (Tony and Sudhesh, 2005). There are different pathways and mechanisms that link obesity with IR, including the distribution of body fat, the role of free fatty acids (FFA), adipokines, pro and anti-inflammatory mediators and genetic factors.

1.5.4 The role of obesity in insulin resistance induction and diabetes

1.5.4.1 Adipose tissue distribution

The distribution of adipose tissue is an important factor in IR, for instance, visceral depots contribute more than peripheral adipose tissues to IR. The reasons include: visceral adipose tissue is metabolically and hormonally more active than other body fat tissue (Ferrannini *et al.,* 2008). The level of FFAs in central fat tissue is higher than in other sites (Fain, 2006). These higher FFAs can flux directly from the central fat tissue into the liver via the hepatic portal vein, compared with other adipose tissues, which drain into the liver via the systemic blood circulation (Kapoor *et al.,* 2005). In addition, the number of macrophages is higher in central body fat than in peripheral fat depots (Trayhurn and Wood, 2005). IL-6, which enhances CRP liver synthesis and induces IR, is also largely expressed in visceral abdominal adipose tissue, compared with subcutaneous abdominal tissue (Fried *et al.,* 1998; Kershaw and Flier, 2004). Moreover, the expression of genes for angiogenesis and fatty acid-binding protein 4 (require for

fatty acid transfer in adipocytes) are higher in visceral adipose tissue than in subcutaneous abdominal tissue (Wagenknecht *et al.,* 2003). Conversely, TNF-α synthesis is similar in both fat sites, whereas leptin secretion is mostly in subcutaneous adipose tissue (Bastard *et al.*, 2006).

1.5.4.2 Free fatty acid and Toll-like receptor 4

FFAs are more resistant to the metabolic effect of insulin and more sensitive to lipolytic hormones. An increased delivery of FFAs to the liver may reduce insulin binding to hepatocytes, and impair insulin action with increased hepatic glucose production (Caballero, 2003). These FFAs can also compete with glucose as an alternative source of energy. FFAs also reduce the response of cells in the liver and adipose tissue to insulin, via their effect on the receptors for insulin, which causes hyperinsulinemia (Boden *et al.,* 2005; Guilherme *et al.,* 2008). FFAs can stimulate TLR4 which belongs to the family of Toll-like receptors that function as pattern recognition receptors that guard against microbial infections as part of the innate immune system. The stimulation of TLRs leads to activation of NFκB, JNK and IkB kinase (IKK) signal pathway and then increased expression of TNF-α and plasma levels of CCL2, which enhance IR (Shi *et al.,* 2006; Huang *et al.,* 2006) (Figure 1.8).

1.5.4.3 Adipocyte-derived factors

Fat cells and macrophages in adipose tissue produce a range of mediators including TNF-α, IL-1β, IL-6 , resistin, PAI-1 ad CCL2, which play a role in the development of IR and T2DM in skeletal muscle, liver and endothelial cells (Caballero, 2003, Olefsky and Glass, 2010, Schenk *et al.,* 2008; Donath *et al.,* 2011). These adipocytokines can impair glucose tolerance through specific intracellular signalling pathways, involving NF-kB, IKK, Activating Protein-1 (AP-1) and JNK signalling pathway. All these pathways could interact with insulin signalling via serine/threonine inhibitory phosphorylation of IRS (Hajer *et al.,* 2008, Pradhan, 2007; Blaschke *et al.,* 2006) (Figure 1.8). On the other hand, a low concentration of anti-inflammatory cytokines such as adiponectin and IL-10 is associated with IR and hyperinsulinaemia (Makki, 2013). Adiponectin and IL-10 both reduced the level of IL-6 and TNF-α, which induce IR and correlate negatively with insulin sensitivity in human (Bruun *et al.,* 2003; Makki, 2013).

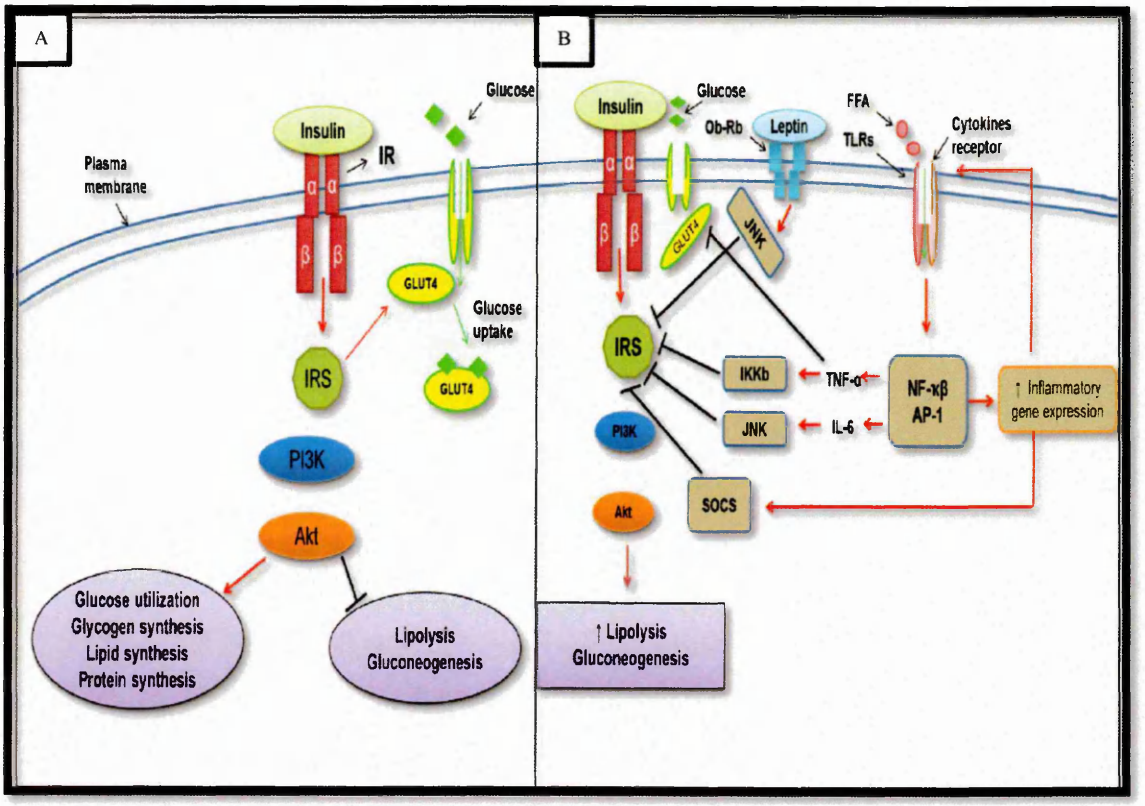


Figure 1.8: The pathway of insulin signalling and its impairment in inflammation. (A) Insulin binds to receptor with both α and β subunits. This ligand binding activates IRS, triggering of PI3K leading to the activation of AKT. The PI3K/AKT pathway regulates the insulin action on metabolic effects including glucose uptake and utilization, lipid synthesis, glycogen synthesis, protein synthesis, and glycogen synthesis). (B) Activation of TLRs by FFA inhibits IRS directly through activation of the NFκB and AP-1 signallings pathways or indirectly via stimulating pro-inflammatory cytokines (IL-6 and TNF-α) production, which prevent action of SOCS. TNF-α supresses GLUT4 or IRS via stimulation of the serine kinases, Ikkb. IL-6 and leptin act as negative regulators via stimulation of the Jnk1. All these action links inflammation to obesity-induced IR. IRS: Insulin Receptor Substrate, GLUT4: glucose transport 4, PI3K: Phosphoinositide 3-kinase, Akt: Protein Kinase B, FFA: free fatty acid, TLRs: Toll-like receptors, NFκB: nuclear factor kappa, AP-I: Activator Protein-I, IKKb: IkappaB kinase, JNK: Jun N-terminal kinases, SOCS: Suppressor of cytokine signalling, (Drawn using information from De Luca and Olefsky, 2008; Frühbeck *et al.*, 2001).

Furthermore, low adiponectin level, as well as leptin, may negatively affect biochemical reactions of glucogenesis and glucose uptake (Kajowaki and Yamauchi, 2005). Leptin as an insulin-sensitizing hormone and its deficiency, or resistance, as a potential link between obesity and diabetes, has been reviewed recently (Martin *et al.,* 2008; Cummings 2013) (Figure 1.8). Additionally, CX3CL1 and CCL2 are significantly increased in obesity and are good predictors for the development of T2DM (Panee 2012). Their role as important immune mediators in physiological and pathological processes might also translate into increased macrophage infiltration into adipose tissue, as observed in obesity (Tateya *et al.,* 2010; Shah *et al.,* 2011).

1.5.4.4 Peroxisome proliferation activated receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors which are ligand-modulated transcription factors that regulate gene expression of numerous proteins involved in lipid metabolism, glucose homeostasis and inflammatory pathway regulation (Shoelson et al., 2006). PPARα, PPARβ/δ and PPARγ are expressed in several tissues includes smooth muscle, vascular wall and adipose tissue (Issemann *et al.,* 1993, Shulman *et al.,* 2005; Filip-Ciubotaru *et al.,* 2011). These receptors are essential for the actions of many insulin sensitizers (Bhatia and Viswanathan, 2006). For example, PPARα null mice demonstrated that PPARα activates genes implicated in lipid metabolism in the liver including fatty acid uptake and β-oxidation taking place in the mitochondria (Im *et al.,* 2011). PPARδ is expressed in metabolically active sites such as liver, muscle, and fat, and has a role in metabolic syndrome (Lee *et al.,* 2006). PPARδ ligands reduce triglyceride accumulation in BAT and liver and enhance fatty acid oxidation in genetically obese mice (Tanaka *et al.,* 2003).

In models of high-fat diet-induced obesity, PPARδ ligands result in retarded weight gain indicating that clinical use of PPARδ activators could be beneficial as anti-obesity agents (Cho *et al.,* 2012). PPARδ is an important regulator of energy expenditure and glucose and lipid metabolism (Billin *et al.,* 2008), where it increases glycolysis/lipogenesis in the liver while activating fat burning in muscle (Lee *et al.,* 2006). PPARγ shows a significant anti-inflammatory action at the level of macrophage-mediated pro-inflammatory responses. It inhibits recruitment of macrophages to sites of inflammation via the suppressor of transcription of CCL2 as well as its receptor CCR2

(Szanto *et al.,* 2008). PPARγ activation has been shown to inhibit macrophage pro-inflammatory cytokines synthesis, TNF-α, IL-1β and IL-6 via an effect on NF-κB (Lefebvre *et al.,* 2006; Szanto *et al.,* 2008). However, it is clearly noted that PPARs contribute to the development of IR or impaired insulin secretion through their role in lipid metabolism, glucose homeostasis and inflammatory factor production, thus, PPARs are potential therapeutic targets for IR.

1.5.5 Low testosterone and the link to T2DM

IR and hyperglycemia are key features of T2DM, which is usually combined with abdominal obesity. Abdominal obesity is also associated with testosterone deficiency; therefore, obesity is linked to testosterone deficiency and T2DM. Overwhelming evidence demonstrates that a reduction in testosterone is associated with the progress of several clinical features linked to T2DM: total testosterone is conversely correlated with IR and insulin concentration in men (Simon *et al.,* 1997 and Jones, 2010). Furthermore, numerous studies indicate that testosterone deficiency is a predictor for the prevalence of IR and T2DM in healthy men (Oh *et al.,* 2002; Jones and Saad, 2009). Testosterone deficiency is more widespread among male diabetic patients (Grossmann *et al.,* 2008) and hypogonadism cases are also coupled with T2DM (Kapoor *et al.,* 2007).

It is known that central obesity is associated with low testosterone and *vice versa*. In a German study, low level testosterone was observed with an inverse correlation with BMI in 155 diabetic patients compared to 155 healthy controls (Zietz, 2000). An improvement in IR and a significant reduction in the level of fasting post-prandial and mean daily blood glucose were initially observed in men with T2DM who were treated with testosterone (Boyanov *et al.,* 2003). Possible explanations for the inverse relationship between testosterone and IR are: firstly, a significant reduction in central obesity following testosterone treatment leads to reduced level of aromatase in adipose tissue, thus inhibiting conversion of testosterone to estradiol and prevention of a negative feedback of estradiol on testosterone secretion via the hypogonadal pituitary axis (Dandona and Dhindsa, 2011). Secondly, FFAs are associated with IR, therefore, testosterone replacement therapy by decreasing abdominal fat mass leads to reduced circulating levels of FAAs and subsequently IR. The effect of testosterone on reduction of central obesity could be by decreasing the uptake of triglycerides to adipocytes

through inhibition of the lipoprotein lipase enzyme activity (Marin *et al.,* 1992; Kapoor *et al.,* 2005). Finally, further actions of testosterone in T2DM include: the action of testosterone as an anti-inflammatory factor that inhibits pro-inflammatory cytokine and chemokine expression especially associated with IR (Pittas *et al.,* 2004; Norata *et al.,* 2006).

It has long been known that testosterone acts as an immune-modulatory factor and its reduction is accompanied by the development of subclinical inflammatory states (Malikan *et al.*, 2004). It is known that an increase in inflammatory factors such as IL-6, TNF-α and IL-1β is accompanied by lowering of testosterone in obesity cases. Therefore, this may demonstrate the presence of an inverse relationship between the pro-inflammatory factors and testosterone level. Accumulating evidence suggests that testosterone can inhibit pro-inflammatory factors secretion and stimulate anti-inflammatory mediator release (Vodo *et al.,* 2013). Furthermore, reduction in the levels of IL-6, IL-1β and TNF-α secreted by monocytes was noted in androgen deficient men with T2DM after testosterone treatment (Corrales *et al.*, 2006). Short-term studies indicated that there was an inverse relationship between testosterone level and CRP, IL-6 and TNF-α, in diabetic and non-diabetic hypogonadal men (Kapoor *et al.*, 2007). However, the reason for this inverse relationship is not fully known, (Beavers *et al.*, 2010) but the reduction of fat mass by testosterone treatment may be the reason for a lower inflammation and explain this inverse relationship. However, the cellular mechanism of testosterone action on inflammatory factors remains unclear.

1.6 Atherosclerosis

Atherosclerosis is the main cause of increased rates of cardiovascular death in the developed world. It is an inflammatory disease, characterized by lesions in the large and middle arteries containing lipids, immune infiltrates, particularly monocytes/ macrophages and T cells, connective tissue elements and debris (Hansson *et al.,* 2006; Erzengin, 2014). This can lead to myocardial infarction in the heart and/or to ischemic stroke (Koelink *et al.,* 2012). The cause of atherosclerosis is a complex combination of genetic factors and metabolic disorders such as obesity and T2DM. Endothelial dysfunction is central to the pathogenesis of atherosclerosis (Tardif, 2009). The result of endothelial injury is an increase in leukocyte adhesion molecules such as intercellular

cell adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Nakashima *et al.,* 1998, Monaco and Paleolog, 2004; Lucas and Greaves, 2001). There is also an increase in pro-inflammatory and pro-atherogenic factors including cytokines, chemokines and their receptors. All these contribute to exacerbation of the damage to the endothelium, with increased monocyte recruitment into the sub-endothelial space, where they differentiate into macrophages. These recruited macrophages take up LDL via scavenger receptors to form foam cells, the hallmark of fatty streak lesions (Qiu *et al.,* 2006 and Stenmark *et al.,* 2013). The lesions develop into fibro-fatty plaques, which contain large numbers of macrophages, fibrous cap and migrated and proliferated smooth muscle cells (Figure 1.9). When plaques are damaged and rupture, pro-thrombotic material is exposed to the coagulation system, with ensuing inhibition of blood flow and induction of CVD (Libby *et al.,* 2011; Frostegård, 2013).

1.6.1 The role of monocyte / macrophage cells in atherosclerosis

In humans, accumulation of foam cells derived from phagocytosis of lipids by monocytes and macrophages is seen in aortic fatty streaks (Auffray *et al.*, 2009, Gordon, 2003; Takahashi *et al.*, 1996) and these are the main leukocyte subset that accumulates at lipid laden vascular sites in atherosclerosis (Ley *et al.,* 2011). The production of leukocyte chemoattractant molecules activates receptors on rolling monocytes leading to integrin-dependent firm adhesion to the endothelium and subsequent diapedesis into the sub-endothelium (Golias et *al.,* 2007). Migration of monocytes into the vessel intima leads to differentiation of these cells into macrophages in response to the local over-expressed macrophage-colony-stimulating factor (M-CSF) (Libby *et al.,* 2002, 2010, Glass and Witztum 2001; Koelink *et al.,* 2012). Inside the arterial intima and under atherogenic conditions, macrophages accumulate in the aorta and show reduced emigration from lesions (Hansson *et al.*, 2001; Ley *et al.,* 2011). In ApoE and MCS-F (op/op) deficient mice, which are deficient in tissue macrophages, are dramatically protected from atherosclerosis, despite high levels of cholesterol (Smith *et al.,* 1995; Linton and Fazio, 2003).

Macrophages secrete TNFα and IL-1β and chemokines including CX3CL1, CCL2 and IL-8 which are associated with local inflammation (Lusis, 2000, Lucas and Greaves, 2001, Braunersreuther *et al.,* 2007, Tabas *et al.,* 2007; Koltsova and Ley, 2011).

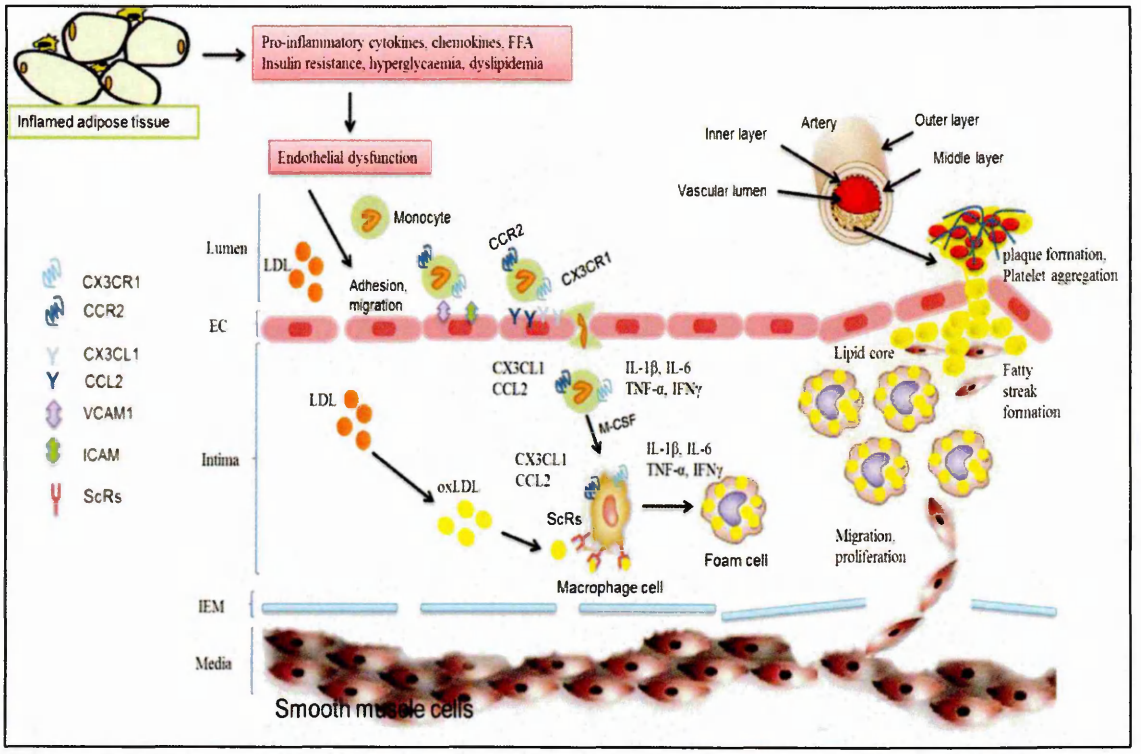


Figure 1.9: The progression of atherosclerotic plaque as a result of obesity and its complications. The conditions associated with obesity including high levels of proinflammatory factors, insulin resistance, hyperglycemia and dyslipidemia can causes endothelial dysfunction. This leads to production of adhesion molcules, VCAM1, ICAM and chemokines by endothelial cells (EC). Monocytes migrate into the arterial wall in response to binding of their chemokine receptors, CX3CR1 and CCR2 with ligands CX3CL1 and CCL2. Monocytes differentiate into macrophages in response to local macrophage colony-stimulating factors (M-CSF). The LDL in the artery wall is modified to oxidized LDL (oxLDL) and loaded onto macrophage scavenger receptors (ScRs) resulting in formation of foam cells. Ox-LDL and chemokines stimulate the production of IL-1β, IL-6, IFNγ and TNF-α by macrophages and foam cells. More advanced stages of atheroseclerosis include smooth muscle cell proliferation, formation of fibrous caps, necrotic cores, calcification, rupture, haemorrhage and thrombosis. IEM: internal elastic membrane. (Drawn using information from Lucas and Greaves, 2001, Barlic and Murphy, 2007, Liu and Jiang, 2011).

Macrophages are considered as the main source of IL-8 in atherosclerotic plaques, which can modulate migration of monocytes and accumulation of macrophages in the endothelial space (reviewed from Apostolakis *et al.,* 2007). Thus, IL-8 is suggested as a proatherogenic chemokine acting as a factor linking immune activity and lipid metabolism (Autieri *et al.,* 2012). Scavenger receptors (ScRs) associated with uptake of modified LDLs by macrophages are under the control of cytokines in lesions (Menno *et al.,* 2000). ScRs regulate the load of oxidised LDL in macrophages and lead to LDL cholesterol accumulation and subsequently the transformation of macrophages into foam cells in early- through to mid-stage atherogenesis (Greaves and Gordon, 2009, Hansson 2005; Williams and Tabas, 1998). Foam cells are generated from the massive uptake of modified LDLs and the intra-cytoplasmic accumulation of cholesteryl esters, a source of growth factors, pro-inflammatory and pro-atherogenic factors in all stages of atherosclerosis. All these factors contribute to smooth muscle cell proliferation, matrix production and actions of metalloproteases, leading to matrix degeneration (Dollery *et al.,* 2006, Libby, 2008; Libby, 2009). The continued accumulation and subsequent apoptosis of plaque cells, leads to the formation of a necrotic core and results in the progressive narrowing of the arterial lumen. This can then trigger thinning of the fibrous cap and its disintegration, with plaque erosion or rupture, leading to thrombus formation and vascular occlusion underlying coronary syndromes, myocardial infarction, or stroke (Glass and Witztum, 2001, Libby, 2002, Hansson, 2005, Hansson and Libby, 2006, Libby, 2008; Weber *et al.,* 2008).

1.6.2 The role of atherogenic biomarkers in atherosclerosis

Measurement of standard lipid cholesterol and TG and lipoproteins are vital for assessing the risk of CVD. These measurements are obtained routinely in clinical practice (Blaha *et al.,* 2008). Lipoproteins are essential in the metabolism and redistribution of lipids such as cholesterol, phospholipids and triacylglycerol. There are several classes of lipoproteins, which are used to transport lipids throughout the body and range in density and content (protein/lipid ratio); chylomicrons (contain dietary lipids), intermediate low density lipoprotein (IDL), VLDL, LDL and the high density lipoprotein (HDL) (Acevedo, 2012). Lipoproteins contain small particles termed apolipoproteins that function as regulators of binding between lipoproteins and receptors. These proteins act as enzyme co-factors during lipid metabolism, helping to

stabilize lipoproteins during transportation from cell or tissue to its destination (Han, 2004). Apolipoproteins are one of the key players in progression of atherosclerosis. Alaupovic (1971) first suggested that measuring apolipoproteins might be more valuable in predicting the risk of CVD than measurement of the lipid components or lipoprotein (Rainwater *et al.,* 1999 and Mora, 2009; Jacobson, 2011). Furthermore, Cholesterol, TG and lipoproteins have been studied extensively for their effect and role in atherosclerosis. Recently, it has been suggested that studies should focus on other factors that have roles in atherosclerosis e.g, studies have investigated the role of oxLDL, fractions of HDL such as HDL2 and HDL3 and apolipoprotein (Parish *et al.,* 2009).

1.6.2.1 The role of oxLDL in atherosclerosis

Animal studies have provided evidence to support the role of oxidative stress in atherosclerosis, particularly through oxidative modification of LDL (Fraley and Tsimikas, 2006; Bossola *et al.,* 2011). oxLDL is a useful marker for CVDs (Itabe *et al.,* 2011, Fraley and Tsimikas, 2006, Itabe and Ueda, 2007; Holvoet *et al.,* 2008). The measurement of oxLDL correlates with CVDs and indicates that oxLDL is a potential prognostic marker for future health events (Itabe *et al,* 2011). oxLDL has the ability to stimulate a number of pro-atherosclerotic effects, including endothelial cell activation, smooth muscle proliferation and monocyte/macrophage recruitment (Ahmed *et al.,* 2009).

Berliner and co-workers (1990) stated that the oxidative modification of LDL, through co-culture with endothelial cells, had stimulatory effects on many cell types and that these effects were due to the oxidized phospholipids generated by oxLDL. For example, oxLDL and its free lipid constituents have been shown to promote monocyte recruitment and inhibit macrophage motility. The effect of oxLDL has been clearly seen through control of monocyte gene expression (Tontonoz *et al.,* 1998). OxLDLs induce functional alteration of monocytic regulation of monocyte chemoattractant receptors, by inducing specifically differentiation of $CCR2^{high}$ $CX3CR1^{low}$ monocytes to $CCR2^{low}$ $CX3CR1^{high}$ macrophages that strongly adhere to $CX3CL1^{+}$ expressed by primary human coronary artery smooth muscle cells (CASMCs) under static conditions (Barlic *et al.,* 2006). This observation indicates that in atherogenesis, there is oxidized lipid-driven activation of macrophage PPARγ in the intima which results in a pro-adhesive chemokine receptor switching CCR2 off, CX3CR1 on, causing cessation of CCR2-

dependent migration and activation of CX3CR1-dependent retention mechanisms, which together promote macrophage accumulation in the vessel wall (Wong *et al.,* 2002; Lesnik *et al.,* 2003). Moreover, oxLDL can increase migration of monocyte cells by enhancing VCAM-1 expression by endothelial cells, which leads to adhesion of these cells within the endothelial space (Calara *et al.,* 199; Aikawa *et al.,* 2002). In addition, oxLDL is recognized to induce foam cell formation and inflammatory responses, where it stimulates expression of a wide diversity of pro-inflammatory cytokines and chemokines in macrophages (Lee *et al.,* 2000). Finally, it has a role in promoting endothelial cell expression of M-CSF leading to differentiation of monocytes to macrophages (Cushing *et al.,* 1990 and Lee *et al.,* 2000). OxLDL also helps to induce release of CCL2 from endothelial cells (reviewed by Pirillo *et al.,* 2013). Although the pathological features of OxLDL have been well considered, the formation, distribution, and overall fate of OxLDL *in vivo* remains unclear (Itabe *et al.,* 2011).

1.6.2.2 The role of Apo A1 and HDL in atherosclerosis

Apolipoprotein A1 is a protein (264 amino acids) that in humans is encoded by the APOA1 gene (Eriksson *et al.,* 2009). It has a specific role in lipid metabolism, is a major protein constituent of HDL particles, has pleiotropic biological functions such as stimulating macrophage cholesterol efflux within artery walls, making the macrophage cells less likely to become fat overloaded, transform into foam cells, die and contribute to progressive atheroma. It also functions by motivating reverse lipid transport, inhibiting LDL oxidation and scavenging toxic phospholipids. Furthermore, it has the ability to improve pancreatic β-cell health and function, can act as an anti-thrombotic factor as well as having anti-inflammatory properties (Navab *et al.,* 2011, Shah 2011). HDL cholesterol and its major protein constituent, (ApoA1), have been shown through clinical and epidemiological studies to have a strong inverse correlation with the development of atherosclerosis and myocardial infarction (reviewed by Chapman *et al.,* 2010).

HDL particles have multiple functions including cholesterol transport (Dastani *et al.,* 2006) and modulation vascular endothelial function by promoting the production of the atheroprotective signalling molecule NO (Mineo *et al.,* 2006), protection against LDL particle oxidation and anti-inflammatory properties (Genest *et al.,* 2003; Marcil *et al.,* 2004). Their role in reverse cholesterol transport, which is transport of cholesterol from peripheral tissues to the liver for secretion into the bile for excretion, made HDL

particles the most important protective factor against the development of atherosclerotic cardiovascular disease (Maeda *et al.,* 2011). Two pathways are involved in these processes. A first pathway is the production of apoA1 from liver and intestine which binds to the cellular ATP-binding cassette transporter (ABCA1) and forms of nascent HDL particles (Wang *et al.,* 2008). Nascent HDL becomes mature HDL following loading by cholesterol from peripheral, extra-hepatic tissues, and arterial tissue (potentially including cholesterol-loaded foam cell macrophages of the atherosclerotic plaque). The esterification of free cholesterol in HDL occurs by lecithin-cholesterol acyl transferase (LCAT) leading to spherical HDL3 and HDL2 particle formation. These subfractions mediate transport of cholesterol to the liver for excretion (Natarajan *et al.,* 2010). The second pathway is by the esterification of free cholesterol in HDL which is made by cholesterol ester transfer protein (CETP), exchanging the core cholesteryl esters of HDL for triglycerides to Apo B-containing lipoproteins mainly VLDL and LDL and then subsequent uptake primarily by hepatic LDL receptors or into the circulation (Kontush *et al.,* 2006; Chapman *et al.,* 2010). Therefore, defects in the gene encoding Apo A1 are associated with HDL deficiencies (Alexander *et al.,* 2009).

HDL can be separated by ultra-centrifugal methods into two main subfractions: lipid-rich HDL2 and lipid-poor HDL3. In addition, it is thought that one or both of the HDL fractions is more related to the risk of CVD than total HDL cholesterol (Bakogianni *et al.,* 2001; Superko *et al.,* 2012). Prospective studies were reported that lower HDL2 and HDL3 is predicted risk for CVD, one showed lower HDL2 cholesterol is powerful indicators of CHD (Williams *et al.,* 2012). A low HDL level in subjects with insulin resistance primarily results from a decrease in the HDL2 and, to some extent, HDL3 levels (Muth *et al.,* 2010). However, suggestions that all subfractions of HDL particles display atheroprotection, through one or more mechanisms, are plausible (Superko *et al.,* 2012). Furthermore, high circulating levels of ApoA1 of HDL have been shown to predict decreased risk of CHD (Chapman *et al.,* 2010).

1.6.2.3 The role of Apo E in atherosclerosis

Apolipoprotein E (ApoE) is a glycoprotein (299 amino acids) and a constituent of chylomicrons, intermediate-density lipoprotein (ILD), LDL, HDL and VLDL. ApoE is encoded by APOE gene and mainly produced by the liver, monocytes and macrophage cells (Hara *et al.*, 2009). It acts as a ligand for lipoproteins with receptors for clearance of lipid from the circulation and for cholesterol metabolism (Singh *et al.,* 2002).

According to studies in mice lacking ApoE (Wu and Huan, 2007) there was accumulation of remnant lipoproteins (VLDL and chylomicron) with total plasma cholesterol levels exceeding 400 mg/dl, even though mice were fed on low fat chow (Kunjathoor *et al.,* 1996). Similarly, in humans with deficiency in ApoE there is increased plasma cholesterol and triglycerides, which are the consequence of impaired clearance of chylomicron, VLDL and LDL remnants (Tennert *et al.,* 2007).

The role of ApoE in atherosclerosis development relates to its synthesis and metabolism by macrophages within vessels (Rosenfeld *et al.,* 1995), which has a local effect on cholesterol homeostasis and on inflammatory reactions in atherosclerotic vessels. In macrophages, the anti-atherogenic effects of endogenous and exogenous ApoE are the induction of cholesterol efflux, thereby stimulating reverse cholesterol transport (Greenow *et al.,* 2005, Curtiss and Boisvert, 2000, Burt *et al.,* 2008). An additional significant atheroprotective effect of ApoE in macrophages was observed through its inhibition of LDL-oxidation and stimulation of enzymes that are associated with lipoprotein metabolism (Walpola *et al.,* 1993, Miyata and Smith, 1996). Furthermore, ApoE has anti-inflammatory actions by regulation of inflammation and cell proliferation, inhibiting expression of adhesion molecules, decreasing the migration of monocytes into the lesion (Stannard *et al.,* 2001) and preventing the migration and proliferation of smooth muscle cells (Kothapalli *et al.,* 2004; Zhu and Hui, 2003).

To assess the role of ApoE in atherogenesis, a number of approaches have been taken including: bone marrow transplantation and transgenic overexpression. Previous studies reported that transplanting bone marrow from mice with normal Apo E gene onto Apo E deficient recipients led to normalization of cholesterol level in the serum and prevented atherosclerosis. This was a result of an increase in ApoE in the serum enhancing lipoprotein clearance (Linton *et al.,* 1995; Huang *et al.,* 2013). Hepatic overexpression of ApoE in ApoE knockout mice prevented the development of atherosclerotic lesions, suggesting plasma ApoE has a role in protecting the arterial intima (Stevenson *et al.,* 1995; Kashyap *et al.,* 1995). This was noted through accumulation of ApoE within preexisting atherosclerotic lesions and also through the induction of morphological changes in lesions, including decreased foam cells and increased smooth muscle cells and extracellular matrix content (Tsukamoto *et al.,* 1999).

In further research, transplantation of ApoE−/− bone marrow onto C57BL/6 (B6) mice did not lead to changes in plasma lipid and lipoprotein levels, but increased atherosclerosis and foam cell formation compared to controls transplanted with ApoE+/+ bone marrow. The athors showed that the expression of ApoE in macrophages was responsible for the anti-atherogenic effect of transplanted bone marrow (Fazio *et al.,* 2002). Moreover, Shimano *et al.* (1995) established transgenic mice that expressed human ApoE in the vessel wall and identified a reduction in atherosclerotic lesions, in the absence of any change in plasma cholesterol. In addition, $ApoE^{h/h}Ldlr^{-/-}$Mx1-cre mice develop spontaneous hyperlipidemia and atherosclerosis on a chow diet (Gaudreault *et al.,* 2012) but following inducible repair of the hypomorphic ApoE alleles in these mice had decreased plasma lipids and ApoB lipoprotein levels, rise in plasma HDL-cholesterol and ApoA1 levels (Eberlé *et al.,* 2013) supporting the role that of hepatic ApoE in the clearance of remnant lipoproteins in the absence of the LDL receptor (Linton *et al.,* 1995) and in promoting HDL expansion and remodelling (Mahley *et al.,* 2006).

1.6.2.4 The role of Apo B in atherosclerosis

Apolipoprotein B (ApoB) is a large protein (4536 amino acids) structural component of all lipoproteins including LDL, intermediate density lipoprotein (IDL), very low-density lipoprotein (VLDL), and LP (a) with the exception of HDL (Kappelle *et al.,* 2011; Lee *et al.,* 2011). ApoB is encoded by APOB gene and has two forms, ApoB-48 and ApoB-100. In humans, ApoB-48 is synthesized in the intestine, where it is complexes with dietary TG and free cholesterol, absorbed from the gut lumen, to form chylomicron particles. ApoB-100 is synthesized in the liver and is found in LDL, IDL and VLDL particles (Walldius and Jungner, 2004). In mice, both ApoB-48 and ApoB-100 are secreted from the liver (Dallinga-Thie, 2010). ApoB is absolutely required for formation of LDL and assist with binding to its receptor in different tissues, allowing cells to internalize LDL and thus absorb cholesterol (Parish *et al.,* 2009).

Increasing evidence indicates that ApoB is a superior marker of vascular disease compared with LDL cholesterol, as ApoB represents the total amount of potentially atherogenic circulating lipoproteins (Walldius and Jungner, 2004, Barter *et al.,* 2006, Ley *et al.,* 2010; Kappelle *et al.,* 2011). Control studies for patients with CHD have found plasma Apo B levels to be more characteristic of disease than other plasma lipids and lipoproteins (Kwiterovich *et al.,* 1992). A further study showed that ApoB was a

stronger predictor of risk than LDL-cholesterol in both men and women (Walldius *et al.,* 2001). This study found that ApoB and the Apo B/Apo A1 ratio should be taken into account as it is highly predictive in evaluating cardiac risk.

In recent years, several groups have used homologous recombination in mouse embryonic stem cells to generate Apo-E-deficient mice and LDL receptor-deficient mice (Maeda, 2011). Both of these targeted mutations interfere with the clearance of Apo-B-containing lipoproteins from the plasma; in both of these animal models, the plasma levels of the Apo-B-containing lipoproteins are elevated and there is increased susceptibility to the development of atherosclerosis. In Apo-B transgenic mice, the high levels of LDL in chow-fed mice were clearly due, at least in part, to the over-production of Apo-B in the liver (Linton *et al.,* 1993). However, mice with modified ApoB-100 had decreased, not increased, LDL particles, in part because of a reduced secretion of modified-ApoB100 particles (Toth *et al.,* 1996, Johnson *et al.,* 2008).

The inconsistencies in the literature when considering Apo B as a good predictor risk of atherosclerosis may be due to the absence of standardised assays for measuring plasma ApoB levels and attempts to develop these methods are ongoing (Carmena *et al.,* 2004; Contois *et al,* 2011).

1.6.2.5 The role of lipoprotein (a) in atherosclerosis

Lipoprotein (a) LP(a) is a protein that is encoded by LPA gene. LP(a) consists of an LDL-like particle and is bound to the highly glycosylated Apo(a) and Apo B in a 1:1 molar ratio, through one or more disulphide bridges (Carmena *et al.,* 2004). Apo (a) is detected in the liver where it is expressed by hepatocytes. The assembly of Apo (a) and LDL particles occurs at the outer hepatocyte surface (Saba and Oridupa, 2012). The half-life of LP (a) in the circulation is about 3 to 4 days, (Rader *et al.,* 1993) and there is limited information about its metabolism. Similarly, the physiological function of LP (a) is largely unknown (Itabe *et al.,* 2011).

LP(a) levels are genetically determined and are an independent risk factor for atherosclerosis (*Enas et al.,* 2006). Moreover, its level in serum has been found to be an inherited risk factor for IHD (Dembinski *et al.,* 2000) and myocardial infarction (Kamstrup *et al.,* 2008; Kamstrup *et al.,* 2011). The relationship between elevated LP (a) levels and CHD was confirmed by a number of retrospective case-control studies. A meta-analysis of prospective studies showed that plasma LP (a) concentration is an

independent risk factor for CHD in both men and women (Craig *et al.,* 1998). Another prospective study found that LP (a) is an independent risk factor for MI and CHD in elderly men (Gaw *et al.,* 2005).

However, four studies found no relationship between LP(a) and CVD (Seman *et al.,* 1999). These discrepancies may result from the lack of standardization and the failure of some immunoassays to measure all Apo (a) isoforms equally (Seman *et al.,* 1999). However, this was finally clarified with the data obtained from Epidemiological Study of MI (PRIME) which included 9133 men with no history of CHD or use of hypolipidemic medicines, that indicated LP (a) as a strong predictor of CHD risk (Luc *et al.,* 2002). In addition, Apo (a) in LP (a) has a similar structure to plasminogen and can compete with plasminogen for binding to the plasminogen receptor. This leads to inhibited conversion of plasminogen to plasmin. Plasmin decreases fibrin blood clots therefore with less plasminogen to degrade fibrin, the concentration of fibrin increases and promotes thrombogenesis (Caplice *et al.,* 2001; Sofi *et al.,* 2007). Because of the unique structure of LP(a) combined with the potential atherogenic risk associated with LDL particles and the thrombogenic risk attributed with Apo (a), LP(a) is considered as a risk factor for atherosclerosis and CVD (Nordestgaard, *et al.,* 2010). Moreover, Lp(a) transports the more atherogenic pro-inflammatory oxidized phospholipids, which attract inflammatory cells to vessel walls (Gouni-Berthold *et al.,* 2011; Tsimikas *et al.,* 2008) and causes smooth muscle cell proliferation (Ichikawa *et al.,* 2002).

1.6.3 The role of pro-inflammatory cytokines in atherosclerosis

Pro-inflammatory cytokines including IL-1β, IL-6, TNF-α and IFN-γ together or individually play a role in development of atherosclerosis. These cytokines that are produced at a distance from adipose tissue or locally in the artery wall can affect atherosclerosis and potentially play a role in various stages of atherosclerosis (Kleemann *et al.,* 2008). In the early stages, TNF-α and IFN-γ can cause imbalance in barrier function of endothelial cells and promote leukocyte transmigration by changing the distribution of vascular endothelial cadherin-catenin complexes and, inhibiting the formation of F-actin stress fibres (Kleinbongard *et al.,* 2010). TNF-α is able to transiently induce a rise in calcium and stimulation of myosin light chain kinase, which causes endothelial junction impairment (Komarova *et al.,* 2010; Ait-Oufella *et al.,* 2011).

Pro-inflammatory cytokines can stimulate attraction of monocytes to endothelial cells by enhancing expression of adhesion molecule, such as ICAM-1 and VCAM-1 and enhancing the up-regulation of selectins and integrin ligands, which are part of the leukocyte adhesion cascade (Weber *et al.,* 2008; Ait-Oufella *et al.,* 2011). Furthermore, IL-1β, IL-6, (Bazan *et al.,* 1997; Garcia *et al.,* 2000), TNF-α (Ahn *et al., 2*004; Ollivier *et al.,* 2003; Lesnik *et al.,* 2003), IFN-γ (Bazan *et al., 1*997, Imaizumi *et al.,* 2000, Ollivier *et al.,* 2003; Lesnik *et al.,* 2003) mediate the migration of monocytes through endothelial cells by activating expression of chemokines (e.g. IL-8, CCL2 and CX3CL1) on endothelial cells, which bind to their receptors on monocytes and macrophages. Similarly, these locally synthesised cytokines, produce by macrophages cells, SMCs and ECs, can regulate and accelerate the transformation of macrophages into foam cells by increasing the expression of scavenger receptors and enhancing cell-mediated oxidation (Ait-Oufella *et al.,* 2011). While IFN-γ has the ability to amplify the expression of pro-inflammatory cytokines including TNFα and IL-1β by monocytes (Butler *et al.,* 1994; Haworth *et al.,*1991) and macrophages (Mallat *et al.,* 2001), TNF-α can trigger the release of IL-1β, IL-6, IL-8 from monocytes, macrophages and neutrophils (Le Page *et al.,* 1999 and Hayes *et al.,* 1995).

TNF-α, IL-6 and IL-1β strongly up-regulate PAI-1 expression at the protein and mRNA level in different cell types, including endothelial cells, smooth muscle cells and monocytes (Wiesbauer *et al.,* 2002, Alessi *et al.,* 2006, Dong *et al.,* 2007). This effect is as a result of altering the fibrinolytic modulator of ECs, reducing the secretion of t-PA and increasing the production of PAI-1(Kruithof *et al.,* 2008). Thus, pro-inflammatory cytokines increase thrombus formation and stimulate the progress of acute coronary syndromes (Ait-Oufella *et al.,* 2011). Furthermore, IFN-γ and IL-1β can act as pro-atherogenic modulators through inhibition of the ATP-binding membrane cassette transporter A1 (Yin *et al.,* 2010), whereas IL-8 is considered as an important pro-atherogenic cytokine due to enhancing lesion formation by expediting leukocyte extravasation and EC adhesiveness (Gerszten *et al.,* 1999).

In the advanced stage of atherosclerotic plaques, cytokines IL-1, TNF-α, and IFN-γ stimulate apoptosis of SMCs, ECs and macrophages (Clarke *et al.,* 2006, Stoneman *et al.,* 2009; Ait-Oufella *et al.,* 2011). Robaye et al. (1991) established apoptosis induction of ECs by TNF-α. LPS-induced apoptosis in macrophages is mediated mostly through

the autocrine production of TNF-α (Xaus *et al.,* 2000). The consequence of this is endothelial dysfunction and macrophage apoptosis, which leads to the accumulation of cellular debris contributing to an increase in the lipid core. Plaque SMC apoptosis leads to thinning in the fibrous cap, leading to its rupture (Mallat and Tedgui, 2000, Tabas *et al.,* 2005; Clarke *et al.,* 2006).

The involvement of pro-inflammatory cytokines in the pathogenesis of atherosclerosis is supported by their detection in human atherosclerotic plaques. Furthermore, high circulating levels of these factors in individuals with atherosclerosis reflect this association. Evidence also comes from the study of genetically modified mice (Ohta *et al.,* 2005; Kamari *et al.,* 2007) or mice with genetic mutations in these cytokine genes (Schieffer *et al.,* 2004; Koga *et al.,* 2007) which show inhibition of the atherosclerotic lesion stages. Anti-inflammatory cytokine, IL-10, can inhibit the production of these pro-inflammatory cytokines. IL-10 is synthesised by T lymphocytes and macrophages and has anti-atherogenic properties (Stoner *et al.,* 2013). These facts are based on studies of atherosclerosis mouse models (Eefting *et al.,* 2007), in which the expression of IL-10 increased in both coronary arteries in atherosclerosis (Satterthwaite *et al.,* 2005). Individuals with atherosclerosis have greater serum levels of IL-10 compared to healthy people. However, it is the balance between anti-inflammatory and pro-inflammatory cytokines which is as a major determinant of plaque stability (Tedgui *et al.,* 2006).

1.6.4 The role of chemokines in atherosclerosis

During the process of vascular inflammation, chemokines play a central role, mediating the recruitment and activation of inflammatory cells (Keane *et al.,* 2000; Liu and Jiang, 2011). Chemokines and their receptors can up-regulate selectins and integrin ligands, supporting leucocyte arrest, either directly or involving their presentation by binding to proteoglycans. In addition, they provide important anti-apoptotic survival cues to leucocytes (Weber, 2008; Zernecke and Weber, 2010). A number of chemokines are expressed by monocytes, macrophages, SMCs and ECs in response to inflammatory mediators in human atherosclerotic plaques (Lucas and Greaves, 2001; Braunersreuther *et al.,* 2007).

1.6.4.1 The role of CCL2 and its receptors CCR2 in atherosclerosis

CCR2 is a member of the G-protein coupled receptor family and is expressed on the cell surface of monocytes and macrophages (Singh and Sobhia, 2013). Two independent mouse models of atherosclerosis have illustrated the role of CCL2 in atherosclerosis (reviewed by Koenen and Weber, 2010). These animal models have a genetic deletion of CCL2 or its receptor CCR2 in atherogenic LDL receptor-deficient (LDLR-/- or apolipoprotein E-deficient (ApoE-/-), mice. These genetic deletions led to reduced atherosclerotic lesions, together with a decrease in macrophage infiltration (Dawson *et al.,* 1999). In addition, CCL2 has been identified in macrophage-rich areas bordering the lipid core, on endothelial cells, SMCs in human and mouse atherosclerotic lesions (Yla-Herttuala *et al.,* 1991, Nelken *et al.,* 1991; Rayner *et al.,* 2000). This was confirmed by other studies reporting that CCL2 and CCR2 interaction accounted for most of the macrophage accumulation in atherosclerotic arteries (Peeters *et al.,* 2009). Blockade of CCL2-CCR2 interaction by using gene therapy in atherosclerosis-prone mice caused inhibition of the formation of fatty streak lesions and limited the progression of pre-existing plaques, without affecting serum lipid concentrations (Coll *et al.,* 2007). Furthermore, the overexpression of CCL2 in $ApoE^{-/-}$ transgenic mice accelerated atherosclerosis by increasing the number of macrophages in artery lesions (Aiello *et al.,* 1999).

In humans, numerous clinical studies have shown elevation of CCL2 levels in serum of patients with CVD (De Lemos *et al.,* 2007, Martinovic *et al.,* 2005, Arakelyan *et al.,* 2005; Herder *et al.,* 2006). A positive correlation was observed between CCR2 expression on circulating monocytes and serum CCL2 with carotid intima-media thickness and cardio-ankle vascular index, measures of atherosclerosis, in chronic haemodialysis patients (Okumoto *et al.,* 2009). Other genetic human studies indicated that men with (SNP)-2518G (alternatively–2578G) in the regulatory region of CCL2, which causes increased promoter activity, is associated with elevated, circulating levels of the CCL2 gene and increased risk of MI (McDermott *et al.,* 2005; Szalai *et al.,* 2001).

1.6.4.2 The role of CX3CL1 and CX3CR1 in atherosclerosis

CX3CL1 is the only known chemokine expressed in both a soluble/shed and a membrane-tethered form. The soluble form of CX3CL1, cleaved from the membrane by the enzyme TACE (TNF-α converting enzyme), is involved in chemotaxis, while the membrane-anchored form promotes adhesion and retention of leukocytes (Fong *et al.,* 1998; Shah *et al.,* 2011).

The expression of fractalkine and its receptor CX3CR1 which, is a G-protein coupled receptor, are up-regulated in atherosclerosic lesions (Umehara *et al.,* 2001, Landsman *et al.,* 2009, Wong *et al.,* 2002; Combadiere *et al.,* 2003). The importance of CX3CL1/CX3CR1 in atherosclerosis was supported by two common coding polymorphisms which are associated with a lower risk of CVD (Moatti *et al.,* 2001; McDermott *et al.,* 2001) while alternative study (Niessner *et al.,* 2005) did not support these findings. However, the significance of CX3CR1 in atherosclerosis was clearly observed through reduction of plaque size and increased lesion stability after using an inhibitory antibody against CX3CL1 in animal models (Bursill *et al.,* 2004). Deletion of the gene encoding CX3CR1 or CX3CL1 in mice prevented the formation of atherosclerosis and reduced monocyte infiltration in murine disease models (Combadiere *et al.*, 2003, Lesnik *et al.,* 2003; Teupser *et al.,* 2004; Saederup *et al.,* 2008).

According to epidemiologic studies, patients with CVD have increased expression of CX3CR1 in peripheral blood mononuclear cells (PBMCs) (Fraticelli *et al.,* 2001; Damas *et al.,* 2005) and elevated serum CX3CL1 levels (Damas *et al.,* 2005). CX3CL1 levels in patients with unstable angina pectoris are even higher than in those patients with stable angina pectoris (Ikejima *et al.,* 2010). However, neither CX3CL1 nor CX3CR1 have been found in normal mouse or human arterial wall (Barlic *et al.,* 2007). CX3CR1 expression has been found on numerous different cell types associated with atherosclerosis, both *in vivo* and *in vitro*, including monocytes, macrophages, T cells, NK cells, dendritic cells and vascular SMCs (Imai *et al.,* 1997; Combadiere *et al.,* 1998). Its ligand, CX3CL1, is only detected in advanced lesions, where it is expressed by SMCs, ECs and macrophages (Imai *et al.,* 1997, Lucas *et al.,* 2003, Cheng *et al.,* 2007, Wong *et al.,* 2002; Lesnik *et al.,* 2003). Therefore, CX3CL1 may act by attracting macrophages that express CX3CR1; while CX3CR1 expressed by SMCs may facilitate

the function of macrophages as well as the migration of SMCs towards endothelium that express high levels of CX3CL1 during atherosclerosis (Wong *et al.,* 2002, Lesnik *et al.,* 2003; Liu and Jiang, 2011). Furthermore, the CX3CL1/CX3CR1 axis possibly leads to the development of atherosclerosis by stimulating monocyte recruitment, as well as enhancing pro inflammatory cytokine production and inducing SMC proliferation (Koenen and Weber 2011).

This interaction between CX3CL1 and CX3CR1 besides directly regulating migration and adhesion, also acts as a signal transduction pathway to coordinate the function of CX3CL1 and other cytokines (Liu and Jiang, 2011). Additionally, one study reported that soluble CX3CL1 stimulates extracellular signal-related kinases and stress-activated protein kinases to enhance the interaction between monocytes and vascular cells (Cambien *et al.,* 2001). Landsman *et al.,* (2009) found that the absence of CX3CL1-CX3CR1 interaction led to increased macrophage apoptosis and inhibited plaque development, which indicted that CX3CR1 is essential for macrophage survival.

1.6.5 Obesity and atherosclerosis

The number of atherosclerosis and CVD patients and their rate of death are increased among obese people. Moreover, excess weight was an independent predictor of CVD death and congestive heart failure, after adjusting to other known recognised risk factors (Sucharda, 2010; Rocha and Folco, 2011). The site of adipose tissue deposition such as central obesity also is important in the incidance of atherosclerosis and ultimately CVD (Kershaw and Flier, 2004). There are a number of mechanisms by which expansion in adipose tissue could critically affect the vessel wall. An enlargement in adipose tissue is associated with hypertension, IR, hyperglycaemia, lipid/lipoprotein metabolism changes and an inflammatory state, including macrophage infiltration. These conditions lead to increased pro-inflammatory and decreased anti-inflammatory profiles, 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 by several mechanisms leading to progression of CVD, stroke and myocardial infarction (Rocha and Folco, 2011).

1.6.6 Low testosterone and atherosclerosis

The importance of studying the relationship between testosterone and the development of atherosclerosis and CVD is highlighted by: (a) the diversity between genders in the rate of CVD and atherosclerosis may be related to sex hormones as estrogen is protective factor (Ng *et al.,* 2003). (b) the rate of mortality from these diseases is higher among men than in women (Jones and Saad, 2009) (c) A previous study reported that the incidence of death from heart disease was higher among patients with low testosterone compared to normal individuals (Nettleship *et al.,* 2007). Others studies suggested that this increase in cardiovascular risk is not due to male gender, but is due to low testosterone levels associated with age (Jones and Saad, 2009) or obesity (Kelly and Jones, 2013). Undoubtedly, testosterone deficiency in men is accompanied with several cardiovascular risk factors including IR and MetS (Kapoor and Jones *et al.,* 2008). The features of these states including hyperglycemia, hypertension, an atherogenic lipid profile, inflammation, a pro-thrombotic fibrinolytic profile and hyperinsulinemia can be individually responsible for the development of CVD, atherosclerosis (Jones, 2010), congestive heart failure and stroke (Ma and Tong, 2010). Notably, some short-term studies found that testosterone replacement therapy (TRT) improved these conditions (reviewed by Jones and Saad, 2009). Furthermore, IR is linked to the classical cardiovascular risk factor, T2DM where the most diabetic patients are at risk of CVD (Hossain *et al.,* 2007). Normalisation of testosterone after a period of treatment improved insulin sensitivity and T2DM via reduction of visceral adiposity and IR in diabetic patients (Kapoor *et al.,* 2006). Testosterone treatment may exert its beneficial actions through an effect on cellular components and mediators of atherosclerosis and CVD.

Dyslipidemia is postulated as a mechanism linking low testosterone with CVD in men, Testosterone has a role in lipid and lipoprotein metabolism (Fahed *et al.,* 2012). Previous cross-sectional studies reported that endogenous low plasma testosterone levels are associated with increased total and LDL cholesterol (Saad *et al.,* 2008; Barud *et al.,* 2002). Epidemiological studies also indicated that testosterone levels in serum are inversely linked with the serum concentrations of triglyceride and LDL-C (Wu, 2003) while other studies found no notable link to TG (Isidori, 2005). Interestingly, testosterone treatment was shown to reduce total cholesterol in 27 hypogonadal men who were at risk of CHD and who were treated with statins (Jones and Saad, 2010).

Testosterone therapy in earlier studies reduced the levels of total and LDL cholesterol, (Saad *et al.,* 2007; 2008). In contrast, a positive relationship was seen between testosterone level and HDL level and Apo A1 in healthy and diabetic men (Uyanik *et al.,* 1997; Van *et al.,* 2003). Contradictory results related to this association have also been reported. For example, Malkin *et al.,* (2004a) and Kapoor *et al.,* (2006) detected no change in HDL, whereas other studies showed a decrease in HDL with testosterone supplementation in hypogonadal men with and without T2DM (Wittert *et al.,* 2003; Jones *et al.,* 2009). Another study reported that the different doses of testosterone had no effect on plasma lipids and apolipoproteins, only the highest dose of testosterone (600 mg/wk) was associated with a reduction in plasma HDL and Apo A1 in young, healthy men (Singh, *et al.,* 2002) and in serum HDL, HDL2, and HDL3 in elderly men (Herbst *et al.,* 2003). Testosterone treatment has also been shown to reduce Lp (a) level in normal men (Zmunda *et al.,* 1997).

In animal studies, hypotestosteronemia and hypertestosteronemia increased significantly the total LDL cholesterol, TG, and Apo B while physiological levels of testosterone had a beneficial effect on serum lipids, lipoproteins, and apolipoproteins in castrated rabbits (Qiang, 2003). Similar beneficial effects were observed in the testicular feminization mouse (Tfm) model, which is considered a model of atherosclerosis, where testosterone treatment decreased the plaque area and aortic intimal thickness (Nettleship *et al.,* 2007). A recent study indicated that the physiological concentration of 5α-dihydrotestosterone (5α-DHT) reduced the progression of atherosclerosis via inhibiting intimal foam cell formation from macrophages in a New Zealand white rabbit model (Qiu *et al.,* 2010). Physiological testosterone treatment also attenuated atherosclerosis in orchidectomised $LDL^{-/-}$ mice and significantly attenuated aortic cholesterol accumulation in orchidectomised male rabbits fed a pro-atherogenic diet for 120 days (Nathan *et al.,* 2001). Despite differences in the association of testosterone treatment with lipid, lipoprotein and apolipoprotein levels in human studies, animal experiments offer important data on the protective effect of testosterone on these parameters. The mechanism by which testosterone acts is unclear. The effect of testosterone on atherogenic factors may be by reducing the lipid uptake through inhibiting lipoprotein-lipase (LPL) activity in adipocytes and activating lipolysis through increasing the number of lipolytic beta-adrenergic receptors (Divers *et al.,* 2010). Furthermore, testosterone can also inhibit the differentiation of adipocyte precursor cells (Shiyama

and Adrian, 2004). Moreover, as a result of obesity-related testosterone decline, the liver is exposed to products of adipocyte metabolism, FFAs, in high concentrations and may induce metabolic disorders due to perturbation of lipid metabolism of the liver (Kelly and Jones 2012). Elevated plasma FFA enhances TG synthesis, leading to hypertriglyceridemia and high level of VLDL. Additionally, testosterone has a role in regulation of hepatic lipoprotein lipase (HLP) activity which catalyses the hydrolysis of triacylglycerols and phospholipids, mediating the removal of lipoproteins from plasma such as LDL, HDL, HDL2 and HDL3 (Herbst *et al.,* 2003). Hypogonadism causes dysregulation in the processes of lipoprotein metabolism and increases atherogenic risk (Deeb *et al.,* 2003).

Testosterone, as mentioned previously, has an inhibitive effect on some pro-inflammatory cytokines and chemokines, associated with atherosclerosis. Especially *in vitro* human and muose studies, reduction of TNF-α, IL-6, IL-1 and IFNγ and increase in IL-10 production was seen after incubation of vascular cells such as monocytes (Li, 1993), macrophages (D'Agostino, 1999), and endothelial cells (Hatakeyama, 2002) with testosterone. In *in vivo* studies, the production of TNF-α was augmented after LPS administration into castrated male mice, which was attenuated by testosterone treatment (Spinedi *et al.,* 1992). Testosterone replacement also significantly reduced TNF-α and CRP and increased IL-10 in a cohort of hypogonadal men with coronary heart disease (Malkin *et al.,* 2004a).

1.7 Summary

Obesity is associated with elevated pro-inflammatory modulators such as cytokines and chemokines, reduction in anti-inflammatory factors such as adiponectin and IL-10 and dyslipidemia. These alterations link obesity with other disorders such as T2DM, atherosclerosis and testosterone deficiency in men. The latter condition, which increases with age, also causes central obesity in men (figure 1.10). However, improvements have been seen in T2DM and atherosclerosis conditions after normalizing testosterone levels, using testosterone replacement treatment. Some studies report that, testosterone displays beneficial effects on levels of pro-inflammatory cytokines and chemokines, while others showed a positive effect on reduction in body composition or reduced dyslipidemia by changes in lipid profile including lipoprotein and apolipoprotein.

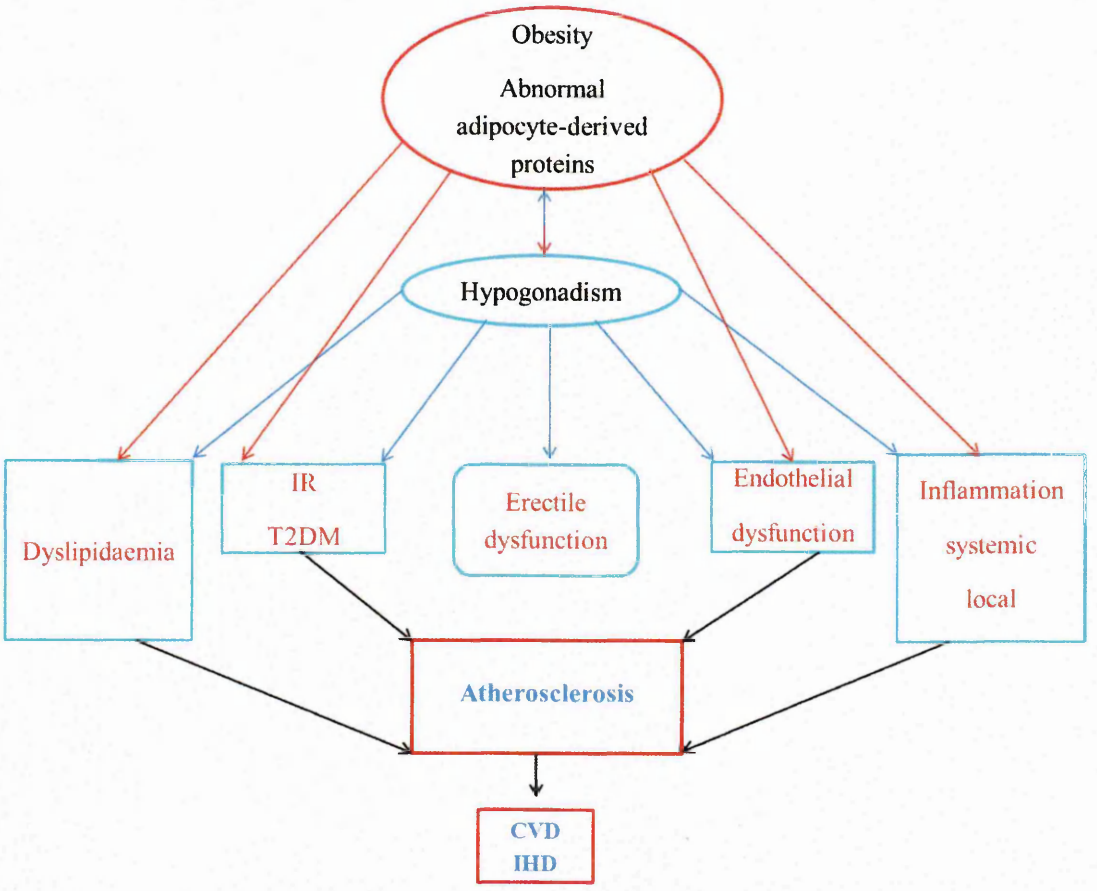


Figure 1.10: Association of obesity and hypogonadism with risk factors of T2DM and atherosclerosis. Obesity can cause low testosterone leading to hypogonadism (red line) and hypogonadism is associated with abdominal obesity which contributes to risk factor for atherosclerosis, CVD and IHD, whether through a direct effect of pro-inflammatory factors on the vasculature or through increasing the prevalence of further risk factors of atherosclerosis such as, IR, T2DM, dyslipidemia and endothelial dysfunction (blue line). T2DM: type 2 diabetes mellitus, CVD: cardiovascular disease, IHD: ischemic heart disease, IR: insulin resistance.

Although several pharmacological blockers of different testosterone receptors have been used to identify the mechanisms behind these beneficial effects, this is still not fully elucidated. Therefore, there needs to be further work to elucidate the beneficial role played by testosterone as a potential treatment of T2DM and atherosclerosis in obese men.

1.8 Aims of this thesis

The aim of the research is to investigate the effect of testosterone on biomarkers of diabetes, atherosclerosis and obesity and to elucidate the mechanisms controlling these interactions.

A combined *in vitro* and *in vivo* strategy was taken with the following objectives:

1. Investigate the effects of physiological testosterone replacement on body composotion, biomarkers and parameters associated with inflammation using two clinical patient cohort studies, one short term and one long term.

2. Investigate whether testosterone modulates the expression of anti/ pro-inflammatory markers and anti-atherogenic factors in liver tissue from animal model of low testosterone.

3. Investigate the effects of testosterone on the modulation of inflammatory and adipocyte drived associated proteins in cell culture models of macrophages and adipocytes, respectively.

Chapter 2

The effect of testosterone treatment in hypogonadal men with T2DM in short and long-term studies

2.1 Introduction

The association of low testosterone with T2DM and CVD is well recognized (Muraleedharan and Jones, 2010; Kelly and Jones, 2013). Epidemiological studies have indicated that up to 40% of men with T2DM have testosterone deficiency (Corona *et al.,* 2006, 2009, Ding *et al.,* 2006; Kapoor *et al.,* 2007a). Furthermore, it is clear that the prevalence of diabetes increases following induction of a hypogonadal state during treatment for prostate cancer (Keating *et al.,* 2010).

Other studies established that endogenous testosterone (total and free) was lower in subjects with MetS compared with those without (Brand *et al.,* 2010, Corona *et al.,* 2011). In addition, low testosterone is associated with impaired insulin sensitivity, increased percentage of body fat, truncal obesity, dyslipidaemia, hypertension and CVD (Wang *et al,* 2011). Decrease in biologically available testosterone in aging men could be associated with the increased prevalence of CVD. This evidence suggests that TRT for hypogonadal men, with diabetes or CVD may be favourable in decreasing the risk or the consequence of these diseases. One of these risk factors is increased inflammatory factors and decreased anti-inflammatory factors in serum in T2DM and atherosclerosis as a result of obesity. Pro-inflammatory cytokines such as TNFα, IL-1 and IL-6 have an essential role in T2DM and atherogenesis development, while IL-10 and adiponectin are anti-diabetic and anti-atherogenic factors (Malkin *et al,* 2004a; Saad, 2009). Testosterone has immunomodulatory actions, and current *in vitro* evidence indicates that testosterone could suppress the expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 and promote the expression of the anti-inflammatory cytokine IL-10 (Malkin *et al.,* 2004a; Corrales *et al.,* 2006).

However, inflammation and infection decrease testosterone concentration as a consequence of the inhibitive action of inflammatory cytokines on the hypothalamic-pituitary-testis axis (Saad, 2009; Muraleedharan and Jones, 2010). The effective role of testosterone within the immune system is clearly documented, where the higher rate of immune-mediated disease in women and androgen deficient men has been associated with the immunosuppressive action of androgens compared with estrogens (Cutolo and Wilder, 2000). Furthermore, administration of testosterone to hypogonadal men with CHD reduced serum TNFα and IL-1β, but not IL6 levels and raised levels of IL-10 (Saad, 2009). In testosterone deficient men suffering from T2DM, baseline testosterone levels were inversely correlated with IL-6 and CRP levels (Kapoor *et al.,* 2007).

Dyslipidaemia is another important risk factor which has an impact on atherosclerosis development and is a consequence of diabetes and obesity (Kelly and Jones, 2013). Lipid profiles including total cholesterol and TG and lipoproteins play a central role in the development of atherosclerotic cardiovascular disease in humans. The plasma concentrations of lipoproteins and their metabolic fates are regulated by apolipoproteins on the surface lipid-rich particles (Davignon *et al.,* 1988). The HDL-C and HDL2-C levels are known to increase in response to the elimination of obesity and smoking, the adoption of habitual exercise, and alcohol consumption (reviewed from Moriyama *et al.,* 2014). The suggestion has been made that investigations in LP (a), apolipoproteins A-I and B (Apo A-I, ApoB), or HDL density subfractions may identify improved drug treatment and lifestyle changes to lower lipid levels, prevent atherosclerosis and limit the consequence of T2DM.

Strong evidence has been provided by several studies for a relationship between plasma lipoprotein and their apolipoproteins, and development of atherosclerosis (Olofsson *et al.,* 2007). In addition, diabetic dyslipidemia is characterized by hypertriglyceridemia; increased LDL, decreased HDL, high Apo B and low ApoA1, which can be the most important cause of atherosclerosis in diabetic patients (Hashemi *et al.,* 2012). Furthermore, previous studies suggest that Apo B might be of greatest value in the diagnosis and treatment of persons with normal or low concentrations of LDL cholesterol (Walldius *et al.,* 2001) For example, patients with T2DM frequently have hypertriglyceridemia together with hyper-apo B, an atherogenic lipid profile that is often unrecognised because of concomitant low or normal levels of LDL cholesterol (Sniderman *et al.,* 2001). The lack of Apo E in the Apo E-deficient mouse model leads them to develop lesions ranging from lipid-laden fatty streaks to advanced fibroproliferative lesions by the age of 30 weeks (Candido *et al.,* 2002). Increased levels of Lp (a) is an associated with coronary atherosclerosis (Armstrong *et al.,* 1986) and are as an independent risk factor of atherosclerosis. Therefore, it is thought that the one mechanism of testosterone as a protective therapy would be its beneficial influence on serum apolipoproteins as androgens are known to affect lipid metabolism. In addition, because the effects of TRT on apolipoprotein levels in both atherosclerotic and diabetic patients are not yet defined, the effects of testosterone on lipid and its carrier lipoproteins, generally and apolipoprotein especially remain controversial. Therefore, an investigation of the effect of testosterone on inflammatory factors on the one hand and

on apolipoprotein on the other, could be important to point out by which mechanism testosterone treatment has improved T2DM conditions and prevents atherosclerosis.

This part of the study involved two patients groups. (1) A double-blinded placebo-controlled group of hypogonadal men with T2DM treated with intramuscular testosterone injection, compared to a control group of patients who received a placebo; this study was a short-term study over 6 months. (2) A group of hypogonadal men with T2DM treated with testosterone in a longitudinal study. For the data analysis these patients were divided into 3 groups 1) normal testosterone, 2) low testosterone without testosterone treatment, 3) low testosterone with testosterone treatment. In addition, for some analyses the patients were divided into four subgroups according to the patient IHD status at initial diagnosis.

2.1.1 Aims

This study had two key aims:

1. To investigate the effects of physiological TRT on body composition (BMI, WC, W/HR), anti/pro-inflammatory cytokines (adiponectin, IL-1β, IL-6, IL-10, CRP and TNFα) and atherogenic factors (HDL, HDL2, HDL3, HDL2/HDL3 ratio, ApoA1, ApoB, ApoE and LP(a)) in serum of hypogonadal men with T2DM at risk of CVD in a short duration placebo-controlled study (6 months).

2. To investigate the difference between groups of diabetic patients (normal testosterone, low testosterone no treatment, low testosterone with treatment +/- IHD) by measuring body composition (BMI, WH, WHR), anti/pro-inflammatory cytokines (adiponectin, IL-1β, IL-6, IL-8, IL-10, IFNγ and TNFα), atherogenic factors (HDL, HDL2,HDL3, HDL2/HDL3 ratio, ApoA1, ApoB, ApoE and LP(a)) levels in serum in a longitudinal observational study (follow up after 5-6 years).

2.2.1 Patients of the double-blinded placebo-controlled study

Testosterone therapy was given to group of hypogonadal men with T2DM in a randomized, double-blind, placebo-controlled study. This study was conducted at the Andrology Outpatient Department, Sheffield Cardiology Research Department, Royal Hallamshire Hospital Sheffield (RHH) and the Centre for Diabetes and Endocrinology, Barnsley District General Hospital, Barnsley, UK. The study included a total of 24 male patients, over the age of 40 years (range, 52-73 years; mean ± SEM 59.6 ± 11.9 years) with T2DM at risk of CVD. The local regional ethics committee approved the protocol (05/Q2308/140), and the patients provided their written informed consent. The inclusion criteria were Type 2 diabetic men with HbA1c (the term HbA1c refers to glycated haemoglobin) up to 9.5%, reflecting no significant symptoms of hyperglycaemia. Hypogonadism was defined as the total testosterone level <12 nmol/l and bioavailable testosterone < 4 nmol/l with symptoms of hypogonadism. Hypogonadal men were referred to the andrology clinic; they were androgen deficient and androgen replacement was deemed clinically indicated by a consultant endocrinologist. Patients were excluded if they had current or previous breast or prostate cancer or an elevated prostate specific antigen (PSA) or irregular digital rectal examination suspicious for prostate cancer. Other exclusion criteria were strict symptoms of benign prostatic hypertrophy, treatment with testosterone in the 3 months prior to the trial, investigational drug treatment in the 3 months prior to the trial and known allergy to Sustanon or peanuts. Participants were also excluded who had peripheral vascular disease defined as either confirmed in a previous diagnostic assessment by a specialist vascular surgeon or an ankle brachial pressure index (ABPI) less than 0.92 and ischaemic leg pain or distal complications such as ulceration or gangrene.

2.2.1.1 Randomisation and drug treatment

The participants were randomly selected by a computer-generated assignment to testosterone treatment or placebo. 11 patients received a total of 12 testosterone injections intramuscularly over 6 months, (i.m. injection was given once every 2 weeks). Testosterone was given as 0.8 ml Sustanon 250 injection (200mg testosterone esters; Organon Laboratories Ltd, Cambridge, UK). This regimen is commonly used as

a standard physiological TRT in men with androgen deficiency. The same protocol was followed with 13 control patients who were given placebo. The placebo was 0.8 ml of 0.9% normal saline. Drugs were drawn in identical syringes and were prepared by research staff in a separate clinical room away from the patient and the doctor assessing the patients.

2.2.1.2 Assessment of patients and sample collection in double-blinded placebo-controlled study

Blood samples were taken from all participants at baseline, 3 and 6 months. All patients and controls were required to complete a detailed questionnaire recording their medical history and their current medications at the screening visit in the morning before 10.00 am and in the fasted state at screening/ baseline, 3 and 6 months, having only taken water to drink since midnight the night before. Smokers were asked not to smoke on the morning of an assessment visit. All patients were instructed to follow their usual diet and physical activity as well as taking supplements including anti-diabetic treatment, anti-hypertensive and lipid-lowering medications, without dose adjustment for the duration of the study. Their height and weight were recorded and the BMI was calculated using the equation (BMI = weight (kg)/height $(m)^2$. The waist circumference was measured; a waist was defined as the point midway between the iliac crest and the costal margin (lower rib). Glucose level, HbA1c and blood pressure were available from the research database.

2.2.2 Patients of the longitudinal study

This study involved 120 men with T2DM who had testosterone levels determined at RHH between 2002 and 2005. Subjects were allocated to two groups based on the Endocrine Society Guidelines recommended cut-off level: i) Total testosterone levels ≤10.4 nmol/l and ii) Total testosterone >10.4 nmol/l. This study involved participants who were assessed and managed routinely within the district-wide diabetic retinopathy screening clinic, as well as the hospital diabetic clinic, and provided a representative sample from the general community. In addition, patients were identified with T2DM from the hospital database. The study was approved by the South Yorkshire Research Ethics Committee (05/Q2308/140). For the purpose of data analysis carried out in the

current study this group were divided into patients with normal testosterone, low testosterone without treatment and low testosterone plus TRT. All the patients were initiated and monitored within the routine clinics in the diabetes and endocrinology department at RHH. There were a number of reasons for patients with low testosterone not receiving testosterone treatment, including patient choice, or the patient declined to attend for further clinical assessment, however all patients still remained in contact with the clinic, and responded when requested to provide a follow-up sample. For the present analysis this group of patients was also divided into 4 subgroups based on IHD status into normal testosterone, IHD with normal testosterone, IHD with low testosterone not receiving testosterone treatment and IHD with low testosterone treated with testosterone.

2.2.2.1 Drug treatment

TRT was given to men with testosterone levels less than the local laboratory normal assay range (<8.4 nmol/l) without a concomitant diagnosis of prostate cancer or other contra-indications to TRT. This group received testosterone treatment as 2% gel testosterone, (average treatment time 5.8±1.3 years). The choice of initial therapy was based on the combined decision between patient and doctor. Doses were adjusted to achieve testosterone levels within the mid to upper normal range (>18nmol/l). The duration of treatment and dosage were based on each individual patients and their needs.

2.2.2.2 Assessment of patients and sample collection in longitudinal study

All blood samples were taken between 0800 and 1100 am. Baseline data on age, height, weight, BMI, smoking, glycaemic control (HbA1c), CVD were obtained from the hospital records.

2.2.3 Determination of pro and anti-inflammatory biomarkers in patients of double-blinded placebo-controlled study by Bead Cytometric Array Assay

BD™ Cytometric Bead Array (CBA) is a flow cytometry application assay that offers a powerful approach to quantify a variety of soluble proteins simultaneously in a single sample. This applies the principles of flow cytometry and considerably decreases sample requirements and time to results. The method uses the same principle as ELISA and applies it to uniquely identifiable antibody-coated beads. These beads are coated

with specific antibody for a single analyte and have an unique fluorescence intensity (http://www.bdbiosciences.com/research/cytometricbeadarray/formats/index.jsp).

In addition, the beads have an internal colour with two different fluorescent dyes (red and infrared). Different concentrations of red and infrared dyes are used to generate up to 100 distinct bead regions by flow cytometry. Each defined bead is conjugated to a specific target analyte followed by binding with a biotinylated detection antibody and a reporter dye, streptavidin-conjugated phycoerythrin. Following incubation and subsequent washing, the samples are analysed on a flow cytometer where these beads are transported in a fluid stream in the cytometer to the laser beam for interrogation. The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. These detectors convert light signals that can be processed by the computer, to electronic signals (voltages) and then are assigned a channel number in an appropriate position on a data plot. List mode data are collected on each particle of bead or event. This data can be analysed to provide information about subpopulations within the sample (Figure 2.1) by using the FCAP Array analysis software that gates on each individual bead population and defines the median fluorescence intensity (MFI) for each analyte in the array. It creates a standard curve and allows interpolation of sample concentrations compared to the standard curve and creates an analysis report in tabular format.

Method

All reagents were obtained from BD Biosciences (UK). All sample and reagent preparations were performed on the day and stored at 4°C until used. Suspension of the capture bead stock solutions by vortex was carefully performed. The preparation of capture bead working solutions was made by adding capture bead reagent (by using each bead for target analyte IL1β, IL-6, IL-10 and TNF-α) to the capture bead diluent. This was in accordance to the number of standards and samples to be tested and to the volume required for each reaction well (25 µl). Similarly, these processes were followed in the preparation of working solution of the PE detection reagent by using PE detection reagent for each target analyte and detection reagent diluent based on required volume (1µl) per reaction and the number of test samples (standard and samples) to generate an ultimate volume of 25µl per well.

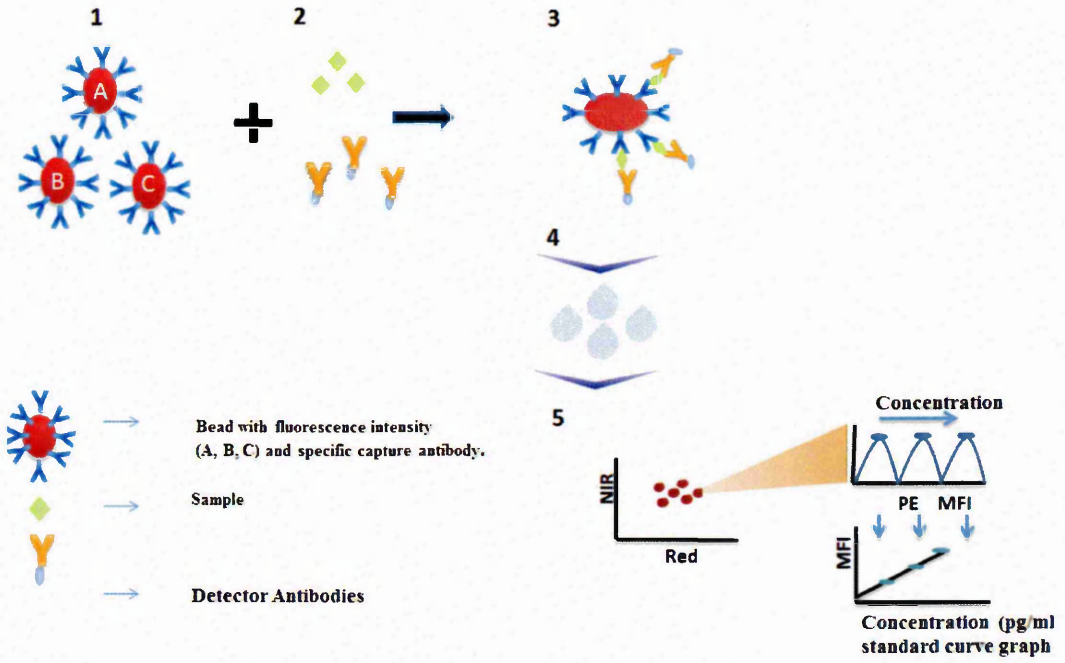


Figure 2.1: Cytometric Bead Array (CBA) principle. The steps of flow Cytometric Bead Array start by adding capture beads which have a distinctive fluorescence intensity and coated with capture antibody specific for the target analyte in a single tube (1) to different beads with a sample or standard and a mixture of detection antibodies (2) that are conjugated to Phycoerythrin (PE). This reaction leads to the formation of a complex of capture bead with PE, conjugated detector antibody and single target analyte (3). Following incubation and consequent washing steps (4), the samples are acquired and analysed (5) on a flow cytometer by the FCAP Array analysis software gates on each distinct bead population and illustrates the median fluorescence intensity (MFI) for each single analyte in the array. It creates a standard curve and determines sample concentrations compared to the standard curve. Adopted and modified from (http://www.bdbiosciencescom/research/cytometricbeadarray/formats/index.jsp).

Following reconstituting the standards with 4 ml of assay diluent and leaving for 15 minutes, serial dilution of standards (0, 10, 20, 40, 80, 156, 312.5, 625, 1250, and 2500 pg/ml) were prepared to create a standard curve for each single bead or multiplex assay.

Micro-plates of 96 wells were pre-wetted by the addition of 100µl of wash buffer into each well, which was then immediately removed. 25 µl of capture bead stock was added per well and then followed by 25 µl of standard (IL-6 IL-1β, IL-10 and TNF-α) and samples. Plate were then mixed by digital shaker for 5 minutes at 500 rpm and incubated for 1 hour at room temperature (RT). 25 µl of PE detection reagent was pipetted into each assay well and mixed by a digital shaker for 5 minutes at 500 rpm and then incubated at RT for 2 hours. Aspiration of buffer in the plate was made and all wells were further drained by vacuum. For bead suspension, 300µl of wash buffer was added to each well and mixed as before. Following insertion of the micro-plate into the BD FACS Array™ flow cytometer, data was acquired and analysis was performed by FCAP Array™ software.

2.2.4 Determination of pro and anti-inflammatory biomarkers in patients of longitudinal studies by Enhanced Sensitivity CBA

The above principle of CBA flow cytometric assay is a standard method to detect target proteins down to 2-10 pg/ml, while, CBA Enhanced Sensitivity Master Buffer Kits are applicable for difficult to detect very low concentrations. The working assay can detect as low as 0.274 pg/ml concentration (from 274 to 200.000 fg/ml) by using a two-step detection system of PE detection reagent (a) and (b). This ensures binding and detection further target analyte and offers reliable quantification and a highly sensitive method for generating accurate results (http://www.bdbiosciences.com/research/cytometricbeadarray/formats/enhanced.jsp).

Method

All reagents were obtained from (BD Biosciences, UK). The principle of this method was as described in the previous section (2.2.3). Following reconstituting the standards with 4 ml of assay diluent for 15 minutes, serial dilution of standards (0, 274, 823, 2,469, 7,407, 22,222, 66,667, 200,000 fg/ml) were prepared to create a standard curve for each analyte. Furthermore, the same steps such adding capture bead and PE

detection reagent were performed as in previous section (2.2.3) (by using each bead and PE detection for target analyte, TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and IL-10). An additional step in this method was made by adding 100μl of the Enhanced Sensitivity Detection Reagent (Part B) to each assay well. Mixing the plate for 5 minutes at 500 rpm using a digital shaker was performed and the plate was incubated at RT for 1 hour. For bead suspension, 200μl of wash buffer was added twice to each well in plate and mixed on a digital shaker for five minutes at 500 rpm. The assay thenfollowed the same protocol as previously described.

2.2.5 Determination of adiponectin by ELISA in patients on both of double-blinded placebo-controlled and longitudinal studies

Enzyme-Linked Immuno-Sorbant Assay (ELISA) is utilized to distinguish a specific antigen in the target analyte by employing the quantitative sandwich enzyme immunoassay technique. This technique depends on the specificity of antigen-antibody type reaction. Due to the high degree of its sensitivity and specificity, it is considered a fast and accurate method to investigate the concentration of antigen in a sample. The binding of two antibodies with the target antigen is usually performed with serial steps of adding multiple liquid reagents, incubations, washes, which are followed by a visual change in the colour of liquid (figure 2.2A). The primary antibody is adsorbed (capture antibody) to the micro-well, the sample to be analysed is added and then after incubation, unbound materials are washed away. Then the second antibody is added and binds to the primary antibody producing an antibody-antigen-antibody complex. The second antibody is attached to an enzyme (horseradish peroxidase) following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added which produces colour in proportion to the amount of antigen present in the sample. The colour development is stopped by stop solution and the intensity of the colour is measured by spectrophotometry on a microplate reader. The antigen concentrations in the samples can be determined by creating a standard curve.

Method

Adiponectin was investigated by using the Human total adiponectin/Acrp30 Quantikine ELISA kit (R&D Systems, UK). The plate was pre-coated with a mouse monoclonal antibody against adiponectin antigen. Serum samples were diluted to 100-fold dilution

with buffered protein base (Calibrator Diluent RD6-39). 100 µl of assay diluent (Calibrator Diluent R1DW) was added to each well followed by 50µl of different adiponectin standards (0, 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 ng/ml). Test samples were pipetted into the wells in duplicate and incubated for 2 hours at RT. Following aspiration and washing each well four times with 400 µl wash buffer, the plate was inverted and blotted against clean paper towels. 200µl of adiponectin antibody-enzyme conjugate was added to each well and the plate incubated for 2 hours at RT. After that washing was repeated and then 200 µl of substrate solution (Tetramethylbenzidine, TMB) was added to each well and the plate incubated and protected from light for 30 minutes at RT. Following adding 50 µl of stop solution (Sulphuric Acid) per well to end the reaction, the plate was read in a microplate reader (Wallac victor^2 1420 multilabel counter, UK) at 450 nm after 30 minutes.

2.2.6 Determination of CRP by ELISA in patients of double-blinded placebo- controlled

Method

All reagents and 96 well microplate contained in the Human total CRP Quantikine ELISA kit (R&D Systems, USA) were brought from 4°C to RT. The microplate was pre-coated with a mouse monoclonal anti-body against CRP antigen. Serum samples were diluted 100-fold with buffered protein base (Calibrator Diluent RD-P). 100 µl of assay diluent (Calibrator Diluent RD1F) was added to each well followed by 50µl of CRP standards (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 ng/ml) and test samples were pipetted into the appropriate wells in duplicate and then the plate incubated for 2 hours at RT. The assay followed the same protocol as described above for adiponectin.

2.2.7 Determination of TNF-α by High Sensitivity ELISA in patients of double-blinded placebo-controlled

The principle of this kit is the same as the standard ELISA that was previosly described however, there was a further step for the improvement of sensitivity (figure 2.2B). This is for the detection of low protein concentrations from serum or cell culture

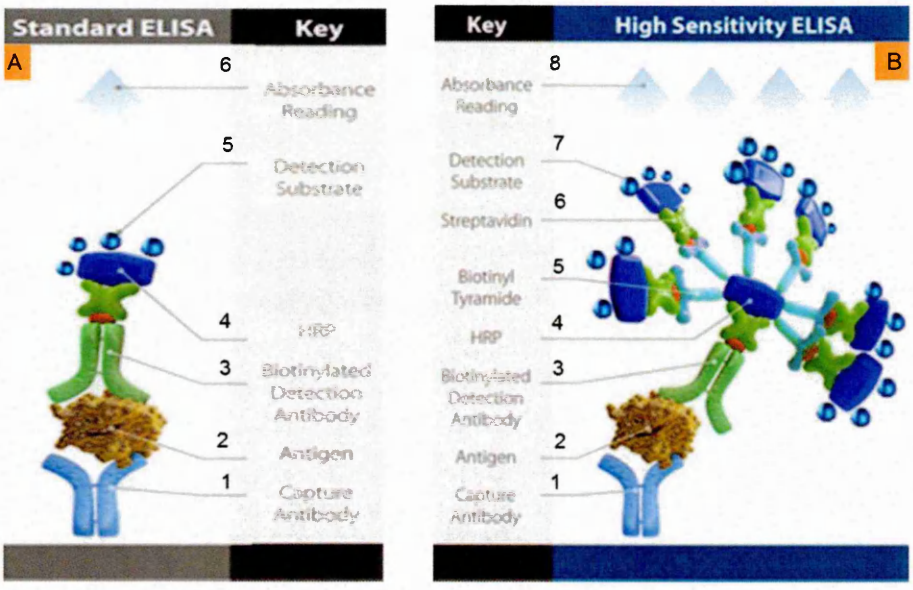


Figure 2.2: The principle of standard ELISA and high sensitivity ELISA method. Capture antibody (1, A&B) (primary antibody) binds to antigen in the target analyte (2, A&B). Biotinylated detection antibody (3, A&B) with streptavidin (4, A&B). In the standard ELISA method detection substrate is then added (5, A) and absorbance measured (6, A). In the high sensitivity ELISA method, following the addition of biotin-streptavidin-HRP as amplification reagent I and further streptavidin-HRP and amplification (6, B) is added to bind to the additional biotin tyramide sites (5, B). Substrate detection is added (7, B) following washing steps to measure absorbance reading (8, A&B) by microplate reader. Modified from http://www.ebioscience.com/knowledge-center/product-line/elisa/high-sensitivity-elisa kits.htm.

samples, by further enhancing the signal obtained from conventional HRP-based ELISA. The signal amplification step uses biotinylated Tyramide resulting in an increased sensitivity without loss of resolution or increase in background. This assay detects low cytokine concentrations ≤ 1.0 pg/ml with reliable quantification.

Method

All reagents and 96 well microplate contained in the Human TNF-α High Sensitivity ELISA kit (eBioscience, UK) were brought from 4°C to RT. The TNF-α microplate was coated with monoclonal antibody to human TNF-α antigen. Serum samples were diluted to 100-fold dilution with buffered protein base. 100µl of TNF-α standards (0, 0.031, 0.063, 0.125, 0.250, 0.500, 10.0 and 20.0 pg/ml) were added to appropriate wells. 50 µl of sample diluent added to wells and then 50 test samples were pipetted into the appropriate wells in duplicate. Biotin- conjugated anti-body was added to all wells and the plate incubated for 2 hours at RT on a shaker (100 rpm). Following aspiration and washing each well six times with 400 µl wash buffer, the plate was inverted and blotted against clean paper towels. 100µl of streptavidin-HRP was added to each well and the plate incubated for 1 hour at RT on a shaker. Washing steps were then repeated and then 100 µl of Amplification Solution I (biotin tyramide) was added to each well and protected from light for 15 minutes at RT on a shaker. Plate was washed again and 100 µl of Amplification Solution II (streptavidin-HRP) was added to each well and protected from light for 15 minutes at RT on a shaker (100 rpm). Washing steps were then repeated and then 100 µl of substrate solution (TMB) was added to each well and protected from light for 10 minutes at RT. Following adding 100 µl of stop solution (phosphoric acid) per well to end the reaction, the plate was subjected to a microplate reader at 450 nm during 30 minutes.

2.2.8 Determination of apolipoprotein by ELISA in patients of double-blinded placebo-controlled and longitudinal studies

2.2.8.1 Apo A1 ELISA

Method

All reagents and microplate of 96 well contained in the apolipoprotein A1 Human

ELISA kit (abcam, UK) were brought from 4°C to room temperature. The ApoA1 microplate was pre-coated with a mouse polyclonal antibody against the Human Apo A1 antigen. Serum samples were diluted 400 fold with buffered protein base. 25µl of ApoA1 standards (0, 0.625, 1.25, 2.50, 5, 10, 20 and 40 µg/ml), biotinylated antibody and test samples were pipetted into the appropriate wells in duplicate and the plate incubated for 2 hours at RT. Following aspiration and washing each well five times with 200 µl wash buffer, the plate was inverted and blotted against paper towels. 50µl of enzyme conjugate was added to each well and the plate incubated for 30 minutes at RT. Washing steps were then repeated and then 50 µl of substrate solution (TMB) was added to each well and protected from light for 10 minutes at RT. Following adding 50 µl of stop solution (hydrochloric acid) per well to end the reaction, the plate were subjected to a microplate reader at 450 nm during 30 minutes.

2.2.8.2 Apo B ELISA

Method

All reagents and microplate of 96 well contained in the MaxDiscovery apolipoprotein B ELISA kit Manual (BIO SCIENTIFC, USA) were brought from 4°C to RT. the Apolipoprotein B microplate was pre-coated with an antibody to the Human Apo B antigen. Serum samples were diluted 10.000-fold with buffered protein base. 100µl of Apo E standards (0, 3, 6, 12, 24, 50, 100 and 200 ng/ml) and test samples were pipetted into the appropriate wells in duplicate and the plate incubated for 1 hour at 37°C. Following aspiration and washing each well five times by 250 µl wash buffer, the plate was inverted and blotted against paper towels. 100µl of ApoB antibody-enzyme conjugate was added to each well and the plate incubated for 1 hour at 37 °C. After this point the assay followed the same protocol as described as before.

2.2.8.3 Apo E ELISA

Method

All reagents and 96 well microplate contained in the apolipoprotein E Human ELISA kit (abcam, USA) were brought from 4°C to RT. The Apolipoprotein E microplate was pre-coated with a mouse polyclonal antibody against the Human Apo E antigen. Serum

samples were diluted 400-fold with buffered protein base. 50µl of different Apo E standards (0, 0.031, 0.063, 0.125, 0.250, 0.500, 10.0 and 20.0 µg/ml) and test samples were pipetted into appropriate wells in duplicate and incubated for 2 hours at RT. Following aspiration and washing each well five times with 200µl wash buffer, the plate was inverted and blotted against paper towels. 50µl biotinylated antibody was added to each well and the plate was incubated for 1hour at RT. Washing steps were then repeated and then 50µl of enzyme conjugate was added to each well and the plate incubated for 30 minutes at RT. After this point the assay followed the same protocol as described as before.

2.2.8.4 Lp (a) ELISA

Method

All reagents and 96 well microplate contained in the Human Lp (a)/ Apo A1 ELISA kit (DRG instruments GmbH, Germany) were brought from 4°C to RT. Lp (a) microplate was pre-coated with a mouse monoclonal anti-Apo (a). Samples of serum were diluted to 1: 202 with Sample Buffer (provided as part of the kit). 25 µl of Lp(a) Calibrator (0, 0.32, 1.1, 2.8, 4.4 U/L) and test serum were pipetted into the suitable wells in duplicate and then 50µl of enzyme conjugate mouse monoclonal anti-Apo(a) was added and the plate incubated on shaker for 1 hour at RT. After this point the assay followed the same protocol as described above for adiponectin.

2.2.9 Determination of HDL subfractions in serum from patients of double-blinded placebo-controlled and longitudinal studies

To measure the level of HDL3 and HDL2 with HDL2/HDL3 ratio in serum, this firstly required determination of the level of total HDL in serum. Secondly, a single precipitation procedure for selectively separating HDL3 from both the apo B-containing lipoproteins and HDL2 is needed. Heparin, manganese chloride ($MnCl_2$) and dextran-sulphate (DS) are usually used for precipitating lipoprotein and HDL2 in the supernatant (Hirano *et al,* 2008).

2.2.9.1 Precipitation method with Heparin/MnCl2/DS to separate HDL3 and HDL2 patients serum of double-blinded placebo-controlled and longitudinal studies

Method

All reagents and samples were brought from 4°C to RT before using. For preparing the precipitation reagent heparin (Sigma Aldrich, UK) (8.25 mg/ml), $MnCl_2$ (Sigma Aldrich, UK) (98.7 mg/ml), and DS (Sigma Aldrich, UK) (12 mg/ml) were mixed together in 1ml of dH_2O. After that, 15µL of the precipitation reagent was added to 75µL of serum and incubated at RT for 30 min, and then centrifuged at 10,000g for 10 min at 4°C. After the centrifugation step the pellet contained the HDL2 and the HDL 3 remained in the supernatant which was then ready for measurement by the method below (2.2.9.2).

2.2.9.2 Determination of total HDL and HDL3 subfraction by using Amplex Red cholesterol assay

Method

Following preparation of the supernatant containing HDL3, all reagents of the Amplex® Red Cholesterol Assay Kit, (Invitrogen™, UK) were brought from 4°C to RT. Serial dilutions of standards were made (0, 2, 4, 6, and 8µg/ml) to create the standard curve. In duplicate, 50µl of standard, control and samples of total HDL and fractionated HDL3 serum were pipetted into each a 96 well microplate (Fisher Scientific, UK) and incubated with 50µl of Amplex Red reagent /HRP/cholesterol oxidase/cholesterol esterase working solution for 30 minutes at 37°C. This working solution was 300µM Amplex® Red reagent containing 75µl of Amplex® Red reagent stock solution, 50µl of the HRP stock solution, 50µl of the cholesterol oxidase stock solution and 5 µl of the cholesterol esterase stock solution, which was added to 4.82 ml of Reaction Buffer. Following incubation, the plate was read in a microplate reader at 570nm within 30 minutes. The HDL3 values were multiplied by 1.2 to correct for dilution by the precipitation reagent. HDL2 was calculated by subtracting the HDL3 value from the total HDL value. Moreover, the ratio of HDL2/HDL3 was calculated by dividing HDL2 by HDL3.

Patients	Hypogonadal men with T2DM	Hypogonadal men with T2DM
Number	24	120
Type of treatment	intramuscular testosterone injection	Gel treatment
Period	for 6 months	5-7years
Investigated parameters	Body composition, apolipoproteins, TNF-α, IL-6, IL-1β, IL-10, adiponectin, CRP and HDL3, HDL2 and HDL2/HDL3	Body composition , apolipoproteins, TNF-α, IL-6, IL-1β, IL-10, IFN-γ 10, adiponectin, IL-8 and HDL3, HDL2 and HDL2/HDL3
Assay technique	ELISA, Cytometric Bead Array and Amplex Red cholesterol assay	ELISA, Cytometric Bead Array and Amplex Red cholesterol assay

Table 2.1: Summary table of human in vivo study. The details of samples patients, analysis method, target parameters, period and of type treatment used in this study

2.2.10 Statistical analysis of assay data

Data is presented as mean ± SEM, unless otherwise stated. In a short-term study, the data were analysed with a statistical software package (SPSS version 20, SPSS Inc, Chicago, Illinois USA), Prism Graphpad 5 software and StatsDirect. Comparison baseline data for treatment groups (placebo and testosterone) was analysed by using *t*-test. Generalized Linear Model (GLM) was used to compare the difference between groups for data followed by a post-hoc Tukey's test. *t*-test was also used to compare the mean change from 0 to 3 and 6 months between groups. Two way ANOVA was used with a post-hoc Tukey's test for HDL and its fraction. In the longitudinal study, the normal distribution of data was assessed by the D'Agostino test for normality. Kruskal-Wallis test was used for non-parametric data followed by a post-hoc Conover-Inman test. All results were considered as statistically significant at $P < 0.05$.

2.3 Results

2.3.1 Patients in double-blinded placebo-controlled study

The baseline clinical characteristics are displayed in Table 2.2. The study population included a group of 24 hypogonadal men with T2DM treated with testosterone (n=11) and a group treated with placebo (n=13). The baseline data of the treatment group and the placebo-treated groups were not statistically different by using the *t* test.

2.3.1.1 Analysis of change in body composition in the double-blinded placebo-controlled study patients

No significant change in BMI, WC and W/H R was seen in either treatment group during the study. There was not significant diffrerence in BMI, WC and W/H R in patients with testosterone treatment compared to patients with placebo from baseline to 6 months (Table 2.3A) (Table 2.3B).

2.3.1.2 Analysis of pro and anti-inflammatory biomarkers by CBA assay

The results measuring IL-1β, IL-6, IL-10, and TNF-α from CBA method were all under the detection limit. Therefore, they were not available for analysis.

2.3.1.3 Analysis of adiponectin by ELISA in the double-blinded placebo-controlled study patients

There was a reduction but not significant in the level of adiponectin in both groups from baseline to 6 months. However, there was a significant difference in adiponectin levels in patients with testosterone treatment, which were lower after 3 months compared to the placebo treated group (2978.10 ± 672 vs 5452.48 ± 836 P=0.03) (Table 2.4A) and the reduction in adiponectin with testosterone by analysis of thechange of levels over time remained significant (-16.90±11.33 vs. 9.85±6.85 ng/ml; P=0.04) (Table 2.4B).

2.3.1.4 Analysis of TNF-α by high Sensitivity ELISA in the double-blinded placebo-controlled study patients

Testosterone treatment tended to lead to a decrease in TNF-α level in serum from

Parameter	Placebo(n=13) Mean ± SEM	Testosterone(n=11) Mean ± SEM
Age year	61.7 ± 11.8	56.6 ±11.9
BMI Kg/m²	32.65 ± 1.1	34.36 ± 1.5
W/H (cm)	1.06 ± 0.01	1.07 ± 0.01
WC (cm)	115.96 ± 3.6	119.12 ± 3.4
Adipo (ng/mL)	5502.28 ± 1186	3800.87 ± 764
CRP (ng/mL)	5685.43 ±741	4271.96±621
TNF-α (pg/ml)	0.56 ± 0.11	0.55 ± 0.11
LP(a) (U/L)	41.14 ± 8.1	57.14 ± 13
ApoB (mg/ml)	1.08 ± 0.17	1.21 ± 0.11
Apo A1(µg/ml)	753 ± 120	482 ± 87
Apo E (µg/ml)	123.2 ± 8.8	131.9 ± 11
HDL mmol/l	0.91 ± 0.06	0.94 ± 0.06
HDL2 (mmo/l)	0.791 ± 0.06	0.742 ± 0.08
HDL3 (mmol/l)	0.138 ± 0.02	0.197 ± 0.03
HDL2/HDL3 Ratio	5.731 ± 1.2	5.7 ± 1.6
HbA_{1C} (%)	7.82 ± 1.52	7.46 ± 1.12
Glucose (mmol/l)	8.85 ± 4.42	10.64 ± 4.59
Systolic BP (mmHg)	138.4 ± 20.7	135.7 ± 16.3
Diastolic BP (mmHg)	70.8 ± 10.0	72.6 ± 9.6

Table 2.2: Baseline characteristics (mean ± SEM) of hypogonadal men with T2DM administered testosterone treatment or placebo groups. The baseline values of each parameter between the placebo and testosterone groups were not statistically different. BMI: body mass index. WC: waist circumference. W/H R: waist to hip ratio. Adipo: adiponectin. CRP: C-reactive protein. TNF-α: tumour necrosis factor. LP (a): Lipoprotein (a). Apo B: apolipoproteins B. Apo A1: apolipoprotein A1. HDL: Hig density lipoprotein cholesterol. Hb1A1c: glycated haemoglobin. Bp: blood pressure. SEM: Standard error mean.

Parameter	Placebo (n=13) Mean ± SEM			Testosterone (n=11) Mean ± SEM			Baseline comparison
	Baseline	3	6	Baseline	3	6	P-value
BMI Kg/m²	32.65 ± 1.1	32.74 ± 1.2	32.64 ± 1.3	34.36 ± 1.5	34.65 ± 1.6	34.82 ± 1.6	0.3
W/H (cm)	1.06 ± 0.01	1.06 ± 0.01	1.06 ± 0.01	1.07 ± 0.01	1.08 ± 0.01	1.07 ± 0.02	0.7
WC (cm)	115.96 ± 3.6	115.20 ± 3.6	115.12± 3.9	119.36 ± 3.4	119.72 ±3.6	120.04 ± 3.9	0.4

Table 2.3A: Comparison of the effect of placebo and testosterone treatment on body composition in hypogonadal men with T2DM at baseline, 3 and 6 months. BMI: body mass index. WC: waist circumference. W/H R: waist to hip ratio. SEM: Standard error mean. GLM: generalized linear model. Baseline comparison analysed by *t*-test.

Parameters	Placebo (n=13) % change Mean ± SEM		Testosterone (n=11) % change Mean ± SEM		p-value (0-3)	p-value (0-6)
	(0-3)	(0-6)	(0-3)	(0-6)		
BMI Kg/m²	0.72 ±0.46	0.33 ± 0.84	0.86 ± 0.48	1.31 ± 0.52	0.8	0.6
W/H (cm)	0.38 ± 0.40	0.41 ± 0.44	0.94 ± 0.71	0.37 ± 0.97	0.4	0.9
WC (cm)	0.72 ± 0.46	0.41±0.85	0.25±0.30	0.45 ± 0.69	0.5	0.9

Table 2.3B: Comparison of the effect of placebo and testosterone treatment on body composition in hypogonadal men with T2DM at 3 and 6 months expressed as percentage from baseline. BMI: body mass index. WC: waist circumference. W/H R: waist to hip ratio. SEM: Standard error mean. Group comparison analysed by *t*-test.

baseline to 6 months. There was no significant difference in TNF-α levels in the testosterone group compared to placebo treated group at any time point (Table 2.4A)(Table 2.4B).

2.3.1.5 Analysis of CRP by ELISA in the double-blinded placebo-controlled study patients

The serum levels of CRP increased from baseline to 6 months in the testosterone-treated group whereas it decreased in the placebo-treated. However, there was not a significant difference between the two groups at any time point (Table 2.4A) (Table 2.4B).

2.3.1.6 Analysis of apolipoproteins by ELISA in the double-blinded placebo-controlled study patients

Neither serum LP (a) nor Apo B concentration changed during the course of the study in either treatment group, and values for the testosterone-treated group were not significantly different from the placebo-treated group. Similarly, no significant change was seen in level of Apo A1 and Apo E in patients treated with testosterone, compared to the placebo treated patient from baseline to 6 months (Table 2.4A) (Table 2.4B).

2.3.1.7 Analysis of HDL subfractions by Amplex Red cholesterol assay in the double-blinded placebo-controlled study patients

There was no effect of testosterone on level of HDL2, HDL3 and HDL2/HDL3 ratio in both groups from baseline to 6 months and in patients with testosterone treatment compared to patients with placebo over six months (Table 2.5A) (Table 2.5B).

Parameters	Placebo Mean ± SEM (n=13)			Testosterone Mean ± SEM (n=11)			Baseline comparison
	Baseline	3	6	Baseline	3	6	(P)
Adipo (ng/ml)	5502 ±1186	5452 ± 836	4880 ± 957	3800 ± 764	2978 ± 672*	3191 ± 526	0.2
CRP (ng/ml)	5685 ± 741	4705 ± 801	4336 ± 992	4271 ± 621	4906 ± 881	5173 ± 992	0.1
TNF-α (pg/ml)	0.56 ± 0.11	0.67 ± 0.08	0.47 ± 0.17	0.55 ± 0.11	0.474 ± 0.10	0.426 ± 0.09	0.9
LP(a) (U/L)	41.14 ± 8.1	43.74 ± 9.9	51.21 ± 11	57.14 ± 13	69.84 ± 12	78.19 ± 16	0.3
Apo B (mg/ml)	1.08 ± 0.11	1.23 ± 0.21	1.46 ± 0.21	1.21 ± 0.11	1.30 ± 0.21	1.26 ±0.21	0.5
Apo A1(µg/ml)	753 ± 120	1018 ± 442	848 ± 101	482 ± 87	676 ± 138	577 ± 35	0.09
Apo E (µg/ml)	123.2 ± 8.8	125.6 ± 12.0	104.8 ± 10.4	131.9 ± 11	129.6 ± 18	102.9 ± 8.6	0.5

Table 2.4A: Comparison of the effect of placebo and testosterone treatment on serum factors in hypogonadal men with T2DM at baseline, 3 and 6 months. Adipo: adiponectin. CRP: C-reactive protein. TNF-α: tumour necrosis factor. LP (a): Lipoprotein (a). Apo B: apolipoproteins B. Apo A1. SEM: Standard error mean. P: P-value. *P: < 0.05; treated testosterone group (3 months) vs placebo treated group, GLM: generalized linear model. Baseline comparison analysed by *t*-test.

Parameters	Placebo (n=13) % change Mean ± SEM		Testosterone (n=11) % change Mean ± SEM		P-value (0-3)	P-value (0-6)
	(0-3)	(0-6)	(0-3)	(0-6)		
Adipo (ng/ml)	9±6.85	-4 ± 6.9	-16±11.33*	-3 ± 11.19	0.04	0.9
CRP (ng/ml)	-5 ± 14.83	-97 ± 58.34	44 ± 38.77	35 ± 24.43	0.2	0.06
TNF-α (pg/ml)	35.11 ±49.72	10.59 ± 105.45	-56.46 ± 29.11	-52.21 ± 36.06	0.1	0.5
LP(a) (U/L)	9.20 ± 9.96	29.30 ± 12.27	64.25 ± 43.64	80.72 ± 62.11	0.2	0.4
Apo B (mg/ml)	25.34 ± 21.38	48.68 ± 27.53	9.16 ± 17.57	-0.26 ±13.10	0.1	0.5
Apo A1(µg/ml)	28.44 ± 28.84	48.11 ± 25.29	52.77 ± 22.34	42.99 ± 17.95	0.8	0.5
Apo E (µg/ml)	5.58 ± 11.56	-12.67 ± 10.02	-15.99 ± 13.25	-19.44 ± 6.36	0.2	0.5

Table 2.4B: Comparison of the effect of placebo and testosterone treatment on serum factors in hypogonadal men with T2DM at, 3 and 6 months epxressed as percentage from baseline. Adipo: adiponectin. CRP: C-reactive protein. TNF-α: tumour necrosis factor. LP (a): Lipoprotein (a). Apo B: apolipoproteins B. Apo A1. SEM: Standard error mean. P: P-value. *P: < 0.05; treated testosterone group (3 months) vs placebo treated group, Group comparison analysed by *t*-test.

Parameters	Placebo: (n=13) Mean ± SEM		Testosterone (n=11) Mean ± SEM		Baseline comparison
	Baseline	6	Baseline	6	*(P)*
HDL (mmol/l)	0.91 ± 0.06	0.97 ± 0.08	0.94 ± 0.06	0.91 ± 0.06	0.7
HDL2 (mmol/l)	0.791 ± 0.06	0.820 ± 0.09	0.742 ± 0.08	0.743 ± 0.07	0.6
HDL3 (mmol/l)	0.138 ± 0.02	0.168 ± 0.02	0.197 ±0.03	0.156 ± 0.07	0.2
HDL2/HDL3 Ratio	6.98 ± 1.2	5.38 ± 0.6	5.77 ± 1.6	5.5 6± 0.94	0.5

Table 2.5A: Comparison of the effect of placebo and testosterone treatment on serum of HDL and its fractions in hypogonadal men with T2DM at baseline and 6 months. HDL: High density lipoprotein. SEM: Standard error mean. P: P-value. Two way ANOVA. Baseline comparison analysed by *t*-test.

Parameters	Placebo: (n=13) % change Mean ± SEM	Testosterone (n=11) % change Mean ± SEM	p-value
	(0-6)	(0-6)	
HDL (mmol/l)	5.62± 4.91	-2.80 ±2.94	0.1
HDL2 (mmol/l)	0.90 ± 5.13	5.65 ± 7.43	0.6
HDL3 (mmol/l)	32.02 ±13.99	4.77 ± 20.47	0.2
HDL2/HDL3 Ratio	-13.49 ± 12.89	84.51 ± 65.91	0.1

Table 2.5B: Comparison of the effect of placebo and testosterone treatment on serum of HDL and its fractions in hypogonadal men with T2DM at 6 months expressed as percentage from baseline. HDL: High density lipoprotein. SEM: Standard error mean. Group comparison analysed by *t*-test.

2.3.2 Patients in longitudinal study

Table 2.6 shows the baseline characteristics of the 120 diabetic males included in the longitudinal study with an average age of 66 years (range 40-79 years). This group was divided based on the baseline level of serum testosterone into normal testosterone participants (n=76), low testosterone participants without testosterone treatment (n=29) and low testosterone with testosterone treatment participants (n=15). Within the groups these were also divided into four subgroups according to IHD status. Patients with IHD and normal testosterone (n=14), IHD with low testosterone without testosterone treatment (n=6) and others had IHD and treated with testosterone therapy (n=4) compared to a further subgroup of patients with normal testosterone without IHD (n=52) and used as untreated (no testosterone) control group.

2.3.2.1 Analysis of change in body composition in the longitudinal study patients

There was no significant difference in BMI, W/H ratio and WC between patients with low testosterone and patients with low testosterone treated with testosterone compared to control samples who had normal testosterone (Table 2.7). Likewise, no significant difference was seen in body composition in IHD subgroups compared to untreated control samples who had normal testosterone without IHD (Table 2.8).

2.3.2.2 Analysis of pro and anti-inflammatory biomarkers by Enhanced Sensitivity CBA in the longitudinal study patients

The level of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ in serum of patient in this study was measured by Enhanced Sensitivity CBA assay. The data for IL-8 was reliable (the coefficient of variation between sample measures was below 10%), however the obtained data for IL-1β, IL-6, IL-10, TNF-α and IFN-γ were not reliable and excluded, due to the much higher variability among these data.

2.3.2.3 Analysis of IL-8 levels by Enhanced Sensitivity CBA assay in the longitudinal study patients

No significant difference was observed in the mean serum IL-8 level in patients with low testosterone either with or without treatment compared to untreated control samples

Parameter	Normal testosterone Group (n=76)	Low testosterone Group (n=29)	Low testosterone + TRT Group (n=15)
Age year[a]	**56.13 ± 1.00**	**56.55 ±1.68**	**57.13±1.19**
BMI Kg/m²[a]	**32.22 ± 0.71**	**33.16 ±1.05**	**35.03±1.12**
W/H (cm)[a]	**0.62 ± 0.01**	**0.64± 0.01**	**0.66 ± 0.01**
WC (cm)[a]	**108.97 ± 1.68**	**112.32 ± 2.58**	**116.85±2.46**
HbA_{1C}[a,c]	**7.19± 0.15**	**7.21 ± 0.19**	**7.61±0.31**
IHD[b,c]	**26.31%**	**24.13%**	**13.3%**
Insulin treatment[b, c]	**34.21%**	**24.14%**	**60%**
Metformin[b, c]	**60.52%**	**41.37%**	**40%**

Table 2.6: Baseline characteristics for patients and untreated controls groups in the longitudinal study. BMI: body mass index. W/H R: waist to hip ratio. WC: waist circumference. [a]Mean with standard error of mean. [b]Number as a percentage, [c] data were obtained from the hospital records. IHD: ischemic heart disease.

Parameter	NT (n=76) Mean ± SEM	LT (n=29) Mean ± SEM	LT+TRT (n=15) Mean ± SEM
BMI Kg/m²	32.42 ± 0.8	32.91 ± 1.0	34.19 ± 1.4
W/H R(cm)	0.56 ± 0.01	0.54 ± 0.02	0.59 ± 0.02
W C (cm)	97.73 ± 2.4	99.01 ± 4.2	104.4 ± 3.7

Table 2.7: Comparison in body composition between patients and untreated controls with normal levels testosterone at the end of the longitudinal study. NT: normal testosterone. LT: low testosterone. LT+TRT: low testosterone with testosterone replacement treatment. BMI: body mass index. W/H R: waist to hip ratio. WC: waist circumference. SEM: Standard error mean. Kruskal-Wallis test.

Parameter	NT (n=52) Mean ± SEM	IHD+NT (n=14) Mean ± SEM	IHD+LT (n=6) Mean ± SEM	IHD+TRT (n=4) Mean ± SEM
BMI Kg/m²	32.06 ± 0.9	31.6 ± 1.4	29.9 ±1.8	33.55 ± 2.5
W/H R(cm)	0.55 ± 0.01	0.55 ± 0.02	0.50 ± 0.03	0.57 ± 0.04
WC (cm)	95.9 ± 2.9	96.29 ± 4.6	83.9 ± 5.8	98.03 ± 7.7

Table 2.8: Comparison in body composition between IHD patients and untreated controls with normal levels of testosterone at the end of the longitudinal study. NT: normal testosterone. IHD+NT: ischemic heart disease with normal testosterone. IHD+LT: ischemic heart disease with low testosterone. IHD+TRT: ischemic heart disease with testosterone replacement treatment. BMI: body mass index. W/H R: waist to hip ratio. WC: waist circumference. SEM: Standard error mean. Kruskal-Wallis test.

(Table 2.9). Similarly, there was no significant difference in levels of IL-8 among all IHD subgroups compared to untreated control samples ($p>0.05$) as shown in table 2.10.

2.3.2.4 Analysis of adiponectin levels by ELISA in the longitudinal study patients

As shown in table 2.9, no significant change was seen in mean serum levels of adiponectin between patients with low testosterone with or without treatment and untreated control samples. In the same way, there was no significant difference in adiponectin level between the 3 subgroups and untreated control samples (Table 2.10).

2.3.2.5 Analysis of apolipoprotein factors by ELISA in the longitudinal study patients

There was no significant difference in all apolipoprotein concentrations (Apo A1, Apo B, Apo E and LP (a)) between patients with low testosterone who were treated with or without testosterone therapy as seen in table 2.9. Similarly, no significant change was seen in the level of these apolipoprotein among all IHD subgroups compared to untreated control samples as shown in table 2.10.

2.3.2.6 Analysis of HDL subfractions by Amplex Red cholesterol assay in longitudinal study

As shown in table 2.11, the mean serum level of HDL ($p=0.05$) in patients with low testosterone was approaching significantly lower than in the untreated control group. Although not statistically significant, an increase in the level of HDL was seen in patients treated with testosterone compared to patients with low testosterone. In contrast, no significant difference was observed in the mean serum HDL2 levels in patients with low testosterone and the untreated control group, whilst the mean serum HDL2 levels in patients treated with testosterone was approaching significantly ($p=0.06$) higher than in the untreated control group. There was a significant increase in the level of HDL2 ($p=0.01$) in patients treated with testosterone compared to patients with low testosterone. It is worthy to note that while there was no a significant difference in the level of HDL3 in patients with low testosterone and the untreated control group, there was significant reduction in its level ($p=0.004$) in patients treated with testosterone

Parameter	NT (n=76) Mean ± SEM	LT (n=29) Mean ± SEM	LT±TRT (n=15) Mean ± SEM
Adipo (ng/ml)	10731 ±941.8	10812 ± 1460	10220 ± 1847
IL-8 (pg/ml)	1.96 ±0.26	2.40 ± 0.68	2.51 ± 0.33
LP(a) (U/L)	175.28 ±25.3	209.1 ± 51.1	144.7 ± 53.5
Apo B (mg/ml)	1.59 ±0.69	1.69 ± 0.11	1.49 ± 0.16
Apo A1(µg/ml)	1725 ±104.1	1734 ± 131.2	1549 ± 136.6
Apo E (µg/ml)	121.3 ±7.8	119.2 ± 11.97	112.9 ± 13.34

Table 2.9: Comparison of adiponectin, IL-8 and apolipoprotein between patients and untreated controls with normal levels testosterone in the longitudinal study. NT: normal testosterone. LT: low testosterone. LT+TRT: low testosterone with testosterone replacement treatment. IL-10: interleukin10. IL-8: interleukin 8. LP (a): lipoprotein. Apo: apolipoprotein. SEM: Standard error mean. Kruskal-Wallis test.

Parameters	NT (n=52) Mean ± SEM	IHD+NT (n=14) Mean ± SEM	IHD+LT (n=6) Mean ± SEM	IHD+TRT(n=4) Mean ± SEM
Adipo (ng/ml)	11559 ± 1241	8836 ± 1281	14134 ± 2984	13813 ± 5928
IL-8 (pg/ml)	1.52 ± 0.22	1.67 ± 0.26	2.51 ± 0.73	2.07 ± 0.60
LP(a) (U/L)	194.9 ± 31.82	181.1 ± 68.09	198.8 ± 142.8	329.8 ± 147.6
Apo B (mg/ml)	1.62 ± 0.07	1.56 ± 0.17	1.44 ± 0.28	1.85 ± 0.53
Apo A1(µg/ml)	1835 ± 133	1649 ± 227	1803 ± 268	2061 ± 798
Apo E (µg/ml)	110.5 ± 7.1	140.4 ± 25.9	107.6 ± 23.6	123.2 ±45.1

Table 2.10: Comparison of adiponectin, IL-8 and apolipoprotein between IHD patients and untreated controls with normal levels testosterone in the longitudinal study. NT: normal testosterone. IHD + NT: ischemic heart disease with normal testosterone. IHD + LT: ischemic heart disease with low testosterone. IHD+TRT: ischemic heart disease with testosterone replacement treatment. IL-8: interleukin 8. LP (a): lipoprotein (a). Apo: apolipoprotein. SEM: Standard error mean. Kruskal-Wallis test.

Parameters	NT (n=76) Mean ± SEM	LT (n=29) Mean ± SEM	LT+TRT (n=15) Mean ± SEM
HDL(mmol/l)	1.40 ± 0.02	1.31 ± 0.04	1.38 ± 0.05
HDL2 (mmol/l)	1.09 ± 0.02	1.01 ± 0.05	1.17 ± 0.05 ¤¤
HDL3 (mmol/l)	0.31 ± 0.01	0.30 ± 0.03	0.21 ± 0.02*
HDL2/HDL3 Ratio	4.24 ± 0.25	4.07 ± 0.34	5.90 ± 0.50 **, ¤¤

Table 2.11: Comparison of HDL, HDL2, HDL3 and HDL2/HDL3 ratios between patients untreated controls with normal levels testosterone in the longitudinal study. NT: normal testosterone. LT: low testosterone. LT+TRT: low testosterone with testosterone replacement treatment. HDL: High density lipoprotein cholesterol. SEM: Standard error mean. * P< 0.05, ** P<0.01 vs NT, ¤¤ P<0.01 vs LT. Kruskal-Wallis test.

compared to the untreated control group. In addition, there was an almost significant decrease in concentration of HDL3 ($p<0.05$) in patients treated with testosterone compared to patients with low testosterone. No significant difference was seen in the HDL2/HDL3 ratio between patients with low testosterone and the untreated control group. Conversely, there was a significant increase in the HDL2/HDL3 ratio in patients treated with testosterone ($p=0.003$) compared to the untreated control group and ($p=0.01$) compared to the patients with low testosterone. As shown in table 2.12, no significant change was seen in levels of HDL in all IHD subgroups. A significant decrease was only seen in the level of HDL2 ($p=0.02$) in IHD patients with normal testosterone compared to the untreated control group. However, no significant difference was seen in levels of HDL3 and H2/H3 ratio in all IHD subgroups compared to the untreated control subjects.

Parameters	NT (n=52) Mean ± SEM	IHD+NT (n=14) Mean ± SEM	IHD+LT (n=6) Mean ± SEM	IHD+TRT (n=4) Mean ± SEM
HDL (mmol/l)	1.43 ± 0.01	1.36 ± 0.04	1.37 ± 0.11	1.29 ± 0.12
HDL2 (mmol/l)	1.12 ± 0.02	1.02 ± 0.04*	1.07 ± 0.09	1.08 ± 0.11
HDL3 (mmol/l)	0.31 ± 0.02	0.34 ± 0.03	0.30 ± 0.05	0.21 ± 0.06
HDL2/HDL3 Ratio	4.31 ± 0.30	3.60 ± 0.51	4.11 ± 0.61	6.15 ± 1.80

Table 2.12: Comparison of HDL, HDL2, HDL3 and HDL2/HDL3 ratios between IHD patients and untreated controls with normal levels testosterone in the longitudinal study. NT: normal testosterone. IHD + NT: ischemic heart disease with normal testosterone. IHD + LT: ischemic heart disease with low testosterone. IHD+TRT: ischemic heart disease with testosterone replacement treatment. HDL: High density lipoprotein cholesterol. SEM: Standard error mean. * $p < 0.05$ vs NT. Kruskal-Wallis test.

Parameters	Double-blinded placebo-control study	Longitudinal study	
		- IHD	+ IHD
Body composition	NC	NC	NC
Adiponectin	Reduction after 3 months	NC	NC
TNF-α	NC	NR	NR
CRP	NC	NI	NI
IL-8	NI	NC	NC
Apolipoprotein	NC	NC	NC
HDL	NC	NC	NC
HDL2	NC	Increased	NC
HDL3	NC	Decreased	NC
HDL2/HDL3 Ratio	NC	Increased	NC

Table 2.13; Summary of results the effect of testosterone treatment on serum factors in hypogonadal men with T2DM in Double-blinded placebo-control and longitudinal studies. NC: no change, NR; no result, NI=not investigated, IHD: ischemic heart disease, Adipo: adiponectin, CRP: C-reactive protein, TNF-α: tumour necrosis factor, IL-8: interleukin 8, HDL: high density lipoprotein.

2.4.1 Patients in double-blinded placebo-controlled study

This study investigated the effect of testosterone replacement therapy over a six month period in men with low testosterone and T2DM.

2.4.1.1 The effect of testosterone on body composition in patients in a double-blinded placebo-controlled study

All of the patients were suffering from obesity as defined by the baseline of BMI criteria and central adiposity, as defined by waist or waist-hip ratio. No significant changes in BMI, WC and W/HR following TRT were found in this study. In a similar study, no significant changes in BMI or waist circumference were observed in diabetic Japanese men, following administration of the testosterone enanthate for 6 months (Ueshiba, 2013). Furthermore, no significant changes were observed in the BMI of diabetic patients following 6 months of testosterone treatment (Kapoor *et al.,* 2007). Similar results were obtained in a study by Frederisen et al. (2012) in that, there was no change in visceral fat mass in aged men with low testosterone levels following 6 months of transdermal testosterone treatment, However, subcutaneous fat mass was significantly reduced in both the high and the abdominal areas when analysed by MRI. A further study found that testosterone treatment for 8 months in 23 middle aged, abdominally obese men was followed by a decrease in visceral fat mass, without changes in body mass, subcutaneous fat and lean body mass (Marin *et al*, 1992a).

The beneficial effects of testosterone on body composition have been reported in other studies. Rebuffe-Scrive *et al.* (1991) showed a reduction in the W/HR in 9 of 11 middle aged men who were moderately obese, after intramuscular injection of testosterone over a 6 weeks period. Furthermore, Saad et al. (2009) reported that a decline in WC was observed in both groups treated with a high dose testosterone undecanoate (TU; 1000 mg) and testosterone gel (50 mg/day) for 9 months in two cohorts of elderly men with late-onset hypogonadism. In a study by Kapoor et al. (2006, 2007) testosterone therapy (200mg sustanon) for 3 months reduced WC and W/HR, improved IR and glycemic control in men with testosterone deficiency and T2DM. Moreover, testosterone therapy for 52 weeks reduced visceral fat accumulation in proportion to the increase in

testosterone levels in 60 non-obese aging men (Allan *et al.,* 2008). Other studies have similarly demonstrated an improvement in WC (Saad *et al.,* 2007, 2008) and decreased fat mass (Agledahl *et al.,* 2008) with TRT.

The dose, duration of treatment, route of administration of testosterone and design of study (Alexandersen and Christiansen, 2004, Jones and Saad, 2009; Traish and Kypreos ., 2011; Kapoor *et al.,* 2007) or the way of measuring regional distribution of fat such as ultrasound, which may not give reliable result (Frederiksen *et al.*, 2012a) are known factors which impact on any change in body composition in response to testosterone.

The latter points may demonstrate why TRT did not show beneficial effects on BMI, WC and W/RH in the current study. However, the mechanisms by which testosterone decreases body composition are still poorly understood, improved understanding of how testosterone is associated with beneficial effects in T2DM is needed.

2.4.1.2 The effect of testosterone on adiponectin in patients in a double-blinded placebo-controlled study

It was found that testosterone treatment reduced serum adiponectin levels significantly after three months of treatment, in comparison to levels in samples from those with the placebo. This agrees with findings by Kapoor et al. (2007) who reported a significant reduction in the level of adiponectin after 3 months testosterone treatment in men with T2DM. Similarly, two other studies found that testosterone treatment reduced adiponectin plasma levels following 6 months, in young hypogonadal non-diabetic men (Lanfranco *et al.,* 2004) and in ageing men with low to normal testosterone levels (Frederiksen *et al.,* 2012). Furthermore, it has been reported that the percentage composition of the high molecular weight (HMW) fraction of adiponectin was reduced by TRT in both animal models (mice) and hypogonadal men (Xu *et al.,* 2005). A previous study that induced low testosterone levels in normal men experimentally, resulted in an increase in adiponectin levels following a supplement of testosterone this increase was prevented (Page *et al.,* 2005b). This was strongly supported by an animal study whereby castration of mice was associated with an increase in adiponectin which was reversed following TRT in both sham-operated and castrated mice (Nishizawa *et al.,* 2002). According to Kapoor et al. (2007), the suppressive action of testosterone on the adiponectin levels is thought to be as a result of a reduction in body fat composition related to TRT, which led to a decrease in adiponectin production from adipocytes. In

support of this hypothesis, previous studies reported that plasma adiponectin levels showed a more significant correlation with the amount of visceral adipose tissue than the subcutaneous adipose tissue (Yatagai *et al.,* 2003; Park *et al.,* 2004; Kwon *et al.,* 2005), while another study stated that the subcutaneous adipose tissue was independently correlated with the HMW adiponectin levels in aging men (Fujikawa *et al.,* 2008). A further study provides contrasting evidence supporting the beneficial effect of testosterone on body composition without affecting the level of adiponectin (Page *et al.,* 2005a). The latter studies reported opposite findings to this current study with no significant change in body composition being seen in patients treated with testosterone. Moreover, Heufelder et al. (2009) found that testosterone therapy in combination with diet and exercise in T2DM men resulted in increase in adiponectin, demonstrating that other factors in addition to testosterone can contribute to a positive outcome. However, the mechanism involved in this action is not fully understood. Therefore, further studies important to determine the mechanism through which this occurs.

2.4.1.3 The effect of testosterone on pro-inflammatory factors in patients in the double-blinded placebo-controlled study

The present study showed no effect of TRT on patient serum CRP levels for 6 months. Similar findings were observed in several studies with different types of testosterone treatment. One of these demonstrated that treatment with anastrozole, which inhibits the conversion of testosterone to estradiol, did not significantly affect inflammatory markers CRP or IL-6 in elderly men with mild hypogonadism (Dougherty *et al.,* 2005), this study demonstrates that when testosterone is acting via the AR no change in these markers is seen. Additional studies reported that there were no effects on CRP levels at any dose of testosterone in young eugonadal men, who received an analogue of gonadotrophin-releasing hormone to block gonadal steroid production, followed by supplementation with graded doses of testosterone (Singh *et al.,* 2002). Similarly, no change was seen in levels of CRP and IL-6 following 3 months of androgen treatment with either dihydrotestosterone or recombinant human chorionic gonadotropin in elderly healthy men with partial androgen deficiency (Ng *et al.,* 2002). At the level of the gene *in vivo*, Corcoran and co-workers, (2010) found that both physiological and supraphysiological concentrations of testosterone had no effect on macrophage CRP mRNA expression in older men. Additionally, a significant inverse correlation between baseline CRP and total testosterone was observed in diabetic men (Kapoor *et al.,* 2007)

while in a second study, free testosterone had a negative correlation with CRP in older age males (Yang *et al.,* 2005). Haider et al. (2007) found that ADT was associated with an unfavourable increase in CRP levels in diabetic men with advanced prostate cancer. However, in the present study the short time of treatment (6 months) might explain the lack of effect of testosterone on anti-inflammatory actions, and long-term treatment would be required to clarify the effect of testosterone on CRP.

In this current study, testosterone had no effect on serum levels of TNF-α patients, this is consistent with the data reported in human and animal studies that testosterone did not show an effect on TNF-α levels (Kapoor *et al.,* 2007; Kelly *et al.,* 2012). In contrast, Malkin et al. (2004) indicated that testosterone treatment induced a small but significant reduction in TNF-α in hypogonadal men, compared with the placebo group. Similarly, another study found that transdermal testosterone reduced serum TNF-α but not IL-6 in normal elderly men (Khosla *et al.,* 2002). A further study stated that while TRT caused a decrease or complete abolition of spontaneous *ex vivo* production of IL-1, IL-6 and TNF-α by antigen-presenting cells in diabetic patients with partial androgen deficiency, testosterone, in *in vitro,* had no effect on the secretion of inflammatory cytokines by these cells after stimulation with LPS plus IFN-γ this suggests that APCs preserve their constitutive machinery to produce inflammatory cytokines under androgen treatment (Corrales *et al.,* 2006). In support of testosterone as an anti-inflammatory agent, induction of acute hypogonadism in normal elderly men performed by administration of GnRH which led to the contrary effect, where this increased the circulating levels of TNF-α and IL-6. In this regard, testosterone showed immune modulatory action through its effects on different inflammatory diseases including rheumatoid arthritis and systemic lupus erythematous, in which androgens have improved the clinical status and decreased inflammatory factors (Cutolo *et al.,* 1991).

However, the lack of change in TNF-α and CRP levels after TRT might be as a result of the lack of influence of testosterone on the mean BMI, WC and W/HR in the group treated with testosterone. The present study data indicated a high degree of obesity among these patients, which did not change with testosterone treatment, and obesity is known to be correlated positively with these inflammatory factors.

2.4.1.4 The effect of testosterone on atherogenic factors in patients in double-blinded placebo-controlled study

Testosterone treatment had no significant effect on HDL, or its fractions HDL2, HDL3 and HDL2/HDL3 ratio. Similarly it had no effect on serum apolipoproteins levels including Apo A1, Apo B, Apo E and LP (a). This is supported by previous findings, where Agledah et al. (2008) found that after normalising for testosterone levels in elderly men with subnormal testosterone levels, no significant change in levels of HDL was noted. Further studies reported that testosterone treatment, for one month, did not affect triglycerides or HDL, in 27 hypogonadal men (Malkin *et al.,* 2004a). Similarly, two other studies in which men with Leydig cell insufficiency, firstly as a result of cytotoxic chemotherapy (Howell *et al.,* 2001) and secondly as a result of age > 65 years of age (Snyder *et al.,* 2001) were treated with transdermal testosterone replacement, reported no change in HDL. These studies showed that the route of administration is a vital factor, because transdermal testosterone seemingly did not affect plasma lipoproteins in healthy elderly men. Others thought that TRT in hypogonadal and elderly men may have a beneficial effect on lipid metabolism, through decreasing total cholesterol and the atherogenic fraction of LDL, without significant alterations in HDL levels or its subfractions HDL2 and HDL3 (Zgliczynski *et al.,* 1996). However, other studies showed contrasting findings with regards to HDL or its fractions following testosterone treatment. An increase (Ozata *et al.,* 1996), or reduction (Bagatell *et al.,* 1992, Behre *et al.,* 1994, Lapauw *et al.,* 2009, Bagatell *et al.,* 1994; Herbst *et al.,* 2003) in HDL and its fraction have both been reported. Hence, although testosterone treatment had no clear effect on HDL and their fractions, it also did not associate negatively with their levels. Especially, as there were previous data related to the inhibiting effect of testosterone treatment on HDL (Frederikse *et al.,* 2012a) in men causing concern for using testosterone as therapy.

Similarly, controversial data have been stated for LP (a) which is considered as risk factor for CVD. Previous studies reported an increase in LP (a) levels with testosterone treatment (Berglund *et al.,* 1996; Von Eckardstein *et al.,* 1997) while a control study (Snyder *et al.,* 2001) found no link between testosterone and LP (a). Similarly, two studies reported no change in LP (a) after testosterone treatment in hypogonadal men (Ozata *et al.,* 1996) and in elderly obese men (Herbst *et al.,* 2003). An uncontrolled study established a 25–59% decline in Lp(a) following testosterone injection in men

with a LP(a) concentration less than 25 nmol/l, but no effect on LP (a) when the level was above 25 nmol/l, prior to testosterone administration (Marcovina *et al.,* 1996). A further study indicated that a decrease in LP (a) levels was associated with GnRH analogue therapy in patients with prostate cancer (Arrer *et al.,* 1996). However, more recently, in a double blind, placebo controlled study over a 6-month period, transdermal testosterone treatment was associated with favourable effects on IR, total and LDL-cholesterol, and LP(a), in hypogonadal men with T2DM and/or MetS (Jones *et al.,* 2011)

Regarding Apo B, while two studies found no alteration in levels of Apo B (Herbst *et al.,* 2003; Tan *et al.,* 1998), others found that androgens were associated with decreased ApoB levels and LDL cholesterol (Dickerman *et al.,* 1996). In an animal study, Zhongguo and Li, (2000) showed testosterone, at physiological serum levels, had a positive effect on lipid profiles, lipoprotein, ApoB, in rabbits whilst hypotestosteronemia and hypertestosteronemia have a negative effect. Testosterone treatment had no effect on ApoE. Similarly, Snyder et al. (2001) found no change in ApoE after testosterone administration. However, at the level of the gene *in vitro*, testosterone therapy increased ApoE mRNA in the liver of Tfm mice (Kelly *et al.,* 2012a) and in human macrophages (Kelly *et al.,* 2012b). Limited information is available regarding ApoE and correlation with testosterone treatment.

In most of the previous studies, there was a reduction in both the lipid profiles and body composition (Malkine *et al.,* 2004, Saad *et al.,* 2008; 2009) whereas in the present study, there was no change in body composition based on the average BMI and W/HR, this may explain why testosterone had not effect on HDL and its fraction or in apolipoprotein. However, these studies were also for a longer time period than the present study or used route of administration of testosterone different from in the current study. This may explain the difference results found in the current study.

2.4.2 Patients in the longitudinal study

This part of the study was conducted on 120 men between the ages of 40 and 79 who had T2DM and received gel or intramuscular testosterone treatment. These patients were initially assessed and diagnosed between 2002 to 2005 years, then followed up 6-7 years later when a serum sample was taken, this sample was analysed in the

current study. In the current study 120 men were divided into a normal testosterone group and groups with low testosterone with or without testosterone treatment. This was based on the testosterone levels at the time of the original assessment (2002-2005). Additionally, these groups were divided based on IHD status into 4 subgroups; normal testosterone subject, normal testosterone subject with IHD, low testosterone subject with IHD with and without testosterone treatment.

2.4.2.1 The effect of testosterone treatment on body composition in patients in the longitudinal study

The results demonstrated that at the end of the period of study there was no significant difference in body composition (BMI, W/HR ratio and WC) between subgroups treated with testosterone compared to untreated control subjects. This was similar to the findings in the double-blinded placebo-controlled study where six months testosterone intramuscular injection had no effect. In the similar way, two studies did not find a change in VAT after treatment with a transdermal testosterone patch for 1 year in nonobese aging men (Allan *et al.,* 2008) and in elderly men (Svartberg *et al.,* 2008). This involved non-obese patients in the first study and measured regional distribution of fat by computed tomography (CT) in the second study, which may not give reliable results, this may explain the lack of effect of testosterone on VAT. This is despite the fact that in previous studies, an inverse correlation between the total testosterone levels of 110 men and accumulation of visceral fat, but not other fat deposition, was seen after a 7.5-year follow-up (Tsai *et al.,* 2000). In long term studies (5-6 years) using a high dose of parenteral testosterone undecanoate 1000 mg, reduction in WC and BMI was observed in obese men with testoserone deficiency (Saad *et al.,* 2013; Yassin *et al.,* 2013), in obese hypogonadal men with T2DM (Haider *et al.,* 2013, 2014) and in ageing men with metabolic syndrome (Francomano *et al.,* 2014). Therefore, the contrast of the current data with the above studies may be attributed to differences dose/duration of treatment and in age of men in these studies.

2.4.2.2 The effect of testosterone treatment on adiponectin in patients in the longitudinal study

The mean serum adiponectin level was not affected by testosterone treatment in

patients with low testosterone with or without treatment compared to untreated control group. There was also no difference in adiponectin levels between IHD subgroups. This is in agreement with a previously published study in which 200mg testosterone enanthate had no effect on adiponectin levels over 36 months period in older men (Page *et al.,* 2005a). This was in contrast to the effect of intramuscular testosterone treatment for 3 months that decreased the level of adiponectin in diabetic patients in a double-blinded placebo-controlled study in this thesis. Similarly, Frederiksen *et al.* (2012a) reported that gel testosterone treatment over 6 months significantly decreased adiponectin levels in aging men and suggested this action was as result of reduction in subcutaneous fat in the abdomen and on the lower extremities. However, in both groups, testosterone treatment as intramuscular injection or gel had no effect on body composition. Thus, it could be beneficial to investigate the effect of testosterone treatment on adiponectin gene expression *in vitro*.

2.4.2.3 The effect of testosterone treatment on IL-8 levels in patients in the longitudinal study

There was no effect of testosterone on the level of IL-8 in patients treated with testosterone compared to the untreated control group and between all IHD subgroups. IL-8 acts as a pro-inflammatory factor and has a role in progression of atherosclerosis (Boekholdt *et al.,* 2004) and inflammation in adipose tissue (Trayhurn *et al.,* 2004). Therefore, testosterone did not show modulation of other inflammatory factors. Furthermore, there is limited evidence on the role of testosterone on IL-8 thus; it is difficult to explain the lack effect of testosterone treatment on IL-8.

2.4.2.4 The effect of testosterone on atherogenic factors in patients in the longitudinal study

Testosterone treatment increased the level of HDL, but not significantly, compared to patients with low testosterone without treatment. Testosterone significantly increased HDL2, and the HDL2/HDL3 ratio compared to patients with low testosterone without treatment. In addition, testosterone significantly decreased HDL3 in the low testosterone group compared to the untreated control group and tended to decrease it compared to patients with low testosterone who were not treated with testosterone. This decrease appeared to be due to the effects of testosterone, as the HDL3 levels in both

the untreated groups (normal and low testosterone) were very similar, whereas the levels in the treated group were lower. Additionally, testosterone treatment was not associated with any change in HDL and its fractions between all IHD sub-groups. However, it was recognized that both total HDL and HDL2 levels have inverse associations with the risk of acute myocardial infarction and may thus be protective factors in IHD, whereas the role of HDL3 remains unclear (Salonen *et al.,* 1991). Another recent study suggested that subjects with higher HDL2 levels were better protected from atherosclerosis, while a shift towards HDL3 was reported in T2DM and MetS. This shift might be due to defect in production of HDL3 or in LCAT which is responsible for convertion of HDL3 to HDL2 (Maeda *et al.,* 2011). Moreover, the HDL2/HDL3 ratio is correlated positively and strongly with the HDL-C level, but negatively and moderately with BMI, WC, and TG (Moriyama *et al.,* 2014). Therefore, the result of the current study demonstrating an increase in HDL2 and HDL2/HDL3 ratio and a decrease in HDL3 in patients treated with testosterone may reflects the anti-atherogenic action of testosterone. An additional study found that androgens significantly increased the level of HDL2 with no change in HDL and HDL3 (Ozata *et al.,* 1996). Moreover, Tan (1998) found that there was a reduction in HDL3 sub-fractions and a decrease in HDL, after treatment with testosterone as a parenteral testosterone ester for 4-weeks.

In the current study, there was no significant change in HDL even though there was a numerical increase in HDL in patients treated with testosterone, compared to untreated patients with low testosterone. Further a single-blind, randomised design study without placebo control, with patients either receiving diet and exercise advice alone, or diet and exercise advice in conjunction with 50mg testosterone gel once daily was conducted by Heufelder and colleagues (2009) in men with T2DM. In that study, testosterone significantly increased levels of HDL and decreased LDL and TG compared with diet and exercise advice alone. In Tfm mice, physiological testosterone replacement augmented HDL levels and this effect was independent of the androgen receptor or ERα-dependent pathways after conversion of testosterone to 17β-estradiol (Nettleship *et al*, 2007a). Saad et al. (2008) reported that testosterone undecanoate decreased the levels of cholesterol and LDL and increased HDL in men with sexual dysfunction and MetS.

In studies carried out by Singh et al. (2002), the highest dose of testosterone (600 mg/wk) caused a reduction in plasma levels of HDL and Apo A1 in healthy young men. Suppression of testosterone with GnRH antagonists increased plasma HDL, HDL2 and HDL3. A meta-analysis indicated that intramuscular testosterone injection to hypogonadal men caused a small, dose-dependent decrease in HDL (Whitsel *et al.,* 2001). Androgens were associated with decreases in HDL cholesterol and ApoA1 levels in 12 competitive bodybuilders who used anabolic steroids (Dickerman *et al.,* 1996). According to Tan (1998), this reduction might come as a result of the effect of testosterone predominantly on hepatic lipase activity. For this, it concluded that the effects of androgen on HDL and its fraction, nevertheless, depend on the formulation and type of androgen used, and the dose and route of administration. Supraphysiological doses of androgens, especially orally administered, nonaromatizable, androgen steroids, will decrease plasma HDL levels significantly (Schwarcz *et al,* 2010).

In the present study, testosterone had no effect on the levels of LP (a) in patients with low testosterone who were treated with testosterone compared to the untreated patients with low testosterone or untreated control group. This is supported by previous studies (Ozata *et al.,* 1996 and Herbst *et al.,* 2003). In contrast to the current data, testosterone treatment has been shown to decrease the levels of LP (a) (Klentze *et al.,* 2005 and Lapauw *et al.,* 2009). No significant change was seen in the levels of ApoA1, ApoB, and ApoE in low testosterone patients with or without testosterone treatment, compared to untreated control group with normal testosterone. Similarly, testosterone treatment had no effect on levels of any apolipoproteins in the IHD subgroups. However, as in the double-blinded placebo-controlled patient study, testosterone did not associate with a change in level of apolipoprotein in diabetic patients. This might be due to the lack of the effect of testosterone treatment on body composition.

2.5 Limitations of the study

Limitations of this study include the small number of patients in both studies and the short duration of treatment in the double-blinded placebo-controlled study which may limit in the benefit of testosterone treatment, thus using a longer study period both before and after treatment would better control more variables, match for age, body composition, stage of disease and interaction with other medications e.g. statins,

metformin. An additional factor limiting the ability of the trial to detect changes in inflammatory cytokines, following testosterone treatment, was the lack results of these parameters by using CBA array assay, therefore alternative methods of assessing these parameters will be considered for future studies.

2.6 Summary

The short term double-blinded placebo-controlled patients study extended previous findings by providing further support for the notion that testosterone therapy decreases adiponectin levels as seen in diabetic patients after three months. However, no alteration in body composition, levels of pro-inflammatory factors or apolipoproteins after testosterone therapy was found in this group of patients. Similarly, testosterone did not affect HDL levels and its fractions, whereas previous studies found a decrease. In the longitudinal study, testosterone treatment also was not associated with any changes in body composition, the level of pro-inflammatory factors and apolipoproteins in diabetic patients. Beneficial effects on HDL fraction were seen in patients with low testosterone were treated with testosterone, but not in IHD subgroups. However, the effect of testosterone supplementation may be elucidated when larger populations and longer study period of testosterone treatment trials are carried out in double-blinded placebo-controlled studies patients or using the same group of patients both before and after treatment in a longitudinal study, as well as for estimating the longer term outcome of testosterone treatment.

Chapter 3

The effect of testosterone treatment on the expression of metabolic markers in the liver of the Tfm mouse model

3.1 Introduction

Animals have been used to study the causes, impact and complications of many diseases affecting humans including T2DM and heart disease related to obesity and have been widely used as models of metabolic syndrome, obesity and androgen insensitivity (Jones and Saad, 2009). These studies have relied on various approaches to explore the mechanisms by which diseases develop or to discover the appropriate strategies that may help protect against these diseases (Malkin *et al.,* 2004). Animal studies have employed different approaches such as using varying degrees of reduction or an increase in nutrition, pharmacological methods, genetic manipulation or surgical techniques to produce useful animal models (Bertram and Hanson, 2001). The ability to modify the genetic make-up of mice and to create 'transgenic' or 'knockout' mice has allowed the progress of several interesting and suitable obesity, T2DM and CVD models (Gajda *et al,* 2007). Additionally, to achieve a better understanding of human obesity, rabbits, rats and mice are used as models as they readily gain weight when supplied with a high-fat diet and this increases also other risk factors related to MetS. Moreover, atherosclerotic lesions comparable to those established in humans can be developed after longer feeding times in Testicular feminized mice (Tfm) which are considered as a model of effect of testosterone.

3.1.2 Testicular feminization identification

An inherited syndrome of testicular feminization has been described in several species in addition to man, including the cow, the rat and the mouse (He *et al.,* 1991). It is also called androgen insensitivity syndrome (AIS). This syndrome was initially termed testicular feminization but this name at present is used for animals, while androgen resistance or androgen insensitivity is used for humans (Howden, 2004). It is identified as a syndrome resulting from unresponsiveness of the target cell to the action of androgenic hormones due to mutations in the androgen receptor genes (Hughes *et al.,* 2006). The first proof that AIS was caused by AR mutations was published in 1989 by Brown and his group. The androgen receptor is a high-affinity androgen-binding protein that mediates the effect of testosterone and dihydrotestosterone by functioning as a trans-acting inducer of gene expression (Brown *et al.,* 1989).

3.1.3 Tfm mice

Tfm mice are totally insensitive to androgen due to a single base deletion in the N-terminal domain of the androgen receptor, resulting in a frame shift mutation creating a premature, stop codon at amino acid 412 and the expression of non-functional truncated androgen receptors (Rizk *et al.,* 2005, He *et al.,* 1991, Monks *et al.,* 2007; Chang *et al.,* 2013). Furthermore, AR binding is greatly decreased in various tissues. Therefore, Tfm in these mice is considered an X-linked genetic disorder in which males carrying the mutation in the AR gene have female phenotypic characteristics (Gilbert *et al.,* 2000). In addition, the lack of functional ARs results in complete infertility of the Tfm male mice (Rizk *et al.,* 2005). Moreover, the testes of these mice are very small and cryptorchid (Reddy and Ohno, 1981).

The physiological consequence of the genetic disorder and histological structure of the testes in these mice is a low testosterone level in the circulation. The reduction of biosynthesis and production of testosterone are due to reduction in 17α hydroxylase, which is the main enzyme of the steroidogenesis process. The reduction of 17α hydroxylase may be as result of the placement of testes in the intra-abdominal area and a lack of action of testosterone *in utero*. In addition, these mice have high levels of LH hormone which is due to AR dysfunction and the low levels of testosterone. Both AR dysfunction and low testosterone lead to the absence of the negative long loop feedback mechanism of testosterone on the activation of pituitary ARs.

Recent studies have used this type of mouse as a model of atherosclerosis. These studies found that Tfm mice exhibited a significant increase in aortic root lipid deposition compared to littermates, following feeding with a high-fat diet. Furthermore, this fatty streak formation was significantly decreased after physiological testosterone replacement therapy (TRT). The effect was independent of the classic AR androgen receptor and was mediated in part by activation to 17β-estradiol (Nettleship *et al.,* 2007). Similarly, Kelly and colleagues in 2012b reported that the beneficial effect of testosterone (both AR-dependent and AR independent) was seen on reduction of fatty streak formation in the Tfm mice (Kelly *et al.,* 2012b).

However, recent studies also found that Tfm mice can be used as models for studying the relationship of low testosterone with hepatic glucose and lipid homoeostasis (figure 3.1). For instance, Kelly *et al.,* (2012a) reported that a high-cholesterol diet induced

significant hepatic lipid accumulation in Tfm mice compared to wild-type mice, and intramuscular TRT considerably abolished this effect, suggesting AR-independent effects on hepatic steatosis and lipid metabolism in these mice. It is known that, the liver is the most important active metabolic organ together with adipose tissue and muscle. The liver has a role in glucose and lipid metabolism (Emanuelli *et al.,* 2014).

3.1.4 Molecules involved in hepatic metabolism

The regulation of glucose and lipid metabolism is the under the control of a number of hormones including insulin and testosterone which led to elevated glucose oxidation by increasing significantly mRNA expression of the receptor in the Chang human liver cell line (Parthasarathy *et al.,* 2009). In an animal study, castration of mice was associated with elevation of blood glucose, reduced insulin levels and decreased glucose uptake. Furthermore, testosterone deficiency plays a key role in induction of IR in the liver by its association with increased adiposity, which leads to the flow of FFA from adipose tissue to liver and a consequent decrease in insulin sensitivity (see figure 3.1) (reviewed by Kelly and Jones, 2013). FFA can stimulate NFκB, JNK, and IKK activation through TLR-4 in hepatocytes, some of which suppress insulin signalling. Activation of these kinases leads to increase in pro-inflammatory cytokines and chemokine genes expression (Ota *et al.,* 2012). Chemokine-mediated macrophage infiltration into the liver might therefore be related to the pathology of IR. Chemokines such as CCL2-CCR2 (Panee *et al.,* 2013) as well as CX3CL1-CX3CR1 (Shah *et al.,* 211) connect inflammation with IR. PAI-1 is another factor which can be induced in the liver by inflammation (Lagoa *et al.,* 2005). It was noted that the liver of obese mice overexpresses PAI-1 (Westerbacka *et al.,* 2007). PAI-1 is known as a regulator of the fibrinolytic system in blood by inhibiting both urokinase-type and tissue-type plasminogen activators. High levels of PAI-1 are found in patients with T2DM or atherosclerosis, which is associated with imbalance in glucose and lipid homeostasis (Dimova and Kietzmann, 2008; Kietzmann and Andreasen, 2008). In general, IR can impair glucose and lipid metabolism in the liver by different pathways. Increased levels of FFAs transported from adipose tissue to the liver induce gluconeogenesis and VLDL

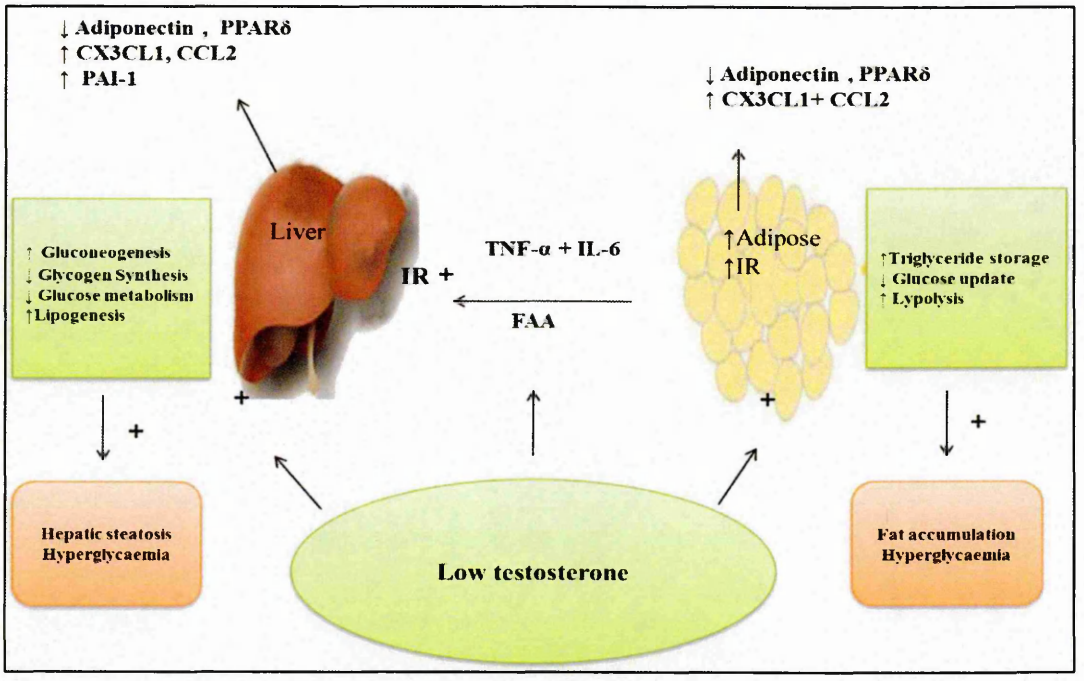


Figure 3.1: The association of low testosterone with inflammation and insulin resistance in adipose tissue and liver. Testosterone deficiency leads to increase adiposity with IR causing fat accumulation and hyperglycaemia. Adipose tissue expansion stimulates the release of FFAs via lipolysis into the circulation, increased pro-inflammatory factors (IL-6, TNF-α, CX3CL1 and CCL2) and decreased anti-inflammatory factors (adiponectin and PPARδ/β). FFAs, IL-6 and TNF-α induce IR in the liver, leading to impairment in glucose metabolism, glycogen synthesis, gluconeogenesis, lipogenesis and ultimately to hepatic steatosis and hyperglycaemia. FFA, free fatty acid. IL-6, interleukin 6. TNF-α, tumour necrosis factor alpha. PPARδ, Peroxisome proliferator-activated receptor delta (Adapted and modified from Kelly and Jones, 2013).

synthesis, that leads to inhibition of lipolysis of chylomicrons and promotes hypertriglyceridemia and decreased of HDL. The latter and the formation of small, dense LDL particules are linked to a higher risk of CVD (Chapman *et al.*, 2010).

The plasma concentration of HDL-cholesterol is to a large extent determined by the production and catabolic rates of its principal apolipoprotein, Apo A1. The plasma concentration of ApoA1 on average reflects the corresponding concentration of HDL-cholesterol (Jung and Choi, 2014). Therefore, Apo A1 is also a powerful predictor of CVD in men, since its role is in reverse transport of cholesterol from peripheral tissues to the liver for clearance. In addition, Apo E is structurally close to Apo A1 (Getz *et al.,* 2009). The liver is the important source for Apo E in plasma (Getz *et al.*, 2009). Apo E has a vital role in the receptor-mediated uptake of lipoprotein by the liver and therefore acts in regulating lipoprotein metabolism (Kolovou *et al.,* 2009). It has also been previously found that ApoE deficient mice accumulate large amounts of triglyceride in their livers (Kuipers *et al.,* 1996). Therefore, hepatic Apo E production is thought to contribute to secretion of VLDL-TG. Notably, the plasma levels of triglycerides are key molcules in the pathogenesis of atherosclerosis (Talayero and Sacks, 2011)

Adiponectin also has a role in lipid and glucose metabolism where it increases lipid catabolism by decreasing TG storage and FFA levels and augmenting HDL concentration in the liver. This leads to improved insulin sensitivity and reduced IR and T2DM (Vázquez-Vela *et al.,* 2008). An additional function of adiponectin is to inhibit the effects of TNF-α, suppressing expression of adhesion molecules in vascular endothelial cells, thus lowering atherogenic risk. The effects of adiponectin are mediated by its receptors, AdipoR1 and AdipoR2 that are expressed located in mouse liver (Kadowaki *et al.,* 2005). It is found that the adiponectin levels are decreased in obesity, T2DM and atherosclerosis. Administration of adiponectin in mice improves these conditions (Chandran *et al.,* 2003).

PPARβ/δ is another beneficial factor regulating the expression of specific target genes involved in lipid metabolism, insulin sensitivity, energy homeostasis, obesity, and inflammation. In addition, PPARβ/δ acts as an anti-inflammatory mediator through reducing, TNF-α, adhesion molecules, decreasing triglycerides and increasing HDL (Fan *et al.,* 2008, Karpe *et al.,* 2009; Di Paola *et al.,* 2010). The use of newly developed selective agonists and genetic approaches showed the substantial association of PPARβ/δ in lipid homeostasis and IR (Karpe *et al.,* 2009).

Therefore, in this chapter, cDNA samples of liver tissue from Tfm mice with and without testosterone treatment were investigated. The effect of testosterone treatment on expression of anti-inflammatory modulators (adiponectin, PPARβ/δ), pro-inflammatory factors (CX3CL1, CX3CR1, CCL2, CCR2 and PAI-1) and anti-atherogenic parameters (Apo A-1 and Apo E) was assessed in these mice due to all these factors being implicated in MetS, T2DM and atherosclerotic conditions. Samples of cDNA from Tfm mice treated with placebo and XY littermate mice were used as controls.

3.1.5 Aim

To investigate whether testosterone modulates the expression of anti and pro-inflammatory markers (adiponectin, PPARβ/δ, PAI-1, CX3CL1, CX3CR1, CCL2, and CCR2) and anti-atherogenic factors (Apo A-1 and Apo E) in liver tissue from Tfm mice fed a high-cholesterol diet.

Tfm and littermate mice were used at age 8 weeks at which time point they were fed on a high fat diet for a period of 28 weeks, *ad libitum.* This diet contained 42% butterfat and 1.25% cholesterol, along with 0.5% cholate, which is required for cholesterol absorption in the mouse (Special Diet Services, UK).

Prior to receiving this high-cholesterol diet for the duration of the study, treated Tfm mice with testosterone, control Tfm mice and littermate received a normal chow diet. This part of the study was performed by Dr. Daniel Kelly at University of Sheffield. All procedures were carried out under the jurisdiction of UK Home Office personal and project licences (project licence number 40/3165, personal licence number 60/11754), governed by the Animals Scientific Procedures Act 1986. Mice received intramuscular injections of testosterone or saline treatment. A sterile 0.3mL 30G needle (BD, UK) was introduced at a right angle to the skin surface into the centre of the muscle mass and 10μL of either Sustanon® 100 (20mg/mL testosterone propionate,40mg/mL testosterone phenylpropionate, 40mg/mL testosterone isocaproate; equivalent to 74mg/mL testosterone) or physiological saline was injected. Mice were injected once fortnightly, from 7 weeks of age, alternating the leg injected to minimise discomfort or irritation (Kelly *et al.,* 2012b).

Hepatic complementary DNA (cDNA) samples from Tfm mice were obtained from animals sacrificed at 36 weeks. The steps of extraction of RNA and synthesis of cDNA were performed by Dr. Daniel M Kelly and Samia Akhtar. 20 cDNA samples were from Tfm mice that were treated with testosterone, 4 cDNA samples were from Tfm mice that were treated with placebo and 12 cDNA samples were from XY littermates controls.

3.2.1 Molecular investigation of gene expression in liver tissue of Tfm mice using semi-quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Real-time reverse transcription quantitative PCR (qRT-PCR) results are based on several steps, including cell processing, RNA extraction, RNA storage and assessment

of RNA concentration, cDNA synthesis and selection of an appropriate constitutively expressed control gene.

qRT-PCR is a development of the basic polymerase chain reaction (PCR), which is used for amplifying a DNA sequence specific to a particular gene, into multiple DNA copies in a quantitative manner. The cDNA can be run on an agarose gel to assess the size and purity of the sample. PCR involves three-steps which are denaturation, annealing and extension, known as a cycle that is repeated a specified number of times in a thermal cycler. The PCR reaction usually includes four phases that are shown in the amplification plot (figure 3.2) the linear-ground phase, the exponential phase, the linear phase and the plateau phase. Through the ground-linear phase, only background fluorescence is detected. The cycle at which the amplification fluorescence exceeds a chosen threshold above the background fluorescence is called the Cycle threshold or Ct value and this indicates the initial exponential phase. During the exponential phase, the amount of DNA is theoretically doubled with each cycle and it is at this point that measurements are made, i.e. when the CT value is obtained. Once the plateau phase is reached, all of the reagents are consumed and no further product is generated.

3.2.1.1 qRT-PCR using SYBR® Green dye methodology

RT-PCR monitors the amplicon amount as the reaction take place. Commonly, the amount of product is directly related to the fluorescence of a reporter dye. Since it distinguishes the amount of product as the reaction develops, RT-PCR offers a wide linear dynamic range, reveals high sensitivity and is quantitative. The preliminary amount of template DNA is inversely related to a parameter measured for each reaction and the Ct value. SYBR Green-based detection is the least expensive and flexible procedure available for RT- PCR (Wang *et al.,* 2006). Other methods (such as TaqMan) need an expensive third primer labelled with a dye and a quencher (Bustin *et al.,* 2005). SYBR Green dye binds to all double-stranded DNA present in the sample by inserting between base pairs (Wang *et al.,* 2006). Signal is generated during the PCR cycle at the end of either the annealing or the extension stage, when the highest amount of double-stranded DNA product is generated. Therefore, the result is an increase in fluorescence intensity proportional to the amount of PCR product created, (figure 3.3).

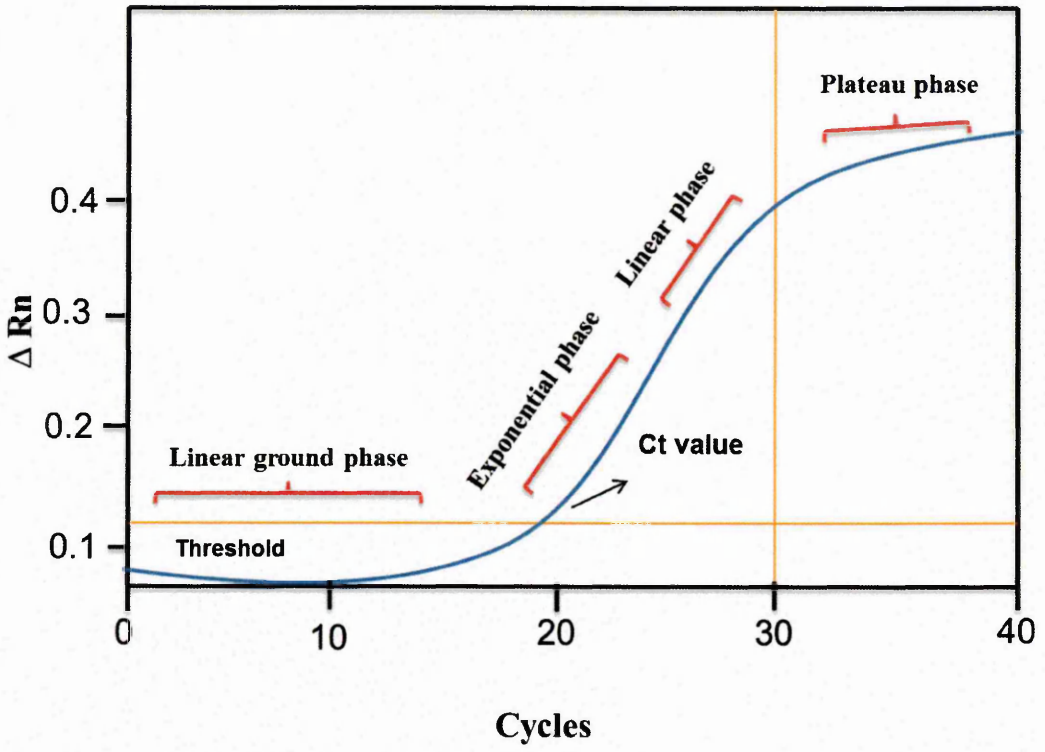
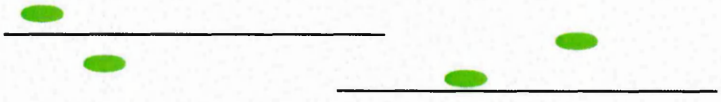


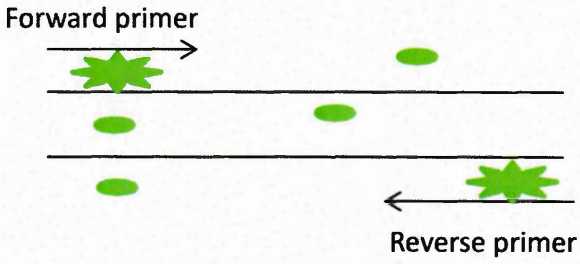
Figure 3.2: Amplification plot of qRT-PCR. Four phases are displayed in the amplification curve the linear-ground phase, the exponential phase, the linear phase and the plateau phase. In the exponential phase the level of fluorescence can be detected whereas the linear phase represents the doubling of PCR product every cycle and plateau phases indicate the end of the reaction. The orange horizontal line represents the threshold above background fluorescence; the cycle at which the amplification fluorescence crosses this line is termed the cycle threshold or Ct value.

A) Reaction setup: The SYBR Green I dye fluoresces when bond to double-stranded DNA.

B) Denaturation: when the DNA is denatured, the SYBR Green I dye is released and the fluorescence is drastically reduced.



C) Polymerization: During extension, primers anneal and PCR product is generated.



D) Polymerization completed: When polymerization is completed. SYBR Green I dye binds to the double-stranded product resulting in a net increase in fluorescence detected by 7900HT PCR system.

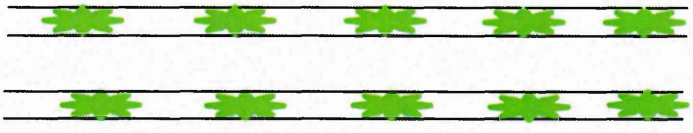


Figure. 3.3: Shows the principle of SYBR Green dye detection in qRT-PCR assay. The steps from A-D demonstrate the action of SYBR Green I dye in three phases of the PCR reaction which are annealing, extension and denaturation. The signal is only detectable during annealing and extension, since the denaturation step contains predominantly single-stranded DNA. It was adapted and redrawn from http://www.lifetechnologies. com/uk/en/home/lifescience/pcr/real-time-pcr/qpcr-education/what-can-you-do-with-qpcr/introduction-to-gene-expression.html.

Method

Each 20µl PCR reaction contained 10µl of SYBR green PCR master mix (Qiagen, UK), 5µl of a 1:10 dilution of cDNA of the sample or control, 1µl of target primer (PrimerDesign, UK) and 4 µl of RNAse free water. All of these amounts were pipetted in duplicate for each sample into 96 well-plates (Applied Biosystems, UK) on ice. Plates were covered with an adhesive film, centrifuged to ensure that all solutions were collected at the bottom of the wells. The analysis of these samples was performed by using the 7900HT PCR system or StepOne™ real-time PCR system (Applied Biosystems, UK) which was according to the cycles below.

Initialization 95°C for 5 min

Denaturation 94°C for 15 seconds
Annealing 60°C for 30 seconds
Final elongation 72°C for 30 seconds
} 40 cycles

Hold 4°C

Melt curve
95°C 60 seconds

55°C for 30 seconds increasing in 10°C increments.

3.2.1.2 Selection of endogenous control reference genes

In qRT-PCR, the selection of the housekeeping gene is important for the normalization of quantitative gene expression results. The candidate housekeeping gene is usually used as reference in qRT-PCR assays to control for error between samples and to measure variations in the levels of a target gene mRNA compared to a housekeeping gene. The level of reference gene expression has to be stable in the same tissues or cell types under the defined experimental conditions. Genes most generally used as internal controls in qRT-PCR studies include: beta actin (ACTB), glyceraldeyde-3-phosphate dehydrogenase (GAPDH), beta glucuronidase (GUSB), hypoxanthine guanine phosphoribosyl transferase (HPRT1) and ribosome small subunit (18S) ribosomal RNA (Choesmel *et al.,* 2007). However, some studies have reported these classical housekeeping genes as appearing to give irregular expression levels in diverse

experimental states (Ohl *et al.,* 2005; Silver *et al.,* 2008). Therefore, it is essential that reliable control genes are specific for the sample group and does not alter under different experimental treatments under investigation. Validation of the control avoids misinterpretation of gene expression data and identifying the most stable housekeeping gene has to be performed from a set of tested candidate reference genes. For this identification, a comprehensive range of bioinformatically validated assays such as geNorm (Vandesomple *et al.,* 2002); NormFinder (Andersen *et al.,* 2004) and BestKeepe (Haller *et al.,* 2004) software programs for housekeeping genes can be used.

Method

RT-PCR was applied to 12 mouse housekeeping genes (ACTB, SDHA, RPL13, ATPSB, EIF4A, 18S, YWHAZ, CYC1, B2M, CANX, UBC, and GAPDH) (PrimerDesign, UK) on 8 biological samples with different experimental conditions used in this study. Each reference gene was tested with each sample in duplicate. This was done by mixing in each well of a 96 well-plates on ice (1µl of 1:10-dilution of sample or control cDNA, 1µl of target reference gene primer, 8µl of RNA free water and 10µl of SYBR Green master mix). Threshold (Ct) values were used for the expression ratio that was calculated by the comparative Ct method (Livak and Schmittgen, 2001). These values were imported into the GeNorm software (Vandesompele *et al.,* 2002), which determines the most stable housekeeping genes. A gene expression stability measure (M value) was determined for each housekeeping gene. The housekeeping gene with an M value less than 1.5 was identified as the most stable one.

3.2.1.3 Amplification efficiency of PCR primers determination

It is important to determine the amplification efficiency of the qRT-PCR assay to obtain accurate data. This means that during the logarithmic phase of the reaction, the PCR product of the target gene is doubling with each cycle. Perfect amplification efficiency is 100%, (Livak *et al.,* 2001). Amplification efficiency between 90 and 110% is considered as acceptable. 100% PCR efficiency demonstrates a change of 3.3 cycles between 10 fold dilutions of template. This is achieved by a 10-fold serial dilution of the template cDNA ranging (from 1:1 to 1:1000 in RNA free water) for obtaining primer efficiencies. The Ct values obtained for all target and reference genes are plotted against log dilution factor on the graph. A linear trend line was applied, with the slope

of the line used to calculate the percentage efficiency of each primer by the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

The R-squared value ≥ 0.95 of linear correlation demonstrates that the Ct values adequately correspond to the trend line fitted.

Method

To determine the primer efficiency, template cDNA was diluted in a series (neat, 1:10, 1:100 and 1:1000); each PCR reaction was carried out in duplicate for the target gene and reference gene. The Ct values obtained were plotted by using $\log_{10}$ of dilution of the cDNA sample against the Ct values, to create the graphical and linear curve at $R^2 > 0.95$ by Microsoft Office Excel.

3.2.1.4 Validation of primer targets by electrophoresis

Validation can be done for the amplified products of the SYBR® Green qRT-PCR analysis by running each amplified target on an agarose gel for visualisation. A single and sharp band should be observed demonstrating that only one specific sequence has been amplified.

Method

7µl of qRT-PCR product for each target gene was mixed with 3µl of 1x loading dye (Promega, UK) and loaded onto a 1.5% agarose gel (Invitrogen, UK) and separated at 110V for 30min. A 200bp ladder (Promega, UK) was also loaded onto the gel. The agarose gel was made from 0.75g of agarose powder (Bioline, UK) was dissolved in 50ml 1X TAE as electrophoresis buffer (Beckman Coulter, UK) and then stained with 3µl ethidium bromide (10mg/ml) (Sigma-Aldrich, UK). Bands were visualised using a UVP bioimaging system (BioRad, UK) and appeared as a single and sharp band referring to specific target gene.

3.2.1.5 Relative quantification analysis of qRT-PCR data

By measuring the Ct values in qRT-PCR analysis, determination of the relative expression of a target gene in the test sample using a reference gene(s) as the normalizer is calculated by using the $2^{-\Delta\Delta CT}$ method (Livak *et al.,* 2001; Schmittgen and Livak, 2008) as follows:

Normalizing the CT of the target gene to that of the reference (Saunders and Lee, 2013) gene, for both the test sample and the calibrator sample is calculated as follows:

ΔCT (test) = CT (target, test) – CT (ref, test)

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

Then normalizing the ΔCT of the test sample to the ΔCT of the calibrator is by:

$$\Delta\Delta CT = \Delta CT \text{ (test)} - \Delta CT \text{ (calibrator)}$$

Calculation of the expression ratio is by:

$$2^{-\Delta\Delta CT}$$

This method is usually used when the target and reference genes have similar and approximately 100% amplification efficiencies. However, if the target and the reference genes do not have similar amplification efficiencies the Pfaffl method can be used (Pfaffl, 2006) which leads to modification of the $2^{-\Delta\Delta CT}$ method by replacing the 2 in the equation by the actual amplification efficiency as follows:

$$\textbf{Ratio} = \frac{(\text{E target})^{\Delta CT,\ \text{target (calibrator} - \text{test)}}}{(\text{E ref})^{\Delta CT,\ \text{ref (calibrator} - \text{test)}}}$$

Mice	XY littermates	Tfm+Placebo	Tfm+Testosterone
Number	12	4	20
Type of treatment	None	placebo	testosterone
Investigated parameters	Adiponectin, PAI-1, PPARβ, Apo A1, ApoE, CX3CL1 and CX3CR1, CCL2 and CCR2	Adiponectin, PAI-1, PPARβ, Apo A1, ApoE, CX3CL1 and CX3CR1, CCL2 and CCR2	Adiponectin, PAI-1, PPARβ, Apo A1, ApoE, CX3CL1 and CX3CR1, CCL2 and CCR2
Assay technique	qRT-PCR	qRT-PCR	qRT-PCR

Table 3.1: Summary table of Tfm mice *in vivo* study. The details of Tfm mice samples, target genes, analysis method used in this study

3.2.2 Statistical Analysis

Data is presented as mean ± SEM. All statistical tests and applications were performed by using Prism 5 Graph pad software and Stats Direct. Statistical differences between groups were analysed by one way ANOVA or Kruskal-Wallis test to analysis parametric and nonparametric data, respectively. When the tests, (one way ANOVA or Kruskal Wallis test) revealed significant differences, multiple comparisons of groups were performed using Tukey's test or Conover–Inman *post hoc*, respectively. All comparisons were two tailed and p values less than 0.05 were considered statistically significant.

3.3.1 Selection of endogenous control reference genes

12 mouse housekeeping genes were tested (ACTB, SDHA, RPL13, ATPSB, EIF4A, 18S, YWHAZ, CYC1, B2M, CANX, UBC, and GADH) on 8 samples with different experimental conditions using qRT-PCR. GADPH was the most stable housekeeping gene, where its M value was less than 1.5. Therefore GADPH was used as the internal control for calculation of relative expression ratios and comparison with the target genes.

3.3.2 Primer efficiency

Following 10-fold serial dilution of the template cDNA ranging (from 1:1 to 1:1000 in RNase free water) primer efficiencies were determined. The Ct values for each dilution were collected during the exponential phase of the PCR, log-transformed and plotted with the slope of the regression line representing the sample's amplification efficiency. The results related to a slope and percentage efficiencies of target genes and reference gene where some were in the ideal range and others had primer efficiencies less than 90% as seen in (figure 3.4 and table 3.2). Therefore, Pfaffi method was used to determine the relative expression of target genes in this study.

3.3.3 Validation of primer targets

Primer products for target genes and reference genes from the qRT-PCR assay were run on 1.5% agarose gel electrophoresis. As shown in (figure 3.5a, b) a single product was visible and sizes of primers was as excepted between (83-129bp) for all target and reference genes for both Tfm mice (A) and XY littermate mice (B).

Gene Target TFM MICE	Amplification factor	% Efficiency
GADPH	1.968	96.84
Adiponectin	1.941	94.08
PPARβ/δ	1.863	86.33
PAI-1	1.931	93.04
CX3CL1	1.863	86.33
CX3CR1	1.92	91.99
CCL2	1.896	89.57
CCR2	1.86	86.32
APO A1	2.00	99.66
APO E	1.941	94.08

Table 3.2: Primer efficiencies of target and reference genes. Calculated primer efficiency percentage was determined by using $E = 10^{(-1/slope)}-1$. These should be in the range of $100 \pm 10\%$ for optimal PCR.

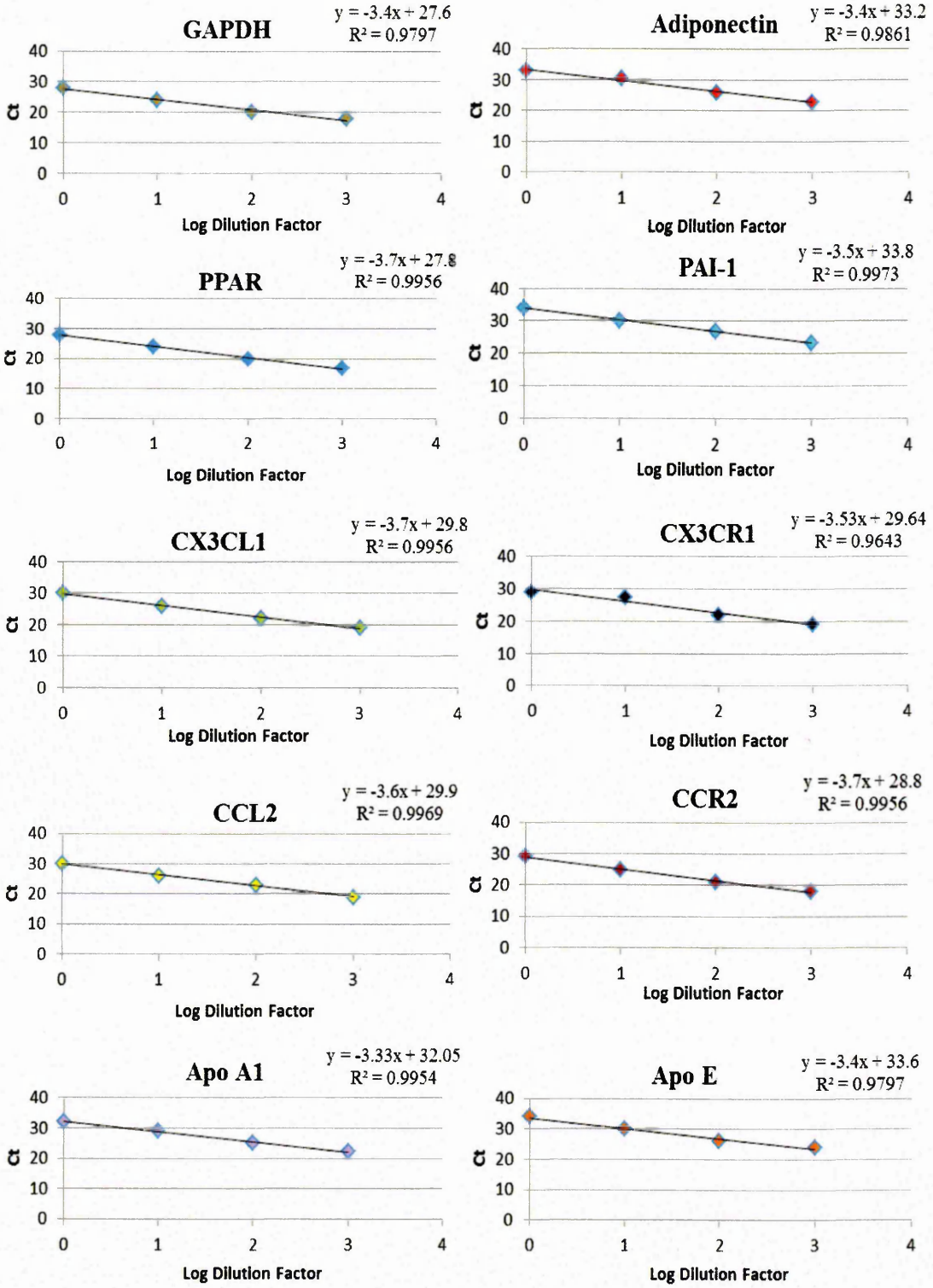


Figure 3.4: Standard-curve plot for calculation of primer efficiency genes. The efficiency of amplification of target genes and internal control (GADPH) was determined using real-time PCR and SYBR Green detection. The Ct values for each dilution were used to calculate slope of the line representing the log of their amplification efficiencies. An ideal slope should be −3.32 for 100% PCR efficiency.

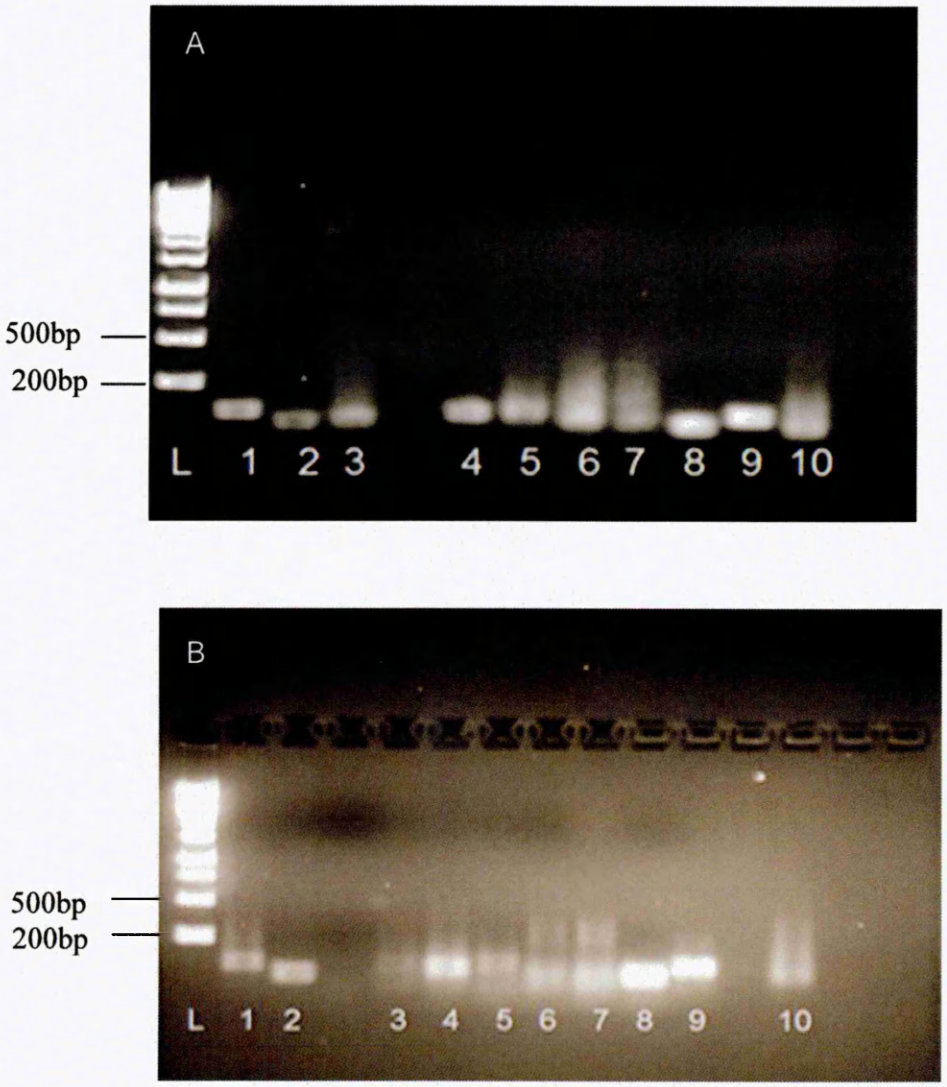


Figure 3.5: Amplification of qRT-PCR products on 1.5% agarose gel electrophoresis for the liver of Tfm mice (A) and XY littermates mice (B). L- Ladder, Lane 1- GADPH Lane 2- Adiponectin, Lane 3- PPARβ/δ, Lane 4- PAI-1, Lane 5- CX3CL1, Lane 6- CX3CR1, Lane 7- CCL2, Lane 8- Apo A1, Lane 9- Apo E, Lane 10- CCR2. All PCR products obtained were between 83 and 129bps which matched the expected sizes as provided by the supplier, PrimerDesign.

3.3.4 Analysis of adiponectin mRNA expression in the liver by qRT-PCR

There was a significant increase (p=0.04) in the expression of adiponectin in the testosterone treated Tfm mice (n=20) compared to the placebo treated Tfm mice (n=4). There was no significant difference in adiponectin expression in either Tfm mouse group compared to XY littermate mice (n=12) (figure 3.6).

3.3.5 Analysis of PPARβ/δ mRNA expression by qRT-PCR

A significant increase was seen in the expression of PPARβ/δ (p=0.04) in Tfm mice treated with testosterone (n=20) compared to Tfm mice with placebo treatment (n=4). Whilst, there was a trend towards an increase in expression of PPARβ/δ in Tfm mice treated with testosterone relative to XY littermate mice (p=0.08), no significant change in expression of hepatic PPARβ/δ in Tfm mice treated with placebo compared to XY littermate mice (n=12) was observed (figure 3.7).

3.3.6 Analysis of PAI-1 mRNA expression by qRT-PCR

In figure 3.8, it can be seen that there was a significant decrease (p=0.03) in expression of PAI-1 in the liver in Tfm mice treated with placebo (n=4) compared to XY littermate mice (n=12) while no significant change in the liver of Tfm mice was seen with testosterone treatment (n=20) compared to the XY control group. In contrast, testosterone treatment increased considerably (p=0.01) expression of hepatic PAI-1 in Tfm mice relative to Tfm mice treated with placebo.

3.3.7 Analysis of CX3CL1 mRNA and its receptors CX3CR1 expression by qRT-PCR

The expression of CX3CL1 was not significantly different in the liver in Tfm mice treated with testosterone (n=20) or with placebo (n=4) compared to XY littermate mice (n=12) (figure 3.9a). There was a decrease in expression of CX3CR1 in the liver of Tfm mice treated with testosterone, compared to the liver of Tfm mice treated with placebo and XY littermate mice, although this did not reach statistical significance (figure 3.9b).

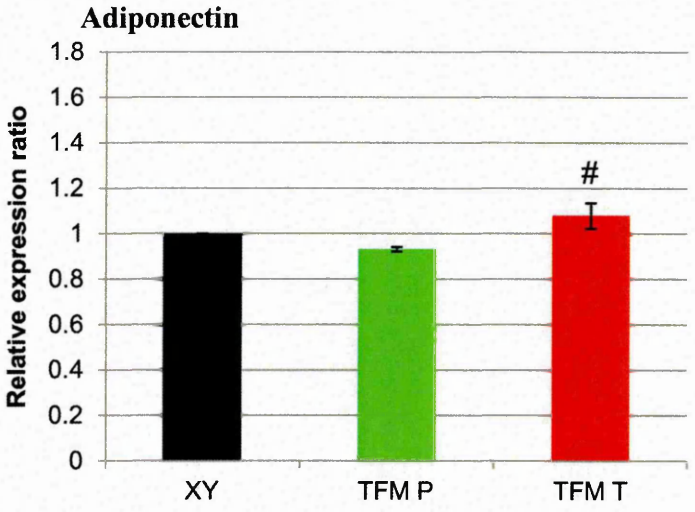


Figure 3.6: Expression of hepatic adiponectin mRNA by qRT-PCR in Tfm mice and XY littermate. A significant increase in its expression in Tfm T compared to Tfm P (Tfm mice with placebo, n=4) whilst, no significant difference was seen in expression of Adiponectin in Tfm T (Tfm mice with testosterone, n=20) compared to XY mice (XY littermate mice, n=12) One Way ANOVA, (n=3). #<0.05 Tfm P vs Tfm T.

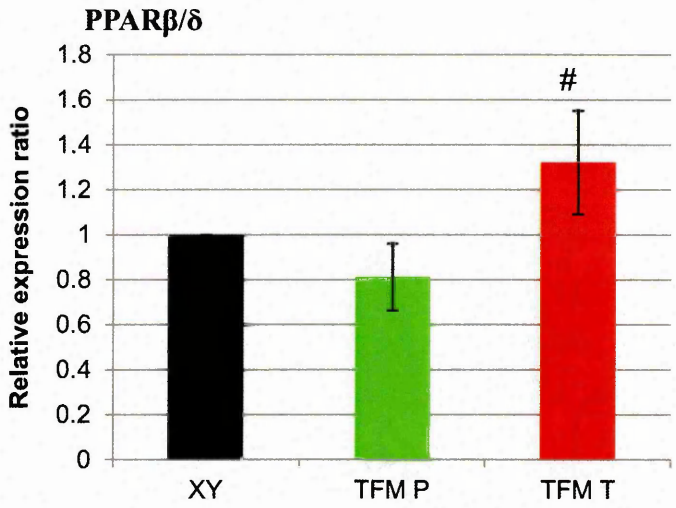


Figure 3.7: Expression of hepatic PPARβ/δ from qRT-PCR in Tfm mice and XY littermate. A significant increase was observed in its expression compared to Tfm P (Tfm mice with placebo, n=4) whereas no significant change was seen in expression of PPAR in Tfm T (Tfm mice with testosterone, n=20) compared to XY mice (XY littermate mice, n=12), Kruskal-Wallis, (n=3). #P<0.05 vs Tfm P.

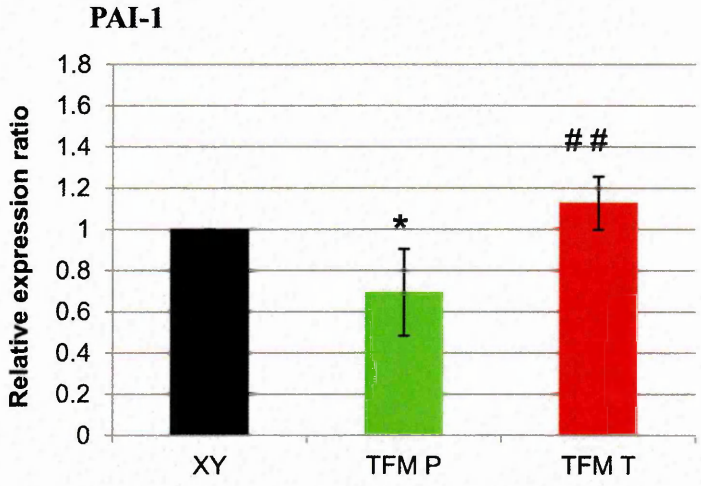


Figure 3.8: Expression of hepatic PAI-1 from qRT-PCR in Tfm mice and XY littermate. A significant reduction was observed in expression of PAI-1 in Tfm P (Tfm mice with placebo, n=4) compared to XY Tfm (XY littermate mice, n=12). A significant increase was seen in Tfm T (Tfm mice with testosterone, n=20) compared to Tfm P (Tfm mice with placebo, n=4). Kruskal-Wallis, (n=3). *P<0.05vs XY mice, ## P<0.01 vs Tfm P.

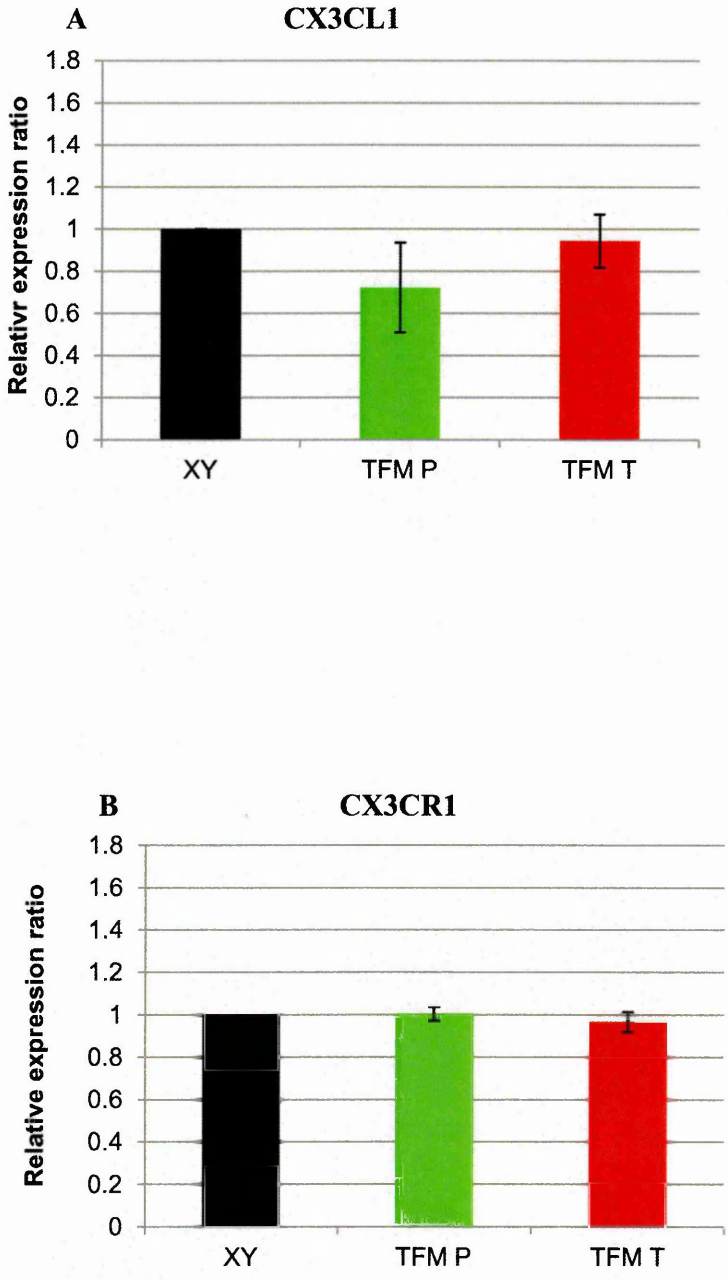


Figure 3.9: Expression of hepatic CX3CL1 (A) and CX3CR1 (B) by qRT-PCR in Tfm mice and XY littermate. No significant change was seen in expression of CX3CL1 (A) and CX3CR1 (B) in Tfm T (Tfm mice with testosterone, n= 20) compared to Tfm P (Tfm mice with placebo, n= 4) and XY mice (XY littermate mice, n= 12), One way ANOVA, (n=3).

3.3.8 Analysis of CCL2 mRNA and its receptors CCR2 mRNA expression in the liver by qRT-PCR

No significant change was seen in expression of CCL2 in the liver Tfm mice treated with testosterone (n=20) or with placebo (n=4) compared to XY littermate mice (n=12) (figure 3.10a). There was a trend to decrease the hepatic expression of CCR2 in Tfm mice with testosterone compared to Tfm mice treated with placebo and XY littermate mice, but this effect did not reach statistical significance (figure 3.10b).

3.3.9 Analysis of apolipoprotein mRNA expression (Apo a1and Apo E) in the liver by qRT-PCR

There was a slight increase in expression of Apo A1 in the liver of Tfm mice treated with testosterone (n=20), but this alteration did not reach statistical significance compared to Tfm mice treated with placebo (n=4) and XY littermate mice (n=12) (figure 3.11a). There was no significant differencea in the expression of Apo E in the liver of both Tfm mice treated with testosterone or with placebo compared to XY littermate mice, as shown in (figure 3.11b).

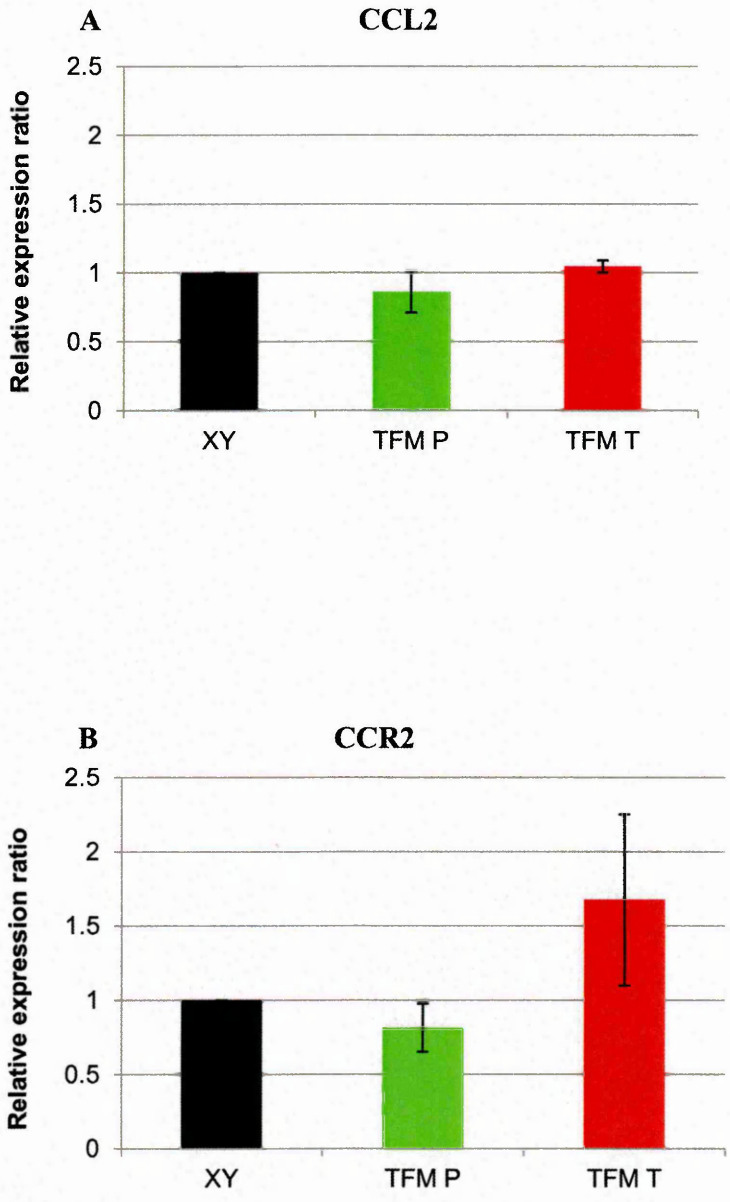


Figure 3.10: Expression of hepatic CCL2 (A) and CCR2 (B) by qRT-PCR in Tfm mice and XY littermate. No significant change was notable in expression of CCL2 (A) and CCR2 (B) in Tfm T (Tfm mice with testosterone, n= 20) compared to Tfm P (Tfm mice with placebo, n= 4) and XY mice (XY littermate mice, n= 12), Kruskal-Wallis, (n=3).

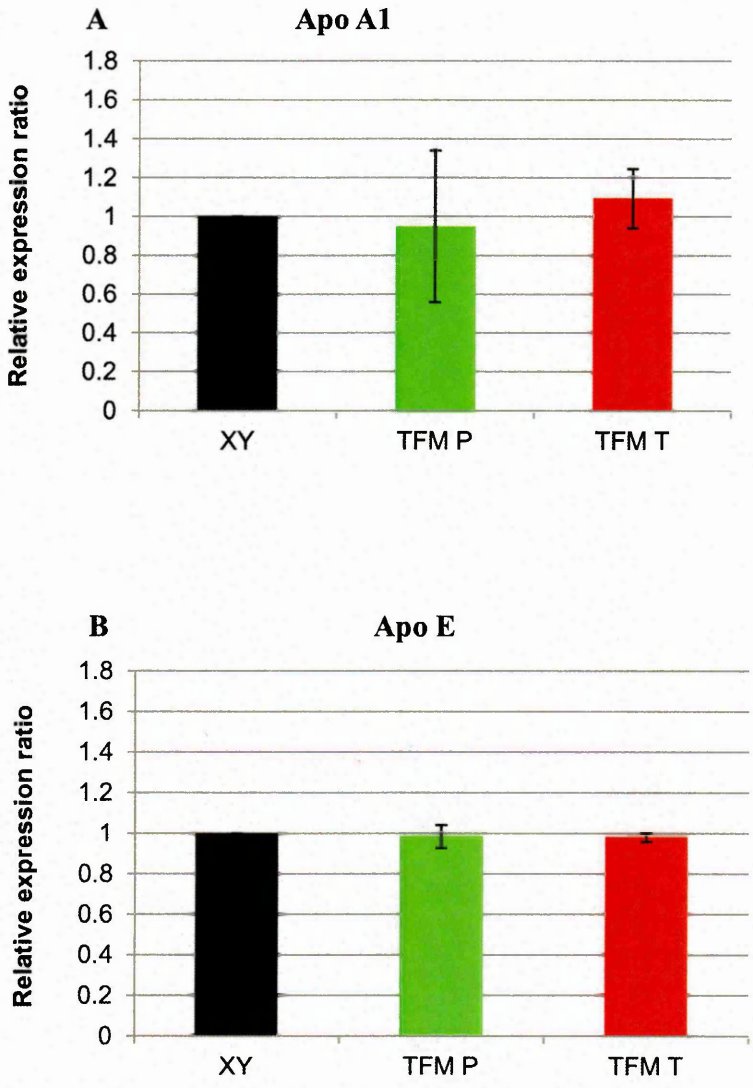


Figure 3.11: Expression of hepatic Apo A1 (A) and Apo E (B) by qRT-PCR in Tfm mice and XY littermate. No significant change was seen in the expression of Apo A1 and Apo E in Tfm T (Tfm mice with testosterone, n=20) compared to Tfm P (Tfm mice with placebo, n=4) and XY mice (XY littermate mice, n=12), One way ANOVA, (n=3).

Parameters	TFM+T
Adiponectin	Increased
PPARβ/δ	Increased
PAI-1	Increased
CX3CL1	NC
CX3CR1	NC
CCL2	NC
CCR2	NC
ApoE	NC

Table 3.3: Summary of results the effect of testosterone treatment on mRNA gene expression of all parameters in Tfm mice study. Tfm: testicular feminization mice, T: testosterone, NC: no change, PPAR: Peroxisome proliferator-activated receptor, PAI-1 Plasminogen activator inhibitor-1, Apo: apolipoprotein.

This part of the study, investigated the gene expression of anti-inflammatory (adiponectin, PPARβ/δ), anti-atherogenic factors (Apo A-1 and Apo E) and pro-inflammatory factors such as PAI-1 and chemokines with their receptors, (CX3CL1, CX3CR1, CCL2 and CCR2) mRNA was qualified using liver tissue following testosterone treatment in Tfm mice compared to XY littermate and placebo Tfm mice controls where all these groups were fed on high fat diet.

As previously mentioned, Tfm mice have low testosterone besides having a non-functioning AR, so they cannot respond to the classical genomic signalling pathways (Monks *et al.,* 2007). Therefore, it was hypothesised that testosterone treatment for these mice may show, firstly whether testosterone can influence the target genes and secondly, whether testosterone is acting via mechanisms other than through the AR.

The first finding was that PCR confirmed the expression of the target genes in hepatic cDNA samples from Tfm mice and XY littermate mice. Secondly, testosterone replacement did have an effect either positively or negatively, on some of the target genes which indicate that testosterone has the ability to act via ER or non-genomic signalling pathways.

3.4.1 The effect of testosterone treatment on hepatic adiponectin mRNA expression in Tfm mice

The current study demonstrated that testosterone significantly increased hepatic mRNA expression of adiponectin in Tfm mice compared to Tfm mice treated with placebo. This is in contrast with the study by Nishizawa et al. (2002) reporting that castration in mice increased plasma adiponectin and testosterone treatment reversed this effect without changes in the mRNA and protein levels of adiponectin in the adipose tissue. These results suggste: 1) changes in adiponectin mRNA expression do not always correspond to changes in plasma adiponectin concentrations, (Combs *et al.,* 2003; Behre *et al.,* 2007). 2) This observation supports the proposal that post-transcriptional and post-translational mechanisms in adiponectin production are differentially regulated (Swarbrick and Havel, 2008).

However, the increase in hepatic mRNA expression of adiponectin may be related to the Tfm mice condition, because it is well recognized that there is an inverse relationship between testosterone and adiponectin (Lanfranco *et al.,* 2004, Page *et al.,* 2005a, Bai *et al.,* 2011; Frederikse *et al.,* 2012) even though both have anti-inflammatory and anti-atherogenic actions (Malkin, *et al.,* 2003; Lanfranco *et al.,* 2004), where adiponectin can improve insulin sensitivity and prevent T2DM by promoting the ability of insulin to suppress glucose production by hepatocytes. Recent studies established that adiponectin increased fatty acid oxidation (Fruebis *et al.,* 2001), increased insulin action in the muscle and liver and additionally improved IR in lipoatrophic and genetically obese mice, both of which had hypo-adiponectinemia (Yamauchi *et al.,* 2001; Berg *et al.,* 2001).

In comparison, testosterone treatment of patients with T2DM in the present study reduced significantly the serum adiponectin concentration after 3 months (chapter 2). Testosterone did not show the same effect on adiponectin expression in the liver of these mice. Further clinical studies on the influence of testosterone therapy have reported a reduction in adiponectin levels in men with T2DM (Kapoor *et al.,* 2007) and in aging men using supraphysiological doses of testosterone (Page *et al.,* 2005a), while no changes were observed in adiponectin levels in aging men treated with physiological doses of testosterone (Page *et al.,* 2005b). The mechanism of the diminution in adiponectin concentration during testosterone therapy is unclear. It is suggested that, the negative effect of testosterone therapy on adiponectin levels may be mediated by factors that are responsible for adiponectin production or synthesis at the same time as their activity is modulated by testosterone. However, this reduction was not observed in the present study with Tfm mice suggesting that testosterone acted by ER or non-genomic pathway. In a study using rats fed a high-fat diet and treated with testosterone it was found that testosterone treatment reduced adiponectin levels in the serum. In this study testosterone decreased fat accumulation in these rats even though they had body weight higher than untreated rats with the same food (Nikolaenko *et al.,* 2013). Tfm mice in the current study had no significant difference in body weight compared to Tfm mice with Placebo or XY mice (Kelly *et al.,* 2012). However, although not statistically significant, Tfm mice receiving testosterone replacement were observed to be heavier and gained more weight over the 28 week experimental period than saline-injected Tfm mice and XY littermates. However, fat accumulation was not investigated in that study by Kelly et al. (2012). Further investigations in these mice are needed to explain why under

certain circumstances, testosterone increases mRNA expression of adiponectin in liver of these mice.

3.4.2 The effect of testosterone treatment on hepatic PPARβ/δ mRNA expression in Tfm mice

A significant increase in hepatic mRNA expression of PPARβ/δ was seen following testosterone treatment in mice compared to those treated with placebo. To the best of current knowledge no studies have been performed on the effect of testosterone on PPARβ/δ compared to another isoforms of PPAR. For example, testosterone treatment increased the sensitivity of PPAR-α receptors in patients with T2DM (Nieschlag *et al.,* 2012). On the contrary, Kilby and colleagues (2013) found that testosterone treatment did not associate with any change in hepatic expression of PPARα and PPARγ in the same mice with the same condition. In an alternative study, testosterone has recently been shown to inhibit PPARγ activity in a transcriptional activity (Du *et al.,* 2009).

PPARβ/δ ligands have been shown to reduce triglyceride accumulation (Tanaka *et al.,* 2003) glucose and lipid metabolism (Billin *et al.,* 2008). According to the anti-inflammatory and anti-atherogenic role of PPARβ/δ in different tissues in the animal models and humans, therefore, the increase of this factor by testosterone may reflect the role of testosterone via AR-independent pathway as anti-inflammatory and anti-atherogenic factor in reduction of atherosclerosis development and T2DM.

3.4.3 The effect of testosterone treatment on hepatic PAI-1 mRNA expression in Tfm mice

Data reported here shows an increase in mRNA PAI-1expression in the liver following testosterone therapy in Tfm mice, compared to Tfm mice treated with placebo. It is known that PAI-1 has a significant role in the development of coronary heart disease (CHD). Additionally, high levels of PAI-1 are associated with IR and lipid abnormalities, (Gruzdeva *et al.,* 2013). Therefore, several studies were conducted to investigate whether testosterone influences the level of PAI-1 in serum or its gene expression in human and mice (Goglia *et al.,* 2010). Cross sectional studies have reported a negative association between testosterone levels and PAI-1 and fibrinogen (Rosano *et al.,* 1999; Webb *et al.,* 1999). Replacement of testosterone in hypogonadal

men and treatment of normal men with dehydroepiandrosterone (DHEA) reduces PAI-1 serum levels (Bavenholm *et al.,* 1998). In addition, Philips *et al.,* (1994) studied the association between testosterone levels and fibrinolytic factors in men with CAD. Results showed that the testosterone levels were correlated negatively with CAD and PAI-1. An additional study investigated the relationship between testosterone levels, fibrinolytic activity and lipid levels, in hyperlipidaemic men (Glueck *et al.,* 1993). It was found that testosterone was inversely associated with PAI-1 activity and fibrinogen levels (Glueck *et al.,* 1993). Similarly a potentially beneficial effect of testosterone on coagulation was reported in men in the period after an acute MI (Pugh *et al.,* 2002). Moreover, there was an increase in fibrinogen, PAI-1 and tPA in diabetic prostate cancer patients during androgen deprivation therapy (Haidar *et al.,* 2007). Former studies using DHT (Norata *et al.,* 2006) or testosterone (Jin *et al.*, 2007) found that the reduction of PAI-1 association with androgen was via AR pathway. This was clearly after blocking these receptors by bicalutamide or flutamide in human endothelial cells, respectively. Therefore, since Tfm mice in the current study have a non-functional AR, the increase in mRNA PAI-1expression of liver might be as result of an AR-independent pathway, in a similar way to the effect on adiponectin mentioned above.

In contrast, Smith and colleagues (2005) found that physiological testosterone replacement did not affect the coagulation system such as PAI-1and fibrinogen level in men with CHD. PAI-1 has the ability to inhibit fibrinolysis processes via suppresses tPA and urokinase (uPA).

The source of PAI- 1 levels in plasma in humans or mice includes: adipocytes, liver cells and endothelial cells (Bastard and Picroni, 1999). Previous reports have revealed that PAI-1 mRNA expression per total RNA mass in adipose tissue was higher in rodents made obese, either genetically or by lesioning the ventromedial hypothalamus, than in their lean counterparts, (Shimomura *et al.,* 1996; Sierra-Honigmann *et al.,* 1998). This finding suggests that the contribution of the mass of adipose tissue is at least as important as upregulation of PAI-1 synthesis in the increase in circulating PAI-1 levels observed in obese mice (Morange *et al.,* 2000). As previously mentioned, Tfm mice with testosterone in the current study were heavier and gained more weight over the 28 week experimental period than Tfm mice with placebo and XY littermates even though this was not statistically significant. Consequently, this could explain the increase in expression of PAI-1 in these Tfm mice regardless of testosterone treatment. However,

evidence from other studies showed that plasma PAI-1 levels are more closely related to fat accumulation and PAI-1 expression in the liver than in adipose tissue in obese mice (Alessi *et al.,* 2003). Therefore, investigation of fat accumulation in the livers of these mice could be valuable in explaining the increase in mRNA PAI-1 expression.

In rat study, results showed that the modifications of aortic expression of PAI-1 were associated with castration and expression returned with the administration of a physiological amount of testosterone but not with a pharmacological dose of the same hormone (Goglia *et al.,* 2010). Supraphysiological doses of testosterone therapy caused a decrease in fibrinogen and PAI-1activity, (Anderson *et al.,* 1995). Nevertheless, Smith et al. (2000) suggested that no changes in the levels of PAI-1 following a physiological dose of testosterone therapy were due to the low dose compared to the effect of supraphysiological doses of testosterone therapy in the previous study. In contrast, Goglia et al. (2010) found that the higher doses of testosterone or DTH resulted in an increase in mRNA for PAI-1 in endothelial cells of both young men and in premenopausal women.

The conflicting results of testosterone increasing simultaneously PAI-1, adiponectin and PPARβ/δ in the liver are hard to explain. It is known that a negative correlation between PA1-1 and both PPARβ/δ and adiponectin is seen in obesity (Garg *et al.,* 2012). Obesity-induced inflammation is associated with increase in PAI-1 and a decrease in both adiponectin and PPARβ/δ in the obese (Dandona *et al.,* 2004; Stienstra *et al.,* 2006). It may be expected therefore, that testosterone treatment would reduce PAI-1 and increase adiponectin and PPARδ/β, however the PAI-1 results do not follow this expectation, this finding may be specific to Tfm mice.

3.4.4 The effect of testosterone treatment on hepatic CX3CL1 and CCL2 mRNA and their receptors expression in Tfm mice

In the present study, testosterone treatment also did not associate with any change in hepatic mRNA gene expression of CX3CL1 and CCL2 or their receptors. Several hepatic cell populations, including hepatocytes, Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells, can secrete chemokines upon activation (Karlmark *et al.,* 2008). Weisberg et al. (2006) studied the role of CCR2 and established that mice lacking CCR2 had decreased adipose tissue macrophage infiltration, decreased hepatic

steatosis, reduced inflammatory profiles and enhanced systemic insulin sensitivity (reviewed by De Luca and Olefsky, 2008). Furthermore, the CX3CL1/CX3CR1 interaction can moderate chronic inflammatory diseases, including atherosclerosis; independent of CCL2/CCR2 (Yao *et al.,* 2014). In addition, Shah *et al.,* (2011) demonstrated that CX3CL1-CX3CR1 is a novel inflammatory adipose chemokine system that moderates monocyte adhesion to adipocytes and is accompanied by obesity, IR, and T2DM. Furthermore, Kanda and co-workers (2006) found that expression of CCL2 receptor, CCR2, was detected in the liver and inhibition of CCL2 function ameliorated both IR and hepatic steatosis as well as reduced the extent of macrophage infiltration into adipose tissue of obese mice.

These data offer evidence for CX3CL1-CX3CR1 as a diagnostic and therapeutic target in cardio-metabolic disease. Furthermore, Kanda *et al.,* (2006) demonstrated that overexpression of CCL2 in adipose tissue caused hepatic steatosis along with adipose tissue inflammation, while systemic deletion of CCL2 inhibited HFD-induced steatosis.

However, a previous study reported that CX3CL1 and its receptor were detected in plaque regions of artery walls of Tfm mice, fed a high fat diet, but were not influenced by testosterone or AR function (Kelly *et al.,* 2012b). In the same study, CCL2 concentrations were significantly elevated in XY littermate and Tfm mice following a high-cholesterol diet compared to normal diet-fed XY littermate, but concentrations of CCL2 were not significantly different in serum of Tfm mice treated with testosterone compared to placebo-treated Tfm mice and XY littermates. Studies investigating the effects of testosterone therapy on chemokines and their receptors in animal models have been limited. However, it is actually difficult to conclude whether testosterone treatment has the ability to influence these receptors and ligands as anti-inflammatory modulator. Therefore, the results presented here are not able to confirm an effect of testosterone.

3.4.5 The effect of testosterone treatment on hepatic Apo A1 and Apo E mRNA expression in Tfm mice

This study has found no significant effect of testosterone therapy on mRNA expression of Apo A1 and ApoE. Both apolipoproteins have anti-atherogenic actions. It is hypothesised that as testosterone has anti-atherogenic action thus testosterone may lead to increase the expression both of Apo A1 and Apo E.

Animal experiments indicated that testosterone influences apo A1 gene activity in inbred strains of mice (Tang *et al.,* 1991), while the outcome of cell culture studies demonstrate that the hormone acts by antagonizing the actions of estrogen, and that androgens have no direct effect on gene transcription in human hepatoma cells (Tang *et al.,* 1991; Tam *et al.,* 1986). However, the outcome of cell culture is not the same as outcomes from whole liver tissues. Pharmacologic doses of estrogen hormone used to treate rodents resulted in larger concentrations of Apo A1 mRNA and gene transcription. While these actions should lead to higher levels of Apo A1 protein, the hormone also had post-transcriptional effects which reduced the abundance of the protein (Seishima *et al.,* 1991; Tang *et al.,* 1991). Clinical studies demonstrate that the mechanism of estrogen increasing the concentration of Apo A1 in post-menopausal women was by inhibiting the activity of hepatic triglyceride lipase (HTGL) (Applebaum-Bowden *et al.,* 1989; Colvin *et al.,* 1991) or by stimulating lipoprotein synthesis (Schaefer *et al.,* 1983; Sacks *et al.,* 1995).

However, the effect of androgens on Apo A1 expression, especially in animal models is uncertain as a result of the lack of information. In human studies, Singh and colleagues (2002) found that a 600mg dose of testosterone enanthate, which was associated with extremely supraphysiological testosterone concentrations, reduced HDL and Apo A1 levels in healthy young men while Tan and co-workers (1998) found that a minimum effective dose of testosterone replacement in the form of parenteral testosterone ester given 4-weekly significantly decreased levels of ApoA1. In line with our findings, previous studies report neither the serum HDL cholesterol nor the ApoA1 concentrations altered following transdermal testosterone treatment in healthy young men (Berglund *et al.,* 1996; Arrer *et al.,* 1996) and following gel testosterone treatment in older men (Rubinow *et al.,* 2012). Nevertheless, other studies found physiological testosterone treatment increased both HDL cholesterol and ApoA1 levels in hypogonadal, elderly men (Zgliczynski *et al.,* 1996) and in normal men (Bagatell *et al.,* 1992).

Testosterone treatment did not show an effect on hepatic mRNA Apo E expression gene in Tfm mice. It is known that Apo E deficient mice (ApoE-/-) mice are severely hypercholesterolemia and develop advanced atheroma independent of diet. These mice are clear evidence of the importance of Apo E as the atheroprotective factor against

atherosclerosis development. Furthermore, injection of cetrorelix, which is used to reduce gonadotropins and sex steroids in treatment of prostate carcinoma and ovarian cancer, into ApoE deficient mice (ApoE-/-) suggests that the associated suppression of testosterone leads to increased atherosclerosis, despite lower cholesterol levels in the male mice (Von Dehn *et al.,* 2001). On the other hand, in female ApoE-/- mice, the reduction in testosterone leads to reduction in estradiol, insulin and HDL levels without effects on atherosclerosis (Von Dehn *et al.,* 2001).

It is recognized that some factors moderate Apo E synthesis such as hormones, dietary cholesterol and fat. Estradiol enhances VLDL production from rat hepatocytes and the VLDLs contain Apo E. Regulation of Apo E gene expression by estradiol occurs both at the transcriptional and post-transcriptional level in rats (reviewed by Ajit *et al.,* 1997). Previous studies report that increases in Apo E synthesis were also observed in castrated C57/BL6 mice given either physiological or pharmacological replacement doses of estradiol, but not testosterone, suggesting that the effect of estradiol was specific to the distribution of Apo E mRNA in the translationally active polysomal pool (Srivastava *et al.,* 1997).

In human macrophage cells, cells were exposed to testosterone 10^{-8} M for 24, 48 and 72h to investigate expression of the LXR-target gene APOE and Apo E gene. LXR-target gene Apo E is responsible for encoding the protein Apo E, involved in binding cholesterol following its removal from the cell. The results showed that testosterone stimulated expression of LXRα and APOE in human macrophages. This suggests that firstly, testosterone activates LXR acting through this nuclear receptor to control the expression of ApoE to aid cholesterol efflux. Secondly, this could explain the role of testosterone as an anti-atherogenic factor (Kilby *et al.,* 2012). However the increase in ApoE seen in other studies compared to the lack of change seen in the currrent study may be due to differenes in the models used for aplication the presene or absence of androgen receptors and the targets studied (transription fators or RNA).

3.5 Limitations of the study

The small sample size of Tfm mice in the present study might be associated with the lack of a clear conclusion. Using adipose tissue samples would be useful to explain the

influence of testosterone on the above genes. Fat distribution was not measured in these mice which made it difficult to draw conclusions on the changes in expression of some genes related to obesity. The absence of receptor blockers for ER or aromatase inhibitors in this study made it difficult to determine the mechanisms by which testosterone exerted its effect.

3.6 Summary

The present study showed that testosterone treatment did not induce significant changes in hepatic expression of mRNA for CX3CL1, CCL2 and their receptors CX3CR1 and CCR2 and atherogenic factors Apo A1 and Apo E in Tfm mice following a high-cholesterol diet compared to placebo controls and XY littermates. However, testosterone showed a beneficial effect by significantly increasing mRNA expression of adiponectin and PPARβ/δ suggesting an effect via the AR-independent pathway. On the other hand, this finding was contradicted by the result for the inflammatory factor PAI-1, where testosterone increased its expression in the liver which was also mediated by the AR-independent pathway. It is possible that more definitive results for the effect of testosterone in Tfm mice may be found with larger numbers of animals thus further work should be performed.

Chapter 4

The *in vitro* effect of testosterone treatment on THP-1 macrophages and 3T3L1 adipocytes

4.1.1 Monocyte/macrophage cell recruitment in atherosclerosis

Increased understanding of the mechanisms underlying atherosclerosis has highlighted prospective targets for new therapeutics. One of these mechanisms involves the contribution of chemokines to inflammation and migration of immune cells into the arterial wall, which is a consequence of interaction between chemokines within the endothelium and their receptors on infiltrating immune cells. Recently, therapeutic approaches to target inhibition of these interactions, with the aim of stopping the recruitment of immune cells into the tissue have been reported (Koenen and Weber, 2011).

The specific immune cells implicated in atherosclerosis are monocyte cells. These cells differentiate into macrophage-derived foam cells, which are the core of the detectable fatty streak in the earliest atherosclerotic lesions in the sub-endothelial space (Zernecke and Weber, 2010). These cells, with their inflammatory mediator production (IL-1β, IL-6, IL-8, TNF-α and IFNγ) can be associated with inflammation in the arterial wall while anti-inflammatory cytokines (IL-10) can reduce the inflammatory condition (see chapter 1). Macrophage cells also have the ability to secrete these factors in the same way, also in the arterial wall. These factors are present in all stages of atherosclerosis. They can (except IL-10) cause dysfunction in endothelial cells and promote leukocyte transmigration into the endothelial space by stimulating secretion of chemokines from immune cells. TNF-α and IFNγ can activate macrophages to secrete TNF-α and IFNγ and other pro-atherogenic factors including IL-8 and CCL2. IL-8 is produced by several different immune cells but mainly by macrophages. It has the ability to attract monocytes at an early stage and accumulate macrophage cells in advancement of atherosclerotic lesions. IL-6 (reviewed by Fernández and Kaski, 2002) and IL-1β (Dinarello *et al.,* 2010) have a role in atherosclerosic events by stimulating the migration and differentiation of the activated macrophages. They also act by triggering the synthesis of metalloproteinases and the expression of LDL receptors in the macrophages, as well as an increase in LDL capture and the secretion of chemoattractants, such as CCL2 and adhesion molecules. However, IL-10 which acts as an anti-inflammatory molecule is also secreted from macrophages. This cytokine has a role in the inhibition of inflammatory cytokines such as TNF-α, IL-6, and IL-1β, and

has been found at high levels in individuals with atherosclerosis in response to the pro-inflammatory environment of atherosclerosis (Huang *et al.,* 2004; Szodoray *et al.,* 2006).

Monocytes and macrophages also express a number of chemokine receptors including: CX3CR1 and CCR2. Each receptor binds its ligand, CX3CL1 and CCL2 respectively, which are secreted from endothelial, and smooth muscle and macrophages cells (Zernecke *et al.,* 2008; Liu and Jiang, 2011). In particular, CX3CR1/CX3CL1 synergizes with CCR2/CCL2 to maximize foam cell formation and the inflammatory response during atherogenesis (Tacke *et al.,* 2007). Blockade of CCR2 and/or CX3CR1 may therefore be beneficial in atherosclerotic cardiovascular disease (Barlic and Murphy, 2007). These chemokine receptors are G protein-coupled receptors containing 7 transmembrane domains that are found predominantly on the surface of leukocytes (Colvin *et al.,* 2006). Many factors can modulate the expression of these receptors including chemokine and proatherogenic and inflammatory cytokines. Pro-inflammatory cytokines, IFN-γ and TNF-α are critical mediators of atherosclerosis in murine models (Boehm *et al.,* 1997) being crucial in the establishment of the inflammatory microenvironment that promotes accumulation and activation of monocytes/macrophages in the arterial wall (Apostolakis *et al.,* 2007). TNF-α and IFNγ induce expression of the CX3CL1/CX3CR1 system in macrophages cells through activating NF-kB, AP-1, STAT and MAPK (Liu and Jiang, 2011). These inflammatory cytokines can also affect the expression of CCL2/CCR2 and thus modulate the recruitment of monocytes and accumulation of macrophges in sites of acute and chronic inflammation, including the developing atherosclerotic lesions (Tangirala *et al.*, 1997 Weber *et al.,* 1999).

4.1.1.1 Monocyte/macrophage cells and testosterone treatment

There was improvement in the health of patients who suffered from CHD following testosterone treatment in short-term studies (Jones and Saad, 2009). Furthermore, according to animal studies, there was a significant reduction in deposition of lipid in the arterial walls of an atherosclerotic mouse model, following testosterone treatment (Nettleship *et al.,* 2007b, 2009; Kelly *et al.,* 2012b). In addition, testosterone had the ability to reduced inflammatory factors including TNF-α, IL-6 and IL-1β and increased anti-inflammatory cytokines in murine macrophages (D'Agostino *et al.,* 1999). These factors have a role in atherosclerosis events. Therefore, replacement therapy for this

testosterone deficiency may lead to reduced atherosclerosis. Moreover, stimulation of the AR influences the expression of chemokine receptors CCR2 (Lai *et al.,* 2009) and CCR1 (Akashi *et al.,* 2006), and consequent chemokine responses, in mice macrophages and in prostate cancer cells, respectively.

It is thought that the effect of testosterone could be through modulating the expression or action of both chemokine receptors, CX3CR1 and CCR2, expressed on macrophages therefore reducing atherosclerosis events. However the effect of testosterone on these receptors has not yet been investigated in macrophages. The action of testosterone could be via stimulation of the AR on macrophages. Furthermore, testosterone can act through an ER mechanism. A previous study found that macrophages had the ability to express aromatase and estrogen receptors (Mor *et al.,* 1998). The same group in 2001 confirmed that monocytic (undifferentiated) THP-1 cells had no aromatase activity, upon differentiation with PMA into tissue macrophages, they express aromatase and have aromatase activity, (Mor *et al.,* 2001). Therefore, testosterone could be converted to estradiol and via ER on macrophages. Additionally, others have observed that testosterone can exhibit its action on immune cells (Wehling *et al.,* 1997, Benten *et al.,* 1999; Heinlein and Chang, 2002) or macrophages (Cutolo *et al.,* 2005) via non-genomic receptors. This effect is on intracellular signaling pathways where testosterone has the ability to rapidly induce an increase in intracellular Ca^{2+} which is linked to a G protein-coupled receptor in immune cells (Gradisnik *et al.,* 2008) and macrophage cells (Benten *et al.,* 1999).

4.1.2 Adipocyte-derived proteins in T2DM and atherosclerosis

The development of adipose tissue is regulated by a complex interaction of transcription factors and adipocytokines. As mentioned in chapter 1, adipose tissue acts as an endocrine organ secreting adipocyte-derived proteins. Adiponectin, PPARβ/δ, leptin, PAI-1, CCL2 and CX3CL1 are involved in the development of adipose tissue in normal and obesity cases. Adiponectin and PPARβ/δ are reduced with obesity and correlated to insulin-sensitizing and anti-inflammatory properties (reviewed from Cefalu *et al.,* 2011; Szanto *et al.,* 2008). In contrast, leptin, PAI-1, CCL2 (Taube *et al.,* 2012; Morton and Schwartz, 2011; Alessi *et al.*, 2007; Guilherme *et al.,* 2008) and CX3CL1 (reviewed by Cefalu *et al.,* 2011; Shah *et al.,* 2011) have been found to be increased in obesity and associated with T2DM and CVD. Moreover, the production of chemokines (CX3CL1

and CCL2) by adipose tissue infiltrated with macrophages is a further cause of endothelial dysfunction in adipose tissue and in the artery wall leading to the development of inflammation (Cefalu *et al.,* 2011; Gustafson *et al.,* 2007; Taube *et al.,* 2012). Recently, it has been reported that CX3CR1 and CCR2, with their ligands, have important roles in monocyte-adipocyte interactions by recruitment of leukocytes to adipose tissue in obesity (Cefalu, 2011). Especially, detection of CX3CR1 (Polyak *et al.,* 2014, Shah *et al.,* 2011; Lumeng *et al.,* 2007) and CCR2 (Kanda *et al.,* 2006) on macrophages as well as their ligand CX3CL1 (Shah *et al.,* 2011) and CCL2 (Kanda *et al.,* 2006) in adipose tissue led to support this concept.

4.1.2.1 Adipocyte cells and testosterone treatment

Adipose tissue expands in obesity cases and this is accompanied by low testosterone levels as clearly documented in an 11-year follow-up patient study (Laaksonen *et al.,* 2005). A further study suggested a strong inverse correlation between body fat and testosterone levels (Kapoor *et al.,* 2005), with hypogonadal men exhibiting a reduced lean body mass and an increased fat mass. Several studies have shown that TRT in obese men reduces BMI and visceral fat mass as well as IR (Rebuffe'-Scrive *et al.,* 1991; Marin *et al.,* 1992a, b, 1995; Saad *et al.,* 2007, 2008; Agledahl*et al.,* 2008; Kelly and Jones, 2013).

Nevertheless, the mechanism by which testosterone affects fat storage and obesity is unclear. There are specific receptors for androgens in adipose tissue, which indicate an important role for these hormones in the regulation and function of this organ. These sex steroid hormones perform their action in adipose tissues via stimulation of both genomic and non-genomic pathways. It is now recognized that there are ER and AR in adipose tissues, so their actions could be direct by AR or by ER after conversion of testosterone to estradiol by aromatase in adipose tissue (Saad, 2009). For instance, in stem cell culture studies, testosterone treatment showed a direct effect on adipogenesis. Singh *et al.,* (2003) stated that treatment of isolated mouse pluripotent stem cells with testosterone stimulated the growth of cells of myocyte lineage rather than adipocytes, whereas testosterone deficiency induced the development of adipocytes over myocytes. Singh *et al.* (2006) reported that testosterone and dihydrotestosterone inhibit adipocyte differentiation *in vitro* through an AR-mediated nuclear translocation of beta-catenin and activation of downstream Wnt signalling. In addition, sex steroid hormones could

stimulate hormone-sensitive lipase leading to lipolysis in adipose tissue by activation of the cAMP cascade (Mayes and Watson, 2004).

In a clinical study, it was shown that testosterone inhibits triglyceride uptake and lipoprotein lipase activity. In this study, testosterone administration returned testosterone levels to mid normal values with a treatment duration of 8-9 months, leading to a decline in the visceral fat mass (Saad, 2009). Therefore, the action of testosterone seems to be implicated in the regulation of both preadipocyte proliferation and differentiation by affecting lipoprotein lipase action. The above studies are examples on the effect of testosterone in modulating adipose tissue and adiposity; however, other studies have been carried out to investigate the correlation between testosterone and adipocyte-derived proteins (Kalinchenko *et al.,* 2010). Changes in these adipocyte-derived proteins as well as in testosterone levels in obesity resulting in MetS were observed (Allan *et al.,* 2007). The role of testosterone is established by findings in men with prostate cancer who undergo androgen ablation therapy (Kalinchenko *et al.,* 2010) particularly in the longer-term, which affects all components of the MetS (Braga-Basaria *et al.,* 2006). The resulting metabolic consequences include abdominal obesity, IR, and atherogenic dyslipidemia along with a pro-thrombotic, inflammatory profile (Maneschi *et al.,* 2012).

Previous studies in hypogonadal men showed that TRT inhibits adiponectin levels, even though both adiponectin and testosterone have an inverse correlation with obesity (Lanfranco *et al.,* 2004). There have been contradictory results related to the effect of testosterone treatment and adiponectin. While in one study, testosterone treatment led to increased levels of adiponectin (Heufelder *et al.,* 2009), in another study a decrease was observed in diabetic patients (Kapoor *et al.,* 2007); a decrease was also seen in studies using high doses of testosterone (Page *et al.,* 2005a) however a further study saw no change (Page *et al.*, 2005b). In contrast to adiponectin, leptin levels increased in direct proportion to the adipose tissue mass (Kapoor *et al.,* 2007). Leptin has a suppressive effect on testosterone production (Saad and Jones 2010). An inverse relationship has been reported between serum testosterone and leptin concentrations in men (Isidori *et al.,* 1999). TRT has been found to decrease serum leptin levels in hypogonadal men (Sih *et al.,* 1997). PAI-1 is also secreted highly in obesity and acts as a pro-thrombotic factor (Russo *et al.,* 2012). TRT was associated with reduction in its level in endothelial cells (Jin *et al.,* 2007), whereas others found no change (Smith *et al.*, 2005). In contrast, to

the best of our knowledge, no research has been performed to investigate the effect of testosterone treatment on CX3CL1, CCL2 and PPARβ/δ in adipose tissue. Sex hormones have been reported to influence the differentiation process of adipocytes (Monjo *et al.,* 2005). However, it is unclear whether testosterone treatment could influence these factors through adipogenesis processes and adipocyte differentiation including adiponectin, PPARβ/δ, PAI-1, leptin, CX3CL1 and CCL2.

4.1.5 Aims of this chapter

1. To investigate the effect of testosterone on CX3CR1 and CCR2 mRNA expression in PMA-differentiated THP-1 cells, as a model of macrophages involved in atherosclerosis, in the presence or absence of cytokines.

2. To investigate the effect of testosterone on the secretion of anti/pro-inflammatory mediators by PMA-differentiated THP-1 cells before and after stimulation with pro-inflammatory cytokines.

3. To investigate the effect of testosterone on mRNA expression of adipocyte derived associated proteins (adiponectin, PPARβ/δ, PAI-1, leptin, CX3CL1 and CCL2) during the differentiation process in the mouse 3T3L1 cell line, as a model of preadipocyte differentiation.

4. To investigate the effect of testosterone on the secretion of adiponectin, CX3CL1 and CCL2 from mature 3T3L1 adipocyte cells.

5. To investigate whether testosterone is acting via an androgen-receptor (AR) or non-androgen receptor mechanism, by blocking the AR with flutamide in each of the above studies.

4.2.1 Cell culture models

Both cell lines (Human acute monocytic leukaemia cell line (THP-1) and Mouse Preadipocyte 3T3L1 cell line) were maintained for indefinite periods in a defined culture medium. Sterile cell culture techniques were used throughout to avoid microbial contamination. All experiments were repeated three times with two replicates for all experimental conditions.

4.2.1.1 THP-1 cell line

THP-1 is a human acute monocytic leukaemia cell line isolated from the peripheral blood of a 1 year old human male with acute monocytic leukaemia (AML) at relapse in 1978 and was originally obtained from the European collection of cell cultures (ECACC, UK). The THP-1 cell line is a valuable model for studying the mechanisms involved in macrophage differentiation, physiological functions and the expression of plasma membrane receptors, and cytokines (Apostolakis *et al.,* 2007). Phorbol myristate acetate (PMA) induces differentiation of THP-1 to macrophage-like cells.

4.2.1.2 3T3-L1 preadipocytes cell line

Mouse 3T3-L1 preadipocyte cell line was developed by clonal expansion from murine Swiss 3T3 cells and was originally obtained from American Type Culture Collection (ATCC, UK). The mouse embryonic fibroblast cell line 3T3-L1 is a favoured model for metabolism and obesity research, because the cells can be chemically induced to differentiate into adipocytes, where they are sensitive to lipogenic and lipolytic hormones and drugs. The mixture of dexamethasone (DEX), 1-methyl-3-isobutylxanthine (MIX), and insulin is used for stimulating cell differentiation of the Mouse 3T3-L1 preadipocyte to mature adipocytes.

4.2.2 Growing and maintaining of cell lines

4.2.2.1 Human monocytic THP-1 cells

THP1 cells were seeded in suspension and maintained in 75 cm^2 tissue culture flasks (Fisher Scientific, UK) containing RPMI 1640 (GIBCO®, UK) media supplemented with 2mM glutamine, 10% foetal bovine serum (FBS), (GIBCO®, UK), 1% (2mM) penicillin/streptomycin (GIBCO®, UK) and 1% (100mM) sodium pyruvate (GIBCO®, UK) at 37°C with relative humidity in a 5%CO_2/ 95% air incubator (Heraeus Instrument, Germany). At 70-90% confluence, medium was changed from the culture flasks and cells were placed into universal 30 ml tubes (Fisher Scientific, UK) and centrifuged for 5 minutes at 200g (Kendro Laboratory, USA). Media was changed approximately every 72 hours. The passage number of this cell line was unknown.

4.2.2.2 Mouse 3T3L1 cells

Cells were maintained in growth medium (preadipocyte medium, PM) containing Dulbecco's modified Eagle's medium (DMEM) (GIBCO®, UK) supplemented with 10% FBS and 1% (2mM) penicillin/streptomycin into 175 cm^2 tissue culture flasks. They were kept at 37°C with 5% CO_2/95% air in a humidified incubator until use and subcultured every 5-7 days. The passage number of this cell line was 7-10 in all experiments.

At 70-90% confluence, medium was removed from the culture flasks and cells were washed twice with phosphate buffered saline (PBS) (Invitrogen, UK) and then aspirated. One ml of trypsin/EDTA solution (0.5% trypsin and 0.53nM EDTA (GIBCO®, UK)) was added to the cells in a175 cm^2 flask and incubated at 37°C for 3 minutes. The flask was tapped gently to remove cells from the bottom surface and an equal amount of complete medium was added to the flask to neutralise the actions of trypsin/EDTA. The cell suspension was transferred to a sterile 50 ml-Falcon tube and centrifuged for 5 minutes at 200g as above. After removing the supernatant, the cells were suspended in 1ml of fresh medium. Viable cell numbers were determined by adding 10µl of this cell suspension to 10µl of trypan blue solution (Sigma-Aldrich, UK) and incubated for 5 minutes. Viable cells do not stain whereas dead cells stain blue. Cells were counted using an automatic cell counter designed to measure cell count and viability

(Countess™, Invitrogen, UK). Cells were then further diluted to working densities and used in the experimental procedures, sub-cultured or cryopreserved.

4.2.3 Differentiation of THP-1 cells into macrophage cells

Induction of terminal differentiation from monocyte-like state to macrophage-like cells was performed by using PMA. PMA (Sigma-Aldrich, UK) was dissolved in DMSO at 1mg/ml and stored at -20°C as a stock solution. Additional dilutions of PMA were made in medium at 1:1000. Working concentration of PMA 100ng/ml was added to THP-1 cells, which is an effective dose for differentiating these cells. The cells were plated in six-well plates (Fisher Scientific, UK) in duplicate at a density of $3x10^5$ cells/ml (2.5ml per well) for 24, 48, 72 and 96 hours.

4.2.4 Differentiation of 3T3L1 preadipocyte cells

To differentiate mouse preadipocyte cells to mature adipocyte cells, they were seeded into six-well plates in duplicate a density of $4x10^5$ cells/ml (2.5ml per well). Once the cells were confluent, they were incubated for an additional 48 hours before initiating differentiation by incubating in differentiation medium (DM), DMEM supplemented with 0.5µM isobutylmethylxanthine (Sigma-Aldrich, UK), 1µM dexamethasone (Sigma-Aldrich, UK) and 10 µg/ml insulin (Sigma-Aldrich, UK) for 3 days. DM was replaced with adipocyte maintenance medium (AM) containing 10 µg/ml insulin only. The medium was changed every 2–3 days and replaced by AM until ready for use. 3T3-L1 adipocytes were suitable for most assays 7-14 days post differentiation, where more than 80% of the cells had differentiated by 7 days.

4.2.5 Freezing cells protocol

Both cell lines were frozen with freezing medium (70% medium, 20% FBS, 10% Dimethyl sulphoxide, (DMSO), (Sigma-Aldrich, UK)) at $1x10^6$ cells/ml. In brief, cells were collected from tissue culture flasks into Falcon universal tubes and centrifuged at 200g at 20°C for 5 min. The supernatant was discarded and cells were re-suspended and counted, as described above. 3 ml of freezing media was added to the cells following re-suspension of cells and 1ml was aliquoted into labelled cryovials (Nalgene, Fisher, UK)

and packed with plenty of tissue in a polystyrene container (Nalgene, Fisher, UK) and stored at -80 °C freezer overnight. Following this cryovials were transferred into a dewar containing liquid nitrogen (Forma Scientific, USA).

4.2.6 Thawing cells protocol

Cell vials were removed from liquid nitrogen and quickly thawed in a water bath at 37°C until thawed. In a laminar flow hood, 1 ml of warm complete medium was added to cells in the cryovial, and then transferred to 10 ml of medium in a sterile universal tube, gently mixed and then centrifuged at 200g for 5 min. After discarding the supernatant cells were re-suspended and viable cells counted as above.

4.2.7 Collection of supernatants and adherent cells for analysis

Cells were treated as in section 4.2.2.2 and supernatants were collected in 5ml tube (Fisher Scientific, UK) to analyse cytokines production from these cells in media and cell pellets were stored at -80 freezers for extraction of RNA.

4.2.8 Oil Red O staining and 3T3L1 cells

The Oil Red O method is a common staining technique used for demonstrating the presence of fat or lipids in fresh and frozen tissue sections. The basis for staining lipids with an oil-soluble dye lies in its high solubility in fatty substances. For staining 3T3L1 cells, the stain must be freshly prepared from the stock solution and the cells fixed. A stock solution of stain was prepared by dissolving 0.3 g of Oil Red O (Sigma-Aldrich, UK) in 100 ml of isopropanol. A working solution was prepared by dilution of 30 ml of the stock stain with 20 ml of distilled water and left to stand for 10 minutes and then filtered using filter paper (Fisher Scientific, UK) into a Coplin jar, and covered immediately. Fixation of cells was performed by incubation of cells in wells of a 6-well plate in 4% paraformaldehyde (Sigma-Aldrich, UK) for 30-40 minutes at RT. Then, the fixative was aspirated and cells were rinsed three times for 5-10 minutes with PBS. Following aspiration of PBS, cells were washed twice with H_2O and then stained with 1 ml of freshly prepared Oil Red O working solution. The Oil Red O solution was removed and cells were washed three times with H_2O. Once the cells were dried the red

colour of lipid particles was monitored and photographed using a microscope with XIi camera (Leica Microsystems, UK Ltd).

4.2.9 Experimental treatment of THP-1 cell

4.2.9.1 Experimental conditions for testing the effects of testosterone with or without flutamide on CX3CR1 and CCR2 expression in THP-1 cells

Testosterone (Sigma-Aldrich, UK) was dissolved in absolute ethanol (Fisher Scientific, UK) at an initial concentration of 10^{-3}M and stored as a stock solution at 4°C until used. Additional dilution was performed at 1:1000 in medium and then further dilutions were made to prepare working concentrations. Furthermore, flutamide (Sigma-Aldrich, UK) was dissolved in absolute ethanol (Fisher Scientific, UK). Flutamide was stored at 4°C as a stock solution at 10^{-3} M. Dilution was performed in medium at 1:1000 and then further dilutions were made to prepare working concentrations before use.

Cells were cultured at a density of 3×10^5 cells/ml in six well plates (2.5 ml) for 24 h and then differentiated with PMA (100 ng/ml) for 48h. Testosterone was added to cells at 10 and 100 nM per well in duplicate and incubated with testosterone from 24 to 96h alone and in combination with flutamide at concentration of 100 and 1000 nM, respectively. Untreated cells were used as the negative control. Ethanol, at a volume equal to that of the diluted working solution of testosterone or flutamide was not added to control cells as vehicle because previous studies have shown no effect of ethanol at this concentration on these cells (Kilby *et al.,* 2012). Following collection of cells as detailed in section (4.2.7), cells and supernatants were stored at -80°C prior to RNA extraction and flow cytometric bead assay analysis.

4.2.9.2 Experimental conditions for testing the effects of 24h cytokine and testosterone with or without flutamide on CX3CR1 and CCR2 expression in THP-cells

THP-1 cells were seeded in 6 well plates at a density of 3×10^5 cells/ml (2.5 ml per well) and then differentiated with PMA as described in section (4.2.3. In the presence of cytokines TNFα plus IFNγ combined, at concentrations of 10 and 100 ng/ml, cells were treated with testosterone at 10 and 100nM alone and in combination with flutamide at 100 and 1000 nM, respictivley. Untreated cells without cytokines and cells with

cytokines but without testosterone were used as negative controls. Collection and storage of cells and supernatants were performed as described above.

4.2.10 Measurement of pro- and anti- inflammatory concentrations in supernatants of THP-1 macrophages by Human Enhanced Sensitivity Cytometric Bead Array

The principle of this method was described in chapter 2 and was used for investigating levels of pro- and anti-inflammatory biomarkers in serum. Here, this method was used for measuring levels of IL-1β, IL-6, IL-8 and IL-10 TNF-α, and IFN-γ in the supernatants from THP-1 macrophages, following treatment with the previous experimental conditions. The assay followed the same protocol as described in chapter 2 section (2.2.4).

4.2.11 Experimental treatment of mouse 3T3L1 cells

4.2.11.1 Experimental conditions for testing the effects of testosterone with or without flutamide on adipocyte derived associated proteins in mouse 3T3L1 cells

Cells were seeded at a density of 4×10^5 cells/ml (2.5ml per well) in six well plates (for 3, 5 and 9 days following differentiation. Testosterone was added at 10, 100 nM with DM for 3 days followed by AM for 5 and 9 days alone and in combination with flutamide at 100 and 1000 nM to cells per well in duplicate, respectively. Untreated pre-adipocyte and mature adipocyte 3T3L1 cells were used as negative controls and untreated cells were used as the negative control. Ethanol, at a volume equal to that of the diluted working solution of testosterone or flutamide was not added to control cells as vehicle because previous studies have shown no effect of ethanol at this concentration on these cells (Tang *et al.,* 2012). All treatments were applied in duplicate, with experiments that were repeated at least three times for each condition. Collection of cells and supernatants were performed as previously above.

4.2.12 Measurement of the secretion of adiponectin, CX3CL1 and CCL2 in supernatants of mouse 3T3L1 cells by ELISA

Adiponectin, CX3CL1 and CCL2 were measured in supernatants of adipocyte cells following the above treatments using ELISA kits from R&D Systems. The method for each ELISA was the same apart from the volumes of reagents used and the concentrations of standards, as detailed below.

4.2.12.1 Investigation of adiponectin in supernatants of mouse 3T3L1 adipocyte by ELISA

Method

All reagents and 96 well microplate in kits the Mouse adiponectin/Acrp30 Quantikine ELISA kit (R&D Systems, USA) were brought from 4°C to RT. 50µl of assay diluents was added to each well and then 50µl of adiponectin standards (0, 0.16, 0.31, 0.62, 1.25, 2.5, 5 and 10 ng/ml) and test supernatants were pipetted into the wells in duplicate and left at RT for 3 hours. Following aspiration and washing each well five times with 400 µl wash buffer, the plate was inverted and blotted against clean paper towels. 100µl of adiponectin antibody-enzyme conjugate was added to each well and left for 1 hour at RT. After that washing was repeated and then 100 µl of substrate solution (TMB) was added to each well and left protected from light for 30 minutes at RT. Following addition 100 µl of stop solution (hydrochloric acid) per well to end the reaction, the plate was read on a microplate reader within 30 minutes at 450 nm.

4.2.12.2 Investigation of CX3CL1 in supernatants of mouse 3T3L1 cells by ELISA

Method

CX3CL1 was investigated by using the Mouse CX3CL1/fractalkine Quantikine ELISA kit (R&D Systems, USA). Reagents were brought from 4°C to RT. 50 µl of assay diluent was added to each well and then 50µl of fractalkine standards (0, 0.62, 1.25, 2.5, 5, 10, 20 and 40 ng/ml) and test supernatants were pipetted into the wells in duplicate and left for 2 hours. Subsequently, the plate was processed as above for the adiponectin assay.

4.2.12.3 Investigation of CCL2 in supernatants of mouse 3T3L1 adipocyte by ELISA

Method

CCL2 was investigated by using the CCL2/JE/MCP-1 Quantikine ELISA kit (R&D Systems, USA). 50µl of assay diluent was added to each well and 50µl of CCL2 standards (0, 15.6, 31.2, 62.5, 125, 250, 500 and 1000ng/ml) and test supernatants were pipetted into the wells in duplicate and left for 2 hours at RT. The assay then followed the same protocol as described above for adiponectin

4.2.13 Investigation of gene expression in THP-1 macrophages and mouse 3T3L1 cells using qRT-PCR

The qRT-PCR principle was previously described in chapter 3 section (3.2.1) using SYBR Green Dye I.

4.2.13.1 Isolation of RNA

RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen, UK). This method is designed to inhibit RNases with the ability to remove contamination by using QlAzollysis reagent (Qiagen UK), which is a monophasic solution of phenol and guanidine thiocyanate. Adding chloroform (Sigma-Aldrich, UK) leads to protein, DNA and RNA separating into phases of aqueous and organic layers in a RNeasy spin column.

Method

According to the manufacturer's protocol, RNA was extracted by adding 1ml of QlAzollysis reagent (Qiagen UK) to cell pellets in Eppendorf tubes and incubated for 5min at RT. 200µl of chloroform was added and the tubes were vigorously vortexed for 15s, then samples were centrifuged (Heraeus Fresco 17 Refrigerated Micro Centrifuge, USA) at 12,000 g for 15min at 4°C to separate protein, DNA and RNA into three layers. The upper aqueous layer containing RNA was separated and collected (about 600µl) into a clean 1.5ml Eppendorf tube and mixed with 600µl of 70 % ethanol. Samples were

removed to the RNeasy spin column in two steps, in each one about 600µl were transferred, and centrifuged for 15s at ≥ 8000g. The supernatant was discarded and the precipitated RNA pellet was washed with 700 µl of buffer RW1 in the spin column and centrifuged at 8000g for 15s. The supernatant was discarded and the precipitated RNA pellet was washed with 500 µl of buffer RPE in the spin column and centrifuged at 8000g for 15s. Again, the supernatant was discarded and the precipitated RNA pellet was washed with 500µl of buffer RW1 in the spin column and centrifuged at 8000g for 2min. The RNeasy column was transferred to a clean 1.5ml Eppendorf tube and centrifuged for 1min at 8000g. Finally, the RNeasy column was transferred to a clean 1.5ml tube and mixed with 30-40µl of RNA free water and then centrifuged for 1min at 8000g. The precipitated RNA pellet was stored at -20°C initially and then at -80°C prior to use in cDNA synthesis and q-RT-PCR.

4.2.13.2 Quantification of RNA concentration

Quantity and high quality of RNA extraction from samples are essentially required for reduing the variation among these samples when reverse transcription of RNA applied for cDNA synthesis. For this the determination of exact of RNA concentration without contamination is advantageous and was performed by using The NanoDrop® ND1000 (Labtech International Ltd, UK).

The purity of RNA can be determined via the ratio of 260/280nm. The ratio of 260/280 in close proximity to 2.0 reflects purity of nucleic acids with respect to protein or solvent contaminants but this is not only enough for determination of purity nucleic acids, therefore, estimate the absorbance of nucleic acids at 260nm relation to 230nm can be also used as a further indication of purity ranging between 1.8-2.0 (Gallagher, 2007)

Method

RNA free water was used to blank The NanoDrop® ND1000, 2µl of sample was placed on the measurement pedestal, the measurement column was then drawn between the ends of two optical fibres to establish the measurement path. The measurement is normally finished at 5-10 seconds, and the spectrum and its analysis is shown on the screen of the attached PC. Once the measurement was made, the sample was wiped

from the measurement pedestals. Between each sample measurement, 2 µl of RNA free water was used as a blank and to avoid any contamination between samples.

4.2.13.3 Investigation of RNA quality

An additional method used alongside the Nanodrop to examine the integrity of total RNA was to run an aliquot of the RNA sample on a denaturing agarose gel stained with ethidium bromide. RNA with high integrity is visualized as sharp, clear 28S and 18S rRNA bands. The 28S rRNA band should appear approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is considered an excellent signal demonstrating that the RNA has high integrity (Huggett *et al.,* 2005; Sambrook and Russel, 2001).

Method

In an Eppendorf tube, 3µl of the extracted RNA sample was mixed with 5µl loading buffer, (Invitrogen, USA) and run on a 1.5 % agarose gel pre-stained with ethidium bromide (Sigma-Aldrich, UK). Using TAE as the buffer, the gel was run for 30 minutes at 110 v and the resulting gel was visualised on a UVP Bioimaging system using Lab Works 4 software (Bio-Rad, UK).

4.2.13.4 Synthesis of cDNA

Process of complementary DNA (cDNA) synthesis is called Reverse Transcription where high quality mRNA template is reverse transcribed into (cDNA) *in vitro.* Preparing cDNA requires mRNA, primers and the Reverse Transcriptase (RT) enzyme. It is advantageous to generate these cDNA samples by using random primers which can efficiently prime any RNA present in the sample. In addition, this type of primer can extend the RNA template at non-specific points and also give higher cDNA yields from limited mRNA. Therefore, random primers were used as a suitable method for cDNA synthesis. It is important that the initial RNA sample is free of genomic DNA. Consequently, RNA samples were mixed with gDNA wipeout buffer to remove contaminating genomic DNA from RNA samples. Then, the RNA template is reverse transcribed into cDNA by the enzyme reverse transcriptase (RT). The RT primer mix

contains a specially optimized mixture of oligo-dT and random primers, RT elongates the sequence using dNTPs to produce highly accurate cDNA.

Method

cDNA synthesis was performed by using a QuantiTect ®Reverse Transcription kit, (Qiagen, Germany). In brief, 2µl of the gDNA wipeout buffer was mixed with template RNA sample and specific RNA free water to give 14µl of total volume in the first microcentrifuge-tube (Sigma-Aldrich, UK). Following 2min incubation at 42°C in a thermocycler, tubes were immediately placed on ice. In the second tube, 1µl of reverse-transcription master mix, 4µl of Quantiscript RT buffer and 1µl of RT Primer Mix with 14µl of RNA template from the first tube and then incubated at 42°C for 15min in a thermocycler followed by 3min at 95°C to inactivate Quantiscript RT. Synthesized cDNA samples were stored at -20°C for RT-PCR analysis.

4.2.13.5 Selection of endogenous control reference genes

For perfect and reliable gene expression analysis, normalization of gene expression data against housekeeping genes (reference or internal control genes) is needed. The steps involved are described in chapter 3 section (3.2.1.2). However, the list of house keeping gene in this part were 12 human housekeeping genes (ACTB, SDHA, RPL13, ATPSB, EIF4A, 18S, YWHAZ, CYC1, B2M, CANX, UBC, and GADH) for THP-1 cell line and similarly 12 mouse housekeeping genes (B actin, β2M, GADPH, TOP1, RPL13A, SDHA, UBC, YWHAZ, 18S, UCB, EFI4A2 and ATP5B) for 3T3L1 cell line on 8 samples from each cell line, at different conditions of experiments that were used in this study. Each reference gene was tested with each sample in duplicate.

4.2.13.6 qRT-PCR using SYBR® Green dye methodology

The same protocol was performed as previously described in chapter 3. Products amplified by the SYBR® Green qRT-PCR analysis for each target were validated by visualisation on an agarose gel with a 100bp ladder (Invitrogen, UK) for THP-1 macrophages and 200bp ladder (Promega, UK) for 3T3L1 adipocyte cells.

Cell line name	Human Monocyte THP-1 cell line	Mouse preadipocyte 3T3L1 cell line
Media	RPMI 1640 containg FBS sodium pyruvate glutamine (2mM) penicillin/streptomycin(2mM)	DMEM containing FBS penicillin/streptomycin (2mM)
Media differentiation	RPMI 1640 + PMA	DMEM + INS/IBMX/Dex
after differentiation	Macrophage cells	Mature adipocyte cells
Investigated parameters	CX3CR1, CCR2, IL-1β, IL-6, IL-8, TNF-α and IFNγ	Adiponectin, PPARβ/δ, PAI-1, CX3CL1, CCL2 and Leptin
Assay technique	qRT-PCR and Cytometric Bead Array	qRT-PCR and ELISA

Table 4.1: Summary table of *in vitro* study. The details of cell lines, media composition, target genes, analysis method used in this study.

4.2.14 Statistical Analysis

Data is presented as mean ± SEM. The statistical analysis was made by using GraphPad Prism 5 and StatsDirect. To determine statistical differences of relative fold increase/decrease in expression of target gene between untreated (control) and treated samples and between groups were analysed by one way, two way ANOVA or Kruskal-Wallis and Friedman test for parametric and nonparametric data, respectively. When these tests revealed significance differences between the groups, then multiple comparison of groups were performed. All comparisons were two tailed and p values less than 0.05 were considered statistically significant.

4.3.1 Differentiation of Human THP-1 cells and Mouse 3T3L1 cells line

4.3.1.1 Human THP-1 cells

In the present study, differentiation of human monocytic cell line THP-1 in response to PMA stimulation at time different periods showed that PMA treatment resulted in remarkable phenotypic changes; according to cell adherence to the bottom of the culture flasks, which increased with incubation time with PMA. In addition, change to the irregular shape of these cells was seen from 24h incubation and this was clearer at 96h treatment with PMA (figure 4.1).

4.3.1.2 Mouse 3T3L1 cells

Figure 4.2 illustrates the morphological changes observed when monitoring the accumulation of lipid droplets in the cytoplasm where the preadipocyte 3T3-L1 cells start to take on adipocyte morphology. Lipid droplets, which are triglycerides, appeared within 4-7 days following induction of differentiation. The size of lipid droplets increased during the experiment period. The droplets extremely small initially and then fuse to form several large ones indicating that the 3T3-LI cells were efficiently differentiated into mature adipocytes based on the Oil Red O staining (figure 4.3).

4.3.2 qRT-PCR in THP-1 and 3T3L1

4.3.2.1 RNA extraction and cDNA synthesis

RNA was extracted for all samples and separated by agarose gel electrophoresis assay. The presence of two sharp bands corresponding to the two ribosomal bands 28S and 18S rRNA subunits that reflect adequate RNA integrity was obtained for the majorty of samples (figure 4.4).

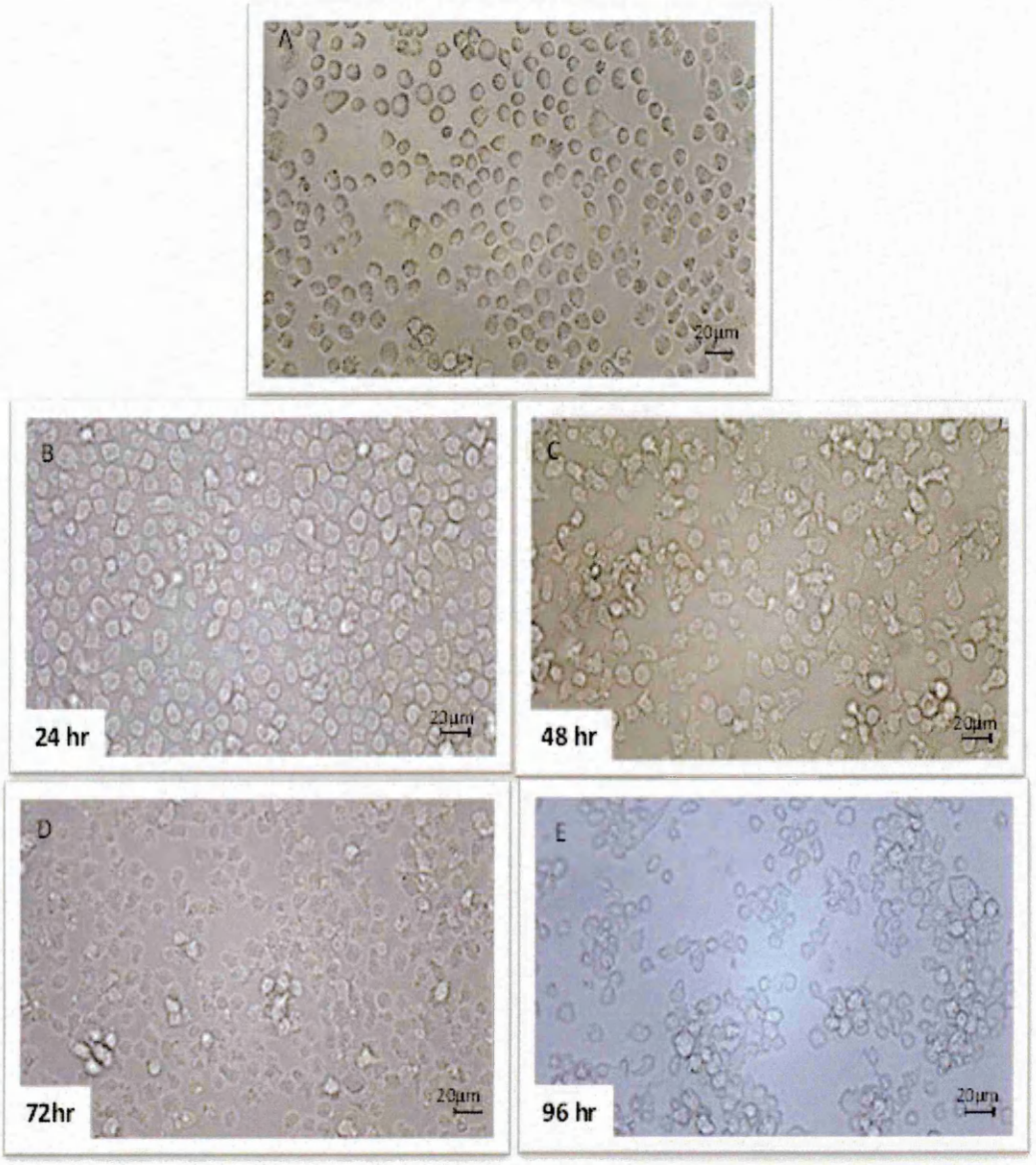


Figure 4.1: Morphological changes of THP-1 cells in response to PMA. Human monocyte THP-1 cell line before differentiation (A) compared to post treatment with PMA (100ng/ml) for 24, 48, 72, 96h (B-E respectively). Cells changes from regular, round cell morphology to irregular shapes over time. PMA (Phorbol 12-myristate13-acetate).

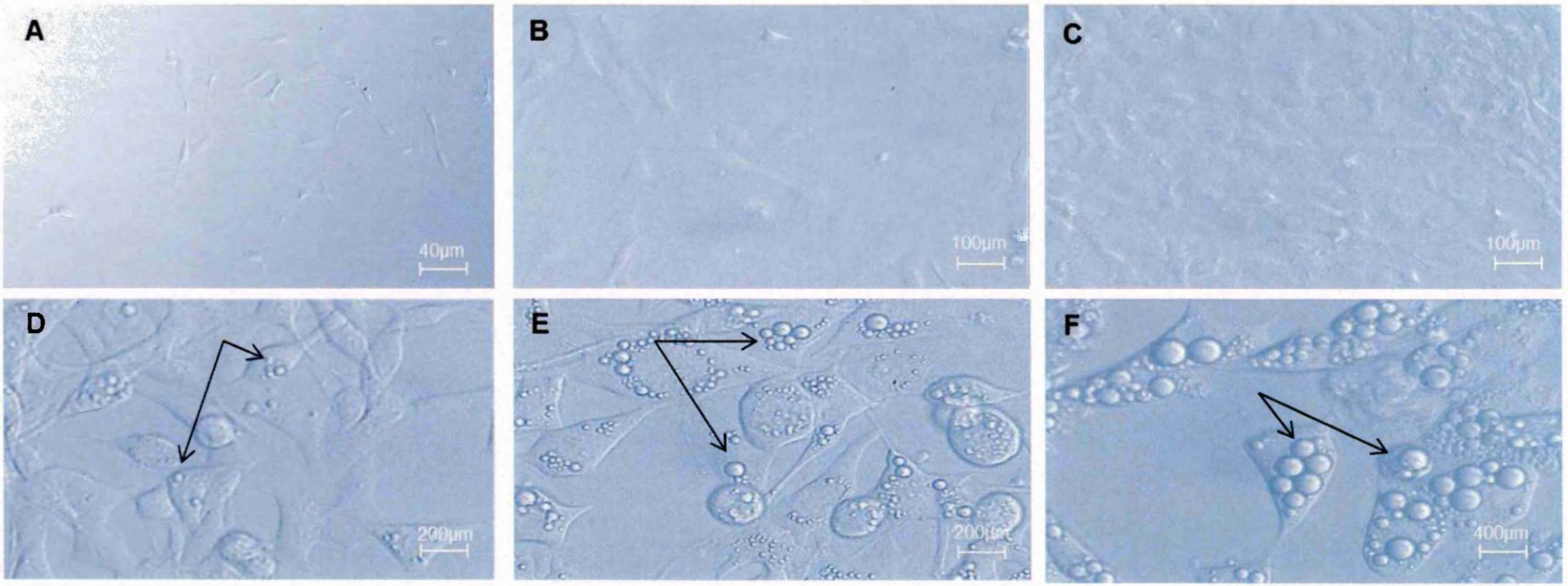


Figure 4.2: Undifferentiated and differentiated mouse preadipocyte 3T3L1 cell line. Preadipocyte cells (A, B) with preadipocyte media (C). Differentiated cells treated with media including insulin, dexamethasone and isobutylmethylxanthine (D) demonstrating small droplets of lipids starting to appear at 3 days (as the arrows indicate) at 5 days (E) and at 9 days (F) indicating increased amount of lipid droplets within the cells.

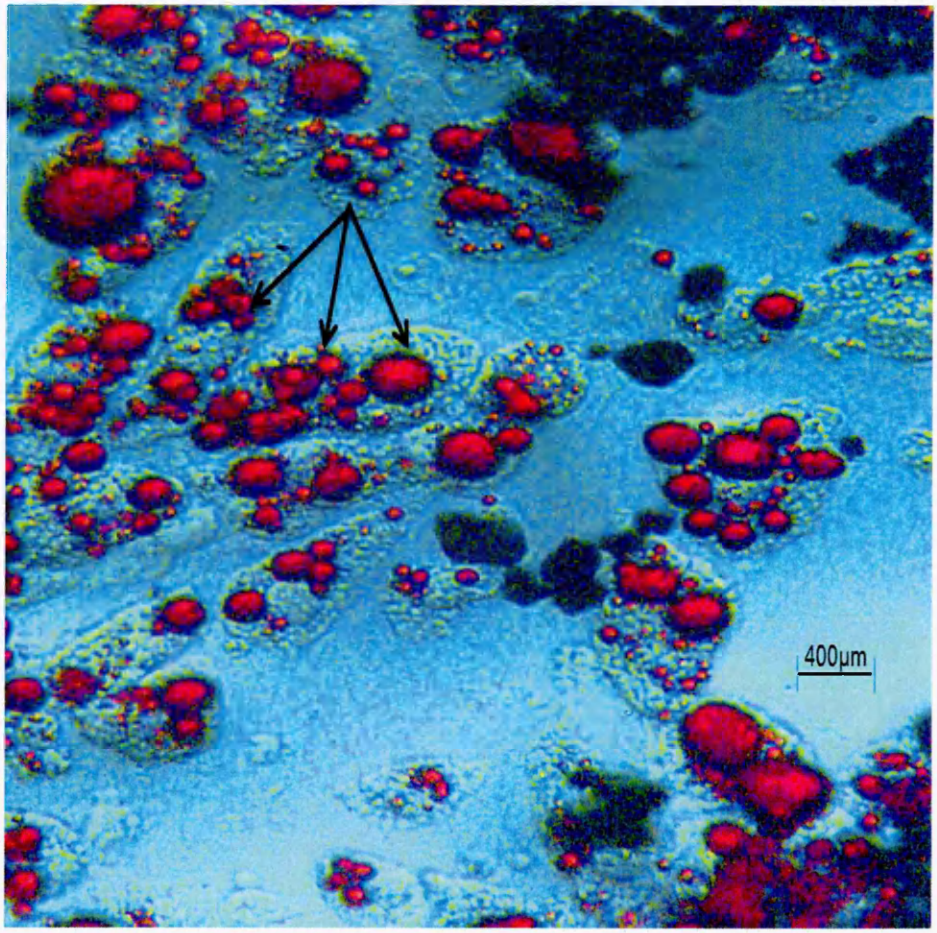


Figure 4.3: 3T3-L1 cells have differentiated into adipocytes and stained with Oil Red O. Visualization of cells after staining for the presence of neutral lipids with Oil Red O.

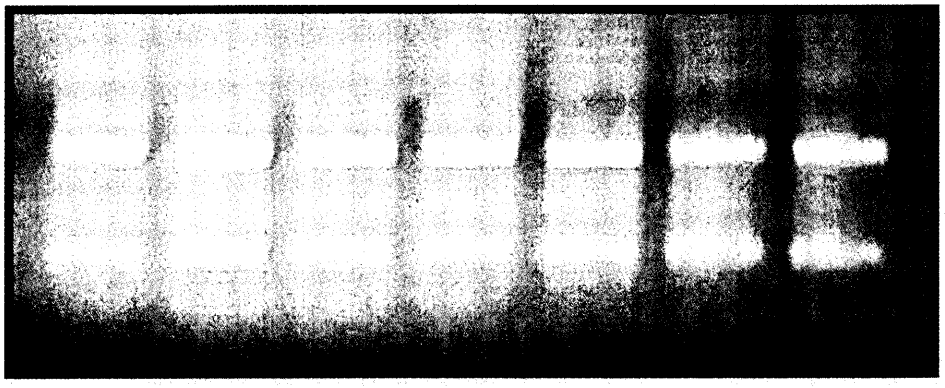


Figure 4.4: Agarose gel electrophoresis of RNA extracted from THP-1 macrophages. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample where each lane represents a separate extracted RNA sample, with perfect quality RNA indicated. The same results were obtained for 3T3-L1 cells (not shown).

4.3.2.2 Selection of endogenous control reference gene

To select the best housekeeping gene for use as an internal control to determine the relative expression change in target genes, in THP-1 macrophages, 12 housekeeping genes (ACTB, SDHA, RPL13, ATPSB, EIF4A, 18S, YWHAZ, CYC1, B2M, CANX, UBC, and GAPDH) were tested on 8 samples at different treatment conditions in experiments using qRT-PCR. The results were calculated by utilising geNorm software analysis. Housekeeping genes with an M-value above 1.5 were removed. Results showed that ACTB was the most stable gene because it had M-value less than 1.5, indicating its constant stability in relative mRNA expression under different experimental conditions. Similarly in the mouse 3T3L1 cell line, 12 mouse housekeeping genes (B actin, β2M, CANX, GADPH, TOP1, RPL13A, SDHA, YWHAZ, 18S, UCB, EFI4A2 and ATP5B) were tested on 8 samples of 3T3L1 cells using qRT-PCR. Following calculation of M values for each housekeeping gene demonstrated that CANX was the most stable housekeeping gene which had a M value less than 1.5.

4.3.2.3 Primer efficiencies and validation of primer targets

Determination of primer efficiency for target genes and reference gene in these cells was undertaken to validate the constant amplification of each PCR product at two cycles each time. In THP-1 macrophages the target and reference genes have similar and nearly 100% efficiencies in the range (93%-110%) as shown in table 4.2A and figure 4.5. Therefore, the $2^{-\Delta\Delta ct}$ (Livak) method was used to determine the relative difference in expression level of target gene in different samples compared to the internal control, reference gene. In contrast, the Pfaffl method was used in the 3T3L1 cell line to determine the relative expression of the target gene in different samples because amplification efficiencies of target and reference genes were not in an acceptable range of ideal amplification efficiencies as shown in table 4.2B and figure 4.6. Data from the two cell lines was compared to control data, which was normalized to a value of 1. Primer products from qRT-PCR revealed a single product and expected sizes when analysed by agarose gel electrophoresis. Figures 4.7 and 4.8 show the expression of target genes AR, CD14, CX3CR1, CCR2 and ACTB, in human THP-1 macrophages, whereas figures 4.9 and 4.10 show target genes AR and adiponectin, PPARβ/δ, leptin, PAI-1, CX3CL1 and CCL2 respectively in mouse 3T3L1 cells.

A

Gene Target	Amplification Factor	% Efficiency
ACTB	1.968	96.84
CX3CR1	1.941	94.08
CCR2	1.931	93.04
CD14	2.102	110.18

B

Gene Target	Amplification Factor	% Efficiency
CANX	2.4	142.00
Adiponectin	2.1	115.00
PAI-1	2.346	134.00
PPARβ/δ	2.2	121.00
Leptin	2.276	127.59
CX3CL1	2.29	128.976
CCL2	2.054	105.35

Table 4.2: **Primer efficiencies of PCR amplification in THP-1 macrophages and 3T3L1 cells**. Determination of primer efficiency percentage for target genes and reference gene in THP-1 macrophages (A) and 3T3L1 cells (B) using the formula, $E = 10^{(-1/slope)} - 1$

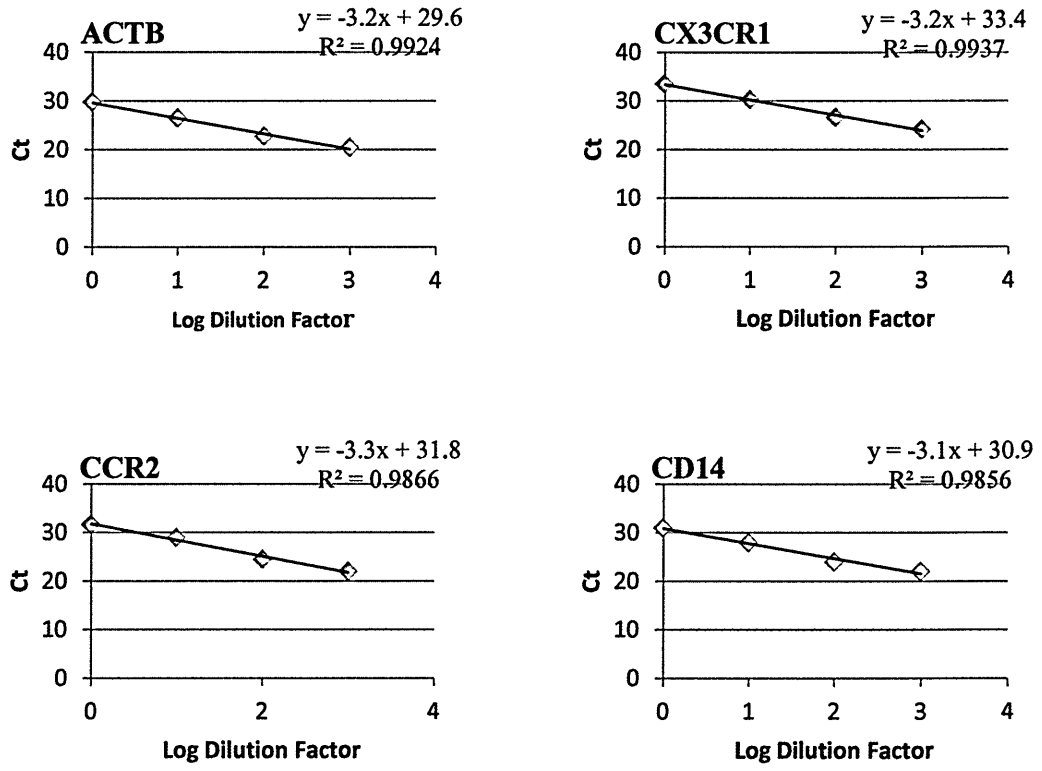


Figure 4.5: Standard-curve plot for calculation of primer efficiency genes in THP-1 macrophages. The efficiency of amplification of target genes and internal control (ACTB) was determined using real-time PCR and SYBR Green detection. The Ct values for each dilution were used to calculate the slope of the line representing the log of their amplification efficiencies. An ideal slope should be −3.32 for 100% PCR efficiency.

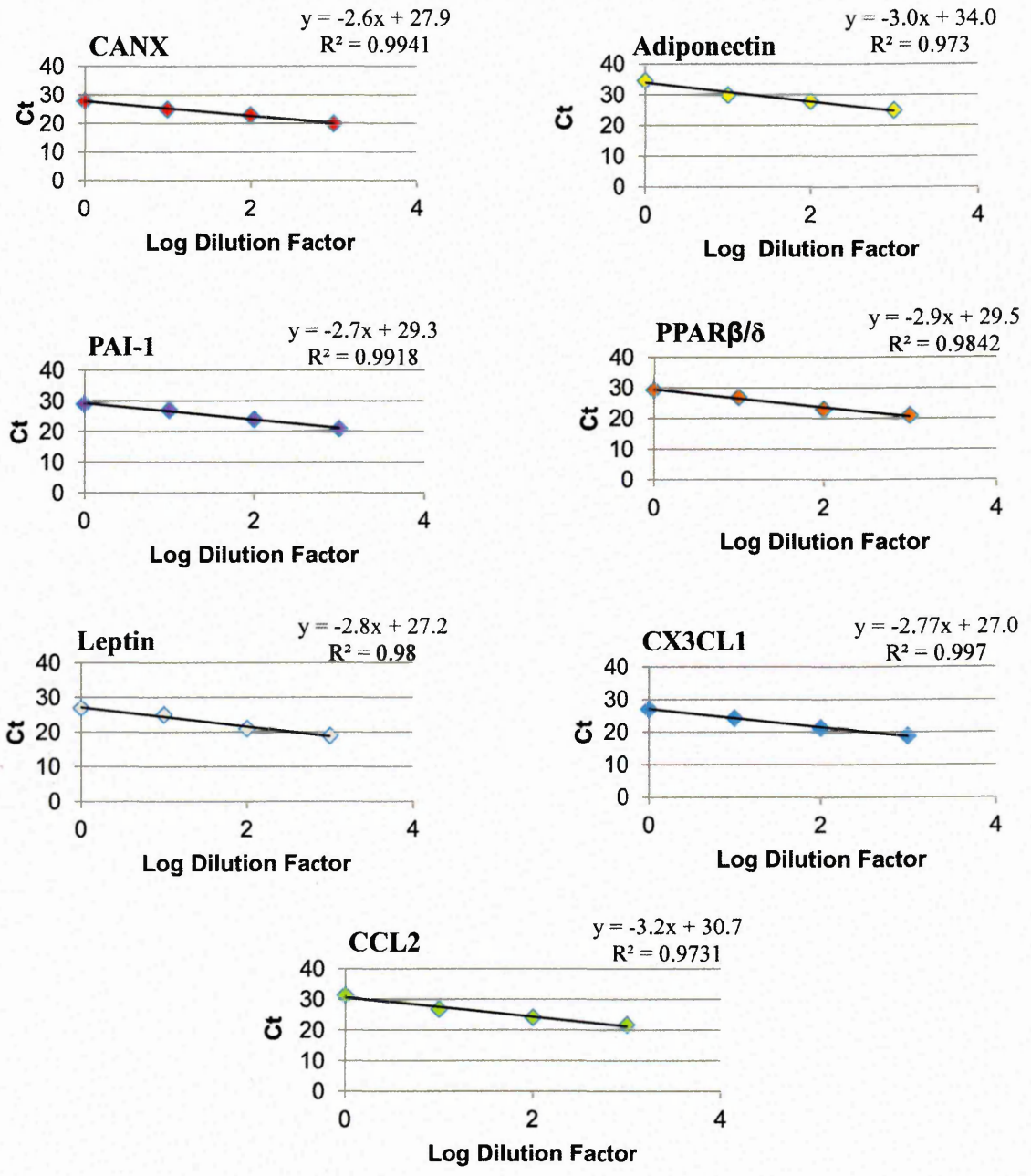


Figure 4.6: Standard-curve plot for calculation of primer efficiency genes in 3T3L1 cells. The efficiency of amplification of target genes and internal control (CANX) was determined using real-time PCR and SYBR Green detection. The Ct values for each dilution were used to calculate the slope of the line representing the log of their amplification efficiencies. An ideal slope should be -3.32 for 100% PCR efficiency.

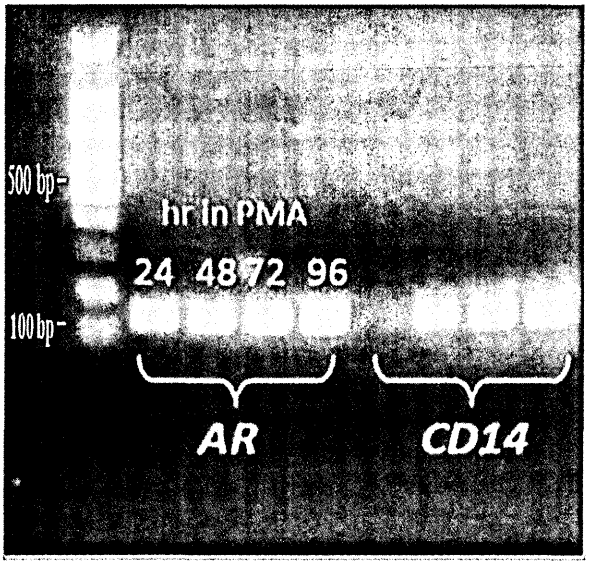


Figure 4.7: Agarose gel electrophoresis for PCR product amplification in human THP-1 macrophages. Androgen receptor (AR) mRNA was expressed from 24h to 96h while CD14 mRNA was observed following 48h treatment with PMA (100ng/ml).

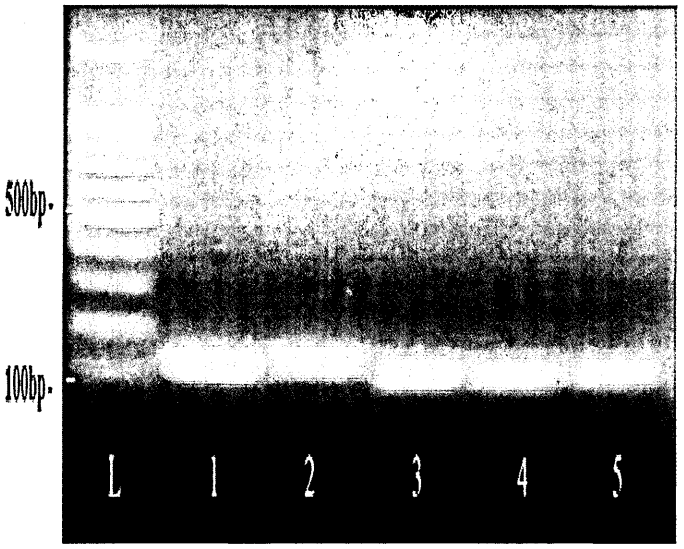


Figure 4.8: Agarose gel electrophoresis showing RT-PCR amplification for primer product in human THP-1 macrophages. Single sharp bands indicate a single detectable product for all target genes. L- Ladder, lane - 1 CX3CR1, lane - 2 CCR2, and lanes 3-5 ACTB.

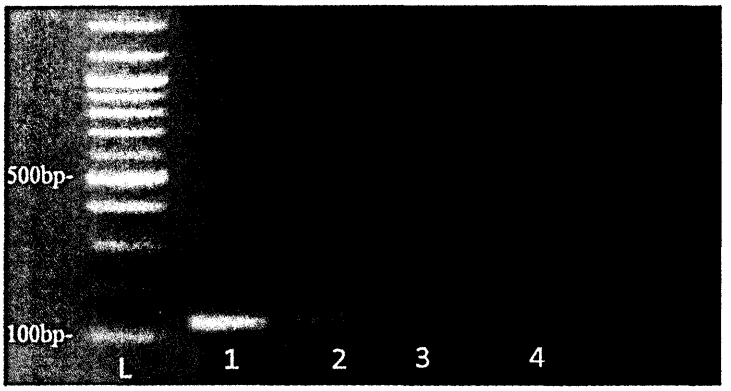


Figure 4.9: Agarose gel electrophoresis showing RT-PCR amplification for primer for androgen receptors in 3T3L1 cell line. Single and sharp bands indicate single detectable product for AR gene. L-ladder, lanes-1 and 2 AR in preadipocytes, lanes-3 and 4 AR in mature adipocyte cells.

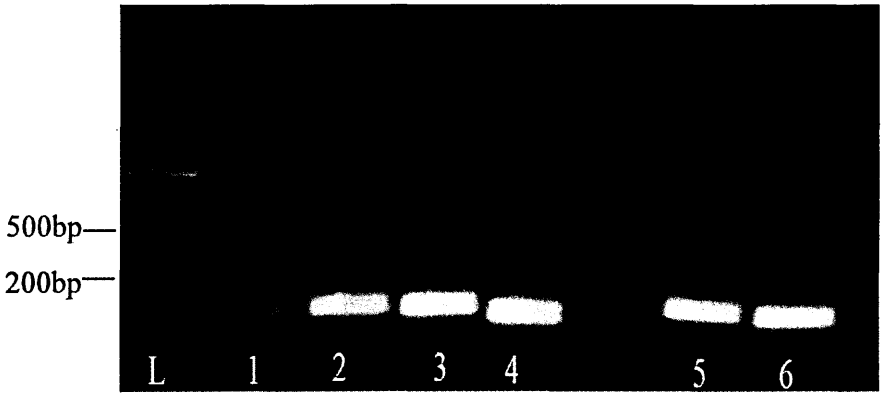


Figure 4.10: Agarose gel electrophoresis showing RT-PCR amplification for primer product in 3T3L1 cell line. Single sharp bands indicate single detectable product for all target genes. L-ladder, lane-1 CCL2, lane-2 CX3CL1, lane-3 adiponectin, lane-4 PPARβ/δ, lane-5 leptin and lane-6 PAI-1.

4.3.3 Analysis of CX3CR1 and CCR2 mRNA expression in human THP-1 macrophages by qRT-PCR

4.3.3.1 Analysis of CX3CR1 and CCR2 mRNA expression in THP-1 macrophages following testosterone treatment, with or without flutamide

At low testosterone concentrations, no significant change was observed in mRNA expression of CX3CR1 following testosterone treatment at 10nM concentration, even though there was a trend to a decrease in expression after 24 and 72h compared to untreated control samples. This effect was reversed, but not significantly by flutamide at 100nM particularly following 24 to 72h (figure 4.11a). Conversely, there was a trend to increase mRNA expression of CCR2 following the same concentration of testosterone treatment. This effect was reversed, but not significantly with flutamide from 24 to 96h at a concentration of 100nM as shown in figure 4.12a.

At high testosterone concentration and compared to untreated control samples, no significant alteration was seen in mRNA expression of both receptors CX3CR1 (figure 4.11b) and CCR2 (figure 4.12b) from 24 to 96h following 100 of testosterone treatment with or without flutamide at concentrations of 1000nM, respectively.

4.3.3.2 Analysis of CX3CR1 mRNA expression in THP-1 macrophages following 24h cytokines and testosterone treatment, with or without flutamide

As shown in figure (4.13a, b), incubation of THP-1 macrophages with TNFα plus IFNγ treatment at 10ng/ml resulted in a decrease, although not significant, in mRNA expression of CX3CR1 after 24h compared to untreated control cells. However, the addition of testosterone treatment at 10 or 100nM had no effect on mRNA CX3CR1 expression in THP-1 macrophages treated with 10ng/ml TNFα plus IFNγ stimulation for 24h (4.13a, b). A significant difference was seen in mRNA expression of CX3CR1 ($p<0.05$) after co-treatment with 1000nM flutamide (figure 4.13b).

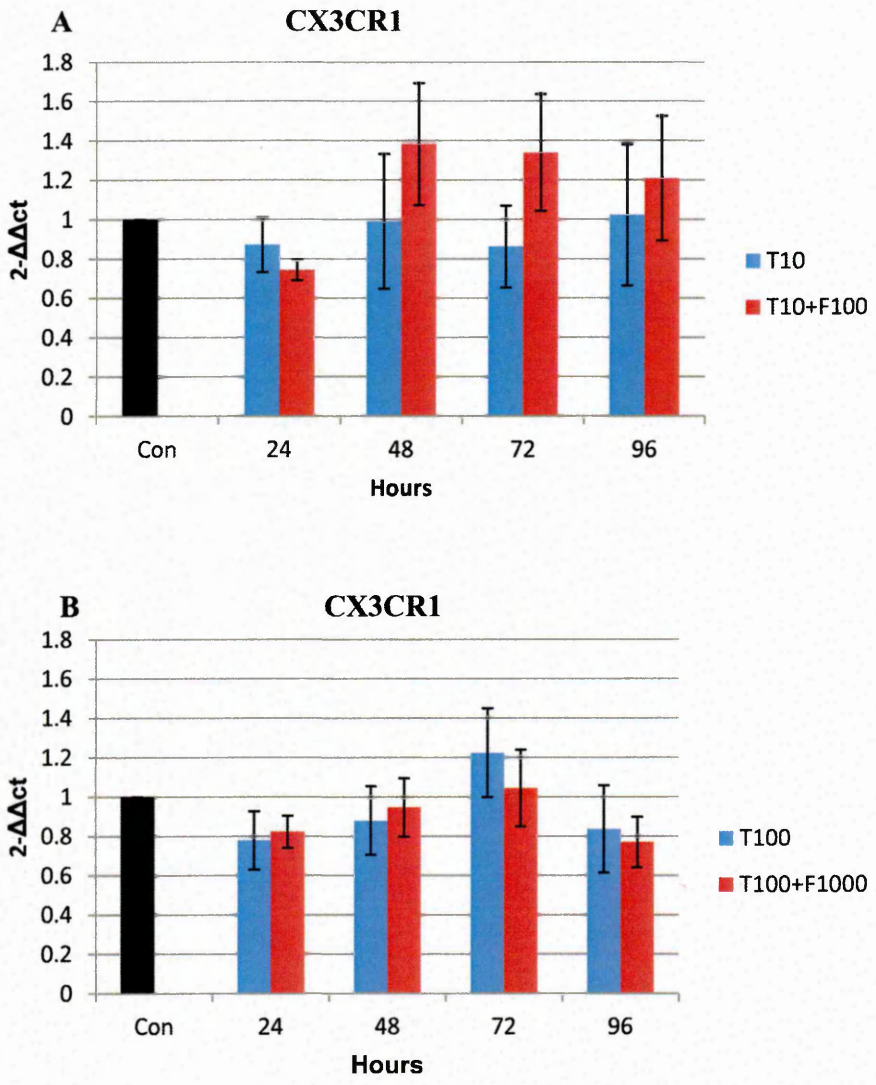


Figure 4.11: CX3CR1 mRNA expression in THP-1 macrophages following testosterone treatment, with or without flutamide from 24 to 96h by qRT-PCR. Results shown as mean±SEM from three separate experiments. Con=control, (A) T10=Testosterone 10nM, F100=Flutamide 100nM, (B) T100=Testosterone100nM, F1000=Flutamide 1000nM. Two way ANOVA.

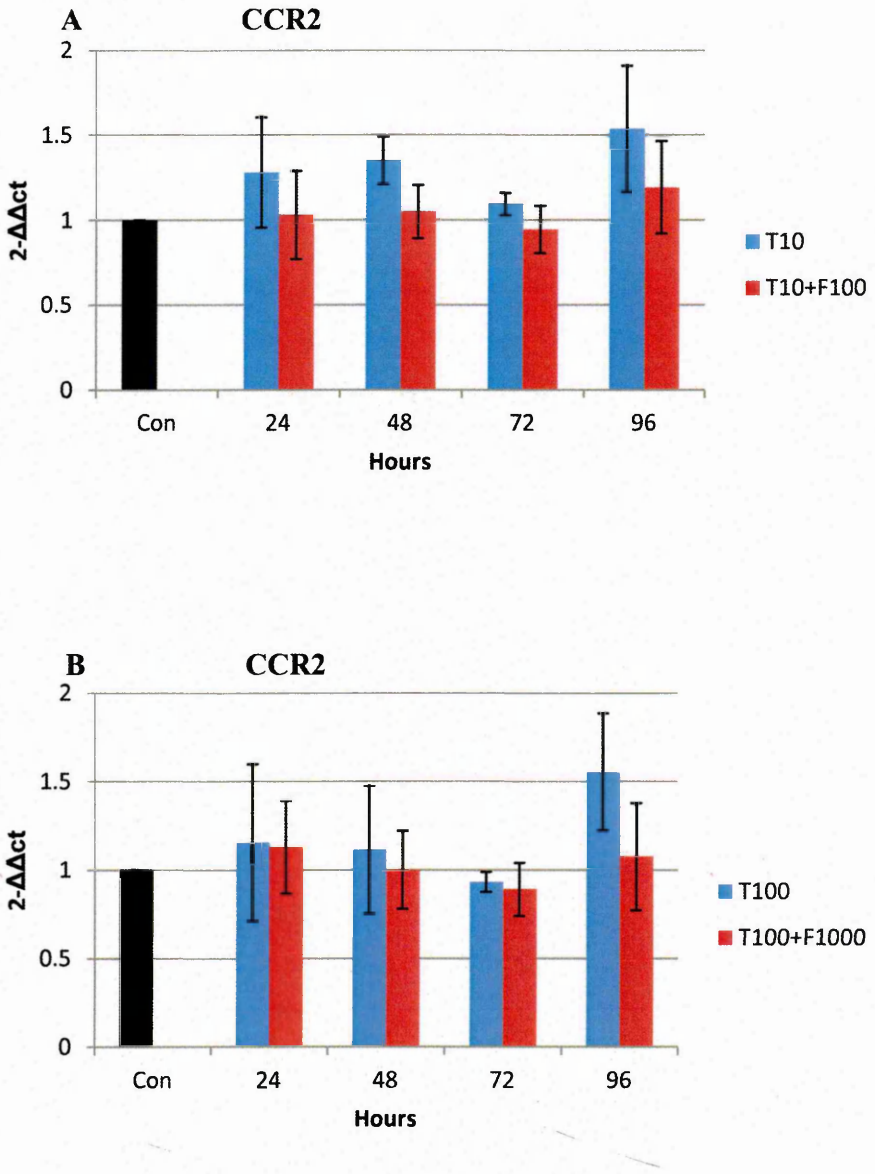


Figure 4.12: CCR2 mRNA expression in THP-1 macrophages following testosterone treatment, with or without flutamide from 24 to 96h by qRT-PCR. Results shown as mean±SEM from three separate experiments. Con= control, (A) T10=Testosterone 10nM, F100=Flutamide 100nM, (B) T100=Testosterone 100nM, F1000=Flutamide 1000nM. Two way ANOVA.

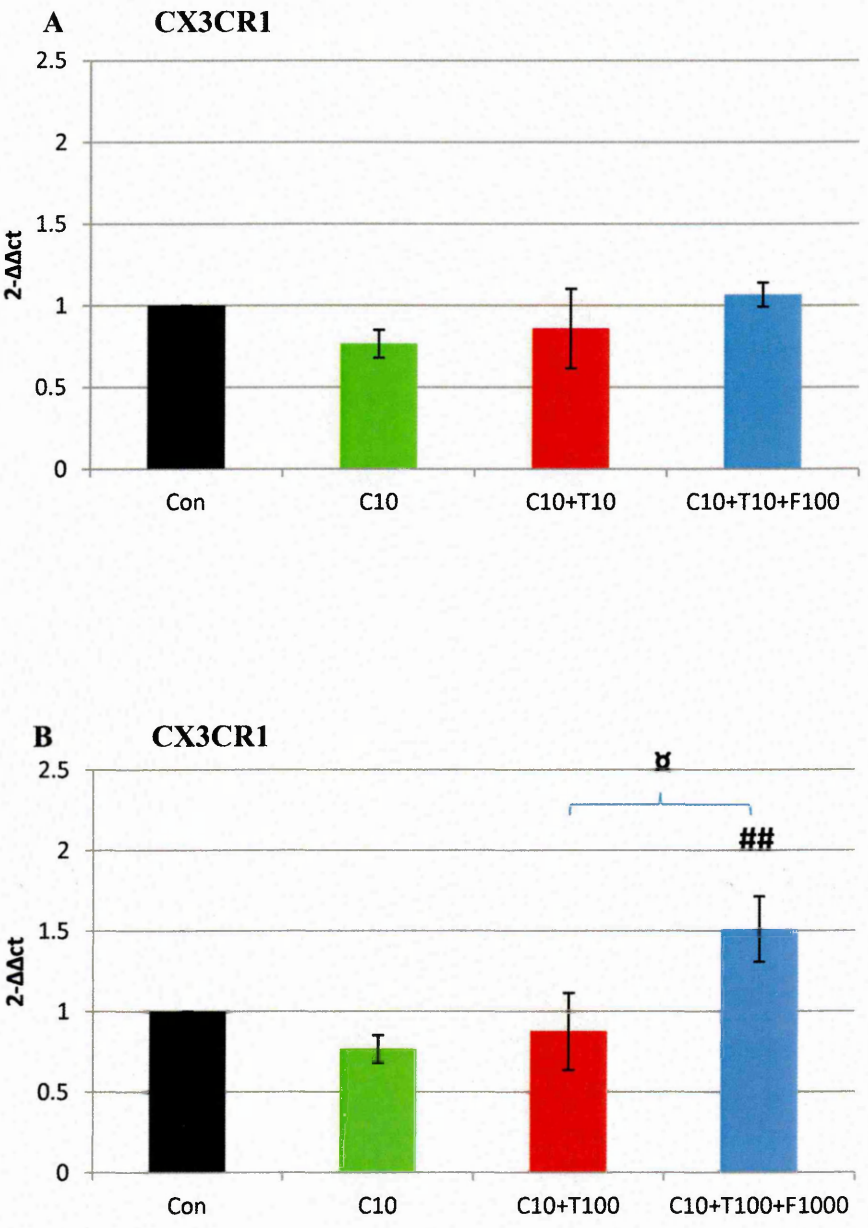


Figure 4.13: CX3CR1 mRNA expression in THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide by qRT-PCR. Results are shown as mean±SEM from three separate experiments. Con=control, C10=cytokine (TNF-α+IFN-γ) 10ng/ml, (A) T10=testosterone 10nM, F100=flutamide 100nM, (B) T100=testosterone 100nM, F1000=flutamide 1000nM. $^{\#\#}P<0.01$ vs C10, $^{¤}P<0.05$ vs C10+T100. Kruskal-Wallis.

In addition, as shown in figure (4.14 a, b), stimulation of THP-1 macrophages for 24h with TNFα plus IFNγ treatment at 100ng/ml led to reduced, but not significantly, expression of CX3CR1 mRNA compared to untreated control cells. Although not statistically significant, 10nM testosterone decreased expression of CX3CR1 mRNA following stimulation with 100ng/ml TNFα plus IFNγ compared to cells treated with cytokines alone. This reduction was significantly abolished by flutamide at 100nM ($P<0.05$) (4.14a). In contrast, 100nM testosterone increased, but not significantly, expression of CX3CR1 mRNA, an effect that was not altered by blocking the AR with 1000nM flutamide (figure 4.14b).

4.3.3.3 Analysis of CCR2 mRNA expression in THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide

There was a trend towards a decrease in expression of CCR2 mRNA following incubation of THP-1 macrophages for 24h with cytokines treatment at 10ng/ml compared to untreated control cells ($P=0.06$). Conversely, the combination of 24h cytokine treatment at 10ng/ml and testosterone treatment at 10nM or 100nM with cells tended to increase expression of mRNA for CCR2 ($P=0.08$ and $P=0.06$ respectively) compared to cells stimulated with cytokines at 10ng/ml (figure 4.15 a, b). No significant effect was seen after flutamide treatment at 100 or 1000nM.

In addition, 24h cytokine treatment at 100ng/ml significantly down regulated expression of CCR2 mRNA compared to untreated control cells ($p<0.01$) (4.16 a, b). However, 10nM testosterone treatment significantly increased expression of CCR2 mRNA following stimulation with 100ng/ml cytokines compared to cells treated with cytokine treatment alone ($p<0.001$). This increase was significantly and partly reduced by co-treatment with 100nM flutamide (4.16a). Moreover, combination of testosterone treatment at 100nM with 100ng/ml TNF-α plus IFNγ for 24h significant increased ($p<0.01$) CCR2 mRNA expression compared to cells treated with cytokine treatment alone. This increase in expression of CCR2 mRNA was not altered by 1000nM flutamide (figure 4. 16b).

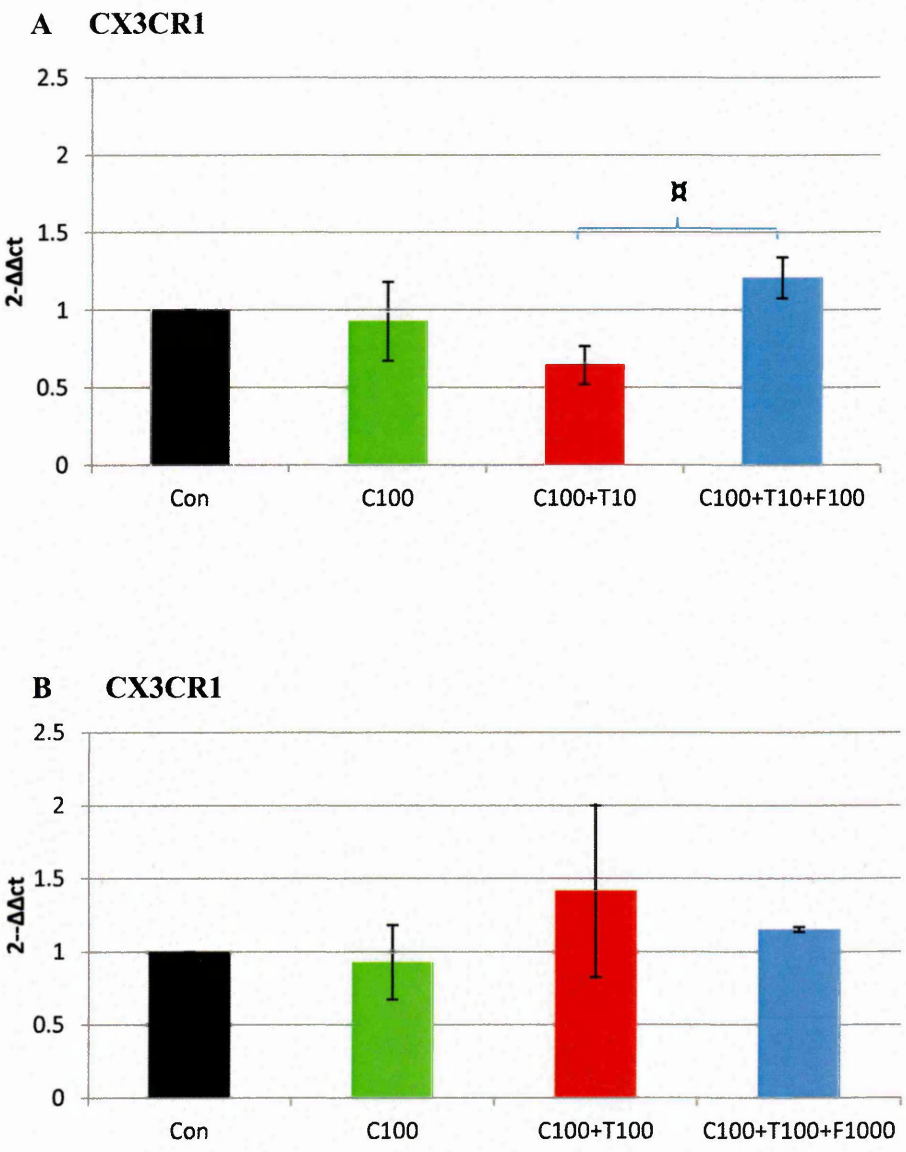


Figure 4.14: CX3CR1 mRNA expression in THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide by qRT-PCR. Results shown as mean±SEM from three separate experiments. Con=control, C100=cytokine (TNF-α+IFN-γ) 100ng/ml, (A) T10=testosterone 10nM, F100=flutamide 100nM, (B) T100=testosterone 100nM, F1000=flutamide 1000nM. $^{\circ}P<0.05$ vs C100+T10, Kruskal-Wallis.

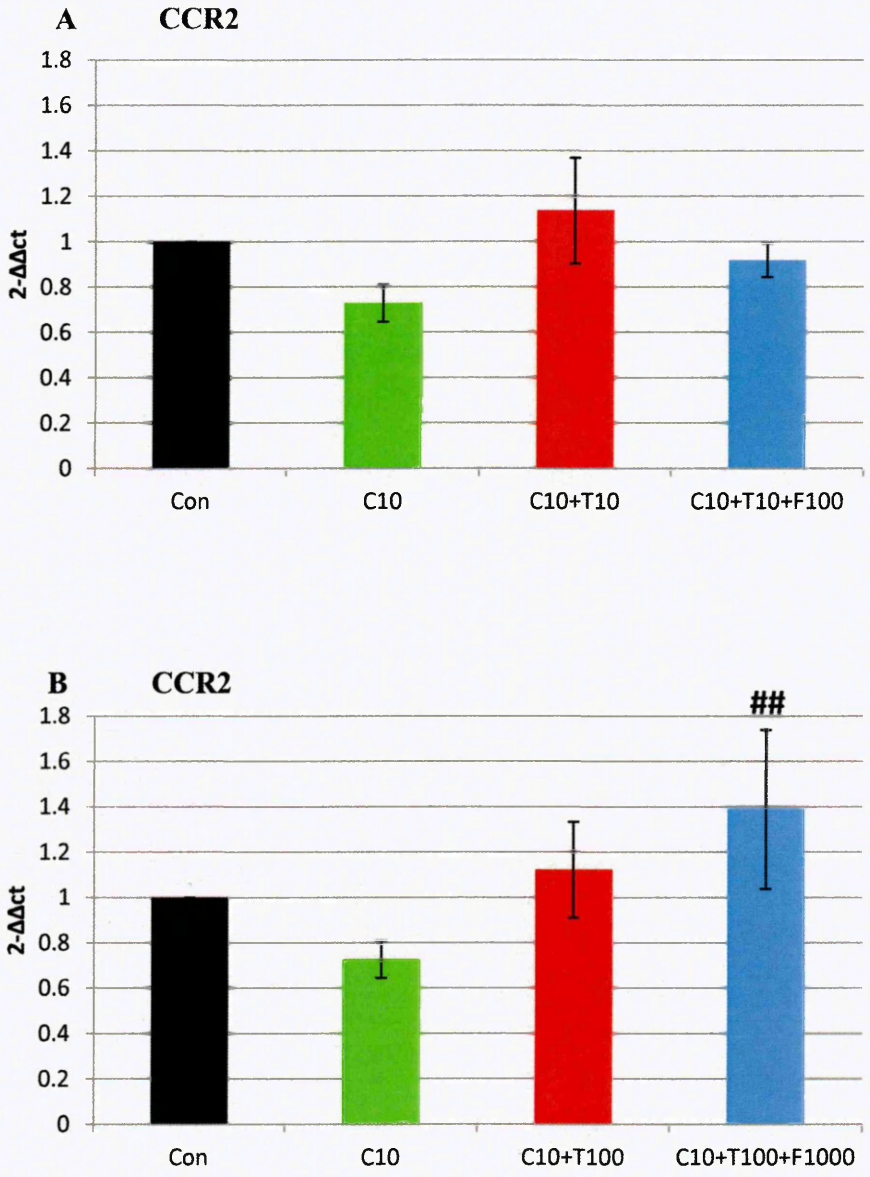


Figure 4.15: CCR2 mRNA expression in THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide by qRT-PCR. Results shown as mean±SEM from three separate experiments. Con=control, C10=cytokine (TNF-α+IFN-γ) 10ng/ml, (A) T10=testosterone 10nM, F100=flutamide 100nM, (B) T100=testosterone 100nM, F1000=flutamide 1000nM. $^{\#\#}P<0.01$ vs C10. Kruskal-Wallis.

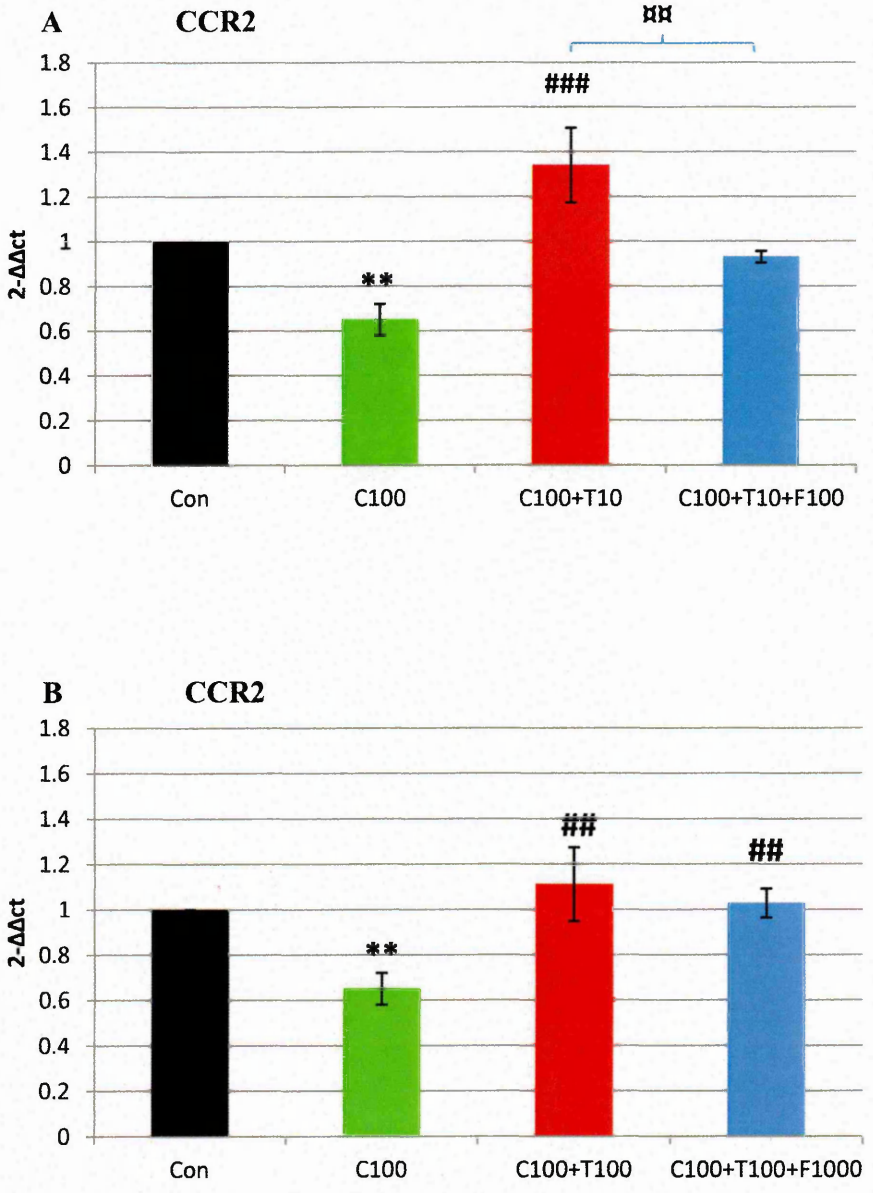


Figure 4.16: CCR2 mRNA expression in THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide by qRT-PCR. Results shown as mean±SEM from three separate experiments. (Con=control, C100=cytokine (TNF-α+IFN-γ) 100ng/ml, T10=testosterone 10nM, F100=flutamide 100nM, T100=testosterone 100nM, F1000=flutamide 1000nM. **P<0.01 vs Con, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs C100, $^{¤¤}$P<0.01vs C100+T10, Kruskal-Wallis.

4.3.4 Investigation of pro and anti- inflammatory secretion by human THP-1 macrophages measured by CBA

There was high variability in the results of the CBA analysis. For IL-1β, IL-6, IL-10, TNF-α and IFN-γ the results were not reliable due to high coefficients of variation in the supernatant of THP-1 macrophages. IL-8 was the only analyte determined by this method in the supernatant of the unstimulated cells. This is probably due to very low levels of the other cytokines, with higher levels of IL-8 detected. In addition, TNF-α and IL-8 were detected reliably following incubation with 24hr cytokines and testosterone treatment, with or without AR blocked, again this is due to the higher levels of these analytes on stimulating the cells with cytokines, compared to the other analytes.

4.3.4.1 Analysis of IL-8 levels in supernatants of THP-1 macrophages following testosterone treatment, with or without flutamide

At the low concentration of testosterone (10nM), there was generally a trend to decrease IL-8 levels from 24 to 48h but this was not significant. Furthermore, there was a large increase but not significant in IL-8 after incubation with testosterone for 96h compared to untreated control cells. This may be due to high levels of variability or small number of samples. An effect that was reversed by flutamide at 100nM but this effect was not significant as shown in (figure 4.17a).

Testosterone treatment (100nM) with or without flutamide (1000nM) had no significant effect on levels of IL-8 from 24 to 96h, compared with untreated control cells (figure 4.17b).

4.3.4.2 Analysis of IL-8 levels in supernatants of THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide

As shown in figures 4.18 a, b cytokine treatment at 10ng/ml led to a significant increase in the production of IL-8 ($P<0.01$) compared to untreated cells. In contrast, testosterone treatment at 10nM or 100nM reduced significantly levels of IL-8 ($P<0.05$) in cells stimulated with cytokines for 24h. However, this reduction did not approach the levels secreted from untreated control cells. The effect was significantly abrogated by flutamide at 100nM ($P<0.05$) and at 1000nM ($P<0.05$) compared to cells treated with

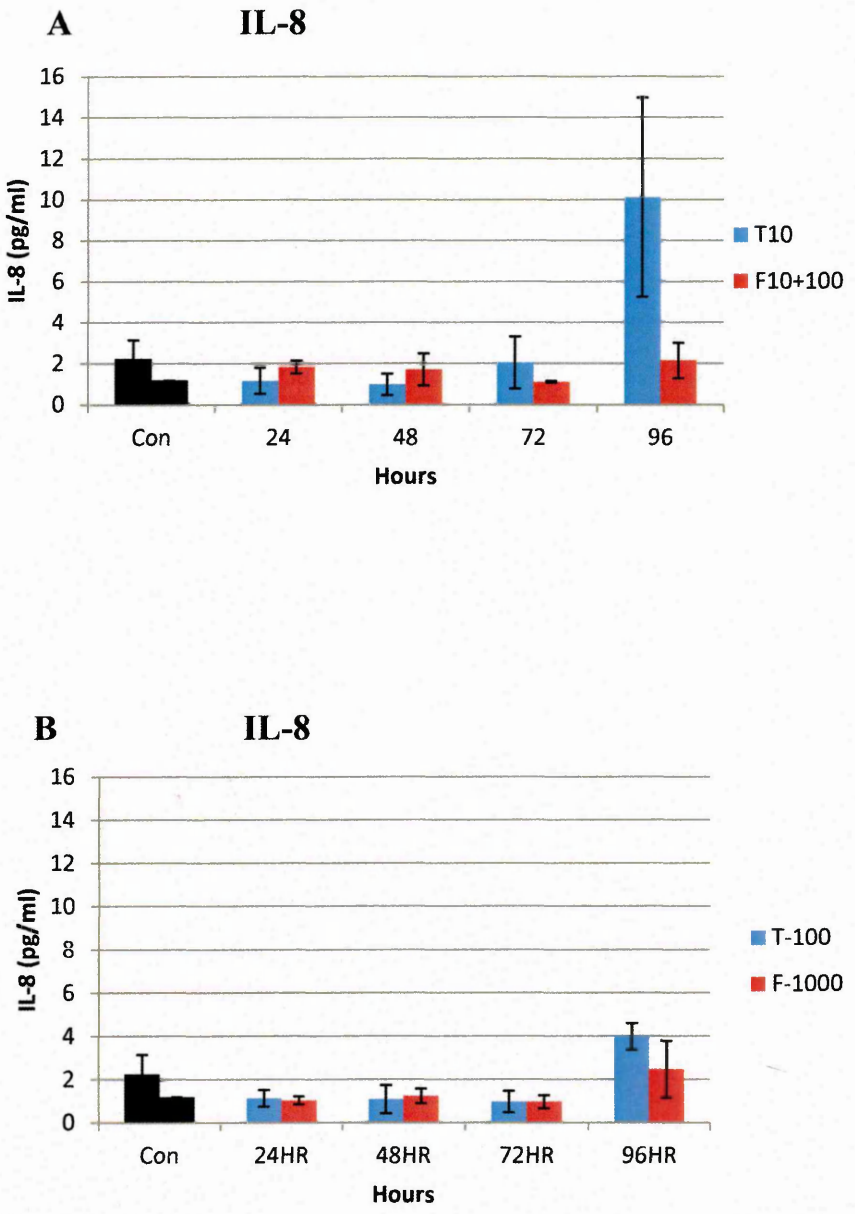


Figure 4.17: CBA analysis of IL-8 level in supernatant of THP-1 macrophages following testosterone treatment, with or without flutamide from 24 to 96h. Results shown as mean±SEM from three separate experiments Con=control, (A) T10=Testosterone10nM, F100=Flutamide 100nM, (B) T100=Testosterone 100nM, F1000=Flutamide 1000nM. Kruskal Wallis.

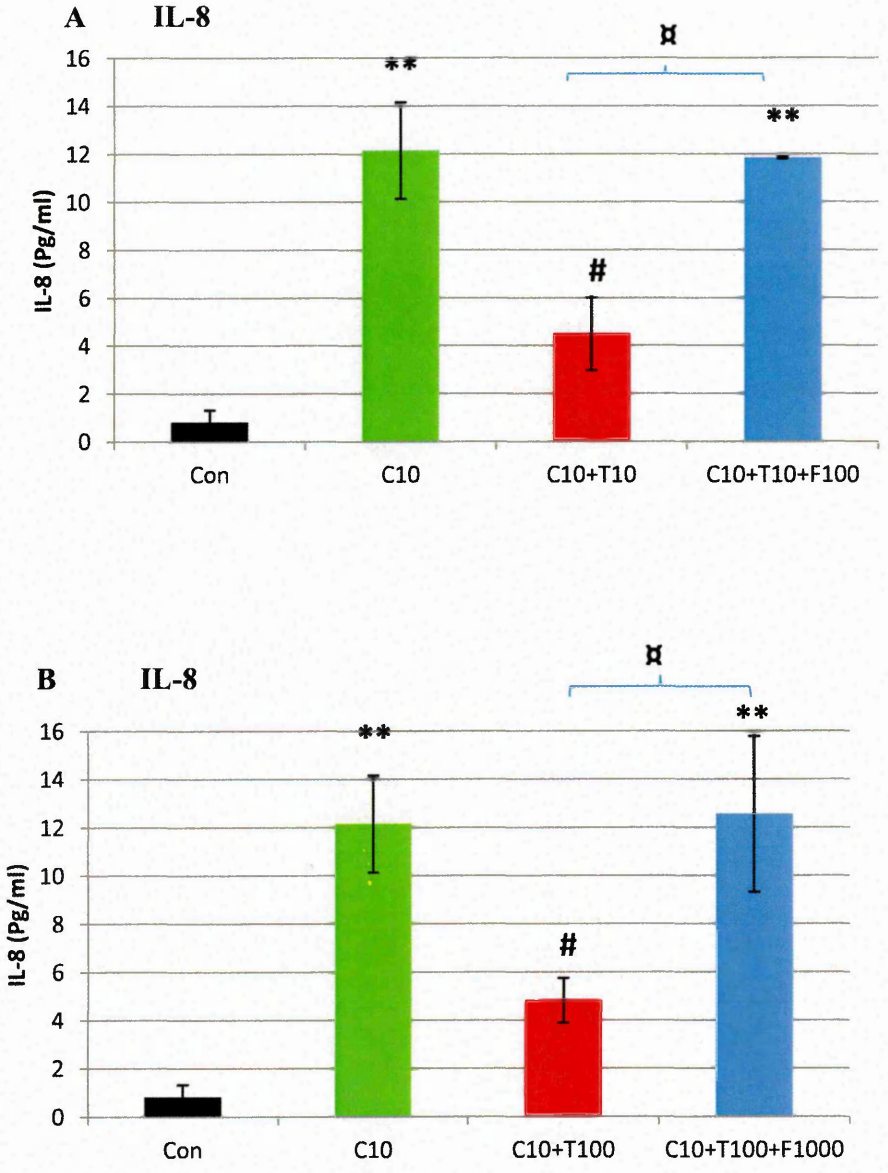


Figure 4.18: CBA analysis of IL-8 levels in supernatant of THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide. Results from three separate experiments shown as mean±SEM. Con=control, C10=cytokine10ng/ml (TNFα+IFN-γ), (A) T10=Testosterone 10nM, F100=Flutamide 100nM, (B) T100=Testosterone 100nM, F1000=Flutamide 1000nM. **P<0.01 vs Con, # P<0.05 vs C10, ¤ P<0.05 vs C10+T10, Kruskal Wallis.

combination of 10ng/ml cytokine and testosterone treatment at 10 and 100 nM, respectively. Furthermore, cytokine treatment at 100ng/ml significantly increased IL-8 levels ($P<0.001$) (figure 4.19 a, b) compared to the untreated control. However, 24h cytokine and testosterone treatment at 10nM significantly reduced ($P<0.001$) IL-8 levels compared to cells treated with cytokine alone (figure 4.19a). Blocked AR with flutamide at 100 nM prevented the decrease in IL-8 levels as a result of 10nM testosterone treatment ($p<0.01$). This increase in IL-8 level due to flutamide was significantly ($P<0.05$) lower than its level in media of cells treated with 100ng/ml cytokine alone (figure 4.19a). In addition, testosterone treatment at 100ng/ml plus 100ng/ml cytokines for 24h down-regulated production of IL-8 compared to cells treated with 100ng/ml cytokines alone ($P<0.01$) (figure 4.19b). Blocked AR with flutamide at 1000nM prevented significantly ($P<0.01$) the reduction in IL-8 levels as a result of testosterone treatment (100nM).

4.3.4.3 Analysis of TNF-α level in supernatants of THP-1 macrophages following 24h cytokines and testosterone treatment, with and without flutamide

TNF-α was not detectable in supernatants of unstimulated THP-1 macrophages controls in all experimental conditions. However, 24h cytokine treatment at 10ng/ml increased markedly levels of TNF-α ($P<0.01$) compared to untreated cells. 10ng/ml cytokines plus 10 or 100nM testosterone treatment was associated with a significant reduction in level of TNF-α ($P<0.01$) compared to cells treated with cytokine alone. However, testosterone treatment reversed the stimulatory effect of cytokine treatment and reduced TNF-α to levels which were not significantly different from untreated controls (figure 4.20 a, b). No effect on levels of TNFα were seen after flutamide treatment at 100 compared to cells treated with 10nM of testosterone (figure 4.20a). Conversely, blocking the AR by flutamide at 1000nM significantly ($P<0.05$) reversed the reduction in TNFα level due to 100 nM testosterone treatment (figure 4.20b).

100 ng/ml cytokine treatment for 24h significantly increased concentrations of TNF-α in the media of THP-1 macrophages ($P<0.01$) compared to untreated cells (figure 4.21a, b). This increase was significantly abrogated with testosterone treatment at 10 or 100nM ($P<0.05$) compared to cells treated with cytokine alone. Flutamide at 100nM did not prevent the decline in TNF due to 10nM testosterone treatment (figure 4.21a) while this decrease was almost significant ($P=0.06$) and partly reversed by flutamide at 1000 nM (figure 4.21b).

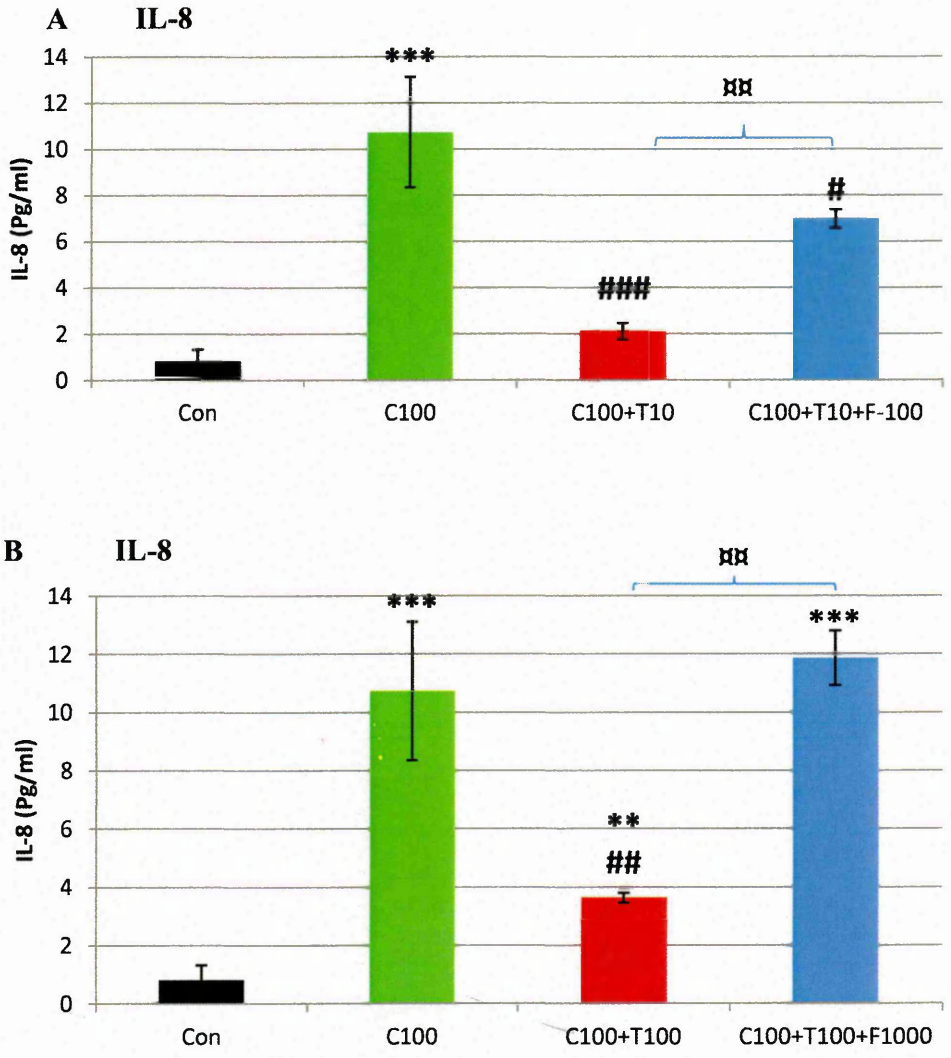


Figure 4.19: CBA analysis of IL-8 levels in supernatants of THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide. Results shown as mean±SEM from three separate experiments. Con=control, C100=cytokine 100 ng/ml (TNFα+IFN-γ), (A) T10=Testosterone 10 nM, F100=Flutamide 100 nM, (B) T100=Testosterone 100 nM, F1000=Flutamide 1000 nM. **P<0.01, ***P<0.001vs Con, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs C100, $^{¤¤}$P<0.01 vs C100+T10 or C100+T100, Kruskal-Wallis.

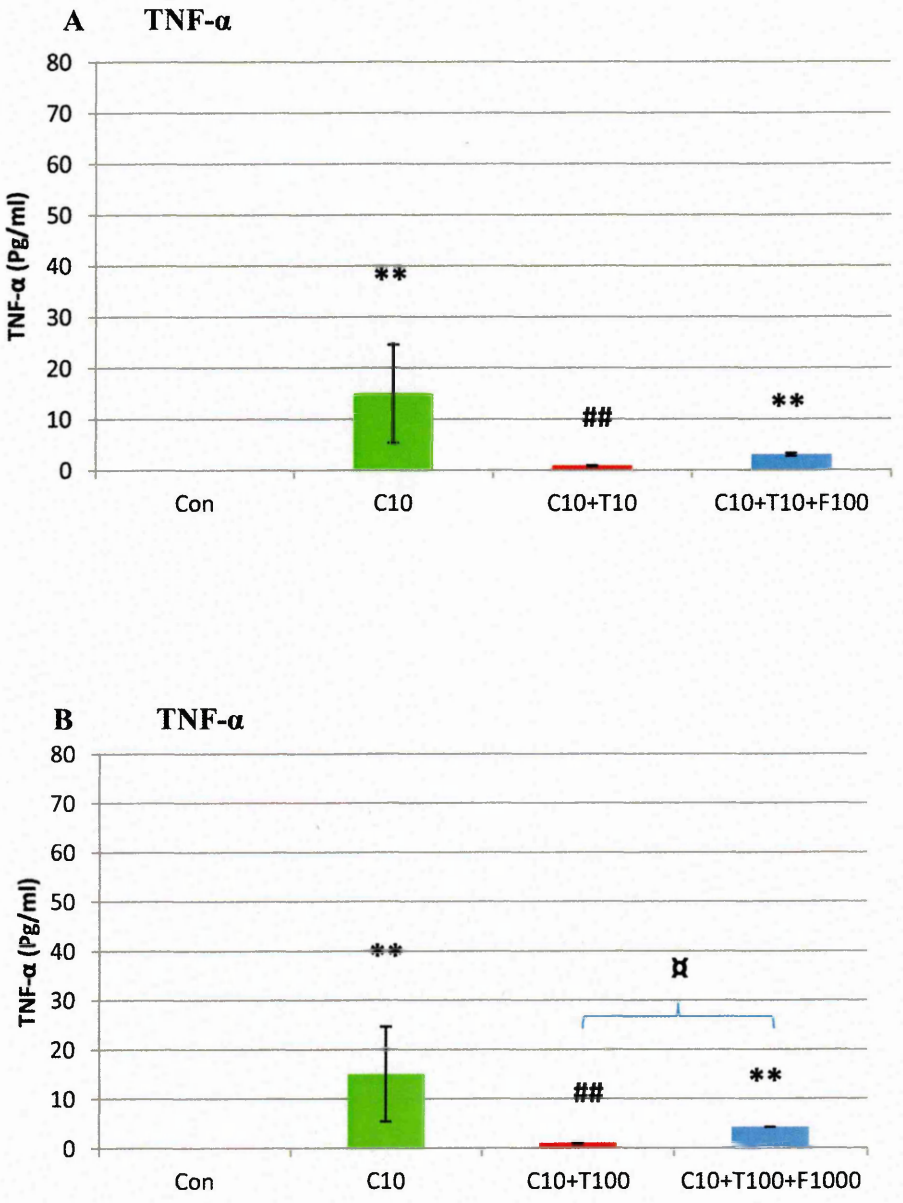


Figure 4.20: CBA analysis of TNF-α levels in supernatants of THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide. Results shown as mean±SEM from three separate experiments. Con=control, C10=Cytokine (TNFα+IFN-γ) 10ng/ml (A) T10=Testosterone 10nM, F100=Flutamide 100nM, (B) T100=Testosterone100nM, F1000=Flutamide 1000nM, **P>0.01vs Con, ## P>0.01 vs C10, ¤ P<0.05 vs C10+T100, Kruskal-Wallis.

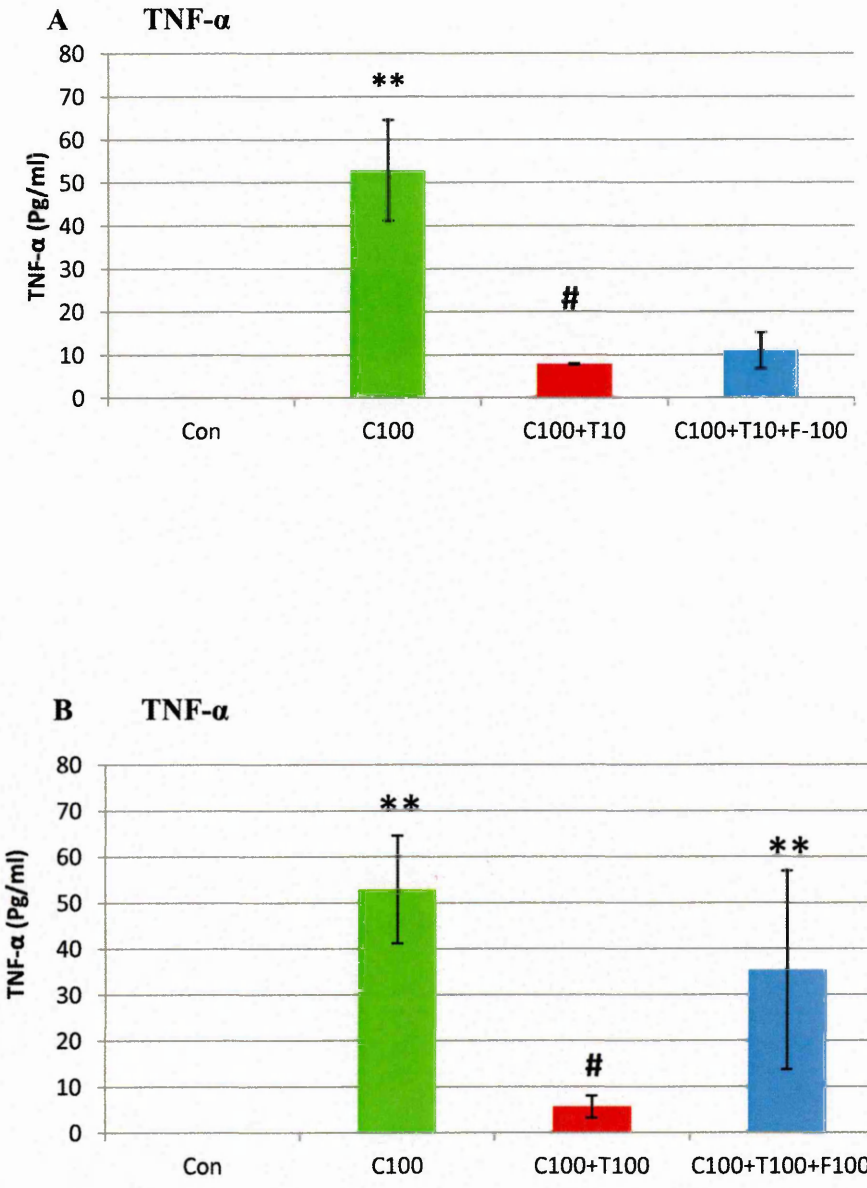


Figure 4.21: CBA analysis of TNF-α levels in supernatants of THP-1 macrophages following 24h cytokine and testosterone treatment, with or without flutamide. Results shown as mean±SEM from three separate experiments. Con=control, C100=Cytokine (TNFα+IFN-γ) 100 ng/ml, (A) T10=Testosterone10 nM, F100=Flutamide100nM, (B) T100=Testosterone100 nM, F1000=Flutamide 1000nM. **P>0.01vs Con, $^{\#}$P>0.05 vs C100, Kruskal-Wallis.

THP-1 Macrophages	Parameters	Testosterone	
		10nM	100nM
mRNA expression	CX3CR1	NC	NC
	CCR2	NC	NC
protein level	IL-8	NC	NC

Table 4.3: Summary of results of effect of testosterone on chemokines receptors expression and level of IL-8 in human THP1 macrophages cells. NC=no change.

THP-1 Macrophages	Parameters	Testosterone+cytokines (TNFα+INFγ)			
		T10nM		T100nM	
		C10ng/ml	C100ng/ml	C10ng/ml	C100ng/ml
mRNA expression	CX3CR1	NC	NC	NC	NC
	CCR2	NC	↑***	NC	↑**
protein level	IL-8	↓*	↓*	↓***	↓**
	TNF-α	↓**	↓**	↓*	↓*

Table 4.4: Summary of results of effect of testosterone on chemokines receptors expression, level of IL-8 and TNF-α following cytokines stimulation in human THP1 macrophages cells. T: testosterone, C: cytokines, NC=no change.AEC. (↑): increase, (↓): decrease, *P<0.05, **P<0.01, ***P<0.001.

4.3.5 Analysis of expression of adipokines and inflammatory factors in mouse 3T3L1 cells by q-RT-PCR

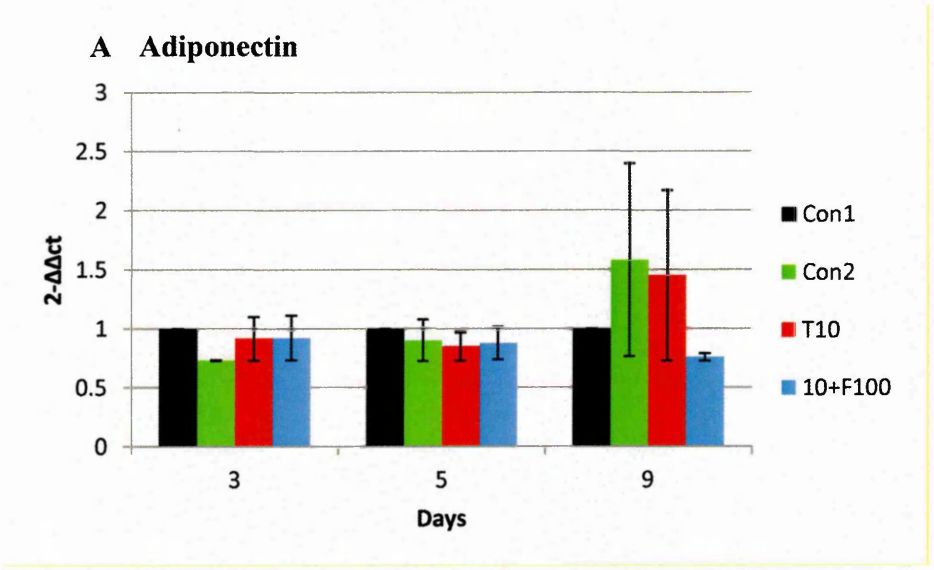
4.3.5.1 Analysis of adiponectin mRNA expression in 3T3L1 cells following testosterone treatment, with or without flutamide

No significant change was seen in mRNA expression of adiponectin in adipocyte cells treated with low or high testosterone, with or without flutamide over time up to 9 days compared to untreated preadipocytes or mature adipocyte control cells as shown in figures 4.22a, b.

4.3.5.2 Analysis of PAI-1 mRNA expression in 3T3L1 cells following testosterone treatment with or without flutamide

In figure 4.23a after 3 days, there was an non-significant increase in expression of PAI-1mRNA in untreated mature adipocyte cells compared to untreated preadipocyte cells. PAI-1 mRNA expression following testosterone at 10 nM decreased but not significant ($p=0.06$) compared to untreated preadipocyte cells and non-significant change was seen compared to untreated mature adipocyte cells after 3 days. No significant difference between the effect of flutamide at 100nM and testosterone at 10nM on the expression of mRNA PAI-1 was seen. Following 5 days, a significant ($P=0.01$) reduction was seen in expression of mRNA PAI-1 in untreated adipocytes compared to untreated preadipocyte cells. Testosterone at 10nM significantly increased ($p<0.01$) PAI-1 mRNA expression compared to untreated mature adipocyte cells; an effect which was significantly ($p<0.01$) abrogated by 100nM flutamide. After 9 days, decrease in PAI-1 mRNA expression ($p=0.05$) was observed in untreated mature adipocyte cells compared to untreated preadipocyte cells. No significant change in expression of PAI-1 mRNA was seen in cells treated with testosterone (10nM), with or without flutamide (100nM) compared to both untreated control cells.

In figure 4.23b, there was no significant change in expression of PAI-1 mRNA in untreated mature adipocyte cells compared to undifferentiated cells or in cells treated with testosterone (100nM), with or without flutamide (1000nM) compared to both untreated cells after 3 and 9 days. However, there was a significant reduction in expression of PAI-1 mRNA after 5 days in untreated mature adipocyte cells ($p<0.01$) and in cells treated with testosterone ($p<0.05$), with or without flutamide ($p<0.05$),



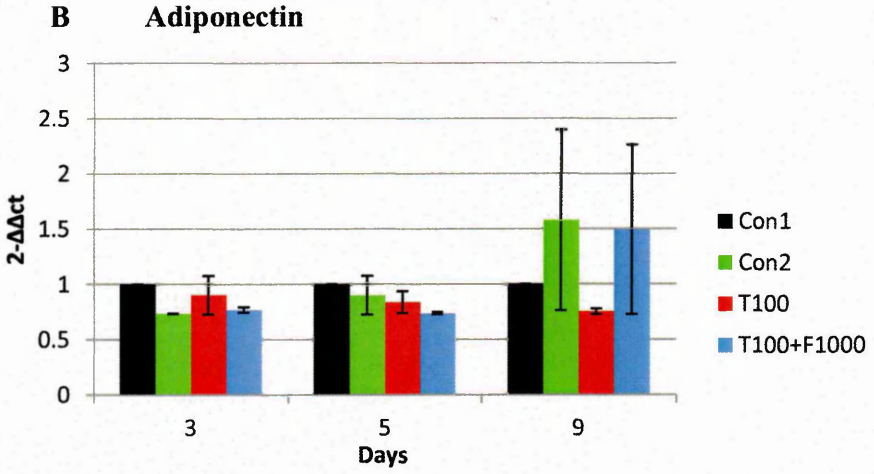


Figure 4.22: Adiponectin mRNA expression in 3T3L1 cell following testosterone treatment, with or without flutamide from 3 to 9 days by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. Friedman test.

Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

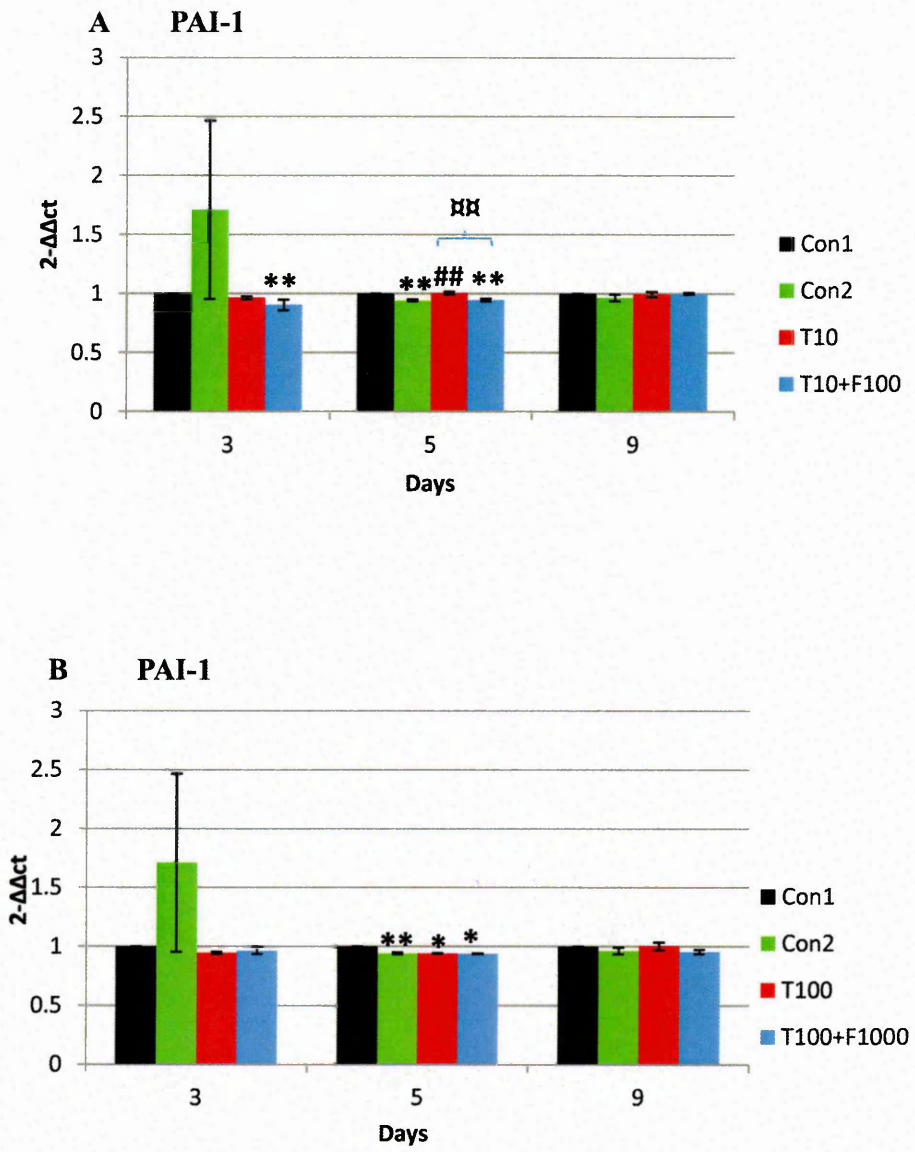


Figure 4.23: PAI-1 mRNA expression in 3T3L1 cell following testosterone treatment, with or without flutamide from 3 to 9 days by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. *P<0.05, ** P<0.01, *** p<0.0001vs Con1. ## P<0.01vs Con2, ¤¤P< 0.01 T10 vs T10+F100 or T100, Friedman test.

Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

compared to only untreated preadipocyte cells; however the changes in expression level were small.

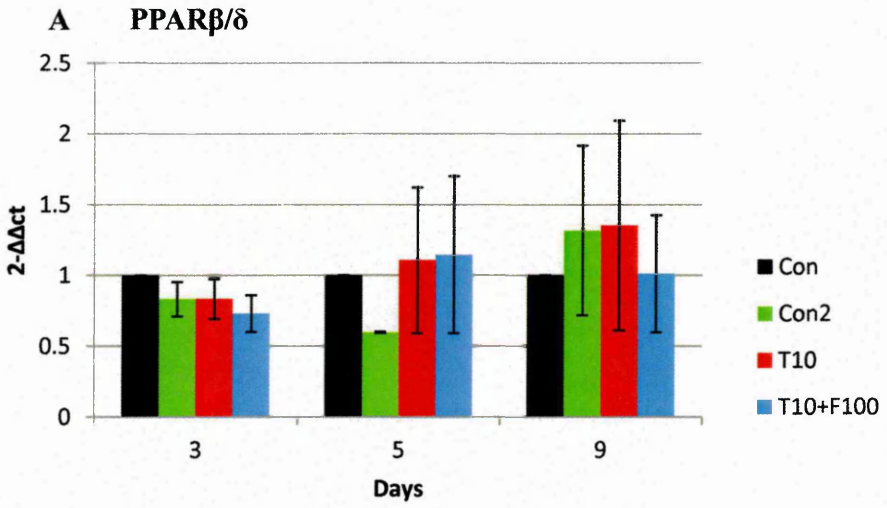
4.3.5.3 Analysis of PPARβ/δ mRNA expression in 3T3L1 cells following testosterone treatment, with or without flutamide

No significant alteration was observed in expression of PPARβ/δ mRNA in untreated mature adipocyte cells and cells treated with testosterone, with or without flutamide at low and high concentrations compared to untreated preadipocyte cell controls as a result of time or treatment (figures 4.24a, b).

4.3.5.4 Analysis of leptin mRNA expression in 3T3L1 cells following testosterone treatment, with or without flutamide

After 3 days, a significant decrease was seen in expression of leptin mRNA ($p<0.001$) in untreated mature adipocyte cells compared to untreated preadipocyte cells (figure 4.25a). Similarly, there was a significant reduction in leptin mRNA expression following testosterone treatment at 10 nM ($p<0.05$) compared to untreated preadipocyte cells, but not untreated mature adipocyte cells. Following 5 days, no significant change was observed in expression of leptin mRNA in untreated adipocyte cells compared to untreated preadipocyte cells. While testosterone treatment at 10nM significantly reduced leptin mRNA expression ($p<0.01$) in mature adipocyte cells compared to untreated preadipocyte cells, testosterone tended to reduce mRNA leptin expression ($p=0.06$) in mature adipocyte cells compared to untreated mature adipocyte cells. At 9 days, there was a significant reduction in expression of leptin mRNA in untreated mature adipocyte cells ($p<0.01$) compared to untreated preadipocyte cells. Testosterone significantly decreased leptin mRNA expression ($p<0.01$) in mature adipocyte cells compared to untreated preadipocyte cells but not untreated mature adipocyte cells. Finally, there was no significant difference between the effect of flutamide at 100nM and testosterone at 10nM on the expression of mRNA leptin in treated cells over all time points.

As shown in figure (4.25b), a significant decrease was seen in expression of leptin mRNA in untreated mature adipocyte cells at 3 days ($p<0.001$) compared to untreated preadipocyte cells. Similarly, significant reduction was observed in cells treated with 100nM testosterone at 3 days ($p<0.01$) compared to untreated preadipocyte cells but not



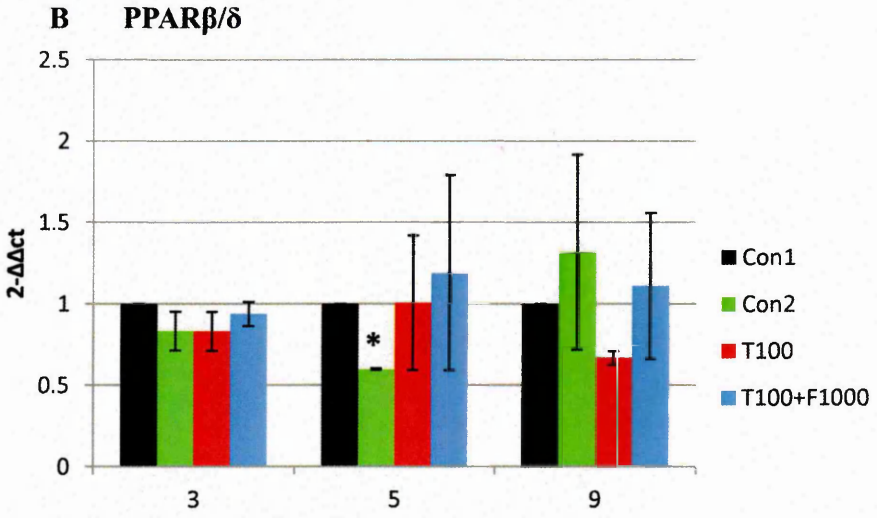


Figure 4.24: PPARβ/δ mRNA expression in 3T3L1 cells following testosterone treatmen, with or without flutamide, from 3 to 9 days by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. *P<0.05 vs Con1. Friedman test.

Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

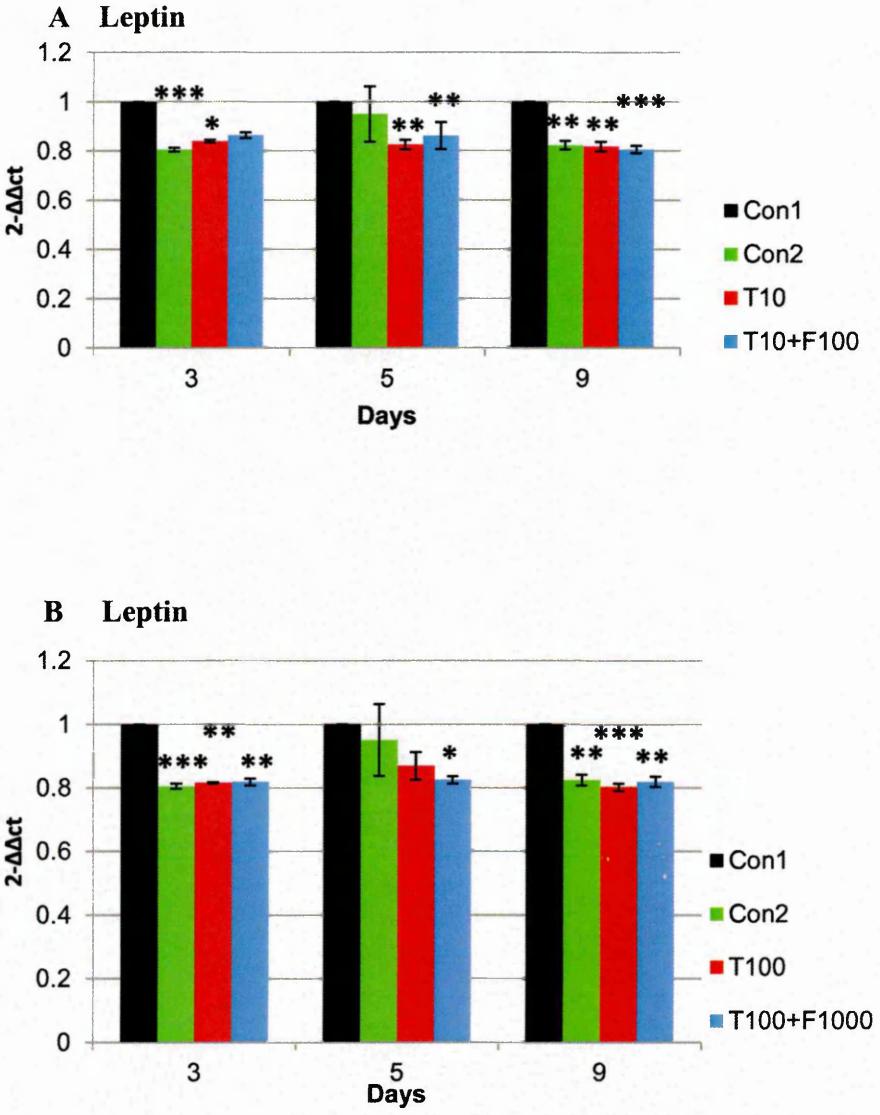


Figure 4.25: Leptin mRNA expression in 3T3L1 cell following testosterone treatment, with or without flutamide, from 3 to 9 days by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM *P<0.05, ** P<0.01, *** P<0.0001vs Con1, Friedman test.

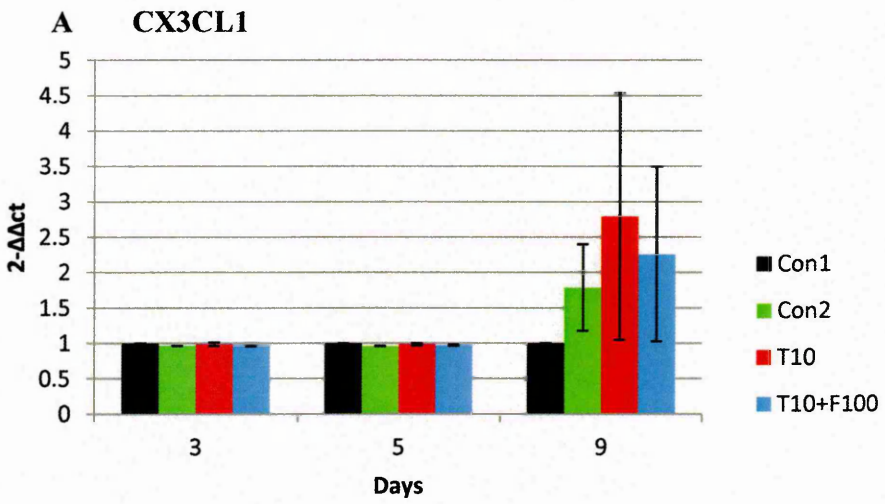
Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

to untreated adipocyte cells. At 5 days, no significant alteration was seen in expression of mRNA leptin in untreated adipocyte cells compared to untreated preadipocyte cells. Testosterone at 100nM tended to reduce ($p=0.08$) expression of leptin mRNA in mature adipocyte cells compared to untreated adipocyte cells. Following 9 days, a significant reduction was observed in expression of leptin mRNA in untreated adipocyte cells ($p<0.01$) and in cells treated with testosterone at 100nM compared to untreated preadipocyte cells. There was no significant change between cells treated with testosterone and untreated adipocyte cells. Flutamide at 1000 nM did not alter the action of testosterone at 100 nM in the expression of leptin mRNA over all time points.

4.3.5.5 Analysis of CX3CL1 mRNA expression in 3T3L1 cells following testosterone treatment, with or without flutamide

Testosterone treatment at 10nM, with or without AR blocked at 100nM, had no effect on expression of CX3CL1 mRNA in mature adipocyte cells compared to both untreated control cells after three and five days (figure 4.26a). While no difference in expression of CX3CL1 mRNA was seen between untreated preadipocyte cells and untreated mature adipocyte cells after 3days, there was a trend to slightly decrease ($p=0.05$) the expression of CX3CL1 mRNA in untreated mature adipocyte cells compared to untreated preadipocyte cells after 5 days. However, there was a non-significant increase in expression of CX3CL1 mRNA in untreated mature adipocyte cells and cells treated with testosterone at 10nM compared to untreated preadipocyte cells following 9 days. Testosterone increased (but not significantly) CX3CL1 mRNA expression in mature adipocyte cells compared to untreated adipocyte cells. This might be as result of high variability among data. Flutamide at 10 nM did not prevent this alteration.

In figure (4.26b), there was a slight but significant decrease in mRNA CX3CL1 expression in untreated adipocyte compared to untreated preadipocyte cells after three($p<0.05$) and five ($p<0.01$) days. Conversely, after 9 days, there was increase (but not significant) in mRNA leptin expression in untreated adipocyte compared to untreated preadipocyte cells. Testosterone at 100nM, with or without flutamide at 1000nM had no effect on CX3CL1 mRNA expression compared to untreated control cells after 3 and 5 days. At 9 days, an increase in expression of CX3CL1 mRNA due to testosterone treatment (100nM) was seen but this was not significant compared to untreated preadipocyte cells. Flutamide at 1000nM abrogated, (but not significantly) this increase.



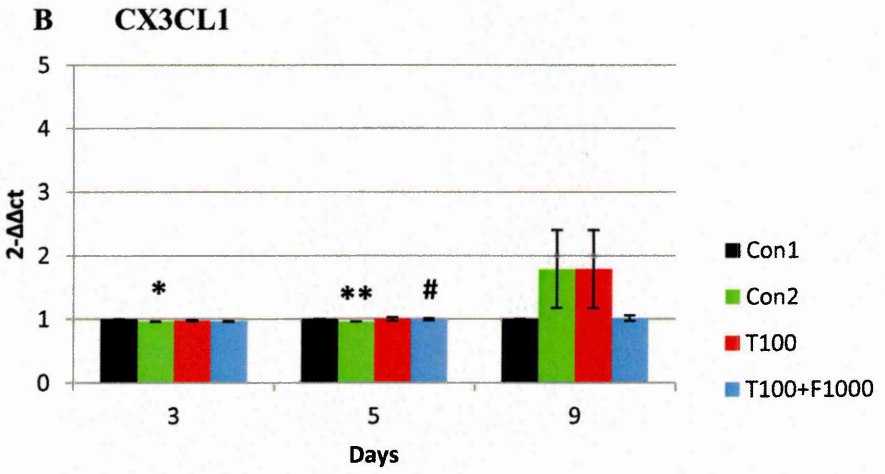


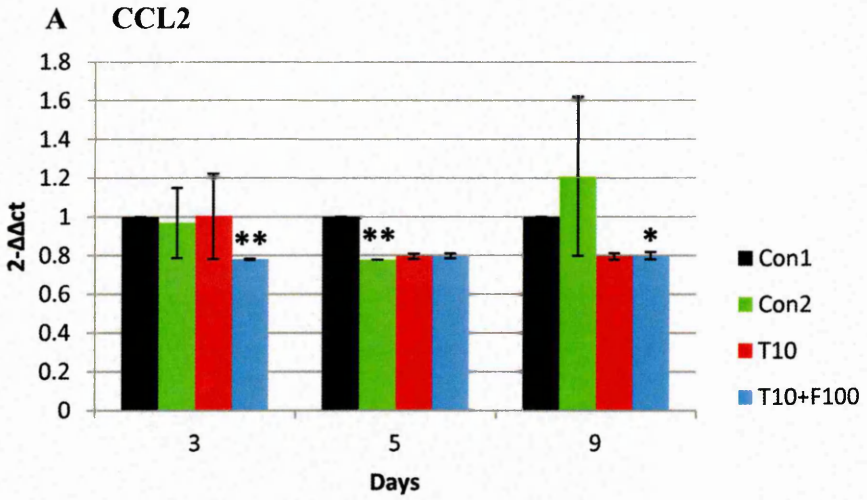
Figure 4.26: CX3CL1 mRNA expression in 3T3L1 cell following testosterone treatment, with or without flutamide from 3 to 9 days by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide. *P<0.05, ** P<0.01, vs Con1. # P<0.05 vs Con2. Friedman test.

Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

4.3.5.6 Analysis of CCL2 mRNA expression in 3T3L1 cells following testosterone treatment with or without flutamide

CCL2 mRNA expression was not changed in untreated mature adipocytes compared to untreated preadipocyte cells and in treated cells with testosterone compared to untreated mature adipocytes at 3 days (figure 4.27a). However, at 5 days, a significant reduction in CCL2 mRNA expression was seen in untreated mature adipocyte cells ($p<0.01$) compared to untreated preadipocyte cells. There was not significant change in CCL2 mRNA expression in cells treated with testosterone compared to untreated mature adipocytes. After 9 days, no significant alteration was seen in mRNA expression of CCL2 in untreated mature adipocyte cells compared to untreated preadipocyte cells. There was trend to decrease mRNA expression of CCL2 in treated cells with testosterone compared to untreated mature adipocyte cells. Flutamide at 100 nM did not alter the action of testosterone at 10 nM on the expression of mRNA CCL2 over all time points.

In figure (4.27b), no significant alteration in expression of mRNA CCL2 expression in untreated mature adipocyte cells compared to untreated preadipocyte cells as well as in treated cells with testosterone treatment (100nM) compared to untreated mature adipocyte cells after 3 days. In contrast, there was considerable reduction in expression of CCL2 mRNA in untreated mature adipocyte ($p<0.01$) and in cells treated with testosterone ($p<0.05$) compared to untreated preadipocyte cells over 5 days. However, there was no significant change in mRNA CCL2 expression in treated cells with testosterone compared to untreated mature adipocyte. Following 9 days, expression of CCL2 mRNA increased but this was not significant in untreated mature adipocyte cells compared to untreated preadipocyte cells. There was a non-significant reduction in the expression of CCL2 mRNA in cells treated with testosterone (100nM) compared to both untreated mature adipocyte cells. Flutamide at 1000 nM did not alter the action of testosterone at 100 nM on the expression of CCL2 mRNA over all time points.



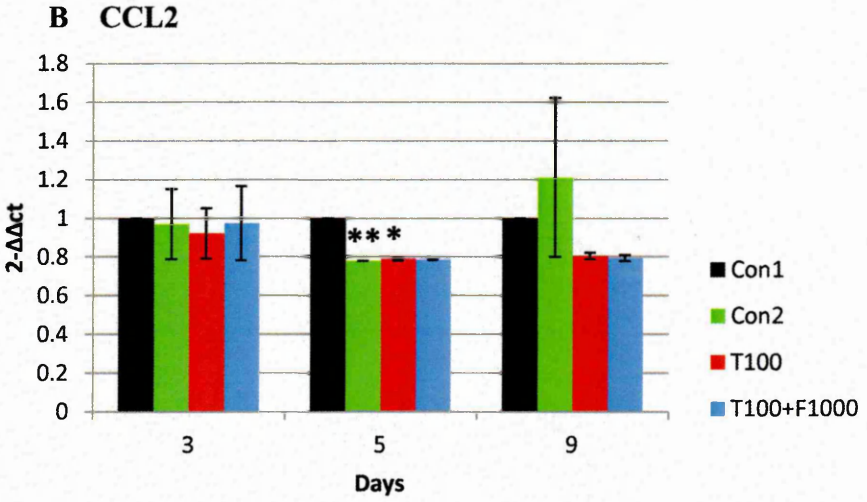


Figure 4.27: CCL2 mRNA expression in 3T3L1 cell following testosterone treatment, with or without flutamide from 3 to 9 by qRT-PCR. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. *P<0.05, ** P<0.01, vs Con1. Friedman test.

Con1=untreated preadipocyte
Con2=untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

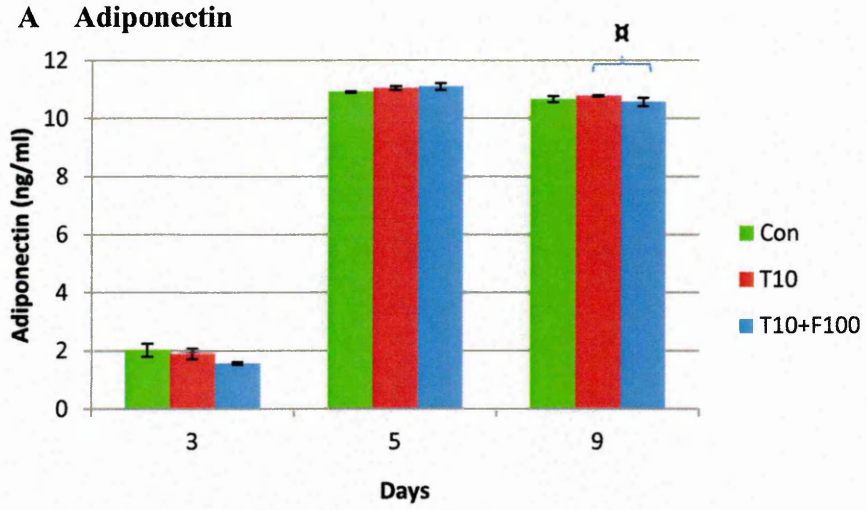
4.3.6 Analysis of adiponectin, CX3CL1 and CCL2 by ELISA in supernatants of adipocyte 3T3L1 cells

4.3.6.1 Analysis of adiponectin levels in supernatants of adipocyte 3T3L1 cells following testosterone treatment, with or without flutamide

There was no significant change in the level of adiponectin in media from cells treated with testosterone (10 or100nM) or after blocking the AR by flutamide (100 or 1000nM) compared to untreated mature adipocyte control following 3, 5 and 9 days (figure 4.28a, b). There were significant differences in the adiponectin levels in media from cells treated with flutamide compared to those treated with testosterone after 9 days at both concentrations, however as there was no difference between the controls and the testosterone treated cells in adiponectin secretion, this finding is unlikely to be of biological significance.

4.3.6.2 Analysis of CX3CL1 levels in supernatants of adipocyte 3T3L1 cells following testosterone treatment, with or without flutamide

There was a significant reduction in the level of CX3CL1 in the media of cells treated with testosterone (10nM) ($p<0.05$) after 3days compared to untreated mature adipocyte cells. Flutamide (10nM) did not prevent this reduction. After 5 days, no significant change in CX3CL1 secretion in cells treated with testosterone, with and without flutamide (100nM). A significant decrease was seen in CX3CL1secretion from cells treated with testosterone (10nM) compared to untreated mature adipocyte cells after 9 days. There was a significant difference in CX3CL1 secretion between cells treated with testosterone and flutamide ($p=0.01$) (figure 4.29a). The reduction due to flutamide was significantly higher than the reduction due to testosterone treatment which is unlikely to be of biological significance. No alteration was observed in CX3CL1 secretion in cells treated with 100nM testosterone, with or without 1000nM flutamide after 3 and 5 days compared to untreated mature adipocyte cell (figure 4.29b). Nevertheless, the level of CX3CL1 in media of cells was reduced by testosterone (but not significantly) and flutamide at 1000nM did not prevent this reduction after 9 days.



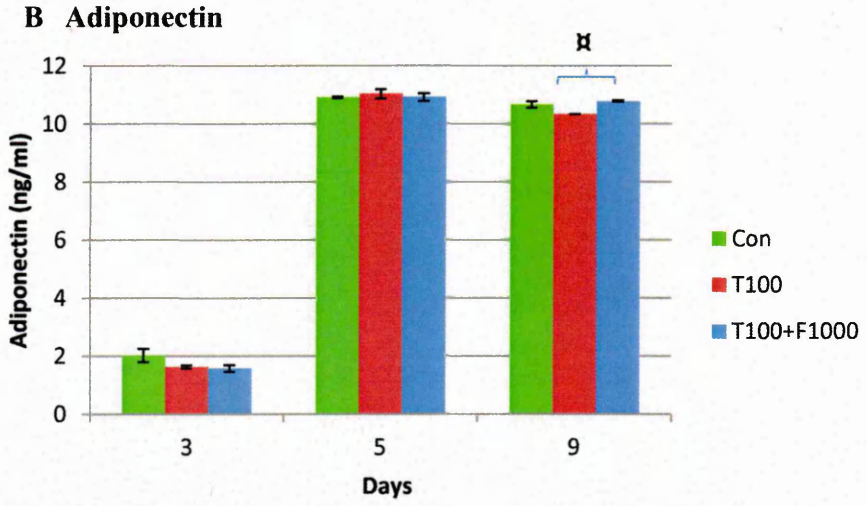


Figure 4.28: ELISA analysis of adiponectin levels in supernatants of 3T3L1 adipocytes following testosterone treatment, with or without flutamide from 3 to 9 days. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone 10nM, F100=Flutamide 100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. $^{\square}P < 0.05$ T10 vs T10+F100 or T100 vs T100+F1000, Friedman test.

Con =untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

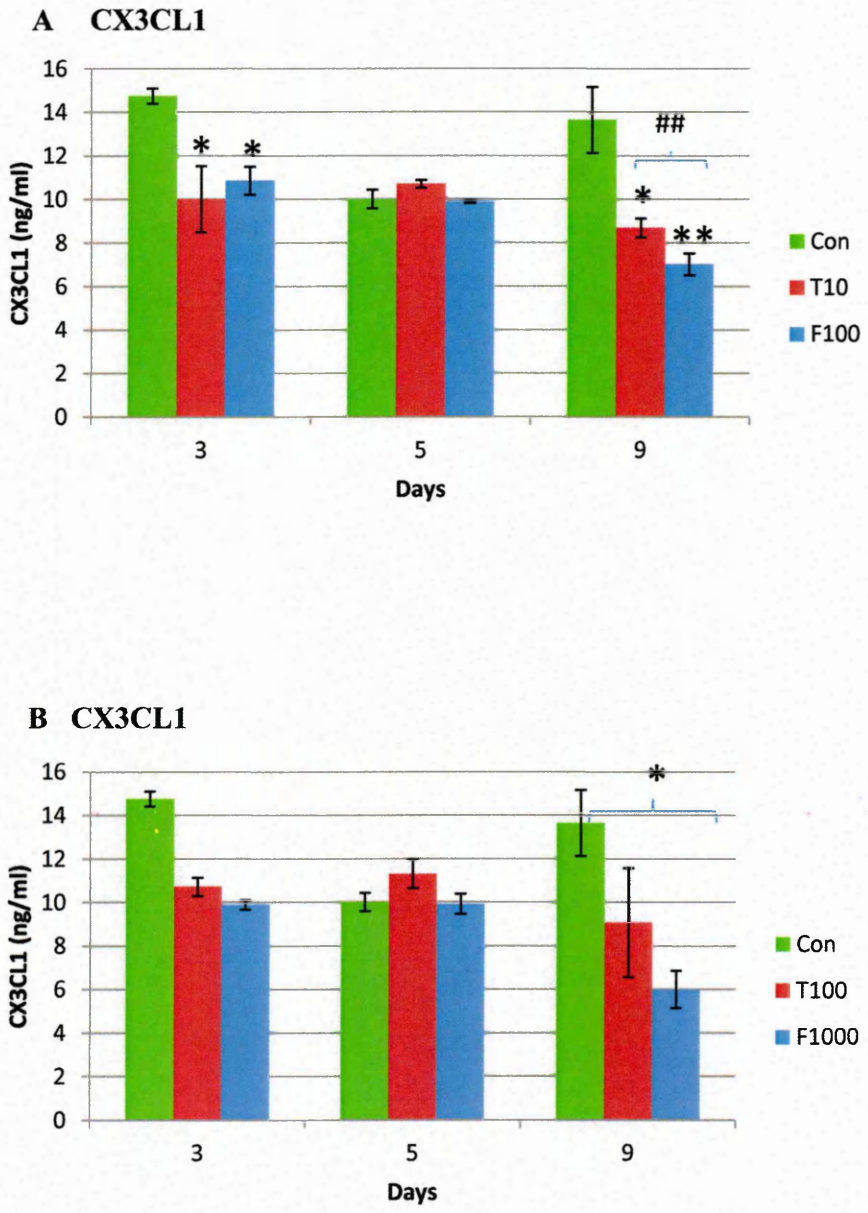


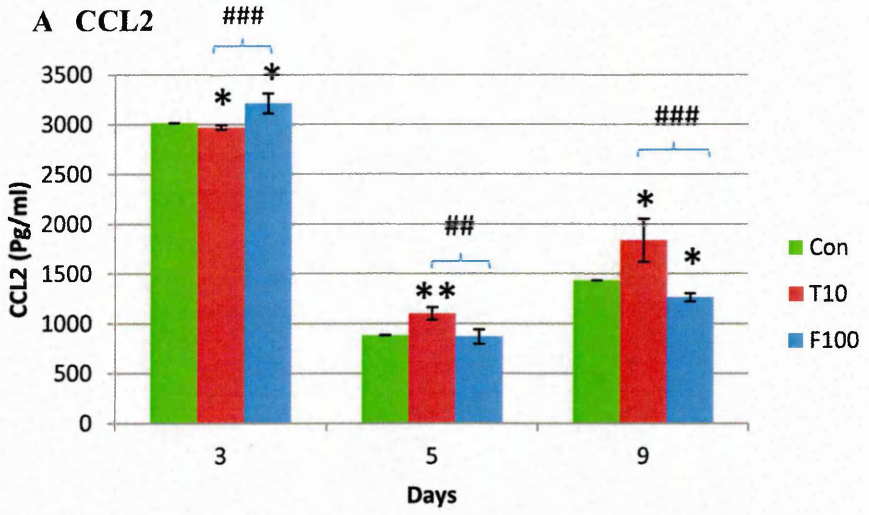
Figure 4.29: ELISA analysis of CX3CL1 level in supernatant of 3T3L1 adipocyte following testosterone treatment, with or without flutamide from 3 to 9 days. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone10nM, F100=flutamide100nM, (B) T100=testosterone100nM, F1000=Flutamide 1000nM. *P<0.05 vs Con, ##P<0.01 T10 vs T10+F100, Two way ANOVA.

Con =untreated mature adipocyte
Mature adipocytes treated with testosterone
Mature adipocytes treated with testosterone and flutamide

4.3.6.3 Analysis of CCL2 levels in supernatants of adipocyte 3T3L1 cells following testosterone treatment, with or without flutamide

After 3 days, a slight but significant reduction in the level of CCL2 ($p<0.05$) was observed in cells treated with testosterone treatment at 10nM compared to untreated mature adipocyte cells, an effect which was markedly ($p<0.001$) abrogated by flutamide at (100nM). In contrast, production of CCL2 was significantly elevated after testosterone at 10nM ($p<0.01$) following 5 days, an effect that was significantly reversed by flutamide at 100nM. Similarly after 9 days, testosterone significantly raised levels of CCL2 in the media of adipocyte cells; an effect that was markedly abolished by flutamide at 100nM ($p<0.001$) (figure 4.30a).

However, no change was seen in levels of CCL2 in media of cells treated with 100nM testosterone, with or without 1000nM flutamide, compared to untreated mature adipocyte cells following 3 and 5 days (figure 4.30b). Conversely, the production of CCL2 in media of cells increased, but not significantly following testosterone at 100nM treatment compared to untreated mature adipocyte cells after 9 days. This increase was reversed significantly by blocking the AR by flutamide at 1000nM ($p<0.05$).



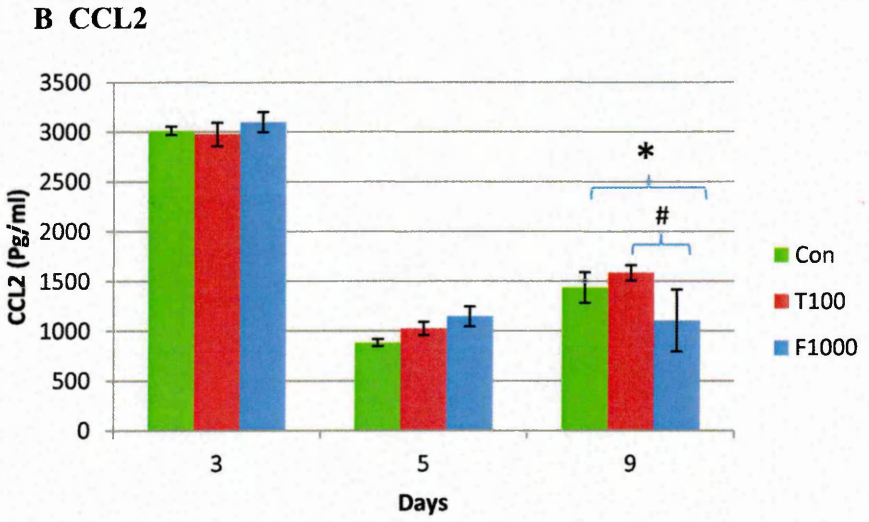


Figure 4.30: ELISA analysis of CCL2 levels in supernatants of 3T3L1 adipocytes following testosterone treatment, with or without flutamide, from 3 to 9 days. Results shown as mean±SEM from three separate experiments. (A) T10=testosterone10nM, F100=flutamide100nM, (B) T100=testosterone 100nM, F1000=Flutamide 1000nM. * $P<0.05$, ** $P<0.01$, vs Con, $^{\#}$ $P<0.05$, T100 vs T100+F1000, $^{\#\#}$ $P<0.01$, $^{\#\#\#}$ $P<0.001$ T10 vs T10+F100, Friedman test.

Con =untreated mature adipocyte

Mature adipocytes treated with testosterone

Mature adipocytes treated with testosterone and flutamide

3T3L1 Adipocyte	Parameters	T10nM	T100nM
mRNA expression	Adiponectin	NC	NC
	PPARβ/δ	NC	NC
	PAI-1	Increased at 5days	NC
	Leptin	NC	NC
	CX3CL1	NC	NC
	CCL2	NC	NC
protein level in supernatant	Adiponectin	NC	NC
	CX3CL1	Decreased at 3 and 9 days	NC
	CCL2	Decreased at 3 days and Increased at 5 and 9 days	NC

Table 4.5: Summary of results of effect of testosterone on expression of gene and protein of all parametersin mouse 3T3L1 adipocyte cells. T: testosterone. NC=no change.

4.4.1 Human THP1 macrophages

As monocytes migrate from circulating blood into the endothelial space they become activated and differentiate into macrophages. This part of the study aimed to investigate the expression of some of the key molecules expressed during this process using PMA-stimulated THP-1 cells as a model of macrophages. It was hypothesized that the interaction of CX3CR1 and CCR2 with their ligands is a crucial step in the migration of monocyte cells from circulating blood into the endothelial space of the artery wall, therefore, testosterone as an immune modulator and anti-inflammatory factor may influence this process by modulating the expression of these receptors and other markers of macrophage activation and inflammation such as IL-8 and TNF-α production. In particular, Veillard and colleagues (2006) suggested that the reduction of inflammation by inhibiting chemokines and their receptors is a novel therapeutic target for CVD. Testosterone can act through a number of mechanisms, including via ARs therefore the AR blocking agent, flutamide, was included in the experiments to investigate the mechanism of action of any effect seen with testosterone.

4.4.1.1 Expression of CX3CR1 and CCR2 mRNA in human THP-1 macrophages

A first set of experiments was conducted to establish whether the CX3CR1 and CCR2 receptors for CX3CL1 and CCL2 respectively, were expressed by THP-1 macrophages. mRNA expression of both chemokine receptors were observed in THP-1 macrophages by using qRT-PCR.

4.4.1.2 The effect of testosterone treatment with or without flutamide on CX3CR1 and CCR2 mRNA expression in THP-1 macrophages

Testosterone treatment, with or without blocked AR using flutamide, did not induce with any significant changes in expression of both receptors over 96h. To our knowledge, there is no other research studying the effect of testosterone on these receptors in THP-1 macrophages. Previous studies were performed to study the expression of chemokine receptors and their ligands in other cell types following androgen treatment with flutamide and other receptor blockers. Some of these studies

found that regulation of these chemokines and their receptors was controlled by androgen, and these actions were reversed by anti-androgen receptors (Dole and Holdsworth, 1997, Akashi *et al.,* 2006; Jamieson *et al.,* 2008) while others found no changes (Kelly *et al.,* 2012b). However, the presence of AR receptors was identified in the present study by qRT-PCR to exclude the fact that loss of its influence was the result of a lack of AR. Testosterone may not have an effect on unstimulated macrophages in absence of inflammation therefore activation of macrophages by cytokines was investigated .

4.4.1.3 The effect of cytokine stimulation on CX3CR1 mRNA expression in THP-1 macrophages for 24h

THP-1 macrophages incubated with cytokines, TNF-α plus IFNγ, at 10 ng/ml concentration for 24h tended to reduce CX3CR1 mRNA expression, whilst at 100ng/ml there was no effect. Previous studies have investigated the effect of TNF-α plus IFNγ on CX3CR1 in THP-1 cells and found that CX3CR1 protein expression was upregulated, however these studies were carried out on undifferentiated cells (Apostolakis, 2007; Sung *et al.,* 2010). Therefore the data presented here implies that the effect of cytokines on chemokine receptor mRNA expression varies depending on the stage of differentiation of the cells.

4.4.1.4 The effect of cytokine stimulation on CCR2 mRNA expression in THP-1 macrophages for 24h

CCR2 mRNA was also down regulated after stimulation for 24h with TNF-α plus IFNγ at 100ng/ml. This was seen in previous studies by Tangirala et al. (1997) and Weber et al. (1999). In these studies TNF-α induced a down-regulation of CCR2 mRNA and surface expression in THP-1 cells, isolated blood monocytes and Mono Mac 6 cells suggesting that pro-inflammatory cytokines can decrease as well as increase chemokine receptor expression. Several *in vitro* studies have established that selective changes in the expression of specific chemokine receptors occur during the differentiation of blood monocytes to macrophages (Opalek *et al.,* 2007). For example, the reduction of CCR2 expression takes place after 4 hours in monocyte cultures (Kaufmann *et al.,* 2001). This decline in CCR2 expression continues for up to seven days, at which time no CCR2 is detected (Fantuzzi *et al.,* 1999). While it is supposed that endogenous maturational

events lead to lack of CCR2 expression in monocytes differentiated *in vitro*, some studies suggest that the lack of CCR2 expression is a direct consequence of binding secreted CCL2 (Mack *et al.,* 2001). In a related study, Sica and colleagues (1997) found that LPS induced inhibition of CCR2 mRNA expression was associated with a reduction of both CCL2 binding and chemotactic responsiveness. Another study established that exposure of monocytes to OxLDL reduces CCR2 expression, causesing a rapid loss of functional CCR2 protein, and inhibition of the physiological response of monocytes to CCL2, suggesting that it acts as a negative regulator of monocyte CCR2 expression (Han *et al.,* 2000). CCR2 may be important to promote the arrest of newly recruited monocytes in the arterial wall, allowing for their cytokine- and growth factor–induced maturation to macrophages (Han *et al.,* 1998). The results presented in the current study support the hypothesis that CCR2 is down-regulated on the surface of monocytes as they undergo *in vitro* differentiation to macrophages (Fantuzzi *et al.,* 1999; Kaufmann *et al.,* 2001) and indicate that PMA-differentiated THP-1 cells stimulated with cytokines can be considered a model of differentiated macrophages at the stage where they have migrated into the arterial wall. The difference in the response to cytokines of CCR2 and CX3CR1, where a greater effect was seen on CCR2 mRNA expression confirms finding by Umehara et al., (2001) who found a differential response of these two chemokine receptors in a THP-1 cell line model of chemotaxis.

4.4.1.5 The effect of testosterone with or without flutamide on CX3CR1 mRNA expression in THP-1 macrophages stimulated with 24h cytokines

This present study showed no significant change in expression of CX3CR1 mRNA in THP-1 macrophages following 24h incubation with cytokines and testosterone, in the presence or absence of flutamide. This finding was observed at all cytokines and testosterone concentrations studied. In a parallel study, Kelly and co-workers (2010) found that testosterone or dihydrotestosterone (DHT) had no effect on the expression of CX3CL1, the ligand of CX3CR1, in vascular cells. This indicates that testosterone appears not to influence mechanisms involved in CX3CR1 regulation in macrophages with or without inflammation.

4.4.1.6 The effect of testosterone with or without flutamide on mRNA CCR2 expression in THP-1 macrophages stimulated with 24h cytokines

In contrast, testosterone induced an increase in CCR2 mRNA expression in TNF-α plus IFN-γ stimulated THP-1 macrophages. This increase was only significant with testosterone treatment at 10nM and 100nM after stimulating with cytokines at 100 ng/ml concentration. This action for testosterone was markedly abrogated by 100nM flutamide suggesting testosterone may act via AR.

The effect of testosterone on CCR2 mRNA expression following stimulation by cytokine treatment demonstrates that testosterone has the ability to influence the regulation of this receptor. In accordance with the hypothesis stated in section (4.4.1.4) (that PMA-differentiated THP-1 cells stimulated by cytokines can be used as a model of macrophages in the arterial wall), the results presented here imply that testosterone may play a beneficial role by inhibiting the conversion of these cells to lipid laden foam cells which are consequently involved in atherosclerotic events. This is demonstrated by the upregulation of CCR2 by testosterone indicating that the cells are arrested at an earlier stage of this process when increased CCR2 is required for chemotaxis of monocytes. Taken together the CCR2 results imply that this action of testosterone comes from its effect on secondary pro-inflammatory factors rather than its direct effect on CCR2 receptor. This is indicated by the lack of an effect of testosterone on CCR2 in THP-1 macrophages that were not previously treated with cytokines.

4.4.1.7 The effect of testosterone on pro and anti-inflammatory cytokines in supernatants of THP-1 macrophages before and after stimulation with cytokine for 24h

The levels of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ in supernatants of THP-1 macrophages are measured by Enhanced Sensitivity CBA assay. The obtained data for IL-1β, IL-6, IL-10, and IFN-γ were not reliable and excluded due to the high variability among these data. The values of IL8 secretion by macrophages following testosterone treatment before and after cytokines stimulation were reliable. Furthermore, TNF values was reliable following treated THP-1macrophage cells with combination of testosterone and cytokine treatment.

4.4.1.8 The effect of testosterone with or without flutamide on IL-8 levels in supernatants of THP-1 macrophages before and after stimulation with cytokine for 24h

Testosterone treatment with or without flutamide was not associated with any change in levels of IL-8 in the media from cells prior to cytokine treatment. However, testosterone treatment and blocking the AR showed an effect on IL-8 following cytokine treatment. 24hr cytokine treatment significantly increased the IL-8 levels at both cytokines concentrations compared to untreated cells. Furthermore, testosterone significantly decreased level of IL-8 in the media of the cells after 24h stimulation with cytokine. Testosterone showed its inhibitive action on IL-8 only after using cytokines; therefore, its action may not be directly on IL-8 but indirectly by inhibiting the action of TNF-α and IFNγ; this agrees with the effect of testosterone on CCR2 discussed in the previous section. This effect of testosterone was via AR as the addition of flutamide reversed the effect of testosterone.

Previous studies using testosterone treatment at pharmacological doses have shown that testosterone suppresses the production of IL-8 in human granulocytes and monocytes stimulated with LPS or zymosan and a similar effect was observed in differentiated HL-60 cells (Boje *et al.,* 2012). In the latter study, flutamide was unable to antagonize testosterone suppression of IL-8 secretion, which is in contrast to the present study.

4.4.1.9 The effect of testosterone with or without flutamide on TNF-α levels in supernatants of THP-1 macrophages before and after stimulation with cytokine for 24h

A significant increase was seen in the level of TNF-α following cytokines treatment at both concentrations compared to untreated cells. Furthermore, testosterone significantly reduced the level of TNF-α in the media after 24h cytokines treatment. Flutamide partially blocked this effect, indicating that the action of testosterone was partly via AR, but also via the AR-independent pathway. The low levels of TNF-α measured in the testosterone treated cells also indicates that most of the added TNF as one of the cytokines used for stimulation was no longer present in the media at the time point studied as the TNF-α added was at ng/ml concentrations and the levels measured by the

assay were at pg/ml levels.. Therefore the effect observed was on TNF-α secretion by the cells, and not due to the added TNF-α.

In previous cell culture studies, testosterone incubated with human monocytes obtained from younger individuals, fibroblasts, and rodent macrophage cell models led to a decline in pro-inflammatory cytokine expression of TNF-α, IL-1β, and IL-6 (Chao *et al.,* 1995, Kanda *et al.,* 1996, Kanda *et al.,* 1997, Parkar *et al.,* 1998, D'Agostino *et al.,* 1999, Gornstein *et al.,* 1999; Corcoran *et al.,* 2010). Previous clinical studies have shown conflicting results about the effect after using physiological and supraphysiological concentrations of testosterone on TNF-α level. An increase (Metcalfe *et al.,* 2008) no change (Kapoor *et al.,* 2007) or a decrease (Hatakeyama *et al.,* 2002, Malkin *et al.,* 2004a, Malkin *et al.,* 2004b; Corrales *et al.,* 2006) in the level of TNF-α have all been reported. In animal studies, no significant change was seen in serum levels of TNF-α following testosterone treatment in Tfm mice on high-fat diet compared to littermates and placebo controls (Kelly *et al.,* 2012b). In contrast, it has been stated that castrated mice show heightened TNF-α responsiveness to the injection of LPS, while testosterone replacement decreased this (Spinedi *et al.,* 1992).

A similar anti-inflammatory effect has been shown for androgen in human endothelial cells, where DHT or testosterone decreased TNFα-induced inflammatory responses through the inhibition of NF-κB signalling pathways (Hatakeyama *et al.,* 2002, Norata *et al.,* 2006; Jin *et al.,* 2009). In an interesting study using human macrophage cells obtained from elderly men and postmenopausal women, testosterone, but not estrogen treatment reduced expression of TNF-α in cells from both men and women (Corcoran *et al.,* 2010). Therefore, the authors suggested that the effect seen with testosterone treatment was not due to aromatization to estrogen but was due to an androgen specific action of testosterone via the AR. When considered with the results of the current study in which flutamide only partially blocked the action of testosterone on TNF-α secretion, it can be suggested that testosterone mediates TNFα secretion in part via AR and also by an AR-independent pathway.

Taking the data presented in the current study together, testosterone treatment increased expression of CCR2 mRNA after stimulation with cytokines via the AR. Therefore testosterone may be exerting its effect by preventing or arresting the process of change to lipid laden foam cells at the early stage when CCR2 expression is higher. Indeed, this was clear from the decreased levels of IL-8 via AR and TNF-α via AR and AR-

independent pathway in media of these cells in response to the addition of testosterone indicating a downregulation of inflammatory processes. All these outcomes will lead to a reduction in atherosclerosis development and reflect the role of testosterone as anti-inflammatory factor. On the other hand, the lack an effect of testosterone on CX3CR1 in macrophage cells can be explained by previous study which indicates that these two receptors are controlled by separate mechanisms (Umehara *et al.,* 2001).

4.4.1.10 Limitations of the study

The *in vitro* conditions used in present study may not reflect the *in vivo* environment in artery wall. Furthermore, research on expression of CX3CR1 or CCR2 by macrophages alone is limited as it does not take into account the interaction with receptors in vascular cells thus it is difficult to address the full role of testosterone in this situation. In addition, THP-1 cells are a cancerous cell line derived from a 3-4 year old boy; consequently, they do not reflect typically normal human monocytes/ macrophage cells. It would be better to use isolated human monocyte-derived macrophage (HMDMS) from, particularly older people who have a high prevalence and severity of atherosclerosis with increasing age (Lloyd-Jones *et al.,* 2010).

4.4.1.11 Summary of finding in THP-1 macrophages

The key finding of the present study demonstrated that PMA-differentiated THP-1 cells stimulated with cytokines are a good model of differentiated macrophages at the stage where they have migrated into the arterial wall and for studying chemokine receptors CX3CR1 and CCR2 expression by these cells. Testosterone had no significant influence on the expression of CX3CR1 and CCR2 and on IL-8 levels secreted by unstimulated THP-1 macrophages cells. Cytokines treatment had no effect on CX3CR1 mRNA expression whereas they significantly decreased expression of CCR2 mRNA. However, while testosterone treatment did not modulate CX3CR1 mRNA expression after cytokines stimulation, testosterone was associated with a significant increase in CCR2 mRNA via AR following cytokines treatment. Testosterone inhibited levels of IL-8 via AR and TNF-α via AR-dependent and or AR-independent pathways indicting the role of testosterone as a modulator of inflammation.

4.4.2 Mouse 3T3L1 cells

In this part of the study, 3T3L1 adipocyte cells were treated with testosterone with or without flutamide to block AR with the aim to investigate whether testosterone can influence expression of adiponectin, PPARβ and leptin or on factors involved in obesity-induced inflammation including: PAI, CX3CL1 and CCL2 during adipogenesis processes and adipocyte differentiation. The presence of AR receptors was identified in these cells by qRT-PCR which was previously reported by (Singh *et al.,* 2006). This also points to a role for testosterone and its receptors in these cells.

4.4.2.1 The effect of testosterone with or without flutamide on adiponectin mRNA expression and secretion of adiponectin in mouse 3T3L1 cells

The current study found that testosterone had no effect on adiponectin mRNA expression or its secretion into the media by 3T3L1 cells with or without the addition of flutamide. There was a significant difference in the levels of adiponectin in the flutamide treated cells, compared to the testosterone treated cells at both testosterone concentrations. However, the fold change was very small and as there was no observed difference between cells treated with testosterone and the control cells this is unlikely to be of biological significance.

In a previous study using the same cells, it was found that testosterone or 5-DHT reduced levels of adiponectin in the media of 3T3L1 without any change in adiponectin protein or mRNA expression (Nishizawa *et al.,* 2002). In that study testosterone therapy reduced plasma adiponectin concentration in both sham-operated and castrated mice without changing mRNA and protein in adipose tissue, indicating that the process of adiponectin secretion from intracellular stores is controlled independently from transcription and it is this secretion mechanism that is influenced by testosterone. Similarly, Bai et al. (2011) found that testosterone decreased adiponectin secretion and mRNA expression in human adipocytes. This was in agreement with the findings of *in vivo* patient studies reported in chapter 3, indicating that testosterone treatment decreased the production of adiponectin in plasma after 3 months. In contrast, Combs *et al.,* (2003) found that castration of mice in neonatal life but not adult life resulted in increased plasma adiponectin concentrations compared with adult females, proposing that additional influences besides gonadal steroids may be responsible for sex

differences in serum adiponectin concentrations (Gui *et al.,* 2004). Furthermore, *in vivo* in Tfm mice in the current study reported in chapter 3, a significant increase was seen in hepatic mRNA adiponectin expression in Tfm mice treated with testosterone compared to Tfm treated with placebo. However, it is unknown whether these testosterone effects on adiponectin expression occur in other tissues. Therefore, this increase may be related to the liver tissue and the Tfm condition.

4.4.2.2 The effect of testosterone with or without flutamide on PAI-1 mRNA expression in mouse 3T3L1 cells

A significant increase was seen in PAI-1 mRNA expression following 10nM testosterone treatment after five days; compared to the untreated mature adipocyte control. This slight increase was reversed by the addition of flutamide (100nM). At 100nM testosterone the only significant effect was a reduction seen at five days compared to the undifferentiated control. These results are consistent with the data presented in chapter 3 in the *in vivo* Tfm mouse study, where testosterone treatment increased hepatic PAI-1 mRNA expression. However, other studies established that testosterone had an inhibitive effect on PAI-1 synthesis (Jin *et al.,* 2007). PAI-1 is known to increase with obesity while testosterone will decrease (Plaisancea *et al.,* 2009). Furthermore, adiponectin is reduced with obesity and has an inverse relationship with PAI-1and leptin (Furukaw *et al.,* 2004; Swarbrick and Havel, 2008). Interestingly, a deterioration of cardiovascular biochemical risk markers is observed after initiation of androgen deprivation therapy, as evidenced by increased fibrinogen, PAI-1 and t-PA (Haidar *et al.,* 2007). In addition, physiological doses of testosterone or DHT, are associated with decreased PAI-1 expression by endothelial cells, where both hormones act on endothelial cells through the AR or via conversion to estradiol. However, these actions are largely lost when higher doses of testosterone were used, (Goglia *et al.,* 2010). The changes in PAI-1 shown in the data presented here, although significant, are small and therefore may not be of biological relevance.

4.4.2.3 The effect of testosterone with or without flutamide on PPARβ/δ mRNA expression in mouse 3T3L1 cell,

In this study, mRNA expression of PPARβ/δ was not affected by testosterone treatment over a 9 day period. On the contrary, as reported in chapter (3) testosterone therapy

significantly increased hepatic expression of PPARβ/δ in the *in vivo* Tfm mouse study. Limited information is available regarding the effect of testosterone therapy on PPARβ/δ especially in adipocytes or generally in adipose tissue. Therefore the lack of the effect of testosterone on mRNA PPARβ/δ expression in this *in vitro* 3T3L1 cell study cannot be fully explained at the present time. Further experiments are needed to see testosterone has an effect after stimulating these cells with pro-inflammatory cytokines.

4.4.2.4 The effect of testosterone with or without flutamide on leptin mRNA expression in mouse 3T3L1 cells

Leptin mRNA expression was decreased in most treatments of mature adipocytes compared to untreated preadipocyte cells. The expression of leptin mRNA was previously reported in 3T3L1 preadipocytes (Chen *et al,* 1998). There was no difference in expression of leptin mRNA between testosterone treated cells and control mature adipocytes at either concentration of testosterone. This is an agreement with a previous study that reported testosterone treatment for 24h was ineffective in modulating leptin gene expression in differentiated 3T3L1 adipocytes (Monjo *et al.,* 2005). In contrast, another study showed that testosterone reduced leptin secretion and mRNA expression in human adipocytes (Bai *et al.,* 2011). A negative relationship between serum leptin and testosterone was observed in previous studies (Luukkaa *et al.,* 1998, Behre *et al.,* 1997; Isidori *et al.,* 1990). Furthermore, it was found that men had lower levels of leptin than women (Saad *et al.,* 1997, Nishizawa *et al.,* 2002). In line with this, human studies reported that testosterone treatment decreased leptin levels in hypogonadal men with T2DM (Kapoor *et al.,* 2007) and in healthy men with testosterone deficiency (Simon *et al.,* 2001) also in young and old hypogonadal men (Jockenhovel *et al.,* 1997; Sih *et al.,* 1997). Conversely, in a study in mice, castration was not associated with any change in levels of leptin (Nishizawa *et al.,* 2002). Furthermore, other clinical studies found no correlation between leptin and testosterone in older hypogonadal men (Sih *et al.,* 1999) in non-diabetic men (Haffner, 1997) or in diabetic patients (Kapoor *et al.,* 2007). Importantly, the use of an aromatase inhibitor which can inhibit conversion of testosterone to estradiol is associated with a decrease in leptin concentrations as well as a rise in testosterone (Blouin *et al.,* 2005). Furthermore, it is known that high levels of leptin are not only associated with increased adipose tissue but also has an undesirable influence on testosterone production via the negative effect of leptin on Leydig cell

function or by reducing the hypothalamic-pituitary-testicular axis (Caprio *et al.,* 1999; Kelly and Jones, 2013).

4.4.2.5 The effect of testosterone with or without flutamide on mRNA expression and secretion of CX3CL1 in mouse 3T3L1 cells

Testosterone had no effect on CX3CL1 mRNA expression, however it significantly reduced its secretion level into the supernatant of adipocyte cells at 10 nM after 3 and 9 days compared to control mature cells. Flutamide at 100nM did not reverse this effect (in fact it significantly increased the effect). There are limited details in the literature about the action of testosterone on chemokines expression in adipocyte cells. However, previous studies have been conducted to investigate the effect of testosterone treatment on these chemokines in other cells. For example, Kelly and colleagues (2010) found no effect of testosterone or DTH on TNFα and IFNγ-induced CX3CL1 mRNA expression in vascular cells. The latter study suggested that this could be due to testosterone's inability to affect the greatly elevated expression of these molecules caused by the combined cytokine stimulation used.

4.4.2.6 The effect of testosterone with or without flutamide on mRNA expression and secretion of CCL2 in mouse 3T3L1 cell

CCL2 mRNA expression decreased significantly in mature adipocytes on day 5 compared to control undifferentiated cells. Testosterone, with or without flutamide, tended to decrease of CCL2 mRNA expression after 5 and 9 days, compared to the control undifferentiated cells. This reduction reached significance with 100nM testosterone after 5 days. However, no significant difference was seen in the testosterone treated cells compared to the mature adipocyte control cells. In contrast, testosterone (10 nM) reduced secretion of CCL2 into the culture media after 3 days and then increased this secretion after 5 and 9 days. The action of testosterone was reversed by flutamide at 100 and 1000nM at the same time, suggesting that testosterone may act through an AR-dependent mechanism. Similarly, a previous study found that elevated secretion of CCL2 by 3T3L1 cells treated with testosterone for 24h, which was dependent on NF-kB signalling (Su *et al.,* 2009). On the contrary, Kelly et al. (2010) reported that no change in expression of CCL2 following testosterone or DTH treatment in human aortic vascular cells and in the level of CCL2 in serum of Tfm mice with

testosterone (2012b). Norata et al., (2006) reported that DTH reduced CCL2 by mediating the AR and this was partly reversed by AR antagonism. The same group in 2010 found that, 17β estradiol did not associated with any changing in CCL2 expression. It is worth mentioning that in latter studies androgen treatment was used after stimulating vascular cells with pro-inflammatory markers such as (TNF-γ and IFN-γ or IL-1) which was not done in the present study.

4.4.2.7 Limitations of the study

3T3L1 preadipocytes are a cell line derived from mice therefore, they do not represent human adipocyte cells. It would be better to investigate the effect of testosterone treatment on adipocyte-derived protein in human adipocyte cells as well as in the absence and presence of inflammatory condition. This could give a clearer picture for the effect of testosterone treatment. Furthermore, using receptor blockers for AR and ER or aromatase inhibitor can demonstrate the mechanism by which testosterone acts.

4.4.2.8 Summary

Considered together, testosterone had no effect at the level of gene expression for adiponectin, PPARβ, leptin, CX3CL1 and CCL2. However, PAI-1 mRNA was increased by testosterone and this varied depending on the dose and time of treatment. Testosterone decreased secretion of CX3CL1 and increased CCL2 in the media of cells and this also varied depending on dose and time of treatment. Furthermore, the effect of testosterone and its action was mediating by both AR-dependent and AR-independent mechanisms. In addition, it is recognized that most of the changes in expression of the target genes were at five days. This may be because these cells contain the enzymes involved in lipolysis and lipogenesis at this time point (Ntambi *et al.,* 2000). It is possible that testosterone can show more clearly its beneficial action after stimulating these cells with pro-inflammatory factors to create inflammatory conditions particularly, leptin and PAI-1 that increase with obesity-induced inflammation. Similarly, pro-inflammatory chemokines CX3CL1 and CCL2 increase with obesity and are involved in the migration of macrophage cells to adipose tissue by binding to their receptors on these cells. Lastly, it would be worth investigating the effect of testosterone on key

transcription factors at various points in the adipogenic and lipogenic processes, to determine the transcription pathways involved.

This study was designed to assess a role for the effect of testosterone on factors associated with the diabetes and atherosclerosis and the obesity, which are associated with low testosterone levels in men. The role of testosterone in these conditions was examined to assess whether it has anti-inflammatory and anti-atherogenic actions through investigating parameters related to these conditions including markers of obesity (BMI, WC and W/HR), adipocyte-derived associated proteins (adiponectin, PPARδ, PAI-1 and leptin), pro-inflammatory cytokines and chemokines and their receptors (TNF-α, CRP, IL-8, CX3CL1, CCL2, CX3CR1 and CCR2) and apolipoprotein and HDL with its fractions (Apo A1, Apo B, Apo E, LP (a), HDL2, HDL3 and HDL2/HDL3). For these investigations different techniques were used including: CBA, ELISA and qRT-PCR in samples from human patients, animal and cell culture models. *In vitro*, testosterone treatment was used at two different concentrations, with and without blocked AR (using flutamide), which helped to define the mechanism by which testosterone acts and whether any actions of testosterone were a direct result of its action on the AR or was through other mechanisms. In patient groups, testosterone was administered either by intramuscular injection or gel application.

5.1.1 Background to the investigations

An association between obesity and low testosterone in men has been reported. Low testosterone is associated with components of MetS including IR, increased central obesity, dyslipidaemia, and CVD (Wang *et al.,* 2011). This relationship between low testosterone and these conditions is not yet fully defined with obesity-induced androgen deficiency and hypogonadism- induced obesity both likely contributing to a bidirectional effect on disease pathology (Kelly and Jones 2013). Cohen (1999) suggested that obesity impairs testosterone levels while low testosterone levels promote increased fat deposition based on the hypogonadal–obesity cycle hypothesis. In this line, many prospective studies on ADT-treated men with prostate cancer have shown increased fat mass and metabolic and cardiovascular perturbations (Choong *et al.,* 2010). A longitudinal study demonstrated association between a low testosterone concentration in men independently predicting the future development of IR and T2DM (Haring *et al.* 2009).

An increase in pro-inflammatory modulators such as cytokines and chemokines, reduction in anti-inflammatory factors such as adiponectin and IL-10 are a link between obesity and CVD, T2DM and testosterone deficiency (Mazur *et al.*, 2014; Malkin *et al.*, 2010). Moreover, there is evidence based on studies involving castration of mice as well as female mice treated with testosterone, that males have a lower incidence of autoimmune diseases compared to females (Liva *et al.,* 2001). In particular, an inhibitive effect of testosterone on pro-inflammatory factors was seen in previous studies (Malkin *et al.,* 2004a; Kalinchenko *et al.,* 2010). Additionally, improvements in central obesity, lipid profile and insulin sensitivity were observed when men were treated with testosterone (Kapoor *et al.,* 2007; Saad *et al.,* 2008). Thus it is important to study the role of testosterone and its use as a treatment to compensate for its deficiency, and its positive role in preventing disease. This role for testosterone may be through an effect on body composition, inflammation factors, and atherogenic markers. It is also necessary to elucidate the mechanism by which testosterone beneficially acts on these factors i.e. whether it is by AR- dependent or AR- independent pathways.

5.1.2 Testosterone as an anti-adipogenic factor and its effect on fat distribution/body composition

It is known that testosterone deficiency is associated with abdominal obesity and *vice versa*. Abdominal obesity is associated with IR. IR is the key central biochemical defect in development of the MetS, T2DM and atherosclerosis (Jones 2010). The present 6 months study found that testosterone had no effect on body composition (BMI, WC and W/HR) in the double-blind placebo study on diabetic patients. This is supported by data from diabetic patients in the longitudinal study where no change was seen in body composition in patients treated with testosterone and in IHD patients compared to the untreated control group. Similarly, previous studies did not find changes in W/HR (Tripathy *et al.,* 1998) and in BMI and W/HR (Lee *et al.,* 2005) after androgen treatment. In contrast to the present studies data, some clinical studies reported that a significant reduction was seen due to testosterone treatment in WC (kapoor *et al.,* 2006; Gooren and Beher, 2008; Saad; Gooren, 2009 and Heufelder *et al.,* 2009) in diabetic patients even though there was no change in BMI in those studies. However, the difference is likely explained by the amount and length of time testosterone was administered or study design. In spite of the fact that BMI has an inverse relationship with total testosterone (reviewed by Dandona *et al.,* 2011).

Furthermore, in cases of treatment of IR by rosiglitazone which works as an insulin sensitizer, by binding to the PPAR receptors in fat cells, this led to restored testosterone levels to mid-normal values in men with T2DM (Kapoor *et al.,* 2008). Similarly, TRT decreases fat mass and increases lean body mass in men in middle-age over 52 weeks (Allan *et al,* 2008) and in older hypogonadal men over 36months (Page *et al.,* 2005). Testosterone therapy also decreased visceral fat accumulation in proportion to the increase in testosterone levels (Allan *et al.,* 2008). However, the lack of change in BMI, WC and W/HR in diabetic patient from the double-blind placebo study undertaken here may be due to the shorter duration and smaller study group size. Testosterone treatment for 6 months may not be sufficient for testosterone to exert its action. Additionally, the small population studied may not show the effect of testosterone treatment in as a larger population. Finally, the diverse and small population especially in the IHD subgroup and small numbers of patients in longitudinal study on diabetic patients could also explain the absence of an observed effect of testosterone on body composition. Therefore, a long-term controlled study with limited variability in patient population would be required to elucidate effect of testosterone on obesity.

The mechanism by which testosterone acts in reduction of adipose tissue mass is thought to be by inhibition of LPL enzyme, a key regulating enzyme for energy metabolism, catabolizing plasma triglycerides into free fatty acids and glycerol. It is known that testosterone acts by binding with AR by genomic and non-genomic mechanisms in adipose tissue. Stimulation of the cAMP cascade by sex steroid hormones, would activate hormone-sensitive lipase leading to lipolysis in adipose tissues (Saad, 2009). A possible beneficial mechanism of testosterone treatment in addition to reduction in fat mass, is, the influence of adipocyte derived associated proteins during the differentiation processes of preadipocyte cells to mature adipocytes (Blouin *et al.,* 2005 and 2008). These proteins include adiponectin, PPARβ/δ, leptin and PAI-1.

Adiponectin has a role as an anti-diabetic, anti-atherogenic and anti-inflammatory factor. In human cross-sectional studies, plasma adiponectin levels are negatively correlated with obesity, W/HR, IR, dyslipidemia, diabetes and cardiovascular disease (reviewed by Matsuzawa 2005). In the current study, testosterone showed contradictory results with regards to adiponectin at the level of genes and protein. In the current clinical study, reduction in levels of adiponectin was observed in diabetic patients in the double-blind

controlled study after 3 months of testosterone treatment, however it increased in the Tfm study and did not change in the other studies. Previous research has shown that testosterone treatment reduced adiponectin level in clinical (Kapoor *et al.,* 2007) and experimental studies (Nishizawa *et al.,* 2002). A decrease in total adiponectin levels was seen in healthy boys during puberty, simultaneously with increasing endogenous androgen production (Böttner *et al.,* 2004). Furthermore, adiponectin levels in hypogonadal men are higher than in eugonadal males, and decreased after testosterone treatment (Lanfranco *et al.,* 2004). Additionally, testosterone deficiency leads to increased adiponectin in hypogonadal men with diabetes (Kapoor *et al.,* 2007) and without diabetes (Page *et al.,* 2005b), despite of the fact that adiponectin and testosterone have anti-inflammatory actions and are both associated with improvement in T2DM and atherosclerosis development (Rasul *et al.,* 2011; Saad *et al.,* 2012). However, other studies have shown that testosterone treatment over one year increased levels of adiponectin (Heufelder *et al.,* 2009). Moreover, a positive correlation between adiponectin and serum levels of total testosterone in the diabetic male (Rasul *et al.,* 2011), in non-diabetic men (Yasui *et al,* 2007) and in men and postmenopausal women (Laughlin *et al.,* 2007) has been established. This is consistent with the data from the *in vivo* Tfm mice in the current study. Testosterone treatment increased hepatic adiponectin mRNA expression compared to mice treated with placebo. However, in the present study there was a lack of testosterone modulation on expression and secretion of adiponectin in the media of 3T3L1 adipocytes in the *in vitro* study. This confirmed a previous report at the level of the gene in this model (Nishizawa *et al.,* 2002). This agreed with data from the longitudinal study on diabetic patients in this thesis where testosterone treatment had no effect on the levels of adiponectin in patients treated with testosterone and in IHD subgroups.

The contrasting effects of testosterone on adiponectin in previous studies have been clearly represented in the current study. However, the reduction in adiponectin in patients after 3 months in the double-blind placebo study may be a temporary effect of testosterone as this was not sustained at 6 months. As Gooren and Bunck (2004) reported, the reduction in circulating adiponectin concentrations might be due to the different routes of testosterone administration used, or after intramuscular testosterone injections, circulating testosterone levels are known to peak above the physiologic range, whereas transdermal testosterone gel produces testosterone levels within the reference range for young adults. This may explain the reduction in adiponectin in diabetic patient

in the double-blind placebo study, who were treated with intramuscular injections while in the second study no effect was seen on adiponectin level in those treated with testosterone gel. Furthermore, other studies indicated that there are further factors associated with the changes in adiponectin levels for instance, smoking (Miyazaki *et al.,* 2003), age (Adamczakt *et al.,* 2005) and dietary factors such as a carbohydrate-rich diet and alcohol consumption (Thamer *et al.,* 2004). Thus, these factors which affect testosterone treatment especially in human *in vivo* studies need to be controlled for.

In the mouse study, the lack of effect of testosterone on adiponectin at the mRNA and protein levels in 3T3L1 adipocytes might be related to this specific mouse strain. Thus, it may be better to study the effect of testosterone on adiponectin by analysing other mouse models or cells from whole adipose tissue. Other possible explainations are that changes in adiponectin can be due to the type of testosterone treatment, the level of dose or its metabolites (Page *et al.,* 2005b). This might explain the increase in hepatic adiponectin mRNA expression in Tfm mice after testosterone treatment. This increase was mediated either by ER after aromatising testosterone to estradiol or via a non-genomic pathway since these mice have non-functional ARs, especially since a recent study found that 17-β estradiol increased adiponectin mRNA expression in adipocyte cells (Capllonch-Amer, 2014). However, this action of testosterone could be specific for the liver of Tfm mice. Alternatively, testosterone may act on adiponectin receptors such as adipo-R1 and adipo-R2, which are involved in production and secretion of adiponectin; it was found that testosterone and E2 increase adiponectin receptor-1 and -2 mRNA and protein in adipose tissue in women with polycystic ovary syndrome (Tan *et al.,* 2006). Thus, it would be worthwhile to carry out further work to investigate whether testosterone influences adiponectin receptors in human adipocyte cells.

PAI-1 is a further factor produced by adipose tissue and has positively correlation with obesity, T2D, CVD and low testosterone (Pergola *et al.,* 2000; Alessi *et al.,* 2007). PAI-1 was investigated in 3T3L1 cells and Tfm mice. Testosterone treatment increased it at a concentration of 10 nM after 5 days and this was mediated by AR, this is supported by the *in vivo* Tfm mice data where hepatic PAI-1 mRNA expression was elevated after testosterone treatment; however this was mediated by AR-independent mechanism. Although, contradicting the data from the mouse model, where the AR is non-functional and therefore not involved in the observed effects, In previous study using human

endothelial cells, it was found that higher doses of DHT or testosterone increased expression of PAI-1 mRNA where DHT exerted these actions through AR, testosterone acted in part through aromatase-dependent conversion to 17β-estradiol (Goglia *et al.,* 2010). In that study, testosterone and DHT also had similar action on expression of PAI-1 mRNA in the aorta of Wistar rats. Therefore, this may explained the action of testosterone on mRNA expression of PAI-1 by mediating ER in the current study of Tfm mice. However, these observations are in opposite of the previous studies in which reduction in PAI-1 was seen after androgen treatment (Bavenholm *et al.,* 1998; Pugh *et al.,* 2002). The changes in PAI-1 seen in in mouse 3T3L1 adipocyte, although significant, are small and therefore may not be of biological relevance. The effect of testosterone on PAI-1 in the Tfm mice may related to the condition of these mice and to other factors such as body weight or HFD as explained in section (3.4.3). The reason behind this increase in both study unclear and thus certain conclusions cannot be made. Since the limited of details related to the effect of testosterone on PAI-1 gene in the liver of Tfm mice, therefore, further research need to be performed with involving receptor blockers for AR and ER or involving aromatase inhibitor. This can demonstrate the mechanism by which testosterone acts.

PPARβ is another factor expressed in adipose tissue and has valuable effect on lipid metabolism, glycaemic control and is a potential target of anti-obesity agents. The current study showed that testosterone had not an effect on PPARβin 3T3L1 adipocyte. These findings are inconsistent with data of *in vivo* Tfm mice in which testosterone increased significantly mRNA PPARβ gene expression of liver. This may due action of testosterone via AR-independent pathway. The lack of influence of testosterone on PPARβ in 3T3L1 cell cannot be elucidated as well as a little is known about the association between PPARβ/δ and testosterone in different tissues such as liver and adipose tissue.

Leptin is an additional factor produced by adipose tissue and increases with obesity. It has an inverse relationship with adiponectin and testosterone. It is associated with reduction of testosterone level in obese men and CVD, T2DM. There was no significant effect for testosterone on mRNA leptin expression compared to untreated mature adipocyte cells. This is in an agreement with previous study where testosterone treatment for 24h was ineffective in modulating leptin gene expression in differentiated 3T3-L1 adipocytes (Monjo *et al.,* 2005). Similarly, in animal study, plasma levels of leptin were similar in male mice with or without castration (Nishizawa *et al.,* 2002).

This is in contrast to previous study that found decrease in leptin following testosterone treatment (Jockenhovel *et al.,* 1997; Sih *et al.,* 1997; Simon *et al.,* 2001 and Kapoor *et al.*, 2007). Furthermore, a considerable decline in serum leptin levels after short-term aromatase inhibition in healthy young and elderly men has been observed (Lapauw *et al.,* 2009). However, the differences in the observations could be due to the different type of studies and multiple factors involved in mRNA expression of leptin.

The conflicting results related to adiponectin, PAI-1, PPARβ/δ and leptin during differentiated processes in *in vitro* 3T3L1 adipocyte and *in vivo* Tfm mice studies make it difficult to draw convinced conclusion that gives the correct interpretation of the action of treatment. However, the effect of testosterone on these parameters could be clear after stimulated 3T3L1 adipocyte with pro-inflammatory mediator as the condition in obesity. Furthermore, measuring the level of secretion of PA-I-1 and leptin in the media of 3T3L1 adipocyte would be valued to elucidate effect of testosterone on obesity. Additionally, using human adipocyte cells would be more useful advantageous for future investigations. Involving of receptor blockers for AR and ER or aromatase inhibitor *in vivo* Tfm mice study, would answer how testosterone acts.

5.1.3 Testosterone as an anti-inflammatory factor

5.1.3.1 Pro-inflammatory cytokines

Testosterone act as an anti-inflammatory factor through its beneficial effect on decreasing levels of inflammatory factors such as IL-1β IL-6, TNF-α, IFNγ and IL-8, where these factors are raised as a consequence of obesity and are involved in the development of T2DM, atherosclerosis and testosterone deficiency (Ferroni *et al.,* 2004; Shoelson *et al.,* 2007; Heufelder *et al.,* 2009; Jones, 2010). CRP which is synthesized in the liver under stimulation of IL-6 and TNFα, is also known as general indicator of inflammation (Fahed *et al.,* 2012).

In the present study, cytokines were measured in serum samples from diabetic patients in the double-blinded placebo-controlled and longitudinal studies, and also were studied in THP-1 macrophages. However, some of these factors (IL-1β, IL-6, IL-10, IFNγ) were not reliably detected. No significant change was seen in TNF-α and CRP levels in serum samples from diabetic patients over six month's testosterone treatment. In addition, there

was no difference in serum levels of IL-8 between patient subgroups in the longitudinal study. Similarly, in human THP-1 macrophages, there was no significant change in levels of IL-8 in media of cells after testosterone treatment in unstimulated cells. However, in cells stimulated with cytokines, testosterone significantly decreased TNF-α, and IL-8 levels in the media. Effects on TNF-α were via both AR and AR-independent pathways whereas IL-8 production was only influenced via AR-dependent pathways. Testosterone therapy had no effect on CRP levels in diabetic patients in either clinical study. A previous study showed that CRP increased in male rats after testosterone treatment (Wataru, 1990). An inverse correlation between CRP and testosterone levels was seen in some clinical studies (Bhatia *et al.,* 2006; Kapoor *et al.,* 2007). Similar to the present clinical study other studies (Ng *et al.,* 2002, Lanfranco *et al.*, 2004; Kapoor *et al.*, 2007) did not report changes in TNF-α and CRP levels. In the present cell culture study, testosterone decreased TNF-α and IL-8, which is in agreement with a previous cell culture study (Hatakeyama, 2002; Boje *et al.,* 2012) and a clinical study (Malkin *et al.,* 2004a).

To summarise, although the clinical study did not show positive effects of testosterone on pro-inflammatory factors, in cell culture however, beneficial effects of testosterone were observed, it may be because the clinical study was more complex due to variability of the patients group.

5.1.3.2 Pro-inflammatory chemokines and their receptor expression

Chemokines, CX3CL1 and CCL2, and their receptors, CX3CR1 and CCR2, are associated with chronic inflammation related to obesity such as IR, T2DM and CVD (Ota *et al.,* 2013). This association is by mediating recruitment and accumulation of leukocytes and trigging low-grade chronic inflammation in adipose tissue, liver and the arterial wall (Panee *et al.*, 2012, Shah *et al.*, 2011; Yao *et al.,* 2014).

This study demonstrated that CX3CR1 mRNA was expressed in the liver of Tfm mice, although testosterone treatment was not associated with any change in its expression. This is in agreement with the data from the *in vitro* study using human THP-1 macrophages. These cells were shown to express CX3CR1 mRNA, however, testosterone had no effect on CX3CR1 mRNA expression, with or without flutamide over 96 hours. Similarly, when cells were treated with TNF-α plus IFNγ and testosterone with or without flutamide there was no effect of testosterone on effect

CX3CR1 mRNA expression. A previous co-culture study reported that CX3CL1 and CX3CR1 are involved in monocyte/macrophage accumulation in the atherosclerotic plaque, where pro-atherogenic factors such as oxLDL induce a chemokine receptor switch: CCR2 ^{off}, CX3CR1 ^{on} (Barlic and Murphy, 2007). In the presence of ox-LDL, the CCR2^{high} CX3CR1^{low} monocytes will be differentiated into CCR2^{low} CX3CR1^{high} subtype and this CX3CR1^{on}-CCR2^{off} regulation is helpful to inhibit CCR-dependent monocyte migration and to enhance the CX3CR1-dependent monocyte recruitment and adhesion (Barlic *et al.,* 2006). This pro-adhesive chemokine receptor switch may prevent CCR2-dependent migration and may induce CX3CR1-dependent retention (Barlic and Murphy, 2007). Therefore macrophages and foam cells are prone to accumulate in vascular wall (Liu and Jiang, 2011). As a result the absence of oxLDL in the media of THP-1 macrophages in the present study, may explain the lack of effect of cytokine stimulation on this receptor. Thus, addition of oxLDL into media in combination with TNF-α plus IFNγ could actually reflect the conditions of the site of inflammation in the artery wall and consequently this system could then be used to test the effect of testosterone on CX3CR1 expression.

Expression of CCR2 mRNA was seen in the liver of Tfm mice and in THP1 macrophages. Testosterone did not show any effect on CCR2 mRNA expression in the liver of Tfm mice. Similarly, testosterone had no effect on CCR2 mRNA expression in the *in vitro* study of unstimulated THP-1 macrophages. However, incubation of these cells with 100ng/ml of TNF-α plus IFNγ for 24h significantly decreased the expression of CCR2 mRNA. The data from the present THP-1 macrophage study extended the previous findings on the reduction of surface expression of CCR2 and mRNA expression in monocyte/ macrophages (Han *et al.*, 2000, Fantuzzi *et al.,* 1999 Opalek *et al.,* 2007). These studies suggested that the down regulation of CCR2 mRNA expression may be vital to promote the arrest of newly recruited monocytes/macrophages in the arterial wall, allowing for their cytokine- and growth factor–induced maturation to macrophages. Therefore, this study provided evidence in agreement with previous findings and indicated that that PMA-differentiated THP-1 cells stimulated with cytokines can be considered a model of differentiated macrophages at the stage where they have migrated into the arterial wall.

High and low concentrations of testosterone treatment significantly increased expression of CCR2 mRNA following stimulation with 100ng/ml of TNF-α plus IFN-γ

in THP-1 macrophages. This increase was abrogated by 100 nM flutamide, suggesting testosterone may function through AR-dependent mechanisms. This is consistent with the data from the Tfm mice which have non-functional AR and no effect for testosterone on CCR2 expression was observed in these mice. The CCR2 results indicated that this action of testosterone comes from its effect on pro-inflammatory factors rather than a direct effect on the CCR2 receptor. This is evidenced by the absence of an effect of testosterone on CCR2 expression in unstimulated THP-1 macrophages. The action of testosterone may be through suppression of the NF-κB signalling pathway which is a master transcription factor in inflammation (Norata *et al.,* 2006; Jin *et al.,* 2009). Testosterone exerted its effects by arresting the monocytets/macrophages in the artery wall preventing the conversion of these cells to lipid laden foam cells which are consequently involved in atherosclerotic events. However, the beneficial effect of testosterone on these receptors or on regulatory of macrophage function remains under debate, especially since recent study has shown that testosterone treatment increase genes related to atherosclerosis in macrophage cells including atherogenic and pro-inflammatory factors (Ng *et al.,* 2003).

The present study is the first study to investigate the effect of testosterone as an anti-inflammatory modulator of CCR2 and CX3CR1 mRNA in THP1 macrophages, therefore, little is known about the effect of testosterone on these receptors an further research is needed. In addition, the expression of CX3CR1 and CCR2 mRNA was only investigated after 24h with cytokines treatment, therefore, it may be advantageous to investigate this expression at earlier time points, particularly, as previous studies showed that the maximal surface expression of CX3CR1 was observed after 15 min after stimulation with pro-inflammatory modulators (Green *et al.,* 2006). Furthermore, as earlier mentioned, oxLDL differentiated macrophages into $CCR2^{low}$ $CX3CR1^{high}$ subtype, thus, investigation of the effects of testosterone treatment on CX3CR1 and CCR2 mRNA expression in the presence of oxLDL plus cytokines in THP-1 macrophages, may actually give a clear picture on this effect.

This study demonstrated that CX3CL1 and CCL2 mRNA were expressed in the liver of Tfm mice and in preadipocyte and mature adipocytes of the mouse 3T3L1 cell line. Testosterone treatment was not associated with any change in CX3CL1 mRNA expression in the liver of Tfm mice. In addition, testosterone treatment had no effect on expression of mRNA CX3CL1 in mouse 3T3L1 cells supporting the data from the

animal model. However, testosterone reduced significantly the level of secreted CX3CL1 at low testosterone concentrations after three and nine days. This reduction was not reversed by AR blockade. This reduction in CX3CL1 in the media may indicate a beneficial effect of testosterone as an anti-inflammatory factor. There is little previous work on CX3CL1 in other cell types e.g. vascular or hepatocyte cells, and this effect may be cell specific. In support of this, previous work in our laboratory found that an apparent reduction of CX3CL1 expression in aortic lipid streaks of Tfm mice receiving testosterone treatment proposing the protective effect of testosterone in atherogenesis is via local anti-inflammatory actions and at least partially via AR-independent mechanisms (Kelly *et al.,* 2010). In that study, testosterone treatment reduced cytokine-induced expression of CX3CL1 at the molecular level in HASMC, an effect not prevented by blockade of the AR. Therefore further work should include cell culture studies on the effect of testosterone on other cell types present in liver and co-culture systems. In addition, the process of CX3CL1 secretion from intracellular stores may be controlled independently from transcription and it is this secretion mechanism that is influenced by testosterone, in a similar way to that of adiponectin as discussed previously.

Testosterone had no effect on expression of CCL2 mRNA in the liver of Tfm mice. Similarly, there was no significant effect of testosterone on CCL2 mRNA expression compared to the untreated mature adipocyte cells in 3T3L1. However, testosterone treatment significantly reduced the level of secreted CCL2 by these cells following 3 days at a low concentration, whereas testosterone increased CCL2 protein level after 5 and 9 days in culture. This effect was prevented by blockade of the AR by increasing CCL2 mRNA expression after 3 days while decreasing it after 5 and 9 days suggesting an AR-dependent mechanism. Although, there are limited studies regarding to testosterone effects on CCL2, the inconsistency of data of testosterone effects on CCL2 at the level of gene or protein in the present study was similarly observed in previous studies *in vivo* in animals (Kelly *et al*, 2012b) and *in vitro* cell culture studies (Su *et al.,* 2009; Norata *et al.,* 2006, 2010) as shown in chapter 3 and 4.

The lack of the effect of testosterone on hepatic CCL2 mRNA expression in Tfm mice might be due to the lack of AR function suggesting that if testosterone does influence CCL2 expression it is via AR-dependent pathways. This was supported through the effect of testosterone on secretion of CCL2 via the AR pathway in mouse 3T3L1 adipocytes. In addition, the modulation of secretion of CCL2 by testosterone was

dependent on the time and treatment dose. Moreover, the contradicting data on the impact of testosterone on mRNA expression and secretion of CCL2 from these cells may be due to the lack of correlation between the mRNA expression of CCL2 and protein level in media. However, it is not always the case that mRNA expression reflects the level of protein expression, the differences between mRNA and protein quantification may be due to some factors including the half-lives of specific mRNAs or proteins, and the intracellular location that can affect the of post-transcriptional processes and the translation rate of the proteins (Gygi *et al.,* 1999). Therefore, the difference in the response of mRNA and protein expression of CCL2 to testosterone may depend on the ability of testosterone action on the level of gene expression or protein secretion.

Since, there is limited published information related to the effect of testosterone on chemokine receptors and ligands in THP-1 macrophages or 3T3L1 adipocytes and Tfm mice respectively, contradictions in the results of the present study cannot be fully explained. However, the presence or the absence of appropriate atherogenic stimulator in ideal media in THP-1 macrophages or pro-inflammatory cytokines in 3T3L1 adipocyte could reveal clearly the influence of testosterone treatment. Furthermore, using further receptor blockers for AR and ER or enzyme inhibitors for aromatase or reductase would be valuable to understand the mechanism by which testosterone works.

5.1.4 Testosterone as anti-atherogenic factor:

Apolipoproteins are considered more sensitive indicators of cardiovascular risk than lipid and lipoprotein levels (Sotiropoulos *et al.,* 2008). These proteins act as enzyme co-factors during lipid metabolism, helping to stabilize lipoproteins during transportation from cell or tissue to their destination (Han, 2004). In the current study, apolipoproteins were investigated after testosterone treatment in human and mice *in vivo*.

Testosterone had no effect on apolipoprotein (Apo A1, Apo B and Apo E) and LP (a) levels in sera of diabetic patient in both studies. This is also supported by the result from the Tfm mice study where testosterone treatment was not associated with any change in apo A1 and apo E at the level of the gene. The absence of action of testosterone on apolipoprotein levels was also observed in another study using physiological testosterone concentrations (Snyder *et al.,* 2001; Herbst *et al.,* 2003) while alternative

studies showed opposing data with a decrease or increase in some of these parameters (Singh *et al.,* 2002, Von Eckardstein *et al.,* 1997, Jones *et al.,* 2011; Dickerman *et al.,* 1996). It was suggested that the mechanism of action of testosterone in modulation of apolipoproteins would be through inhibition of fat mass and this may explain the lack of apolipoprotein changes because there was no change in WC, W/HR and BMI of patients in the clinical study.

In the present study, testosterone similarly did not modulate the level of HDL and its fractions including (HDL2, HDL3 and HDL2/HDL3 ratio) in sera of diabetic patients of the double -blind placebo- controlled study. However, there was an increase, but not significant, in levels of HDL, and a significant increase in HDL2 and HDL2/HDL3 ratio while there was significantly decreased levels of HDL3 in serum of diabetic patients who were treated with testosterone in the longitudinal study. No change was seen in HDL and its fraction in the IHD subgroup in the latter study. HDL has atheroprotective functions and is negatively associated with atherosclerosis (Traish *et al.,* 2009). It is widely accepted that spherical HDL possesses atheroprotective functions such as antioxidant and anti-inflammatory properties, while it also promotes reverse cholesterol transport from peripheral tissues to the liver, where it delivers cholesterol to hepatocytes through binding to the HDL-receptor (Maeda *et al.,* 2011; Traish *et al.,* 2009). HDL subftractions includes HDL2 and HDL3, which play an important role in the function of the HDL molecule. Case control studies have shown that the higher HDL2 levels rather than HDL3 are responsible for the inverse relationship between HDL levels and CAD (Sotiropoulos *et al.,* 2008). Lagos et al. (2009) recently reported that the HDL phenotype includes a greater percentage of small HDL3 and fewer large HDL2, resulting in a lower HDL2/HDL3 ratio which is associated with an increase in MetS components. In this regard, it was previously found that the HDL2/HDL3 ratio correlated positively with the HDL levels, suggesting that when the HDL cholesterol level decreases, a decrease in the large HDL2 subclass also occurs (Moriyama *et al.,* 2014). Thus, Moriyama et al. (2014) suggested that the maintenance of a high HDL2/HDL3 ratio might be important for subjects with MetS. This may explain the finding in the current study where numerical increase in HDL and significant elevated in HDL2 and HDL2/HDL3ratio were seen in patient treated with testosterone.

There were data related to the adverse effect of testosterone on HDL and its fractions which some of them were partly similar to the current data. 250 mg testosterone esters for 3 months reduced significantly the HDL3 subfractions, while it had no effect on

HDL and HDL2 levels in young patients with hypogonadotropic hypogonadism (Taslipinar *et al.,* 2010). An additional study found that androgen increased significantly the level of HDL2 with no change in HDL and HDL3 (Ozata *et al.,* 1996). Moreover, Tan (1998) found that there was a reduction in HDL3 sub-fractions after treatment by testosterone as a parenteral testosterone ester for 4-weeks. In contrast to the present study, previous studies found that reduction in HDL, HDL2 and HDL3 (Thompson *et al.,* 1989, Tan 1998; Bagatell *et al.,* 1992) or in HDL and Apo A1 (Singh, 2001) or in HDL alone (Emmelot-Vonk *et al,* 2008, Lapauw *et al.,* 2009; Frederiksen *et al.,* 2012a) was seen following androgen treatment. High dose, formulation and type of androgen and route of administration may explain these discrepancies. Furthermore, Heufelder et al. (2009) reported a synergistic effect between testosterone treatment and lifestyle in increasing HDL levels and decreasing TG levels, in recently diagnosed T2DM subjects. However, the lack of effect of testosterone on HDL and its fractions may be due to the small duration of intramuscular testosterone injection and small group size of patients in of the double -blind placebo- controlled study. In the longitudinal study, testosterone showed beneficial effects by increasing HDL and HDL2 and HDL2/ HDL3 ratio and reducing HDL3 and this may be related to the gel testosterone treatment and length of treatment.

5.1.5 Limitation

Diverse results found in the present study may be a result of the small number of samples, or also be due to using different samples from animal and human in *in vivo* and *in vitro* conditions. In addition, in the part of *in vitro* studies, it was involved cancerous cells or mouse cells which may not reflect the actual status of the normal and human cells *in vivo*. Further factors could contribute to contradictory data among these studies including differences in study design, doses, formulations and delivery modes of testosterone. In clinical studies, factors such as age, smoking, diet, physical exercise, body fat distribution and hypogonadal state could affect the action of testosterone treatment. Moreover, the lack of normal distribution requiring the use of non-parametric statistics for assessing the differences may lead to the absence of statistical significance of the data that seemed to include significant changes. On other hand, the usage of testosterone as treatment is still subject to controversy. There are many causes for controversy in the practice of medicine. These reasons are related to the risk of

testosterone therapy which previously was observed. Cancer, liver toxicity and reduction of HDL and adiponectin have been the main risks associated with testosterone and causes for concern. Furthermore, the precise mechanistic explanations for the action of testosterone as beneficial or risk treatment remain undetermined. This was a clear in the recent studies where Muraleedharan and co-workers reported that testosterone treatment improved the survival of men with T2DM (2013) while other did not find any beneficial effect (Vigen *et al.,* 2013). However, some contributors factor such as super-physiological dose of testosterone, formulation of testosterone and mode of delivery is thought to be associated with these risks, while physiological doses and balancing testosterone with the relative abundance of estradiol could aid to achieve the beneficial effects while reducing these risks, Indeed, it cannot be ruled out the benefit testosterone in improvement of lean body mass and muscle strength, erythropoiesis and bone mineral density, sexual function, mood and reduction of fat mass insulin sensitivity, vascular tone, blood which have been improved in previous study.

5.1.6 Future work

5.1.6.1 *In vivo* study (human)

A longer study and larger population samples in well-controlled clinical trials may give a clearer explanation of the mechanism of testosterone, taking into account of BMI, age, and treatment with drugs; it was difficult to adjust for these variables with the sample size of the current study. It may be valuable to investigate other ways of analysing testosterone such as the measurement of SHBG, FT and BT which may better reflect biologically relevant concentrations of testosterone and its action. Furthermore, it would be valued to investigate additional parameters including subfractions of adiponectin, leptin and PAI-1.

5.1.6. 2 *In vivo* study (mice)

It would be useful to study adipose tissue from Tfm mice to see the effect of testosterone action on markers and key transcription factors in adipogenic procedures. Furthermore, the effect of testosterone treatment on factors involved in fat accumulation such as LPL could be investigated. These experiments could be extended by investigating the effect of DHT or further blockers of the ER or inhibitors of aromatase or reductase enzymes; this will elucidate the mechanism by which testosterone acts. The effect of testosterone in other mouse models could also be investigated, for example the Apo E knock-out mouse model which is a model of atherosclerosis.

5.1.6.3 *In vitro* study

Testosterone's effect on CX3CR1 and CCR2 expression in macrophage cells may be seen more clearly if these cells are studied in conditions closer to those seen in atherosclerosis, inflammation and atherogenesis. Indeed, there is evidence from different studies showing the role of testosterone in the reduction of fatty streak formation. Therefore, macrophage cells could be stimulated with LDL or native LDL such as oxLDL which will offer a closer model of atherogenesis conditions including the secretion of pro-inflammatory cytokine and chemokine factors. Therefore, this experiment would be worthwhile to investigate the effect of testosterone in a more complete atherosclerosis model. Furthermore, it would be favourable to use isolated

adult human monocyte/macrophage cells from patients with atherosclerosis as an atherosclerosis model to investigate what happens in reality in the artery wall instead of using cancerous cells (monocyte/macrophage THP-1 cell line). These cells could be cultured in conditions which mimic more closely the formation of foam cells. As with the studies in mice the addition of receptor blockers or aromatase enzyme inhibitors would provide further evidence of the mechanisms of testosterone action.

There is no currently available human adipocyte cell line which is why the data presented here was generated using mouse cells. However, the use of isolated human preadipocyte cells could be beneficial to get answers for the unknown questions related to the role of adipocyte derived associated proteins, in the development of T2DM, atherosclerosis and low testosterone in obesity before and after testosterone treatment. Furthermore, similar to the experiments suggested above, the use of different testosterone receptor and enzyme blockers could be used to investigate how testosterone mediates its action. It would be valuable to stimulate these cells by using pro-inflammatory factors including TNF-α, IFN-γ and IL-1β and so on. This would lead to a more adequate model of inflammatory adipocyte cells secreting pro-inflammatory factors such as CX3CL1, CCL2, PAI-1 and leptin. Therefore, the effect of testosterone on the improvement of the development of an inflammatory response with IR could be studied in this model. Moreover, by stimulating these cells, a co-culture model can be used by involving human THP-1 macrophages with human preadipocyte type cells. In addition, it would be advantageous to investigate the effect of testosterone therapy on LPL enzymes as well as key markers of differentiation and transcription factors.

5.1.7 Conclusion

The aim of this study was investigate of the actions of testosterone in diabetes, atherosclerosis and obesity. This was carried out using two patient cohort studies, an animal model and cell culture methods. According to the results presented here, both positive and negative effects of testosterone were found. Positive effects of testosterone included improvement in HDL fraction and HDL2/HDL3 ratio and decreased TNFα, IL-8, CCL2, CX3CL1, However, negative effects included a lack of change in BMI, WC and W/HR in patients and adverse changes in mediators e.g decreased adiponectin, and increased PAI-1. In addition testosterone treatment also had different effects on adiponectin in the three investigations undertaken i.e human, mouse and cell culture. Conflicts in the data indicate the complexity of the relationships and underlying interactions. Therefore the work presented here has yielded positive results indicating that testosterone has a beneficial effect on some parameters, other results are more difficult to explain; it is clear that further detailed and extensive work is needed to clarify the benefits and mechanisms of testosterone treatment for hypogonadism in define patients with diabetes, atherosclerosis or obesity.

References

Abate N. (2013). Role of subcutaneous adipose tissue in the pathogenesis of insulin resistance. *Journal of Obesity* 2013, 1-5.

Abate N., Haffner S. M., Garg A., Peshock R. M., & Grundy S. M. (2002). Sex steroid hormones, upper body obesity, and insulin resistance. *Journal of Clinical Endocrinology & Metabolism 87*, 4522-4527.

Acevedo S. F. (2012). The confounding factor of apolipoprotein E on response to chemotherapy and hormone regulation altering long-term cognition outcomes. *Lipoproteins—Role in Health and Diseases.Rijeka, Croatia: InTech* .

Adamczak M., Rzepka E., Chudek J., & Więcek A. (2005). Ageing and plasma adiponectin concentration in apparently healthy males and females. *Clinical Endocrinology 62*, 114-118.

Adams L. A., Lymp J. F., St Sauver J., Sanderson S. O., Lindor K. D., Feldstein A., & Angulo P. (2005). The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology 129*, 113-121.

Adams M. R., Williams J. K., & Kaplan J. R. (1995). Effects of androgens on coronary artery atherosclerosis and atherosclerosis-related impairment of vascular responsiveness. *Arteriosclerosis, Thrombosis, and Vascular Biology 15*, 562-570.

Affymetrix, ebioscience. (2012). [online]. Last accessed 25 March 2012 at: http://www.ebioscience.com/knowledge-center/product-line/elisa/high-sensitivity-elisa.

Aggarwal A., Schneider D. J., Sobel B. E., & Dauerman H. L. (2003). Comparison of inflammatory markers in patients with diabetes mellitus versus those without before and after coronary arterial stenting. *The American Journal of Cardiology 92*, 924-929.

Agledahl I., Hansen J., & Svartberg J. (2008). Impact of testosterone treatment on postprandial triglyceride metabolism in elderly men with subnormal testosterone levels. *Scandinavian Journal of Clinical & Laboratory Investigation 68*, 641-648.

Ahima R. S. (2006). Adipose tissue as an endocrine organ. *Obesity 14*, 242S-249S.

Ahima R. S. & Flier J. S. (2000). Leptin. *Annual Review of Physiology 62*, 413-437.

Ahmed K. A., Muniandy S., & Ismail I. S. (2009). Nε-(Carboxymethyl) lysine and Coronary Atherosclerosis-Associated Low Density Lipoprotein Abnormalities in Type 2 Diabetes: Current Status. *Journal of Clinical Biochemistry and Nutrition 44*, 14.

Ahn S. Y., Cho C., Park K., Lee H. J., Lee S., Park S. K., Lee I., & Koh G. Y. (2004). Tumor necrosis factor-α induces fractalkine expression preferentially in arterial endothelial cells and mithramycin A suppresses TNF-α-induced fractalkine expression. *The American Journal of Pathology 164*, 1663-1672.

Aiello R. J., Bourassa P. K., Lindsey S., Weng W., Natoli E., Rollins B. J., & Milos P. M. (1999). Monocyte chemoattractant protein-1 accelerates atherosclerosis in

apolipoprotein E-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 1518-1525.

Aikawa M., Sugiyama S., Hill C. C., Voglic S. J., Rabkin E., Fukumoto Y., Schoen F. J., Witztum J. L., & Libby P. (2002). Lipid lowering reduces oxidative stress and endothelial cell activation in rabbit atheroma. *Circulation 106*, 1390-1396.

Aird W. C. (2007). Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circulation Research 100*, 174-190.

Ait-Oufella H., Taleb S., Mallat Z., & Tedgui A. (2011). Recent advances on the role of cytokines in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 31*, 969-979.

Akashi T., Koizumi K., Nagakawa O., Fuse H., & Saiki I. (2006). Androgen receptor negatively influences the expression of chemokine receptors (CXCR4, CCR1) and ligand-mediated migration in prostate cancer DU-145. *Oncology Reports 16*, 831-836.

Alaupovic P. Apolipoproteins and lipoproteins. (1971). *Atherosclerosis* 13,141–146.

Albalat A., Liarte C., MacKenzie S., Tort L., Planas J., & Navarro I. (2005). Control of adipose tissue lipid metabolism by tumor necrosis factor-α in rainbow trout (Oncorhynchus mykiss). *Journal of Endocrinology 184*, 527-534.

Albers J. J., Taggart H. M., Applebaum-Bowden D., Haffner S., Chesnut III C. H., & Hazzard W. R. (1984). Reduction of lecithin-cholesterol acyltransferase, apolipoprotein D and the Lp (a) lipoprotein with the anabolic steroid stanozolol. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism 795*, 293-296.

Alberti K. G., Eckel R. H., Grundy S. M., Zimmet P. Z., Cleeman J. I., Donato K. A., Fruchart J. C., James W. P., Loria C. & M., Smith S. C. (2009). Harmonizing the metabolic syndrome. *Circulation 120*, 1640-1645.

Alessi M. & Juhan-Vague I. (2006). PAI-1 and the metabolic syndrome links, causes, and consequences. *Arteriosclerosis, Thrombosis, and Vascular Biology 26*, 2200-2207.

Alessi M., Poggi M., & Juhan-Vague I. (2007). Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. *Current Opinion in Lipidology 18*, 240-245.

Alessi M. C., Bastelica D., Mavri A., Morange P., Berthet B., Grino M., & Juhan-Vague I. (2003). Plasma PAI-1 levels are more strongly related to liver steatosis than to adipose tissue accumulation. *Arteriosclerosis, Thrombosis, and Vascular Biology 23*, 1262-1268.

Alexander E. T., Tanaka M., Kono M., Saito H., Rader D. J., & Phillips M. C. (2009). Structural and functional consequences of the Milano mutation (R173C) in human apolipoprotein AI. *Journal of Lipid Research 50*, 1409-1419.

Alexandersen P. & Christiansen C. (2004). The aging male: testosterone deficiency and testosterone replacement. An up-date. *Atherosclerosis 173*, 157-169.

Alexanderson C., Eriksson E., Stener-Victorin E., Lönn M., & Holmäng A. (2009). Early postnatal oestradiol exposure causes insulin resistance and signs of inflammation in circulation and skeletal muscle. *Journal of Endocrinology 201*, 49-58.

Ali K., Middleton M., Puré E., & Rader D. J. (2005). Apolipoprotein E suppresses the type I inflammatory response in vivo. *Circulation Research 97*, 922-927.

Allan C., Strauss B., Burger H., Forbes E., & McLachlan R. (2008). Testosterone therapy prevents gain in visceral adipose tissue and loss of skeletal muscle in nonobese aging men. *Journal of Clinical Endocrinology & Metabolism 93*, 139-146.

Allan C., Strauss B., & McLachlan R. (2007). Body composition, metabolic syndrome and testosterone in ageing men. *International Journal of Impotence Research 19*, 448-457.

Allan C. A. & McLachlan R. I. (2010). Androgens and obesity. *Current Opinion in Endocrinology, Diabetes and Obesity 17*, 224-232.

Almer G., Frascione D., Pali-Schöll I., Vonach C., Lukschal A., Stremnitzer C., Diesner S. C., Jensen-Jarolim E., Prassl R., & Mangge H. (2012). Interleukin-10: an anti-inflammatory marker to target atherosclerotic lesions via PEGylated liposomes. *Molecular Pharmaceutics 10*, 175-186.

Aloisi A. M. (2013). Testosterone-Induced Effects on Lipids and Inflammation. *Mediators of Inflammation 2013*.

Altieri P., Spallarossa P., Barisione C., Garibaldi S., Garuti A., Fabbi P., Ghigliotti G., & Brunelli C. (2012). Inhibition of Doxorubicin-Induced Senescence by PPARδ Activation Agonists in Cardiac Muscle Cells: Cooperation between PPARδ and Bcl6. *PloS One 7*, e46126.

American College of Obstetricians and Gynecologists Committee on Gynecologic Practice (2008). Hormone therapy and heart disease. *Obesity Gynecol 112*, 1189-1192.

Amitani M., Asakawa A., Amitani H., & Inui A. (2013). The role of leptin in the control of insulin-glucose axis. *Frontiers in Neuroscience 7*.

Andersen K. K., Frystyk J., Wolthers O. D., Heuck C., & Flyvbjerg A. (2007). Gender differences of oligomers and total adiponectin during puberty: a cross-sectional study of 859 Danish school children. *Journal of Clinical Endocrinology & Metabolism 92*, 1857-1862.

Anderson R., Ludlam C., & Wu F. (1995). Haemostatic effects of supraphysiological levels of testosterone in normal men. *Thrombosis and Haemostasis 74*, 693-697.

Apostolakis S., Krambovitis E., Vlata Z., Kochiadakis G. E., Baritaki S., & Spandidos D. A. (2007). CX3CR1 receptor is up-regulated in monocytes of coronary artery diseased patients: impact of pre-inflammatory stimuli and renin–angiotensin system modulators. *Thrombosis Research 121*, 387-395.

Applebaum-Bowden D., McLean P., Steinmetz A., Fontana D., Matthys C., Warnick G., Cheung M., Albers J., & Hazzard W. (1989). Lipoprotein, apolipoprotein, and lipolytic

enzyme changes following estrogen administration in postmenopausal women. *Journal of Lipid Research 30*, 1895-1906.

Arakelyan A., Petrkova J., Hermanova Z., Boyajyan A., Lukl J., & Petrek M. (2005). Serum levels of the MCP-1 chemokine in patients with ischemic stroke and myocardial infarction. *Mediators of Inflammation 2005*, 175-179.

Araujo A. B., Kupelian V., Page S. T., Handelsman D. J., Bremner W. J., & McKinlay J. B. (2007). Sex steroids and all-cause and cause-specific mortality in men. *Archives of Internal Medicine 167*, 1252.

Arinami T., Hirano T., Kobayashi K., Yamanouchi Y., & Hamaguchi H. (1990). Assignment of the apolipoprotein AI gene to 11q23 based on RFLP in a case with a partial deletion of chromosome 11, del (11)(q23. 3→ qter). *Human Genetics 85*, 39-40.

Arita Y., Kihara S., Ouchi N., Takahashi M., Maeda K., Miyagawa J., Hotta K., Shimomura I., Nakamura T., & Miyaoka K. (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochemical and Biophysical Research Communications 257*, 79-83.

Armstrong V., Cremer P., Eberle E., Manke A., Schulze F., Wieland H., Kreuzer H., & Seidel D. (1986). The association between serum Lp (a) concentrations and angiographically assessed coronary atherosclerosis: dependence on serum LDL levels. *Atherosclerosis 62*, 249-257.

Arrer E., Jungwirth A., Mack D., Frick J., & Patsch W. (1996). Treatment of prostate cancer with gonadotropin-releasing hormone analogue: effect on lipoprotein [a]. *Journal of Clinical Endocrinology & Metabolism 81*, 2508-2511.

Arslanian S. & Suprasongsin C. (1997). Testosterone treatment in adolescents with delayed puberty: changes in body composition, protein, fat, and glucose metabolism. *Journal of Clinical Endocrinology & Metabolism 82*, 3213-3220.

Ashcroft G. S., Mills S. J., Flanders K. C., Lyakh L. A., Anzano M. A., Gilliver S. C., & Roberts A. B. (2003). Role of Smad3 in the hormonal modulation of in vivo wound healing responses. *Wound Repair and Regeneration 11*, 468-473.

Asscheman H., Gooren L., Megens J., Nauta J., Kloosterboer H., & Eikelboom F. (1994). Serum testosterone level is the major determinant of the male-female differences in serum levels of high-density lipoprotein (HDL) cholesterol and HDL< sub> 2</sub> cholesterol. *Metabolism 43*, 935-939.

Assmann G. & Gotto A. M. (2004). HDL cholesterol and protective factors in atherosclerosis. *Circulation 109*, III-8-III-14.

Auffray C., Sieweke M. H., & Geissmann F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annual Review of Immunology 27*, 669-692.

Austin, C. R. & Edwards, R. G. (1981). *Mechanisms of sex differentiation in animals and man.* : Academic Press.

Autieri M. V. (2012). Pro-and anti-inflammatory cytokine networks in atherosclerosis. *ISRN Vascular Medicine 2012.*

Auwerx J. (1991). The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia 47*, 22-31.

Avramoglu R. K. & Adeli K. (2004). Hepatic regulation of apolipoprotein B. *Reviews in Endocrine and Metabolic Disorders 5*, 293-301.

Bagatell C. J., Heiman J. R., Matsumoto A. M., Rivier J. E., & Bremner W. J. (1994). Metabolic and behavioral effects of high-dose, exogenous testosterone in healthy men. *Journal of Clinical Endocrinology & Metabolism 79*, 561-567.

Bagatell C. J., Knopp R. H., Vale W. W., Rivier J. E., & Bremner W. J. (1992). Physiologic testosterone levels in normal men suppress high-density lipoprotein cholesterol levels. *Annals of Internal Medicine 116*, 967-973.

Bai, J., Liu Y., Niu G., Bai L., Xu X., Zhang G., & Wang, L. (2011). Relationship between adiponectin and testosterone in patients with type 2 diabetes. *Biochemia medica*, 21, 1, 65-70.

Bailey C. J. (2007). Treating insulin resistance: future prospects. *Diabetes & Vascular Disease Research : Official Journal of the International Society of Diabetes and Vascular Disease 4*, 20-31.

Bakogianni M. C., Kalofoutis C. A., Skenderi K., & Kalofoutis A. T. (2001). Clinical evaluation of plasma high-density lipoprotein subfractions (HDL2, HDL3) in non-insulin-dependent diabetics with coronary artery disease. *Journal of Diabetes and its Complications 15*, 265-269.

Balkau B., Deanfield J. E., Despres J. P., Bassand J. P., Fox K. A., Smith S. C.,Jr, Barter P., Tan C. E., Van Gaal L., Wittchen H. U., Massien C., & Haffner S. M. (2007). International Day for the Evaluation of Abdominal Obesity (IDEA): a study of waist circumference, cardiovascular disease, and diabetes mellitus in 168,000 primary care patients in 63 countries. *Circulation 116*, 1942-1951.

Banka C. L. (1996). High density lipoprotein and lipoprotein oxidation. *Current Opinion in Lipidology 7*, 139-142.

Barak Y., Liao D., He W., Ong E. S., Nelson M. C., Olefsky J. M., Boland R., & Evans R. M. (2002). Effects of peroxisome proliferator-activated receptor δ on placentation, adiposity, and colorectal cancer. *Proceedings of the National Academy of Sciences 99*, 303-308.

Baranova A., Collantes R., Gowder S. J., Elariny H., Schlauch K., Younoszai A., King S., Randhawa M., Pusulury S., & Alsheddi T. (2005). Obesity-related differential gene expression in the visceral adipose tissue. *Obesity Surgery 15*, 758-765.

Barish G. D., Narkar V. A., & Evans R. M. (2006). PPARδ: a dagger in the heart of the metabolic syndrome. *Journal of Clinical Investigation 116*, 590-597.

Barlic J., Zhang Y., Foley J. F., & Murphy P. M. (2006). Oxidized Lipid-Driven Chemokine Receptor Switch, CCR2 to CX3CR1, Mediates Adhesion of Human Macrophages to Coronary Artery Smooth Muscle Cells Through a Peroxisome Proliferator-Activated Receptor γ–Dependent Pathway. *Circulation 114*, 807-819.

Barlic J., Zhang Y., & Murphy P. M. (2007). Atherogenic lipids induce adhesion of human coronary artery smooth muscle cells to macrophages by up-regulating chemokine CX3CL1 on smooth muscle cells in a TNFα-NFκB-dependent manner. *Journal of Biological Chemistry 282*, 19167-19176.

Barnett, T. & Kumar, S. (2009). Obesity and diabetes. 2th ed. John Wiley & Sons.

Barter P. J. & Kastelein J. J. (2006a). Targeting cholesteryl ester transfer protein for the prevention and management of cardiovascular disease. *Journal of the American College of Cardiology 47*, 492-499.

Barter P., Ballantyne C., Carmena R., Cabezas M. C., Chapman M., Couture P., Graaf J., Durrington P., Faergeman O., & Frohlich J. (2006b). Apo B versus cholesterol in estimating cardiovascular risk and in guiding therapy: report of the thirty-person/ten-country panel. *Journal of Internal Medicine 259*, 247-258.

Barud W., Palusiński R., Bełtowski J., & Wójcicka G. (2002). Inverse relationship between total testosterone and anti-oxidized low density lipoprotein antibody levels in ageing males. *Atherosclerosis 164*, 283-288.

Barzilay J. I., Abraham L., Heckbert S. R., Cushman M., Kuller L. H., Resnick H. E., & Tracy R. P. (2001). The relation of markers of inflammation to the development of glucose disorders in the elderly the cardiovascular health study. *Diabetes 50*, 2384-2389.

Bastard J., Maachi M., Lagathu C., Kim M. J., Caron M., Vidal H., Capeau J., & Feve B. (2006). Recent advances in the relationship between obesity, inflammation, and insulin resistance. *European Cytokine Network 17*, 4-12.

Bastard J. & Pieroni L. (1999). Plasma plasminogen activator inhibitor 1, insulin resistance and android obesity. *Biomedicine & Pharmacotherapy 53*, 455-461.

Bastelica D., Morange P., Berthet B., Borghi H., Lacroix O., Grino M., Juhan-Vague I., & Alessi M. (2002). Stromal Cells Are the Main Plasminogen Activator Inhibitor-1-Producing Cells in Human Fat Evidence of Differences Between Visceral and Subcutaneous Deposits. *Arteriosclerosis, Thrombosis, and Vascular Biology 22*, 173-178.

Bauche I. B., Ait El Mkadem S., Rezsohazy R., Funahashi T., Maeda N., Miranda L. M., & Brichard S. M. (2006). Adiponectin downregulates its own production and the expression of its AdipoR2 receptor in transgenic mice. *Biochemical and Biophysical Research Communications 345*, 1414-1424.

Båvenholm P., De Faire U., Landou C., Efendic S., Nilsson J., Wiman B., & Hamsten A. (1998). Progression of coronary artery disease in young male post-infarction patients is linked to disturbances of carbohydrate and lipoprotein metabolism and to impaired fibrinolytic function. *European Heart Journal 19*, 402-410.

Bazan J. F., Bacon K. B., Hardiman G., Wang W., Soo K., Rossi D., Greaves D. R., Zlotnik A., & Schall T. J. (1997). A new class of membrane-bound chemokine with a CX3C motif.

BD Biosciences. (2013). [online]. Last accessed 25 May 2013 at: (http://www.bdbioscie ncescom/research/cytometricbeadarray/formats/index.jsp).

Beavers K. M., Brinkley T. E., & Nicklas B. J. (2010). Effect of exercise training on chronic inflammation. *Clinica Chimica Acta 411*, 785-793.

Bebo B. F.,Jr, Schuster J. C., Vandenbark A. A., & Offner H. (1999). Androgens alter the cytokine profile and reduce encephalitogenicity of myelin-reactive T cells. *Journal of Immunology (Baltimore, Md.: 1950) 162*, 35-40.

Behre C. J., Gummesson A., Jernås M., Lystig T. C., Fagerberg B., Carlsson B., & Carlsson L. (2007). Dissociation between adipose tissue expression and serum levels of adiponectin during and after diet-induced weight loss in obese subjects with and without the metabolic syndrome. *Metabolism 56*, 1022-1028.

Behre H. M., Böckers A., Schlingheider A., & Nieschlag E. (1994). Sustained suppression of serum LH, FSH and testosterone and increase of high-density lipoprotein cholesterol by daily injections of the GnRH antagonist cetrorelix over 8 days in normal men. *Clinical Endocrinology 40*, 241-248.

Behre H. M., Simoni M., & Nieschlag E. (1997). Strong association between serum levels of leptin and testosterone in men. *Clinical Endocrinology 47*, 237-240.

Bell L. N., Cai L., Johnstone B. H., Traktuev D. O., March K. L., & Considine R. V. (2008). A central role for hepatocyte growth factor in adipose tissue angiogenesis. *American Journal of Physiology-Endocrinology and Metabolism 294*, E336-E344.

Bensinger S. J. & Tontonoz P. (2008). Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature 454*, 470-477.

Benten W. P., Lieberherr M., Stamm O., Wrehlke C., Guo Z., & Wunderlich F. (1999). Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. *Molecular Biology of the Cell 10*, 3113-3123.

Berg A. H. & Scherer P. E. (2005). Adipose tissue, inflammation, and cardiovascular disease. *Circulation Research 96*, 939-949.

Berglund L., Carlström K., Stege R., Gottlieb C., Eriksson M., Angelin B., & Henriksson P. (1996). Hormonal regulation of serum lipoprotein (a) levels: effects of parenteral administration of estrogen or testosterone in males. *Journal of Clinical Endocrinology & Metabolism 81*, 2633-2637.

Berliner J., Territo M., Sevanian A., Ramin S., Kim J. A., Bamshad B., Esterson M., & Fogelman A. M. (1990). Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *Journal of Clinical Investigation 85*, 1260.

Bertram C. E. & Hanson M. A. (2001). Animal models and programming of the metabolic syndrome. *British Medical Bulletin 60*, 103-121.

Bhasin S., Woodhouse L., Casaburi R., Singh A. B., Bhasin D., Berman N., Chen X., Yarasheski K. E., Magliano L., & Dzekov C. (2001). Testosterone dose-response relationships in healthy young men. *American Journal of Physiology-Endocrinology and Metabolism 281*, E1172-E1181.

Bhatia V., Chaudhuri A., Tomar R., Dhindsa S., Ghanim H., & Dandona P. (2006). Low testosterone and high C-reactive protein concentrations predict low hematocrit in type 2 diabetes. *Diabetes Care 29*, 2289-2294.

Bhatia V. & Viswanathan P. (2006). Insulin resistance and PPAR insulin sensitizers. *Current Opinion in Investigational Drugs (London, England : 2000) 7*, 891-897.

Billin A. N. (2008). PPAR-β/δ agonists for Type 2 diabetes and dyslipidemia: an adopted orphan still looking for a home.

Blaha M. J., Blumenthal R. S., Brinton E. A., & Jacobson T. A. (2008). The importance of non–HDL cholesterol reporting in lipid management. *Journal of Clinical Lipidology 2*, 267-273.

Blaschke F., Takata Y., Caglayan E., Law R. E., & Hsueh W. A. (2006). Obesity, peroxisome proliferator-activated receptor, and atherosclerosis in type 2 diabetes. *Arteriosclerosis, Thrombosis, and Vascular Biology 26*, 28-40.

Blouin K., Boivin A., & Tchernof A. (2008). Androgens and body fat distribution. *The Journal of Steroid Biochemistry and Molecular Biology 108*, 272-280.

Blouin K., Després J., Couillard C., Tremblay A., Prud'homme D., Bouchard C., & Tchernof A. (2005). Contribution of age and declining androgen levels to features of the metabolic syndrome in men. *Metabolism 54*, 1034-1040.

Blouin K., Richard C., Brochu G., Hould F., Lebel S., Marceau S., Biron S., & Tchernof A. (2006). Androgen inactivation and steroid-converting enzyme expression in abdominal adipose tissue in men. *Journal of Endocrinology 191*, 637-649.

Blüher S., Kratzsch J., & Kiess W. (2005). Insulin-like growth factor I, growth hormone and insulin in white adipose tissue. *Best Practice & Research Clinical Endocrinology & Metabolism 19*, 577-587.

Boden G., She P., Mozzoli M., Cheung P., Gumireddy K., Reddy P., Xiang X., Luo Z., & Ruderman N. (2005). Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-κB pathway in rat liver. *Diabetes 54*, 3458-3465.

Boehm U., Klamp T., Groot M., & Howard J. (1997). Cellular responses to interferon-γ. *Annual Review of Immunology 15*, 749-795.

Boekholdt S. M., Peters R. J., Hack C. E., Day N. E., Luben R., Bingham S. A., Wareham N. J., Reitsma P. H., & Khaw K. T. (2004). IL-8 plasma concentrations and the risk of future coronary artery disease in apparently healthy men and women: the

EPIC-Norfolk prospective population study. *Arteriosclerosis, Thrombosis, and Vascular Biology 24*, 1503-1508.

Bogan J. S. & Lodish H. F. (1999). Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes defined by endogenous ACRP30 and GLUT4. *The Journal of Cell Biology 146*, 609-620.

Boisvert W. A., Spangenberg J., & Curtiss L. K. (1995). Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. *Journal of Clinical Investigation 96*, 1118.

Boisvert W. A. (2004). Modulation of atherogenesis by chemokines. *Trends in Cardiovascular Medicine 14*, 161-165.

Boisvert W. A., Santiago R., Curtiss L. K., & Terkeltaub R. A. (1998). A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. *Journal of Clinical Investigation 101*, 353.

Boje A., Moesby L., Timm M., & Hansen E. W. (2012). Immunomodulatory effects of testosterone evaluated in all-trans retinoic acid differentiated HL-60 cells, granulocytes, and monocytes. *International Immunopharmacology 12*, 573-579.

Boring L., Gosling J., Cleary M., & Charo I. F. (1998). Decreased lesion formation in CCR2−/− mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature 394*, 894-897.

Bossola M., Tazza L., Luciani G., Tortorelli A., & Tsimikas S. (2011). OxPL/apoB, lipoprotein (a) and OxLDL biomarkers and cardiovascular disease in chronic hemodialysis patients. *J Nephrol 24*, 581-588.

Böttner A., Kratzsch J., Müller G., Kapellen T. M., Blüher S., Keller E., Blüher M., & Kiess W. (2004). Gender differences of adiponectin levels develop during the progression of puberty and are related to serum androgen levels. *Journal of Clinical Endocrinology & Metabolism 89*, 4053-4061.

Boyanov M., Boneva Z., & Christov V. (2003). Testosterone supplementation in men with type 2 diabetes, visceral obesity and partial androgen deficiency. *The Aging Male 6*, 1-7.

Boyle Jr E. M., Kovacich J. C., Hèbert C. A., Canty Jr T. G., Chi E., Morgan E. N., Pohlman T. H., & Verrier E. D. (1998). Inhibition of interleukin-8 blocks myocardial ischemia-reperfusion injury. *The Journal of Thoracic and Cardiovascular Surgery 116*, 114-121.

Braga-Basaria M., Dobs A. S., Muller D. C., Carducci M. A., John M., Egan J., & Basaria S. (2006). Metabolic syndrome in men with prostate cancer undergoing long-term androgen-deprivation therapy. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology 24*, 3979-3983.

Brahimi-Horn M. C. & Pouysségur J. (2007). Oxygen, a source of life and stress. *FEBS Letters 581*, 3582-3591.

Brand J. S., van der Tweel I., Grobbee D. E., Emmelot-Vonk M. H., & van der Schouw, Yvonne T (2011). Testosterone, sex hormone-binding globulin and the metabolic syndrome: a systematic review and meta-analysis of observational studies. *International Journal of Epidemiology 40*, 189-207.

Braunersreuther V. & Mach F. (2006). Leukocyte recruitment in atherosclerosis: potential targets for therapeutic approaches? *Cellular and Molecular Life Sciences CMLS 63*, 2079-2088.

Braunersreuther V., Zernecke A., Arnaud C., Liehn E. A., Steffens S., Shagdarsuren E., Bidzhekov K., Burger F., Pelli G., & Luckow B. (2007). Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology 27*, 373-379.

Brennan A., Li T., Kelesidis I., Gavrila A., Hu F., & Mantzoros C. (2007). Circulating leptin levels are not associated with cardiovascular morbidity and mortality in women with diabetes: a prospective cohort study. *Diabetologia 50*, 1178-1185.

Breslow J., Ross D., McPherson J., Williams H., Kurnit D., Nussbaum A., Karathanasis S., & Zannis V. (1982). Isolation and characterization of cDNA clones for human apolipoprotein AI. *Proceedings of the National Academy of Sciences 79*, 6861-6865.

Brown C., Goss S., Lubahn D., Joseph D., Wilson E., French F., & Willard H. (1989). Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. *American Journal of Human Genetics 44*, 264.

Brown J. D. & Plutzky J. (2007). Peroxisome proliferator–activated receptors as transcriptional nodal points and therapeutic targets. *Circulation 115*, 518-533.

Brown J., Wang H., Hajishengallis G. N., & Martin M. (2011). TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *Journal of Dental Research 90*, 417-427.

Bruck B., Brehme U., Gugel N., Hanke S., Finking G., Lutz C., Benda N., Schmahl F. W., Haasis R., & Hanke H. (1997). Gender-specific differences in the effects of testosterone and estrogen on the development of atherosclerosis in rabbits. *Arteriosclerosis, Thrombosis, and Vascular Biology 17*, 2192-2199.

Bruno G., Fornengo P., Novelli G., Panero F., Perotto M., Segre O., Zucco C., Deambrogio P., Bargero G., & Perin P. C. (2009). C-Reactive Protein and 5-Year Survival in Type 2 Diabetes The Casale Monferrato Study. *Diabetes 58*, 926-933.

Bruserud, Ø. & Kittang, A. O. (2010). *The chemokine system in experimental and clinical hematology.* : Springer.

Bruun J. M., Lihn A. S., Verdich C., Pedersen S. B., Toubro S., Astrup A., & Richelsen B. (2003). Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *American Journal of Physiology-Endocrinology and Metabolism 285*, E527-E533.

Buckley D. I., Fu R., Freeman M., Rogers K., & Helfand M. (2009). C-reactive protein as a risk factor for coronary heart disease: a systematic review and meta-analyses for the US Preventive Services Task Force. *Annals of Internal Medicine 151*, 483-495.

Bulló M., Casas R., Portillo M., Basora J., Estruch R., García-Arellano A., Lasa A., Juanola-Falgarona M., Arós F., & Salas-Salvadó J. (2013). Dietary glycemic index/load and peripheral adipokines and inflammatory markers in elderly subjects at high cardiovascular risk. *Nutrition, Metabolism and Cardiovascular Diseases 23*, 443-450.

Bursill C. A., Channon K. M., & Greaves D. R. (2004). The role of chemokines in atherosclerosis: recent evidence from experimental models and population genetics. *Current Opinion in Lipidology 15*, 145-149.

Burt T. D., Agan B. K., Marconi V. C., He W., Kulkarni H., Mold J. E., Cavrois M., Huang Y., Mahley R. W., & Dolan M. J. (2008). Apolipoprotein (apo) E4 enhances HIV-1 cell entry in vitro, and the APOE ε4/ε4 genotype accelerates HIV disease progression. *Proceedings of the National Academy of Sciences 105*, 8718-8723.

Bustin S. x. & Mueller R. (2005). Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clinical Science 109*, 365-379.

Butler D., Maini R., Feldmann M., & Brennan F. (1994). Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *European Cytokine Network 6*, 225-230.

Dawson T., A Kuziel W., A Osahar T., & Maeda N. (1999). Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. *Atherosclerosis 143*, 205-211.

Caballero A. E. (2003). Endothelial dysfunction in obesity and insulin resistance: a road to diabetes and heart disease. *Obesity Research 11*, 1278-1289.

Cai Y., Li J., & Yan C. (2013). Vinpocetine attenuates lipid accumulation and atherosclerosis formation. *Biochemical and Biophysical Research Communications 434*, 439-443.

Calabresi L., Franceschini G., Sirtori M., Gianfranceschi G., Werba P., & Sirtori C. R. (1990). Influence of serum triglycerides on the HDL pattern in normal subjects and patients with coronary artery disease. *Atherosclerosis 84*, 41-48.

Calara F., Dimayuga P., Niemann A., Thyberg J., Diczfalusy U., Witztum J. L., Palinski W., Shah P. K., Cercek B., & Nilsson J. (1998). An animal model to study local oxidation of LDL and its biological effects in the arterial wall. *Arteriosclerosis, Thrombosis, and Vascular Biology 18*, 884-893.

Cambien B., Pomeranz M., Schmid-Antomarchi H., Millet M., Breittmayer V., Rossi B., & Schmid-Alliana A. (2001). Signal transduction pathways involved in soluble fractalkine–induced monocytic cell adhesion. *Blood 97*, 2031-2037.

Candido R., Jandeleit-Dahm K. A., Cao Z., Nesteroff S. P., Burns W. C., Twigg S. M., Dilley R. J., Cooper M. E., & Allen T. J. (2002). Prevention of accelerated

atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E–deficient mice. *Circulation 106*, 246-253.

Caplice N. M., Panetta C., Peterson T. E., Kleppe L. S., Mueske C. S., Kostner G. M., Broze G. J., & Simari R. D. (2001). Lipoprotein (a) binds and inactivates tissue factor pathway inhibitor: a novel link between lipoproteins and thrombosis. *Blood 98*, 2980-2987.

Capllonch-Amer G., Llado I., Proenza A. M., Garcia-Palmer F. J., & Gianotti M. (2014). Opposite effects of 17-beta estradiol and testosterone on mitochondrial biogenesis and adiponectin synthesis in white adipocytes. *Journal of Molecular Endocrinology 52*, 203-214.

Caprio M., Isidori A. M., Carta A. R., Moretti C., Dufau M. L., & Fabbri A. (1999). Expression of Functional Leptin Receptors in Rodent Leydig Cells 1. *Endocrinology 140*, 4939-4947.

Carmena R., Duriez P., & Fruchart J. (2004). Atherogenic lipoprotein particles in atherosclerosis. *Circulation 109*, III-2-III-7.

Carter K. W., Hung J., Powell B. L., Wiltshire S., Foo B. T., Leow Y. C., McQuillan B. M., Jennens M., McCaskie P. A., & Thompson P. L. (2008). Association of Interleukin-1 gene polymorphisms with central obesity and metabolic syndrome in a coronary heart disease population. *Human Genetics 124*, 199-206.

Casquero A., Berti J., Salerno A., Bighetti E., Cazita P., Ketelhuth D., Gidlund M., & Oliveira H. (2006). Atherosclerosis is enhanced by testosterone deficiency and attenuated by CETP expression in transgenic mice. *Journal of Lipid Research 47*, 1526-1534.

Castrillo A. & Tontonoz P. (2004). Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. *Annu.Rev.Cell Dev.Biol. 20*, 455-480.

Cefalu W. T. (2011). Fractalkine: a cellular link between adipose tissue inflammation and vascular pathologies. *Diabetes 60*, 1380-1382.

Chandel A., Dhindsa S., Topiwala S., Chaudhuri A., & Dandona P. (2008). Testosterone concentration in young patients with diabetes. *Diabetes Care 31*, 2013-2017.

Chandran M., Phillips S. A., Ciaraldi T., & Henry R. R. (2003). Adiponectin: more than just another fat cell hormone? *Diabetes Care 26*, 2442-2450.

Chandrasekar B., Mummidi S., Perla R., Bysani S., Dulin N., Liu F., & Melby P. (2003). Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochem.J 373*, 547-558.

Chang C., Yeh S., Lee S. O., & Chang T. (2013). Androgen Receptor (AR) Pathophysiological Roles in Androgen Related Diseases in Skin, Bone/Muscle, Metabolic Syndrome and Neuron/Immune Systems: Lessons Learned from Mice Lacking AR in Specific Cells.

Chao T., Van Alten P. J., Greager J. A., & Walter R. J. (1995). Steroid sex hormones regulate the release of tumor necrosis factor by macrophages. *Cellular Immunology 160*, 43-49.

Chapman M. J., Le Goff W., Guerin M., & Kontush A. (2010). Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *European Heart Journal 31*, 149-164.

Charest N. J., Zhou Z., Lubahn D. B., Olsen K. L., Wilson E. M., & French F. S. (1991). A frameshift mutation destabilizes androgen receptor messenger RNA in the Tfm mouse. *Molecular Endocrinology 5*, 573-581.

Chean M. H., & Kam P. A. (2005). Obesity: basic science and medical aspects relevant to anaesthetists. *Anaesthesia* 60, 1009–1021.

Chen B., Lam K. S., Wang Y., Wu D., Lam M. C., Shen J., Wong L., Hoo R. L., Zhang J., & Xu A. (2006). Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochemical and Biophysical Research Communications 341*, 549-556.

Chen X., Hausman D., Dean R., & Hausman G. (1998). Hormonal Regulation of Leptin mRNA Expression and Preadipocyte Recruitment and Differentiation in Porcine Primary Cultures of S-V Cells. *Obesity Research 6*, 164-172.

Chen M., Xu W., Su C. L., & Lin J. F. (2008). Testosterone impairs insulin sensitivity in 3T3-L1 adipocytes and the related nongenomic mechanisms. *Zhonghua Yi Xue Za Zhi 88*, 1488-1492.

Cheng C., Tempel D., van Haperen R., de Boer H. C., Segers D., Huisman M., van Zonneveld A. J., Leenen P. J., van der Steen A., & Serruys P. W. (2007). Shear stress–induced changes in atherosclerotic plaque composition are modulated by chemokines. *Journal of Clinical Investigation 117*, 616-626.

Chiesa G., Hobbs H., Koschinsky M., Lawn R., Maika S., & Hammer R. (1992). Reconstitution of lipoprotein (a) by infusion of human low density lipoprotein into transgenic mice expressing human apolipoprotein (a). *Journal of Biological Chemistry 267*, 24369-24374.

Cho S. Y., Jeong H. W., Sohn J. H., Seo D., Kim W. G., & Lee S. (2012). An ethanol extract of Artemisia iwayomogi activates PPARδ leading to activation of fatty acid oxidation in skeletal muscle. *PloS One 7*, e33815.

Choesmel V., Bacqueville D., Rouquette J., Noaillac-Depeyre J., Fribourg S., Cretien A., Leblanc T., Tchernia G., Da Costa L., & Gleizes P. E. (2007). Impaired ribosome biogenesis in Diamond-Blackfan anemia. *Blood 109*, 1275-1283.

Choong K. & Basaria S. (2010). Emerging cardiometabolic complications of androgen deprivation therapy. *The Aging Male 13*, 1-9.

Chu N., Spiegelman D., Hotamisligil G. S., Rifai N., Stampfer M., & Rimm E. B. (2001). Plasma insulin, leptin, and soluble TNF receptors levels in relation to obesity-

related atherogenic and thrombogenic cardiovascular disease risk factors among men. *Atherosclerosis 157*, 495-503.

Clark R. B. (2002). The role of PPARs in inflammation and immunity. *Journal of Leukocyte Biology 71*, 388-400.

Clarke M. C., Figg N., Maguire J. J., Davenport A. P., Goddard M., Littlewood T. D., & Bennett M. R. (2006). Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nature Medicine 12*, 1075-1080.

Clement K., Viguerie N., Poitou C., Carette C., Pelloux V., Curat C. A., Sicard A., Rome S., Benis A., Zucker J. D., Vidal H., Laville M., Barsh G. S., Basdevant A., Stich V., Cancello R., & Langin D. (2004). Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology 18*, 1657-1669.

Cockerill G. W., Rye K., Gamble J. R., Vadas M. A., & Barter P. J. (1995). High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arteriosclerosis, Thrombosis, and Vascular Biology 15*, 1987-1994.

Cohen P. (1999). The hypogonadal–obesity cycle: role of aromatase in modulating the testosterone–estradiol shunt–a major factor in the genesis of morbid obesity. *Medical Hypotheses 52*, 49-51.

Coll B., Alonso-Villaverde C., & Joven J. (2007). Monocyte chemoattractant protein-1 and atherosclerosis: is there room for an additional biomarker? *Clinica Chimica Acta 383*, 21-29.

Colvin P. L., Auerbach B. J., Case L. D., Hazzard W. R., & Applebaum-Bowden D. (1991). A dose-response relationship between sex hormone-induced change in hepatic triglyceride lipase and high-density lipoprotein cholesterol in postmenopausal women. *Metabolism 40*, 1052-1056.

Combadière C., Potteaux S., Gao J., Esposito B., Casanova S., Lee E. J., Debré P., Tedgui A., Murphy P. M., & Mallat Z. (2003). Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation 107*, 1009-1016.

Combadière C., Potteaux S., Rodero M., Simon T., Pezard A., Esposito B., Merval R., Proudfoot A., Tedgui A., & Mallat Z. (2008). Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6Chi and Ly6Clo monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation 117*, 1649-1657.

Combadiere C., Salzwedel K., Smith E. D., Tiffany H. L., Berger E. A., & Murphy P. M. (1998). Identification of CX 3CR1 a chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *Journal of Biological Chemistry 273*, 23799-23804.

Combs T. P., Berg A. H., Rajala M. W., Klebanov S., Iyengar P., Jimenez-Chillaron J. C., Patti M. E., Klein S. L., Weinstein R. S., & Scherer P. E. (2003). Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes 52*, 268-276.

Combs T. P., Pajvani U. B., Berg A. H., Lin Y., Jelicks L. A., Laplante M., Nawrocki A. R., Rajala M. W., Parlow A. F., & Cheeseboro L. (2004). A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology 145*, 367-383.

Combs T. P., Berg A. H., Rajala M. W., Klebanov S., Iyengar P., Jimenez-Chillaron J. C., Patti M. E., Klein S. L., Weinstein R. S., & Scherer P. E. (2003). Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. *Diabetes 52*, 268-276.

Cone R. D. (2005). Anatomy and regulation of the central melanocortin system. *Nature Neuroscience 8*, 571-578.

Considine R. V., Sinha M. K., Heiman M. L., Kriauciunas A., Stephens T. W., Nyce M. R., Ohannesian J. P., Marco C. C., McKee L. J., & Bauer T. L. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine 334*, 292-295.

Contois J. H., Warnick G. R., & Sniderman A. D. (2011). Reliability of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B measurement. *Journal of Clinical Lipidology 5*, 264-272.

Corbould A. (2007). Chronic testosterone treatment induces selective insulin resistance in subcutaneous adipocytes of women. *Journal of Endocrinology 192*, 585-594.

Corcoran M. P., Meydani M., Lichtenstein A. H., Schaefer E. J., Dillard A., & Lamon-Fava S. (2010). Sex hormone modulation of proinflammatory cytokine and C-reactive protein expression in macrophages from older men and postmenopausal women. *The Journal of Endocrinology 206*, 217-224.

Corona G., Mannucci E., Forti G., & Maggi M. (2009). Hypogonadism, ED, metabolic syndrome and obesity: a pathological link supporting cardiovascular diseases. *International Journal of Andrology 32*, 587-598.

Corona G., Mannucci E., Schulman C., Petrone L., Mansani R., Cilotti A., Balercia G., Chiarini V., Forti G., & Maggi M. (2006). Psychobiologic correlates of the metabolic syndrome and associated sexual dysfunction. *European Urology 50*, 595-604.

Corona G., Monami M., Rastrelli G., Aversa A., Tishova Y., Saad F., Lenzi A., Forti G., Mannucci E., & Maggi M. (2011). Testosterone and Metabolic Syndrome: A Meta-Analysis Study. *The Journal of Sexual Medicine 8*, 272-283.

Corrales J., Almeida M., Burgo R., Mories M., Miralles J., & Orfao A. (2006). Androgen-replacement therapy depresses the ex vivo production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men with partial androgen deficiency. *Journal of Endocrinology 189*, 595-604.

Corrales J., Burgo R., García-Berrocal B., Almeida M., Alberca I., Gonzalez-Buitrago J., Orfao A., & Miralles J. (2004). Partial androgen deficiency in aging type 2 diabetic men and its relationship to glycemic control. *Metabolism 53*, 666-672.

Couillard C., Gagnon J., Bergeron J., Leon A. S., Rao D., Skinner J. S., Wilmore J. H., Després J., & Bouchard C. (2000). Contribution of body fatness and adipose tissue distribution to the age variation in plasma steroid hormone concentrations in men: the heritage family study 1. *Journal of Clinical Endocrinology & Metabolism 85*, 1026-1031.

Cousin B., Andre M., Villena P., Casteilla L., & Penicaud L. (2003). Human adipose cells as candidates in defense and tissue remodeling phenomena. *Biochem Biophys Res Commun*, 309: 502

Craig W. Y., Neveux L. M., Palomaki G. E., Cleveland M. M., & Haddow J. E. (1998). Lipoprotein (a) as a risk factor for ischemic heart disease: metaanalysis of prospective studies. *Clinical Chemistry 44*, 2301-2306.

Crawford E. D., Barqawi A. B., O'Donnell C., & Morgentaler A. (2007). The association of time of day and serum testosterone concentration in a large screening population. *BJU International 100*, 509-513.

Cummings B. (2013). Leptin therapy in type 2 diabetes. *Diabetes, Obesity and Metabolism 15*, 607-612.

Curat C. A., Miranville A., Sengenès C., Diehl M., Tonus C., Busse R., & Bouloumié A. (2004). From blood monocytes to adipose tissue-resident macrophages induction of diapedesis by human mature adipocytes. *Diabetes 53*, 1285-1292.

Curtiss L. K. & Boisvert W. A. (2000). Apolipoprotein E and atherosclerosis. *Current Opinion in Lipidology 11*, 243-251.

Cushing G. L., Gaubatz J. W., Nava M. L., Burdick B. J., Bocan T., Guyton J. R., Weilbaecher D., DeBakey M. E., Lawrie G. M., & Morrisett J. D. (1989). Quantitation and localization of apolipoproteins [a] and B in coronary artery bypass vein grafts resected at re-operation. *Arteriosclerosis, Thrombosis, and Vascular Biology 9*, 593-603.

Cushing S. D., Berliner J. A., Valente A. J., Territo M. C., Navab M., Parhami F., Gerrity R., Schwartz C. J., & Fogelman A. M. (1990). Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proceedings of the National Academy of Sciences 87*, 5134-5138.

Cutolo M., Balleari E., Giusti M., Intra E., & Accardo S. (1991). Androgen replacement therapy in male patients with rheumatoid arthritis. *Arthritis & Rheumatism 34*, 1-5.

Cutolo M. & Wilder R. L. (2000). Different roles for androgens and estrogens in the susceptibility to autoimmune rheumatic diseases. *Rheumatic Disease Clinics of North America 26*, 825-839.

D'Agostino P., Milano S., Barbera C., Bella G., Rosa M., Ferlazzo V., Farruggio R., Miceli D., Miele M., & Castagnetta L. (1999). Sex Hormones Modulate Inflammatory Mediators Produced by Macrophagesa. *Annals of the New York Academy of Sciences 876*, 426-429.

Dahlman I., Kaaman M., Olsson T., Tan G. D., Bickerton A. S., Wåhlén K., Andersson J., Nordström E. A., Blomqvist L., & Sjögren A. (2005). A unique role of monocyte

chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *Journal of Clinical Endocrinology & Metabolism 90*, 5834-5840.

Dallinga-Thie G. M., Franssen R., Mooij H. L., Visser M. E., Hassing H. C., Peelman F., Kastelein J. J., Péterfy M., & Nieuwdorp M. (2010). The metabolism of triglyceride-rich lipoproteins revisited: new players, new insight. *Atherosclerosis 211*, 1-8.

Damås J. K., Boullier A., Wæhre T., Smith C., Sandberg W. J., Green S., Aukrust P., & Quehenberger O. (2005). Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, is elevated in coronary artery disease and is reduced during statin therapy. *Arteriosclerosis, Thrombosis, and Vascular Biology 25*, 2567-2572.

Dandona P., Aljada A., & Bandyopadhyay A. (2004). Inflammation: the link between insulin resistance, obesity and diabetes. *Trends in Immunology 25*, 4-7.

Dandona P., Aljada A., Chaudhuri A., Mohanty P., & Garg R. (2005). Metabolic Syndrome A Comprehensive Perspective Based on Interactions Between Obesity, Diabetes, and Inflammation. *Circulation 111*, 1448-1454.

Dandona P. & Dhindsa S. (2011). Update: hypogonadotropic hypogonadism in type 2 diabetes and obesity. *The Journal of Clinical Endocrinology & Metabolism 96*, 2643-2651.

Dastani E. (2006). A novel nonsense apolipoprotein A-I mutation (apoA-IE136X) causes low HDL cholesterol in French Canadians. *Atherosclerosis 185*, 127-136.

Davignon J. (2005). Apolipoprotein E and atherosclerosis beyond lipid effect. *Arteriosclerosis, Thrombosis, and Vascular Biology 25*, 267-269.

Davignon J., Cohn J. S., Mabile L., & Bernier L. (1999). Apolipoprotein E and atherosclerosis: insight from animal and human studies. *Clinica Chimica Acta 286*, 115-143.

Davignon J., Gregg R. E., & Sing C. F. (1988). Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 8*, 1-21.

Daynes R. A. & Jones D. C. (2002). Emerging roles of PPARs in inflammation and immunity. *Nature Reviews Immunology 2*, 748-759.

De Heredia F. P., Gómez-Martínez S., & Marcos A. (2012). Obesity, inflammation and the immune system. *Proceedings of the Nutrition Society 71*, 332-338.

De Lemos J. A., Morrow D. A., Blazing M. A., Jarolim P., Wiviott S. D., Sabatine M. S., Califf R. M., & Braunwald E. (2007). Serial Measurement of Monocyte Chemoattractant Protein-1 After Acute Coronary SyndromesResults From the A to Z Trial. *Journal of the American College of Cardiology 50*, 2117-2124.

De Lemos J. A., Morrow D. A., Sabatine M. S., Murphy S. A., Gibson C. M., Antman E. M., McCabe C. H., Cannon C. P., & Braunwald E. (2003). Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndromes. *Circulation 107*, 690-695.

De Luca C. & Olefsky J. M. (2008). Inflammation and insulin resistance. *FEBS Letters 582*, 97-105.

De Luca C. & Olefsky J. M. (2006). Stressed out about obesity and insulin resistance. *Nature Medicine 12*, 41-42.

De Pergola G. (2000). The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone. *International Journal of Obesity & Related Metabolic Disorders 24*.

De Pergola G., De Mitrio V., Sciaraffia M., Pannaccuilli N., Minenna A., Giorgino F., Petronelli M., Laudadio E., & Giorgino R. (1997). Lower androgenicity is associated with higher plasma levels of prothrombotic factors irrespective of age, obesity, body fat distribution, and related metabolic parameters in men. *Metabolism 46*, 1287-1293.

De Winther M. P., van Dijk K. W., Havekes L. M., & Hofker M. H. (2000). Macrophage scavenger receptor class AA multifunctional receptor in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 20*, 290-297.

Deachapunya C., Palmer-Densmore M., & O'Grady S. M. (1999). Insulin stimulates transepithelial sodium transport by activation of a protein phosphatase that increases Na-K ATPase activity in endometrial epithelial cells. *The Journal of General Physiology 114*, 561-574.

Debing E., Peeters E., Duquet W., Poppe K., Velkeniers B., & Van Den Brande P. (2008). Men with atherosclerotic stenosis of the carotid artery have lower testosterone levels compared with controls. *International Angiology 27*, 135-141.

Deeb S. S., Zambon A., Carr M. C., Ayyobi A. F., & Brunzell J. D. (2003). Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. *Journal of Lipid Research 44*, 1279-1286.

DeFranco D. B. (2000). Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors. *Kidney International 57*, 1241-1249.

Delalla O. F., Elliott H. A., & Gofman J. W. (1954). Ultracentrifugal studies of high density serum lipoproteins in clinically healthy adults. *American Journal of Physiology--Legacy Content 179*, 333-337.

Dembinski T., Nixon P., Shen G., Mymin D., & Choy P. (2000). Evaluation of a new apolipoprotein (a) isoform-independent assay for serum Lipoprotein (a). *Molecular and Cellular Biochemistry 207*, 149-155.

Demirbag R., Yilmaz R., Ulucay A., & Unlu D. (2005). The inverse relationship between thoracic aortic intima media thickness and testosterone level. *Endocrine Research 31*, 335-344.

Denholm E. M. & Stankus G. P. (1995). Changes in the expression of MCP-1 receptors on monocytic THP-1 cells following differentiation to macrophages with phorbol myristate acetate. *Cytokine 7*, 436-440.

Desurmont C., Caillaud J., Emmanuel F., Benoit P., Fruchart J. C., Castro G., Branellec D., Heard J., & Duverger N. (2000). Complete atherosclerosis regression after human ApoE gene transfer in ApoE-deficient/nude mice. *Arteriosclerosis, Thrombosis, and Vascular Biology 20*, 435-442.

Di Paola R., Crisafulli C., Mazzon E., Esposito E., Paterniti I., Galuppo M., Genovese T., Thiemermann C., & Cuzzocrea S. (2010). GW0742, a high-affinity PPAR - beta/delta agonist, inhibits acute lung injury in mice. *Shock (Augusta, Ga.) 33*, 426-435.

Diano S., Horvath T. L., Mor G., Register T., Adams M., Harada N., & Naftolin F. (1999). Aromatase and estrogen receptor immunoreactivity in the coronary arteries of monkeys and human subjects. *Menopause 6*, 21-28.

Dickerman R., McConathy W., Schaller F., & Zachariah N. (1996). Cardiovascular complications and anabolic steroids. *Eur Heart J 17*, 1912.

Dieudonne M., Pecquery R., Leneveu M., & Giudicelli Y. (2000). Opposite Effects of Androgens and Estrogens on Adipogenesis in Rat Preadipocytes: Evidence for Sex and Site-Related Specificities and Possible Involvement of Insulin-Like Growth Factor 1 Receptor and Peroxisome Proliferator-Activated Receptorγ 2. *Endocrinology 141*, 649-656.

Diez J. J. & Iglesias P. (2003). The role of the novel adipocyte-derived hormone adiponectin in human disease. *European Journal of Endocrinology 148*, 293-300.

Digby J. E., McNeill E., Dyar O. J., Lam V., Greaves D. R., & Choudhury R. P. (2010). Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin. *Atherosclerosis 209*, 89-95.

Dimmeler S., Hermann C., & Zeiher A. (1998). Apoptosis of endothelial cells. Contribution to the pathophysiology of atherosclerosis? *European Cytokine Network 9*, 697-698.

Dimmeler S., Haendeler J., Nehls M., & Zeiher A. M. (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1β–converting enzyme (ICE)-like and cysteine protease protein (CPP)-32–like proteases. *The Journal of Experimental Medicine 185*, 601-608.

Dimova E. Y. & Kietzmann T. (2008). Metabolic, hormonal and environmental regulation of plasminogen activator inhibitor-1 (PAI-1) expression: lessons from the liver. *Thromb Haemost 100*, 992-1006.

Dinarello C. A., Donath M. Y., & Mandrup-Poulsen T. (2010). Role of IL-1 [beta] in type 2 diabetes. *Current Opinion in Endocrinology, Diabetes and Obesity 17*, 314-321.

Ding E. L., Song Y., Malik V. S., & Liu S. (2006). Sex differences of endogenous sex hormones and risk of type 2 diabetes. *JAMA: The Journal of the American Medical Association 295*, 1288-1299.

Divers S. L., McQuillan H. J., Matsubara H., Todo T., & Lokman P. M. (2010). Effects of reproductive stage and 11-ketotestosterone on LPL mRNA levels in the ovary of the shortfinned eel. *Journal of Lipid Research 51*, 3250-3258.

Dole E. & Holdsworth M. (1997). Nilutamide: an antiandrogen for the treatment of prostate cancer. *The Annals of Pharmacotherapy 31*, 65-75.

Dollery C. M. & Libby P. (2006). Atherosclerosis and proteinase activation. *Cardiovascular Research 69*, 625-635.

Donath M. Y. & Shoelson S. E. (2011). Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology 11*, 98-107.

Dong J., Fujii S., Imagawa S., Matsumoto S., Matsushita M., Todo S., Tsutsui H., & Sobel B. E. (2007). IL-1 and IL-6 induce hepatocyte plasminogen activator inhibitor-1 expression through independent signaling pathways converging on C/EBPδ. *American Journal of Physiology-Cell Physiology 292*, C209-C215.

Dougherty R. H., Rohrer J. L., Hayden D., Rubin S. D., & Leder B. Z. (2005). Effect of aromatase inhibition on lipids and inflammatory markers of cardiovascular disease in elderly men with low testosterone levels. *Clinical Endocrinology 62*, 228-235.

Du J., Zhang L., & Wang Z. (2009). Testosterone inhibits the activity of peroxisome proliferator-activated receptor γ in a transcriptional transaction assay. *Die Pharmazie-an International Journal of Pharmaceutical Sciences 64*, 694-695.

Dunger A., Cunningham J. M., Delaney C. A., Lowe J. E., Green M. H., Bone A. J., & Green I. C. (1996). Tumor necrosis factor-α and interferon-γ inhibit insulin secretion and cause DNA damage in unweaned-rat islets: extent of nitric oxide involvement. *Diabetes 45*, 183-189.

Dusserre E., Moulin P., & Vidal H. (2000). Differences in mRNA expression of the proteins secreted by the adipocytes in human subcutaneous and visceral adipose tissues. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease 1500*, 88-96.

Eberle D., Luk F. S., Kim R. Y., Olivas V. R., Kumar N., Posada J. M., Li K., Gaudreault N., Rapp J. H., & Raffai R. L. (2013). Inducible ApoE gene repair in hypomorphic ApoE mice deficient in the low-density lipoprotein receptor promotes atheroma stabilization with a human-like lipoprotein profile. *Arteriosclerosis, Thrombosis, and Vascular Biology 33*, 1759-1767.

Eckel R., Yost T., & Jensen D (1995). Alterations in lipoprotein lipase in insulin resistance. *Int J Obes Relat Metab Disord 19*, S16.

Eefting D., Schepers A., De Vries M. R., Pires N. M., Grimbergen J. M., Lagerweij T., Nagelkerken L. M., Monraats P. S., Jukema J. W., & van Bockel J. H. (2007). The effect of interleukin-10 knock-out and overexpression on neointima formation in hypercholesterolemic APOE* 3-Leiden mice. *Atherosclerosis 193*, 335-342.

Egashira K. (2003). Molecular mechanisms mediating inflammation in vascular disease special reference to monocyte chemoattractant protein-1. *Hypertension 41*, 834-841.

Egashira K., Zhao Q., Kataoka C., Ohtani K., Usui M., Charo I. F., Nishida K., Inoue S., Katoh M., & Ichiki T. (2002). Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circulation Research 90*, 1167-1172.

Elkind M. S., Tai W., Coates K., Paik M. C., & Sacco R. L. (2006). High-sensitivity C-reactive protein, lipoprotein-associated phospholipase A2, and outcome after ischemic stroke. *Archives of Internal Medicine 166*, 2073.

Emanuelli B., Vienberg S. G., Smyth G., Cheng C., Stanford K. I., Arumugam M., Michael M. D., Adams A. C., Kharitonenkov A., & Kahn C. R. (2014). Interplay between FGF21 and insulin action in the liver regulates metabolism. *The Journal of Clinical Investigation 124*, 0-0.

Emmelot-Vonk M. H., Verhaar H. J., Pour H. R. N., Aleman A., Lock T. M., Bosch J. R., Grobbee D. E., & van der Schouw, Yvonne T (2008). Effect of testosterone supplementation on functional mobility, cognition, and other parameters in older men: a randomized controlled trial. *Jama 299*, 39-52.

Enas E. A., Chacko V., Senthilkumar A., Puthumana N., & Mohan V. (2006). Elevated lipoprotein (a)—a genetic risk factor for premature vascular disease in people with and without standard risk factors: a review. *Disease-a-Month 52*, 5-50.

Enerbäck S. (2010). Human brown adipose tissue. *Cell Metabolism 11*, 248-252.

Engeland C. G., Sabzehei B., & Marucha P. T. (2009). Sex hormones and mucosal wound healing. *Brain, Behavior, and Immunity 23*, 629-635.

Engeli S., Böhnke J., Feldpausch M., Gorzelniak K., Janke J., Bátkai S., Pacher P., Harvey-White J., Luft F. C., & Sharma A. M. (2005). Activation of the peripheral endocannabinoid system in human obesity. *Diabetes 54*, 2838-2843.

Engström G., Hedblad B., Stavenow L., Lind P., Janzon L., & Lindgärde F. (2003). Inflammation-sensitive plasma proteins are associated with future weight gain. *Diabetes 52*, 2097-2101.

Epstein F. H. & Ross R. (1999). Atherosclerosis—an inflammatory disease. *New England Journal of Medicine 340*, 115-126.

Eriksson M., Schönland S., Yumlu S., Hegenbart U., von Hutten H., Gioeva Z., Lohse P., Büttner J., Schmidt H., & Röcken C. (2009). Hereditary apolipoprotein AI-associated amyloidosis in surgical pathology specimens: identification of three novel mutations in the APOA1 gene. *The Journal of Molecular Diagnostics 11*, 257-262.

Erqou S., Kaptoge S., Perry P. L., Di Angelantonio E., Thompson A., White I. R., Marcovina S. M., Collins R., Thompson S. G., & Danesh J. (2009). Lipoprotein (a) concentration and the risk of coronary heart disease, stroke, and nonvascular mortality. *JAMA: The Journal of the American Medical Association 302*, 412-423.

Erzengin F. (2014). New Hypotheses and Viewpoints for Atherosclerosis in 2013. *General Medicine: Open Access* .

Esposito K., Giugliano G., Scuderi N., & Giugliano D. (2006). Role of Adipokines in the Obesity–Inflammation Relationship: The Effect of Fat Removal. *Plastic and Reconstructive Surgery 118*, 1048-1057.

Esposito K., Pontillo A., Giugliano F., Giugliano G., Marfella R., Nicoletti G., & Giugliano D. (2003). Association of low interleukin-10 levels with the metabolic syndrome in obese women. *Journal of Clinical Endocrinology & Metabolism 88*, 1055-1058.

Esteve E., Ricart W., & Fernandez-Real J. M. (2005). Dyslipidemia and inflammation: an evolutionary conserved mechanism. *Clinical Nutrition 24*, 16-31.

Esteve E., Ricart W., & Fernández-Real J. M. (2009). Adipocytokines and Insulin Resistance The possible role of lipocalin-2, retinol binding protein-4, and adiponectin. *Diabetes Care 32*, S362-S367.

Everett, B., and Ridker, P (2008). Using inflammatory biomarkers to gide lipid therapy. *Current Cardiovascular Risk Reports 2*, 29-34.

Fahed A. C., Gholmieh J. M., & Azar S. T. (2012). Connecting the lines between hypogonadism and atherosclerosis. *International Journal of Endocrinology 2012*.

Fain J. N. (2006). Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. *Vitamins & Hormones 74*, 443-477.

Fain J. N., Madan A. K., Hiler M. L., Cheema P., & Bahouth S. W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology 145*, 2273-2282.

Falkenstein E., Tillmann H., Christ M., Feuring M., & Wehling M. (2000). Multiple actions of steroid hormones—a focus on rapid, nongenomic effects. *Pharmacological Reviews 52*, 513-556.

Fan Y., Wang Y., Tang Z., Zhang H., Qin X., Zhu Y., Guan Y., Wang X., Staels B., Chien S., & Wang N. (2008). Suppression of pro-inflammatory adhesion molecules by PPAR-delta in human vascular endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology 28*, 315-321.

Fantuzzi L., Borghi P., Ciolli V., Pavlakis G., Belardelli F., & Gessani S. (1999). Loss of CCR2 expression and functional response to monocyte chemotactic protein (MCP-1) during the differentiation of human monocytes: role of secreted MCP-1 in the regulation of the chemotactic response. *Blood 94*, 875-883. .

Faris J. E. & Smith M. R. (2010). Metabolic sequelae associated with androgen deprivation therapy for prostate cancer. *Current Opinion in Endocrinology, Diabetes and Obesity 17*, 240-246.

Fasshauer M, Klein J, Neumann S, Eszlinger M & Paschke R (2001). Adiponectin gene expression is inhibited by beta adrenergicstimulation via protein kinase A in 3T3-L1 adipocytes. *FEBS Letters 507*, 142-146.

Fazio S. & Linton M. F. (1996). Murine bone marrow transplantation as a novel approach to studying the role of macrophages in lipoprotein metabolism and atherogenesis. *Trends in Cardiovascular Medicine 6*, 58-65.

Fazio S., Babaev V. R., Burleigh M. E., Major A. S., Hasty A. H., & Linton M. F. (2002). Physiological expression of macrophage apoE in the artery wall reduces atherosclerosis in severely hyperlipidemic mice. *Journal of Lipid Research 43*, 1602-1609.

Fernández R. P. & Kaski J. C. (2002). Interleukin-10 and coronary disease. *Revista Española De Cardiología 55*, 738-750.

Ferrannini E., Sironi A. M., Iozzo P., & Gastaldelli A. (2008). Intra-abdominal adiposity, abdominal obesity, and cardiometabolic risk. *European Heart Journal Supplements 10*, B4-B10.

Ferrante A. (2007). Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *Journal of Internal Medicine 262*, 408-414.

Ferroni P., Basili S., Falco A., & Davì G. (2004). Inflammation, insulin resistance, and obesity. *Current Atherosclerosis Reports 6*, 424-431.

Festa A., D'Agostino R., Howard G., Mykkänen L., Tracy R. P., & Haffner S. M. (2000). Chronic subclinical inflammation as part of the insulin resistance syndrome the insulin resistance atherosclerosis study (IRAS). *Circulation 102*, 42-47.

Fielding C. J. & Fielding P. (1995). Molecular physiology of reverse cholesterol transport. *Journal of Lipid Research 36*, 211-228.

Figaro M. K., Kritchevsky S. B., Resnick H. E., Shorr R. I., Butler J., Shintani A., Penninx B. W., Simonsick E. M., Goodpaster B. H., & Newman A. B. (2006). Diabetes, Inflammation, and Functional Decline in Older Adults Findings from the Health, Aging and Body Composition (ABC) study. *Diabetes Care 29*, 2039-2045.

Filip-Ciubotaru F., Foia L., Manciuc C., & Grigore C. (2011) PPARs: structure, mechanisms of action and control. *Rev Med Chir Soc Med Nat Iasi*, 115; 477-84.

Finch C. E. & Crimmins E. M. (2004). Inflammatory exposure and historical changes in human life-spans. *Science (New York, N.Y.) 305*, 1736-1739.

Fong A. M., Robinson L. A., Steeber D. A., Tedder T. F., Yoshie O., Imai T., & Patel D. (1998). Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *The Journal of Experimental Medicine 188*, 1413-1419.

Ford E. S. (2003). The metabolic syndrome and C-reactive protein, fibrinogen, and leukocyte count: findings from the Third National Health and Nutrition Examination Survey. *Atherosclerosis 168*, 351-358.

Fraley A. E. & Tsimikas S. (2006). Clinical applications of circulating oxidized low-density lipoprotein biomarkers in cardiovascular disease. *Current Opinion in Lipidology 17*, 502-509.

Francis M. C. & Frohlich J. J. (2001). Coronary artery disease in patients at low risk—apolipoprotein AI as an independent risk factor. *Atherosclerosis 155*, 165-170.

Fraticelli P., Sironi M., Bianchi G., D'Ambrosio D., Albanesi C., Stoppacciaro A., Chieppa M., Allavena P., Ruco L., & Girolomoni G. (2001). Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. *Journal of Clinical Investigation 107*, 1173-1181.

Frayn K., Arner P., & Yki-Jarvinen H. (2006). Fatty acid metabolism in adipose tissue, muscle and liver in health and disease. *Essays Biochem 42*, 89-103

Frederiksen L., Nielsen T., Wraae K., Hagen C., Frystyk J., Flyvbjerg A., Brixen K., & Andersen M. (2009). Subcutaneous rather than visceral adipose tissue is associated with adiponectin levels and insulin resistance in young men. *Journal of Clinical Endocrinology & Metabolism 94*, 4010-4015.

Frederiksen L., Højlund K., Hougaard D. M., Brixen K., & Andersen M. (2012a). Testosterone therapy increased muscle mass and lipid oxidation in aging men. *Age 34*, 145-156.

Frederiksen L., Højlund K., Hougaard D. M., Mosbech T. H., Larsen R., Flyvbjerg A., Frystyk J., Brixen K., & Andersen M. (2012b). Testosterone therapy decreases subcutaneous fat and adiponectin in aging men. *European Journal of Endocrinology 166*, 469-476.

Fried S. K., Bunkin D. A., & Greenberg A. S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *Journal of Clinical Endocrinology & Metabolism 83*, 847-850.

Fröhlich M., Imhof A., Berg G., Hutchinson W. L., Pepys M. B., Boeing H., Muche R., Brenner H., & Koenig W. (2000). Association between C-reactive protein and features of the metabolic syndrome: a population-based study. *Diabetes Care 23*, 1835-1839.

Frostegård J. (2013). Immunity, atherosclerosis and cardiovascular disease. *BMC Medicine 11*, 117.

Fruebis J., Tsao T., Javorschi S., Ebbets-Reed D., Erickson M. R. S., Yen F. T., Bihain B. E., & Lodish H. F. (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proceedings of the National Academy of Sciences 98*, 2005-2010.

Fruhbeck G., Gomez-Ambrosi J., Muruzabal F. J., & Burrell M. A. (2001). The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *American Journal of Physiology.Endocrinology and Metabolism 280*, E827-47.

Fu L., Gao Q., & Shen J. (2008). Relationship between testosterone and indexes indicating endothelial function in male coronary heart disease patients. *Asian Journal of Andrology 10*, 214-218.

Fuentes L., Rőszer T., & Ricote M. (2010). Inflammatory mediators and insulin resistance in obesity: role of nuclear receptor signaling in macrophages. *Mediators of Inflammation 2010.*

Fujikawa R., Ito C., Nakashima R., Orita Y., & Ohashi N. (2008). Is there any association between subcutaneous adipose tissue area and plasma total and high molecular weight adiponectin levels? *Metabolism 57*, 506-510.

Furukawa Y., Matsumori A., Ohashi N., Shioi T., Ono K., Harada A., Matsushima K., & Sasayama S. (1999). Anti–monocyte chemoattractant protein-1/monocyte chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. *Circulation Research 84*, 306-314.

Gajda A. M., Pellizzon M. A., Ricci M. R., & Ulman E. A. (2007). Diet-induced metabolic syndrome in rodent models. *Animal Lab News 74*, 775-793.

Gallagher S. R. & Desjardins P. R. (2007). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Human Genetics* , A. 3D. 1-A. 3D. 21.

Gametchu B., Watson C. S., & Pasko D. (1991). Size and steroid-binding characterization of membrane-associated glucocorticoid receptor in S-49 lymphoma cells. *Steroids 56*, 402-410.

Gannon P. O., Godin-Ethier J., Hassler M., Delvoye N., Aversa M., Poisson A. O., Péant B., Fahmy M. A., Saad F., & Lapointe R. (2010). Androgen-regulated expression of arginase 1, arginase 2 and interleukin-8 in human prostate cancer. *PloS One 5*, e12107.

Garaulet M., Perex-Llamas F., Fuente T., Zamora S., & Tebar F. J. (2000). Anthropometric, computed tomography and fat cell data in an obese population: relationship with insulin, leptin, tumor necrosis factor-alpha, sex hormone-binding globulin and sex hormones. *European Journal of Endocrinology / European Federation of Endocrine Societies 143*, 657-666.

Garcia E., Lacasa M., Agli B., Giudicelli Y., & Lacasa D. (1999). Modulation of rat preadipocyte adipose conversion by androgenic status: involvement of C/EBPs transcription factors. *Journal of Endocrinology 161*, 89-97.

Garcia G. E., Xia Y., Chen S., Wang Y., Ye R. D., Harrison J. K., Bacon K. B., Zerwes H., & Feng L. (2000). NF-kappaB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1beta, TNF-alpha, and LPS. *Journal of Leukocyte Biology 67*, 577-584.

Garfagnini A, Devoto G, Rosselli P (1995). Relationship between HDL-C and apolipoprotein A-I and the severity of coronary artery disease . *Eur Heart J 16*, 465-70.

Garg M. K., Dutta M. K., & Mahalle N. (2012). Adipokines (adiponectin and plasminogen activator inhhibitor-1) in metabolic syndrome. *Indian Journal of Endocrinology and Metabolism 16*, 116-123.

Gaudreault N., Kumar N., Posada J. M., Stephens K. B., Reyes de Mochel N. S., Eberle D., Olivas V. R., Kim R. Y., Harms M. J., Johnson S., Messina L. M., Rapp J. H., & Raffai R. L. (2012). ApoE suppresses atherosclerosis by reducing lipid accumulation in circulating monocytes and the expression of inflammatory molecules on monocytes and vascular endothelium. *Arteriosclerosis, Thrombosis, and Vascular Biology 32*, 264-272.

Gaw A., Murray H. M., & Brown E. (2005). Plasma lipoprotein (a)[Lp (a)] concentrations and cardiovascular events in the elderly: evidence from the prospective study of pravastatin in the elderly at risk (PROSPER). *Atherosclerosis 180*, 381-388.

Genest J. & Pedersen T. R. (2003). Prevention of cardiovascular ischemic events high-risk and secondary prevention. *Circulation 107*, 2059-2065.

Gerrity R. (1981). The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *The American Journal of Pathology 103*, 181.

Gerrity R., Goss J., & Soby L. (1985). Control of monocyte recruitment by chemotactic factor (s) in lesion-prone areas of swine aorta. *Arteriosclerosis, Thrombosis, and Vascular Biology 5*, 55-66.

Gerszten R. E., Garcia-Zepeda E. A., Lim Y., Yoshida M., Ding H. A., Gimbrone M. A., Luster A. D., Luscinskas F. W., & Rosenzweig A. (1999). MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature 398*, 718-723.

Gesta S., Tseng Y., & Kahn C. R. (2007). Developmental origin of fat: tracking obesity to its source. *Cell 131*, 242-256.

Getz G. S. & Reardon C. A. (2009). Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. *Journal of Lipid Research 50 Suppl*, S156-61.

Gilbert S. F. (2000). Chromosomal sex determination in mammals. developmental biology, 2th ed,Sunderland: Sinauer; [http://www.ncbi.nlm.nih.gov/books/NBK9967].

Giltay E., Fonk J., Von Blomberg B., Drexhage H., Schalkwijk C., & Gooren L. (2000). In vivo effects of sex steroids on lymphocyte responsiveness and immunoglobulin levels in humans. *Journal of Clinical Endocrinology & Metabolism 85*, 1648-1657.

Glass C. (2002). The macrophage foam cell as a target for therapeutic intervention. *Nat Med 8*, 1235-1242.

Glass C. K. & Witztum J. L. (2001). Atherosclerosis: the road ahead. *Cell 104*, 503-516.

Glueck C., Glueck H., Stroop D., Speirs J., Hamer T., & Tracy T. (1993). Endogenous testosterone, fibrinolysis, and coronary heart disease risk in hyperlipidemic men. *The Journal of Laboratory and Clinical Medicine 122*, 412-420.

Goglia L., Tosi V., Sanchez A., Flamini M., Fu X., Zullino S., Genazzani A., & Simoncini T. (2010). Endothelial regulation of eNOS, PAI-1 and t-PA by testosterone and dihydrotestosterone in vitro and in vivo. *Molecular Human Reproduction 16*, 761-769.

Goh H., Loke D., & Ratnam S. (1995). The impact of long-term testosterone replacement therapy on lipid and lipoprotein profiles in women. *Maturitas 21*, 65-70.

Goldstein J. L. & Wilson J. D. (1972). Studies on the pathogenesis of the pseudohermaphroditism in the mouse with testicular feminization. *Journal of Clinical Investigation 51*, 1647.

Golias C., Tsoutsi E., Matziridis A., Makridis P., Batistatou A., & Charalabopoulos K. (2007). Review. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. *In Vivo (Athens, Greece) 21*, 757-769.

Gooren L. (2003). Androgen deficiency in the aging male: benefits and risks of androgen supplementation. *The Journal of Steroid Biochemistry and Molecular Biology 85*, 349-355.

Gooren J., & Bunck C. (2004). Androgen replacement therapy: present and future. *Drugs* 64,1861-91.

Gooren L. & Behre H. (2008). Testosterone treatment of hypogonadal men participating in competitive sports. *Andrologia 40*, 195-199.

Gooren, A. Haider, F. Saad (2008). Change of waist circumference by baseline testosterone levels in 95 hypogonadal men (T: 5.9–12.1 nmol/L) treated with parenteral testosterone undecanoate. *Endocrine Society Meeting Abstract Book* , 461-462.

Goossens G. H. (2008). The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiology & Behavior 94*, 206-218.

Gonzalez M., del Mar Bibiloni M., Pons A., Llompart I., & Tur J. (2012). Inflammatory markers and metabolic syndrome among adolescents. *European Journal of Clinical Nutrition 66*, 1141-1145.

Gordon S. (2003). Alternative activation of macrophages. *Nature Reviews Immunology 3*, 23-35.

Gornstein R. A., Lapp C. A., Bustos-Valdes S. M., & Zamorano P. (1999). Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *Journal of Periodontology 70*, 604-609.

Gornstein R. A., Lapp C. A., Bustos-Valdes S. M., & Zamorano P. (1999). Androgens modulate interleukin-6 production by gingival fibroblasts in vitro. *Journal of Periodontology 70*, 604-609.

Gosling J., Slaymaker S., Gu L., Tseng S., Zlot C. H., Young S. G., Rollins B. J., & Charo I. F. (1999). MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. *Journal of Clinical Investigation 103*, 773-778.

Gouni-Berthold I. & K Berthold H. (2011). Lipoprotein (a): current perspectives. *Current Vascular Pharmacology 9*, 682-692.

Graham T. L., Mookherjee C., Suckling K. E., A Palmer C. N., & Patel L. (2005). The PPARδ agonist GW0742X reduces atherosclerosis in LDLR< sup>−/−</sup> mice. *Atherosclerosis 181*, 29-37.

Greaves D. R. & Gordon S. (2009). The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. *Journal of Lipid Research 50*, S282-S286.

Greaves D. R., Häkkinen T., Lucas A. D., Liddiard K., Jones E., Quinn C. M., Senaratne J., Green F. R., Tyson K., & Boyle J. (2001). Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus-and activation-regulated chemokine, are expressed in human atherosclerotic lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology 21*, 923-929.

Green S. R., Han K. H., Chen Y., Almazan F., Charo I. F., Miller Y. I., & Quehenberger O. (2006). The CC chemokine MCP-1 stimulates surface expression of CX3CR1 and enhances the adhesion of monocytes to fractalkine/CX3CL1 via p38 MAPK. *Journal of Immunology (Baltimore, Md.: 1950) 176*, 7412-7420.

Greenow K., Pearce N. J., & Ramji D. P. (2005). The key role of apolipoprotein E in atherosclerosis. *Journal of Molecular Medicine 83*, 329-342.

Griggs R. C., Kingston W., Jozefowicz R. F., Herr B. E., Forbes G., & Halliday D. (1989). Effect of testosterone on muscle mass and muscle protein synthesis. *J Appl Physiol 66*, 498-503.

Gropler, R., and Peterson, L. (2010). Adipose Tissue Imaging: The Potential and the Challenge. *JACC Cardiovasc Imaging, 3*, 852.

Grossmann M. (2011). Low testosterone in men with type 2 diabetes: significance and treatment. *Journal of Clinical Endocrinology & Metabolism 96*, 2341-2353.

Grossmann M., Thomas M. C., Panagiotopoulos S., Sharpe K., MacIsaac R. J., Clarke S., Zajac J. D., & Jerums G. (2008). Low testosterone levels are common and associated with insulin resistance in men with diabetes. *Journal of Clinical Endocrinology & Metabolism 93*, 1834-1840.

Gruzdeva O., Uchasova E., Dyleva Y., Belik E., Shurygina E., & Barbarash O. (2013). Plasminogen activator inhibitor-1, free fatty acids, and insulin resistance in patients with myocardial infarction. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 6; 293-301.

Gu L., Okada Y., Clinton S. K., Gerard C., Sukhova G. K., Libby P., & Rollins B. J. (1998). Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor–deficient mice. *Molecular Cell 2*, 275-281.

Guerre-Millo M. (2002). Adipose tissue hormones. *Journal of Endocrinological Investigation 25*, 855-861.

Gui Y., Silha J. V., & Murphy L. J. (2004). Sexual dimorphism and regulation of resistin, adiponectin, and leptin expression in the mouse. *Obesity Research 12*, 1481-1491.

Guilherme A., Virbasius J. V., Puri V., & Czech M. P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature Reviews Molecular Cell Biology 9*, 367-377.

Guillermo E. (2002). Diabetic Ketoacidosis and Hyper glycemic Hyperosmolar Syndrome. *Diabetes Spectrum 15*, 28-36.

Guo J., Van Eck M., Twisk J., Maeda N., Benson G. M., Groot P. H., & Van Berkel T. J. (2003). Transplantation of monocyte CC-chemokine receptor 2–deficient bone marrow into ApoE3–Leiden mice inhibits atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology 23*, 447-453.

Gupta S., Pablo A. M., c Jiang X., Wang N., Tall A. R., & Schindler C. (1997). IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *Journal of Clinical Investigation 99*, 2752.

Gustafson B., Hammarstedt A., Andersson C. X., & Smith U. (2007). Inflamed Adipose Tissue A Culprit Underlying the Metabolic Syndrome and Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 27*, 2276-2283.

Gygi S. P., Rochon Y., Franza B. R., & Aebersold R. (1999). Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology 19*, 1720-1730.

Gyllenborg J., Rasmussen S. L., Borch-Johnsen K., Heitmann B. L., & Juul A. (2001). Cardiovascular risk factors in men: the role of gonadal steroids and sex hormone-binding globulin. *Metabolism 50*, 882-888.

Haffner J. (1996). Androgens in relation to cardiovascular disease and insulin resistance in aging men. *Androgens and the Aging Male.New York, London: Parthenon Publishing Group* , 65-84.

Haffner S. M., Miettinen H., Karhapää P., Mykkänen L., & Laakso M. (1997). Leptin concentrations, sex hormones, and cortisol in nondiabetic men. *Journal of Clinical Endocrinology & Metabolism 82*, 1807-1809.

Haffner S. M., Mykkanen L., Gruber K. K., Rainwater D. L., & Laakso M. (1994). Lack of association between sex hormones and Lp (a) concentrations in American and Finnish men. *Arteriosclerosis, Thrombosis, and Vascular Biology 14*, 19-24.

Haidar A., Yassin A., Saad F., & Shabsigh R. (2007). Effects of androgen deprivation on glycaemic control and on cardiovascular biochemical risk factors in men with advanced prostate cancer with diabetes. *The Aging Male 10*, 189-196.

Haider A., Gooren L., Padungtod P., & Saad F. (2010). Improvement of the metabolic syndrome and of non-alcoholic liver steatosis upon treatment of hypogonadal elderly men with parenteral testosterone undecanoate. *Experimental and Clinical Endocrinology & Diabetes 118*, 167.

Hajer G. R., van Haeften T. W., & Visseren F. L. (2008). Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European Heart Journal 29*, 2959-2971.

Hak A. E., Witteman J. C., de Jong F. H., Geerlings M. I., Hofman A., & Pols H. A. (2002). Low levels of endogenous androgens increase the risk of atherosclerosis in elderly men: the Rotterdam study. *Journal of Clinical Endocrinology & Metabolism 87*, 3632-3639.

Hall S. A., Esche G. R., Araujo A. B., Travison T. G., Clark R. V., Williams R. E., & McKinlay J. B. (2008). Correlates of low testosterone and symptomatic androgen deficiency in a population-based sample. *The Journal of Clinical Endocrinology & Metabolism 93*, 3870-3877.

Haller F., Kulle B., Schwager S., Gunawan B., Heydebreck A. v., Sültmann H., & Füzesi L. (2004). Equivalence test in quantitative reverse transcription polymerase chain reaction: confirmation of reference genes suitable for normalization. *Analytical Biochemistry 335*, 1-9.

Hamilton E., Gianatti E., Strauss B., Wentworth J., Lim-Joon D., Bolton D., Zajac J., & Grossmann M. (2011). Increase in visceral and subcutaneous abdominal fat in men with prostate cancer treated with androgen deprivation therapy. *Clinical Endocrinology 74*, 377-383.

Han K. H., Tangirala R. K., Green S. R., & Quehenberger O. (1998). Chemokine Receptor CCR2 Expression and Monocyte Chemoattractant Protein-1–Mediated Chemotaxis in Human Monocytes A Regulatory Role for Plasma LDL. *Arteriosclerosis, Thrombosis, and Vascular Biology 18*, 1983-1991.

Han X. (2004). The role of apolipoprotein E in lipid metabolism in the central nervous system. *Cellular and Molecular Life Sciences CMLS 61*, 1896-1906.

Han K. H., Chang M. K., Boullier A., Green S. R., Li A., Glass C. K., & Quehenberger O. (2000). Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator-activated receptor gamma. *The Journal of Clinical Investigation 106*, 793-802.

Hansson G. K. (2009). Atherosclerosis—an immune disease: the Anitschkov Lecture 2007. *Atherosclerosis 202*, 2-10.

Hansson G. K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine 352*, 1685-1695.

Hansson G. K. (2001). Immune mechanisms in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 21*, 1876-1890.

Hansson G. K. & Hermansson A. (2011). The immune system in atherosclerosis. *Nature Immunology 12*, 204-212.

Hansson G. K. & Libby P. (2006). The immune response in atherosclerosis: a double-edged sword. *Nature Reviews Immunology 6*, 508-519.

Hara M., Matsushima T., Satoh H., Iso-o N., Noto H., Togo M., Kimura S., Hashimoto Y., & Tsukamoto K. (2003). Isoform-dependent cholesterol efflux from macrophages by apolipoprotein E is modulated by cell surface proteoglycans. *Arteriosclerosis, Thrombosis, and Vascular Biology 23*, 269-274.

Hargrove G., Junco A., & Wong N. (1999). Hormonal regulation of apolipoprotein AI. *Journal of Molecular Endocrinology 22*, 103-111.

Hashemi M., Saadat M., Behjati M., & Kelishadi R. (2012). Comparison of Serum Apolipoprotein Levels of Diabetic Children and Healthy Children with or without Diabetic Parents. *Cholesterol 2012*.

Hatakeyama H., Nishizawa M., Nakagawa A., Nakano S., Kigoshi T., & Uchida K. (2002). Testosterone inhibits tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. *FEBS Letters 530*, 129-132.

Hausman G. J., Campion D. R., & Martin R. J. (1980). Search for the adipocyte precursor cell and factors that promote its differentiation. *Journal of Lipid Research 21*, 657-670.

Haworth C., Brennan F. M., Chantry D., Turner M., Maini R. N., & Feldmann M. (1991). Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: Regulation by tumor necrosis factor-α. *European Journal of Immunology 21*, 2575-2579.

Hayes M. P., Freeman S. L., & Donnelly R. P. (1995). IFN-γ priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and MRNA stability. *Cytokine 7*, 427-435.

He T., Chan T. A., Vogelstein B., & Kinzler K. W. (1999). PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell 99*, 335-345.

He W. W., Kumar M. V., & Tindall D. J. (1991). A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicularfeminized mouse. *Nucleic Acids Research 19*, 2373-2378.

Heeren J., Beisiegel U., & Grewal T. (2006). Apolipoprotein E recycling: implications for dyslipidemia and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 26*, 442-448.

Heeschen C., Dimmeler S., Hamm C. W., Fichtlscherer S., Boersma E., Simoons M. L., Zeiher A. M., & CAPTURE Study Investigators (2003). Serum level of the antiinflammatory cytokine interleukin-10 is an important prognostic determinant in patients with acute coronary syndromes. *Circulation 107*, 2109-2114.

Heinlein C. A. & Chang C. (2002). The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Molecular Endocrinology 16*, 2181-2187.

Henrichot E., Juge-Aubry C. E., Pernin A., Pache J. C., Velebit V., Dayer J. M., Meda P., Chizzolini C., & Meier C. A. (2005). Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arteriosclerosis, Thrombosis, and Vascular Biology 25*, 2594-2599.

Henriksson P., Angelin B., & Berglund L. (1992). Hormonal regulation of serum Lp (a) levels. Opposite effects after estrogen treatment and orchidectomy in males with prostatic carcinoma. *Journal of Clinical Investigation 89*, 1166.

Henriksson P., Einarsson K., Eriksson A., Kelter U., & Angelin B. (1989). Estrogen-induced gallstone formation in males. Relation to changes in serum and biliary lipids during hormonal treatment of prostatic carcinoma. *Journal of Clinical Investigation 84*, 811.

Herbst K. L., Amory J. K., Brunzell J. D., Chansky H. A., & Bremner W. J. (2003). Testosterone administration to men increases hepatic lipase activity and decreases HDL and LDL size in 3 wk. *American Journal of Physiology-Endocrinology and Metabolism 284*, E1112-E1118.

Herder C., Baumert J., Thorand B., Martin S., Löwel H., Kolb H., & Koenig W. (2006). Chemokines and Incident Coronary Heart Disease Results From the MONICA/KORA Augsburg Case-Cohort Study, 1984–2002. *Arteriosclerosis, Thrombosis, and Vascular Biology 26*, 2147-2152.

Hermans P. & Hazelzet J. A. (2005). Plasminogen activator inhibitor type 1 gene polymorphism and sepsis. *Clinical Infectious Diseases 41*, S453-S458.

Hess K. & Grant P. J. (2011). Inflammation and thrombosis in diabetes. *Thrombosis and Haemostasis 105*, S43.

Heufelder A. E., Saad F., Bunck M. C., & Gooren L. (2009). Fifty-two—Week Treatment With Diet and Exercise Plus Transdermal Testosterone Reverses the Metabolic Syndrome and Improves Glycemic Control in Men With Newly Diagnosed Type 2 Diabetes and Subnormal Plasma Testosterone. *Journal of Andrology 30*, 726-733.

Hiipakka R. A. & Liao S. (1998). Molecular mechanism of androgen action. *Trends in Endocrinology & Metabolism 9*, 317-324.

Hirano T., Nohtomi K., Koba S., Muroi A., & Ito Y. (2008). A simple and precise method for measuring HDL-cholesterol subfractions by a single precipitation followed by homogenous HDL-cholesterol assay. *Journal of Lipid Research 49*, 1130-1136.

Hodgin J. B. & Maeda N. (2002). Minireview: estrogen and mouse models of atherosclerosis. *Endocrinology 143*, 4495-4501.

Hoekstra M., Kruijt J. K., Van Eck M., & Van Berkel T. J. (2003). Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells. *Journal of Biological Chemistry 278*, 25448-25453.

Hofbauer L. C., Ten R. M., & Khosla S. (1999). The anti-androgen hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line. *Journal of Bone and Mineral Research 14*, 1330-1337.

Hoffstedt J., Arvidsson E., Sjölin E., Wåhlén K., & Arner P. (2004). Adipose tissue adiponectin production and adiponectin serum concentration in human obesity and insulin resistance. *Journal of Clinical Endocrinology & Metabolism 89*, 1391-1396.

Holme I., Aastveit A. H., Hammar N., Jungner I., & Walldius G. (2010). Inflammatory markers, lipoprotein components and risk of major cardiovascular events in 65,005 men

and women in the apolipoprotein mortality risk study (AMORIS). *Atherosclerosis* 213, 299-305.

Holvoet P., Lee D., Steffes M., Gross M., & Jacobs Jr D. R. (2008). Association between circulating oxidized low-density lipoprotein and incidence of the metabolic syndrome. *JAMA: The Journal of the American Medical Association 299*, 2287-2293.

Hong E., Ko H. J., Cho Y., Kim H., Ma Z., Tim Y. Y., Friedline R. H., Kurt-Jones E., Finberg R., & Fischer M. A. (2009). Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle. *Diabetes 58*, 2525-2535.

Horvath C., Welt F. G., Nedelman M., Rao P., & Rogers C. (2002). Targeting CCR2 or CD18 Inhibits Experimental In-Stent Restenosis in Primates Inhibitory Potential Depends on Type of Injury and Leukocytes Targeted. *Circulation Research 90*, 488-494.

Hosogai N., Fukuhara A., Oshima K., Miyata Y., Tanaka S., Segawa K., Furukawa S., Tochino Y., Komuro R., & Matsuda M. (2007). Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes 56*, 901-911.

Hossain P., Kawar B., & El Nahas M. (2007). Obesity and diabetes in the developing world—a growing challenge. *New England Journal of Medicine 356*, 213-215.

Hotamisligil G. (1999). The role of TNFα and TNF receptors in obesity and insulin resistance. *Journal of Internal Medicine 245*, 621-625.

Hotamisligil. G, Peraldi. P, Budavari .A, Ellis.R, White .M, Spiegelman (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α and obesity-induced insulin resistance. *Science, 271*, 665-668.

Hotamisligil G. S., Shargill N. S., & Spiegelman B. M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science (New York, N.Y.) 259*, 87-91.

Hotamisligil G. S., Peraldi P., Budavari A., Ellis R., White M. F., & Spiegelman B. (1996). IRS-1-Mediated Inhibition of Insulin Receptor Tyrosine Kinase Activity in TNF-α and Obesity-Induced Insulin Resistance. *Science-New York then Washington-* , 665-667.

Hotta K., Funahashi T., Arita Y., Takahashi M., Matsuda M., Okamoto Y., Iwahashi H., Kuriyama H., Ouchi N., & Maeda K. (2000). Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arteriosclerosis, Thrombosis, and Vascular Biology 20*, 1595-1599.

Howden K. J. (2004). Androgen insensitivity syndrome in a thoroughbred mare (64, XY—testicular feminization). *The Canadian Veterinary Journal 45*, 501.

Howell S. J., Radford J. A., Adams J. E., Smets E., Warburton R., & Shalet S. M. (2001). Randomized placebo-controlled trial of testosterone replacement in men with mild Leydig cell insufficiency following cytotoxic chemotherapy. *Clinical Endocrinology 55*, 315-324.

Hu F. B., Meigs J. B., Li T. Y., Rifai N., & Manson J. E. (2004). Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes 53*, 693-700.

Huang Z. H., Reardon C. A., Subbaiah P. V., Getz G. S., & Mazzone T. (2013). ApoE derived from adipose tissue does not suppress atherosclerosis or correct hyperlipidemia in apoE knockout mice. *Journal of Lipid Research 54*, 202-213.

Huang Y., Li T., Sane D. C., & Li L. (2004). IRAK1 serves as a novel regulator essential for lipopolysaccharide-induced interleukin-10 gene expression. *The Journal of Biological Chemistry 279*, 51697-51703.

Huggett J., Dheda K., Bustin S., & Zumla A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity 6*, 279-284.

Hughes IA D. A. (2006). Androgen resistance. *Best Pract Res Clin Endocrinol Metab 20*, 577-98.

Huo Y., Weber C., Forlow S., Sperandio M., Thatte J., Mack M., Jung S., Littman D. R., & Ley K. (2001). The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium. *Journal of Clinical Investigation 108*, 1307-1314.

Huoya M. d. O., Penalva R. A., Alves S. R., Feitosa G. S., Gadelha S., & Ladeia A. M. T. (2009). Comparison of inflammatory biomarkers between diabetic and non-diabetic patients with unstable angina. *Arquivos Brasileiros De Cardiologia 92*, 283-289.

Hwang S., Ballantyne C. M., Sharrett A. R., Smith L. C., Davis C. E., Gotto A. M., & Boerwinkle E. (1997). Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases the Atherosclerosis Risk In Communities (ARIC) study. *Circulation 96*, 4219-4225.

Ichikawa T., Unoki H., Sun H., Shimoyamada H., Marcovina S., Shikama H., Watanabe T., & Fan J. (2002). Lipoprotein (a) promotes smooth muscle cell proliferation and dedifferentiation in atherosclerotic lesions of human apo (a) transgenic rabbits. *The American Journal of Pathology 160*, 227-236.

Ignatenko L. L., Mataradze G. D., & Rozen V. B. (1992). Endocrine mechanisms for the formation of sex-related differences in hepatic estrogen receptor content and their significance for the realization of an estrogen effect on angiotensinogen blood level in rats. *Hepatology 15*, 1092-1098.

Ikejima H., Imanishi T., Tsujioka H., Kashiwagi M., Kuroi A., Tanimoto T., Kitabata H., Ishibashi K., Komukai K., Takeshita T., & Akasaka T. (2010). Upregulation of fractalkine and its receptor, CX3CR1, is associated with coronary plaque rupture in patients with unstable angina pectoris. *Circulation Journal : Official Journal of the Japanese Circulation Society 74*, 337-345.

Im S. S., Kim M. Y., Kwon S. K., Kim T. H., Bae J. S., Kim H., Kim K. S., Oh G. T., & Ahn Y. H. (2011). Peroxisome proliferator-activated receptor {alpha} is responsible for the up-regulation of hepatic glucose-6-phosphatase gene expression in fasting and db/db Mice. *The Journal of Biological Chemistry 286*, 1157-1164.

Imai T., Hieshima K., Haskell C., Baba M., Nagira M., Nishimura M., Kakizaki M., Takagi S., Nomiyama H., & Schall T. J. (1997). Identification and Molecular Characterization of Fractalkine Receptor CX< sub> 3</sub> CR1, which Mediates Both Leukocyte Migration and Adhesion. *Cell 91*, 521-530.

Imaizumi T., Matsumiya T., Fujimoto K., Okamoto K., Cui X., Ohtaki U., Yoshida H., & Satoh K. (2000). Interferon-γ stimulates the expression of CX3CL1/fractalkine in cultured human endothelial cells. *The Tohoku Journal of Experimental Medicine 192*, 127-139.

Inouye K. E., Shi H., Howard J. K., Daly C. H., Lord G. M., Rollins B. J., & Flier J. S. (2007). Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes 56*, 2242-2250.

Inzucchi S., Bergenstal R., Buse J., Diamant M., Ferrannini E., Nauck M., Peters A., Tsapas A., Wender R., & Matthews D. (2012). American Diabetes Association (ADA); European Association for the Study of Diabetes (EASD). Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care 35*, 1364-1379.

Iribarren C., Husson G., Go A. S., Lo J. C., Fair J. M., Rubin G. D., Hlatky M. A., & Fortmann S. P. (2007). Plasma leptin levels and coronary artery calcification in older adults. *Journal of Clinical Endocrinology & Metabolism 92*, 729-732.

Ishibashi S., Goldstein J., Brown M., Herz J., & Burns D. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *Journal of Clinical Investigation 93*, 1885.

Ishibashi S., Brown M. S., Goldstein J. L., Gerard R. D., Hammer R. E., & Herz J. (1993). Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *Journal of Clinical Investigation 92*, 883.

Ishigaki Y., Oka Y., & Katagiri H. (2009). Circulating oxidized LDL: a biomarker and a pathogenic factor. *Current Opinion in Lipidology 20*, 363-369.

Isidori A. M., Caprio M., Strollo F., Moretti C., Frajese G., Isidori A., & Fabbri A. (1999). Leptin and androgens in male obesity: evidence for leptin contribution to reduced androgen levels. *Journal of Clinical Endocrinology & Metabolism 84*, 3673-3680.

Isidori A. M., Giannetta E., Greco E. A., Gianfrilli D., Bonifacio V., Isidori A., Lenzi A., & Fabbri A. (2005). Effects of testosterone on body composition, bone metabolism and serum lipid profile in middle-aged men: a meta-analysis. *Clinical Endocrinology 63*, 280-293.

Isidori A. M., Strollo F., Morè M., Caprio M., Aversa A., Moretti C., Frajese G., Riondino G., & Fabbri A. (2000). Leptin and aging: correlation with endocrine changes in male and female healthy adult populations of different body weights. *The Journal of Clinical Endocrinology & Metabolism 85*, 1954-1962.

Issemann I., Prince R., Tugwood J., & Green S. (1993). The retinoid X receptor enhances the function of the peroxisome proliferator activated receptor. *Biochimie 75*, 251-256.

Itabe H. (1998). Oxidized phospholipids as a new landmark in atherosclerosis. *Progress in Lipid Research 37*, 181-207.

Itabe H., Obama T., & Kato R. (2011). The dynamics of oxidized LDL during atherogenesis. *Journal of Lipids 2011*.

Itabe H. & Ueda M. (2007). Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *Journal of Atherosclerosis and Thrombosis 14*, 1-11.

Izadi V., Farabad E., & Azadbakht L. (2012). Serum adiponectin level and different kinds of cancer: a review of recent evidence. *International Scholarly Research Notices 2012*.

Jackson G. (2011). What makes a good editorial? *International Journal of Clinical Practice 65*, 913.

Jackson T. A., Richer J. K., Bain D. L., Takimoto G. S., Tung L., & Horwitz K. B. (1997). The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Molecular Endocrinology 11*, 693-705.

Jagannathachary S. & Kamaraj D. (2010). Obesity and periodontal disease. *Journal of Indian Society of Periodontology 14*, 96-100.

Jamieson W. L., Shimizu S., D'Ambrosio J. A., Meucci O., & Fatatis A. (2008). CX3CR1 is expressed by prostate epithelial cells and androgens regulate the levels of CX3CL1/fractalkine in the bone marrow: potential role in prostate cancer bone tropism. *Cancer Research 68*, 1715-1722.

Jensen M. D. (2006). Adipose tissue as an endocrine organ: implications of its distribution on free fatty acid metabolism. *European Heart Journal Supplements* 8, B13-B19.

Jiang R., Schulze M. B., Li T., Rifai N., Stampfer M. J., Rimm E. B., & Hu F. B. (2004). Non-HDL cholesterol and apolipoprotein B predict cardiovascular disease events among men with type 2 diabetes. *Diabetes Care 27*, 1991-1997.

Jiangtao L., Dongchen Z., Shudong X., Yunpeng S., Lihong W., Liangrong Z., & Jianhua Z. (2009). Reduced testosterone levels in males with lone atrial fibrillation. *Clinical Cardiology 32*, 43-46.

Jin H., Lin J., Fu L., Mei Y., Peng G., Tan X., Wang D., Wang W., & Li Y. (2007). Physiological testosterone stimulates tissue plasminogen activator and tissue factor pathway inhibitor and inhibits plasminogen activator inhibitor type 1 release in endothelial cells. *Biochemistry and Cell Biology 85*, 246-251.

Jin H., Qiu W. B., Mei Y. F., Wang D. M., Li Y. G., & Tan X. R. (2009). Testosterone alleviates tumor necrosis factor-alpha-mediated tissue factor pathway inhibitor

downregulation via suppression of nuclear factor-kappa B in endothelial cells. *Asian Journal of Andrology 11*, 266-271.

Jockenhövel F., Blum W. F., Vogel E., Englaro P., Müller-Wieland D., Reinwein D., Rascher W., & Krone W. (1997). Testosterone substitution normalizes elevated serum leptin levels in hypogonadal men. *Journal of Clinical Endocrinology & Metabolism 82*, 2510-2513.

Jockenhövel F., Bullmann C., Schubert M., Vogel E., Reinhardt W., Reinwein D., Müller-Wieland D., & Krone W. (1999). Influence of various modes of androgen substitution on serum lipids and lipoproteins in hypogonadal men. *Metabolism 48*, 590-596.

Johnson L. A., Altenburg M. K., Walzem R. L., Scanga L. T., & Maeda N. (2008). Absence of hyperlipidemia in LDL receptor-deficient mice having apolipoprotein B100 without the putative receptor-binding sequences. *Arteriosclerosis, Thrombosis, and Vascular Biology 28*, 1745-1752.

Jones R., Hall J., Pugh P., English K., Channer K., & Jones T. (2002). Circulating steroid levels in the testicular feminised (Tfm) mouse. *Endocrine* 3, P268

Jones R. D., Nettleship J. E., Kapoor D., Jones H. T., & Channer K. S. (2005). Testosterone and atherosclerosis in aging men. *American Journal of Cardiovascular Drugs 5*, 141-154.

Jones T. H., Arver S., Behre H. M., Buvat J., Meuleman E., Moncada I., Morales A. M., Volterrani M., Yellowlees A., & Howell J. D. (2011). Testosterone replacement in hypogonadal men with type 2 diabetes and/or metabolic syndrome (the TIMES2 study). *Diabetes Care 34*, 828-837.

Jones T. H. & Saad F. (2009). The effects of testosterone on risk factors for, and the mediators of, the atherosclerotic process. *Atherosclerosis 207*, 318-327.

Jones T. H. (2009). Advances in the management of testosterone deficiency, *Karger 1th ed*, 1-4.

Jones T. H (2010). Testosterone deficiency: a risk factor for cardiovascular disease? *Trends in Endocrinology & Metabolism 21*, 496-503.

Jones B. A., Beamer M., & Ahmed S. (2010). Fractalkine/CX3CL1: a potential new target for inflammatory diseases. *Molecular Interventions 10*, 263-270.

Jones T. H., Arver S., Behre H. M., Buvat J., Meuleman E., Moncada I., Morales A. M., Volterrani M., Yellowlees A., Howell J. D., Channer K. S., & TIMES2 Investigators (2011). Testosterone replacement in hypogonadal men with type 2 diabetes and/or metabolic syndrome (the TIMES2 study). *Diabetes Care 34*, 828-837.

Joseph S. & Macdermot J. (1992). Thrombin promotes actin polymerization in U937 human monocyte-macrophage cells. Analysis of the signalling mechanisms mediating actin polymerization. *Biochem.J 286*, 945-950.

Jung U. J. & Choi M. (2014). Obesity and its metabolic complications: The role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *International Journal of Molecular Sciences 15*, 6184-6223.

Juge-Aubry C. E., Somm E., Pernin A., Alizadeh N., Giusti V., Dayer J., & Meier C. A. (2005). Adipose tissue is a regulated source of interleukin-10. *Cytokine 29*, 270-274.

Juvonen T., Savolainen M., Kairaluoma M., Lajunen L., Humphries S., & Kesäniemi Y. (1995). Polymorphisms at the apoB, apoA-I, and cholesteryl ester transfer protein gene loci in patients with gallbladder disease. *Journal of Lipid Research 36*, 804-812.

Kadowaki T. & Yamauchi T. (2005). Adiponectin and adiponectin receptors. *Endocrine Reviews 26*, 439-451.

Kalinchenko S. Y., Tishova Y. A., Mskhalaya G. J., Gooren L. J., Giltay E. J., & Saad F. (2010). Effects of testosterone supplementation on markers of the metabolic syndrome and inflammation in hypogonadal men with the metabolic syndrome: the double-blinded placebo-controlled Moscow study. *Clinical Endocrinology 73*, 602-612.

Kamari Y., Werman-Venkert R., Shaish A., Werman A., Harari A., Gonen A., Voronov E., Grosskopf I., Sharabi Y., & Grossman E. (2007). Differential role and tissue specificity of interleukin-1α gene expression in atherogenesis and lipid metabolism. *Atherosclerosis 195*, 31-38.

Kamstrup P. R., Benn M., Tybjærg-Hansen A., & Nordestgaard B. G. (2008). Extreme Lipoprotein (a) Levels and Risk of Myocardial Infarction in the General Population The Copenhagen City Heart Study. *Circulation 117*, 176-184.

Kamstrup P. R., Tybjærg-Hansen A., & Nordestgaard B. G. (2011). Lipoprotein (a) and risk of myocardial infarction-genetic epidemiologic evidence of causality. *Scandinavian Journal of Clinical & Laboratory Investigation 71*, 87-93.

Kanda H., Tateya S., Tamori Y., Kotani K., Hiasa K., Kitazawa R., Kitazawa S., Miyachi H., Maeda S., & Egashira K. (2006). MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *Journal of Clinical Investigation 116*, 1494-1505.

Kanda N., Tsuchida T., & Tamaki K. (1996). Testosterone inhibits immunoglobulin production by human peripheral blood mononuclear cells. *Clinical & Experimental Immunology 106*, 410-415.

Kanda N., Tsuchida T., & Tamaki K. (1997). Testosterone suppresses anti-DNA antibody production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Arthritis & Rheumatism 40*, 1703-1711.

Kanemaki T., Kitade H., Kaibori M., Sakitani K., Hiramatsu Y., Kamiyama Y., Ito S., & Okumura T. (1998). Interleukin 1β and interleukin 6, but not tumor necrosis factor α, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. *Hepatology 27*, 1296-1303.

Kapoor D., Clarke S., Stanworth R., Channer K., & Jones T. (2007). The effect of testosterone replacement therapy on adipocytokines and C-reactive protein in hypogonadal men with type 2 diabetes. *European Journal of Endocrinology 156*, 595-602.

Kapoor D., Malkin C., Channer K., & Jones T. (2005). Androgens, insulin resistance and vascular disease in men. *Clinical Endocrinology 63*, 239-250.

Kapoor D. & Jones T. H. (2008). Androgen deficiency as a predictor of metabolic syndrome in aging men. *Drugs & Aging 25*, 357-369.

Kapoor D., Channer K. S., & Jones T. H. (2008). Rosiglitazone increases bioactive testosterone and reduces waist circumference in hypogonadal men with type 2 diabetes. *Diabetes & Vascular Disease Research : Official Journal of the International Society of Diabetes and Vascular Disease 5*, 135-137.

Kapoor D., Goodwin E., Channer K. S., & Jones T. H. (2006). Testosterone replacement therapy improves insulin resistance, glycaemic control, visceral adiposity and hypercholesterolaemia in hypogonadal men with type 2 diabetes. *European Journal of Endocrinology / European Federation of Endocrine Societies 154*, 899-906.

Kappelle P., Gansevoort R., Hillege J., Wolffenbuttel B., & Dullaart R. (2011). Apolipoprotein B/A-I and total cholesterol/high-density lipoprotein cholesterol ratios both predict cardiovascular events in the general population independently of nonlipid risk factors, albuminuria and C-reactive protein. *Journal of Internal Medicine 269*, 232-242.

Karlmark K. R., Wasmuth H. E., Trautwein C., & Tacke F. (2008). Chemokine-directed immune cell infiltration in acute and chronic liver disease.

Karpe F., Dickmann J. R., & Frayn K. N. (2011). Fatty acids, obesity, and insulin resistance: time for a reevaluation. *Diabetes 60*, 2441-2449.

Karpe F. & Ehrenborg E. E. (2009). PPARdelta in humans: genetic and pharmacological evidence for a significant metabolic function. *Current Opinion in Lipidology 20*, 333-336.

Kasama T., Wakabayashi K., Sato M., Takahashi R., & Isozaki T. (2010). Relevance of the CX3CL1/fractalkine-CX3CR1 pathway in vasculitis and vasculopathy. *Translational Research 155*, 20-26.

Kashyap V. S., Santamarina-Fojo S., Brown D. R., Parrott C. L., Applebaum-Bowden D., Meyn S., Talley G., Paigen B., Maeda N., & Brewer Jr H. B. (1995). Apolipoprotein E deficiency in mice: gene replacement and prevention of atherosclerosis using adenovirus vectors. *Journal of Clinical Investigation 96*, 1612.

Kathiresan S., Gabriel S. B., Yang Q., Lochner A. L., Larson M. G., Levy D., Tofler G. H., Hirschhorn J. N., & O'Donnell C. J. (2005). Comprehensive survey of common genetic variation at the plasminogen activator inhibitor-1 locus and relations to circulating plasminogen activator inhibitor-1 levels. *Circulation 112*, 1728-1735.

Kaufmann A., Salentin R., Gemsa D., & Sprenger H. (2001). Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 alpha during differentiation of human monocytes to macrophages. *Journal of Leukocyte Biology 69*, 248-252.

Keane M. P. & Strieter R. M. (2000). Chemokine signaling in inflammation. *Critical Care Medicine 28*, N13-N26.

Keating N. L., O'Malley A. J., Freedland S. J., & Smith M. R. (2010). Diabetes and cardiovascular disease during androgen deprivation therapy: observational study of veterans with prostate cancer. *Journal of the National Cancer Institute 102*, 39-46.

Keller E. T., Chang C., & Ershler W. B. (1996). Inhibition of NFκB activity through maintenance of IκBα levels contributes to dihydrotestosterone-mediated repression of the interleukin-6 promoter. *Journal of Biological Chemistry 271*, 26267-26275.

Kelly D. M., Sellers D. J., & Woodroofe M. N. (2010). Investigation of the mechanisms underlying the development of atherosclerosis. PHD thesis, Shefield Hallam Unversity.

Kelly D. M., Akhtar S., Brooke J., Channer K., & Jones T. (2012a). Effect of testosterone on hepatic liver X receptor and ApoE expression as a potential mechanism of atheroprotection in the testicular feminised mouse.

Kelly D. M. & Jones T. H. (2013). Testosterone: a metabolic hormone in health and disease. *Journal of Endocrinology 217*, R25-R45.

Kelly D. M., Sellers D. J., Woodroofe M. N., Jones T. H., & Channer K. S. (2012b). Effect of testosterone on inflammatory markers in the development of early atherogenesis in the testicular-feminized mouse model. *Endocrine Research* , 1-14.

Kempen H., Van Gent C., Buytenhek R., & Buis B. (1987). Association of cholesterol concentrations in low-density lipoprotein, high-density lipoprotein, and high-density lipoprotein subfractions, and of apolipoproteins AI and AII, with coronary stenosis and left ventricular function. *J Lab Clin Med 109*, 19-26.

Kershaw E. E. & Flier J. S. (2004). Adipose tissue as an endocrine organ. *The Journal of Clinical Endocrinology & Metabolism 89*, 2548-2556.

Khosla S., Atkinson E. J., Dunstan C. R., & O'fallon W. (2002). Effect of estrogen versus testosterone on circulating osteoprotegerin and other cytokine levels in normal elderly men. *Journal of Clinical Endocrinology & Metabolism 87*, 1550-1554.

Kiechl S., Willeit J., Mayr M., Viehweider B., Oberhollenzer M., Kronenberg F., Wiedermann C. J., Oberthaler S., Xu Q., & Witztum J. L. (2007). Oxidized Phospholipids, Lipoprotein (a), Lipoprotein-Associated Phospholipase A2 Activity, and 10-Year Cardiovascular Outcomes Prospective Results From the Bruneck Study. *Arteriosclerosis, Thrombosis, and Vascular Biology 27*, 1788-1795.

Kiess W., Petzold S., Töpfer M., Garten A., Blüher S., Kapellen T., Körner A., & Kratzsch J. (2008). Adipocytes and adipose tissue. *Best Practice & Research Clinical Endocrinology & Metabolism 22*, 135-153.

Kietzmann T. & Andreasen P. (2008). Plasminogen activator inhibitor-1 (PAI-1): A molecule at the crossroads to cell survival or cell death. *Thromb.Haemost 100*, 965-968.

Kilby E., Akhtar S., & Jones T. (2012). The role of testosterone activation of liver X receptor in a human macrophage cell line and its implications for the treatment of type 2 diabetes and atherosclerosis.

Kim H., Higashimori T., Park S., Choi H., Dong J., Kim Y., Noh H., Cho Y., Cline G., & Kim Y. (2004). Differential effects of interleukin-6 and-10 on skeletal muscle and liver insulin action in vivo. *Diabetes 53*, 1060-1067.

Kim J. K. (2006). Fat uses a TOLL-road to connect inflammation and diabetes. *Cell Metabolism 4*, 417-419.

Kim T. J., Kim T. H., Lee H. J., Peddle L., Rahman P., Hu P., Greenwood C. M., & Inman R. D. (2008). Interleukin 1 polymorphisms in patients with ankylosing spondylitis in Korea. *The Journal of Rheumatology 35*, 1603-1608.

Kirk E. A., Sagawa Z. K., McDonald T. O., O'Brien K. D., & Heinecke J. W. (2008). Monocyte chemoattractant protein-1 deficiency fails to restrain macrophage infiltration into adipose tissue. *Diabetes 57*, 1254-1261.

Kitamoto S. & Egashira K. (2003). Anti-monocyte chemoattractant protein-1 gene therapy for cardiovascular diseases. *Expert Review of Cardiovascular Therapy 1*, 393-400.

Kleemann R., Zadelaar S., & Kooistra T. (2008). Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovascular Research 79*, 360-376.

Kleinbongard P., Heusch G., & Schulz R. (2010). TNFα in atherosclerosis, myocardial ischemia/reperfusion and heart failure. *Pharmacology & Therapeutics 127*, 295-314.

Klentze M. (2004). Testosterone, The Male Hormone Connection: Treating Diabetes and Heart Disease. *Anti-Aging Therapeutics 6*, 59.

Klover, P. Zimmers, T, Koniaris, Land Mooney, R (2003). Chronic exposure to interleukin -6 causes hepatic insulin resistance in mice. Diabetes, *Diabetes 52*, 2784-2789.

Knight B. L., Perombelon Y., Soutar A. K., Wade D. P., & Seed M. (1991). Catabolism of lipoprotein (a) in familial hypercholesterolaemic subjects. *Atherosclerosis 87*, 227-237.

Koelink P. J., Overbeek S. A., Braber S., de Kruijf P., Folkerts G., Smit M. J., & Kraneveld A. D. (2012). Targeting chemokine receptors in chronic inflammatory diseases: an extensive review. *Pharmacology & Therapeutics 133*, 1-18.

Koenen R. R. & Weber C. (2011). Chemokines: established and novel targets in atherosclerosis. *EMBO Molecular Medicine 3*, 713-725.

Koenen R. R. & Weber C. (2010). Therapeutic targeting of chemokine interactions in atherosclerosis. *Nature Reviews Drug Discovery 9*, 141-153.

Koga M., Kai H., Yasukawa H., Yamamoto T., Kawai Y., Kato S., Kusaba K., Kai M., Egashira K., Kataoka Y., & Imaizumi T. (2007). Inhibition of progression and stabilization of plaques by postnatal interferon-gamma function blocking in ApoE-knockout mice. *Circulation Research 101*, 348-356.

Koistinen H., Bastard J., Dusserre E., Ebeling P., Zegari N., Andreelli F., Jardel C., Donner M., Meyer L., & Moulin P. (2000). Subcutaneous adipose tissue expression of tumour necrosis factor-a is not associated with whole body insulin resistance in obese nondiabetic or in type-2 diabetic subjects. *European Journal of Clinical Investigation 30*, 302-310.

Kolovou G. D., Anagnostopoulou K. K., Kostakou P., Giannakopoulou V., Mihas C., Hatzigeorgiou G., Vasiliadis I. K., Mikhailidis D. P., & Cokkinos D. V. (2009). Apolipoprotein E gene polymorphism and obesity status in middle-aged men with coronary heart disease. *In Vivo (Athens, Greece) 23*, 33-39.

Koltsova E. K. & Ley K. (2011). How dendritic cells shape atherosclerosis. *Trends in Immunology 32*, 540-547.

Komarova Y. & Malik A. B. (2010). Regulation of endothelial permeability via paracellular and transcellular transport pathways. *Annual Review of Physiology 72*, 463-493.

Kontush A. & Chapman M. J. (2006). Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacological Reviews 58*, 342-374.

Kothapalli D. (2004). Anti-mitogenic effects of HDL and ApoE mediated by Cox-2 dependent- IP activation. *J Clin Invest 113*, 609-618.

Krey G., Keller H., Mahfoudi A., Medin J., Ozato K., Dreyer C., & Wahli W. (1993). < i> Xenopus</i> peroxisome proliferator activated receptors: Genomic organization, response element recognition, heterodimer formation with retinoid X receptor and activation by fatty acids. *The Journal of Steroid Biochemistry and Molecular Biology 47*, 65-73.

Kruithof E. (2008). Regulation of plasminogen activator inhibitor type 1 gene expression by inflammatory mediators and statins. *Thromb Haemost 100*, 969-975.

Kruithof E., Mestries J., Gascon M., & Ythier A. (1997). The coagulation and fibrinolytic responses of baboons after in vivo thrombin generation: effect of interleukin 6. *Thrombosis and Haemostasis 77*, 905-910.

Kuboki K., Jiang Z. Y., Takahara N., Ha S. W., Igarashi M., Yamauchi T., Feener E. P., Herbert T. P., Rhodes C. J., & King G. L. (2000). Regulation of endothelial constitutive nitric oxide synthase gene expression in endothelial cells and in vivo a specific vascular action of insulin. *Circulation 101*, 676-681.

Kuipers F., Van Ree J., Hofker M. H., Wolters H., Veld G., Havinga R., Vonk R. J., Princen H., & Havekes L. M. (1996). Altered lipid metabolism in apolipoprotein E–deficient mice does not affect cholesterol balance across the liver. *Hepatology 24*, 241-247.

Kumada M., Kihara S., Sumitsuji S., Kawamoto T., Matsumoto S., Ouchi N., Arita Y., Okamoto Y., Shimomura I., & Hiraoka H. (2003). Association of hypoadiponectinemia with coronary artery disease in men. *Arteriosclerosis, Thrombosis, and Vascular Biology 23*, 85-89.

Kunjathoor V., Wilson D., & LeBoeuf R. (1996). Increased atherosclerosis in streptozotocin-induced diabetic mice. *Journal of Clinical Investigation 97*, 1767.

Kuschert G. S., Coulin F., Power C. A., Proudfoot A. E., Hubbard R. E., Hoogewerf A. J., & Wells T. N. (1999). Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry 38*, 12959-12968.

Kwiterovich Jr P. O., Coresh J., Smith H. H., Bachorik P. S., Derby C. A., & Pearson T. A. (1992). Comparison of the plasma levels of apolipoproteins B and A-1, and other risk factors in men and women with premature coronary artery disease. *The American Journal of Cardiology 69*, 1015-1021.

Kwon K., Jung S. H., Choi C., & Park S. (2005). Reciprocal association between visceral obesity and adiponectin: in healthy premenopausal women. *International Journal of Cardiology 101*, 385-390.

La Merrill M., Emond C., Kim M. J., Antignac J. P., Le Bizec B., Clement K., Birnbaum L. S., & Barouki R. (2013). Toxicological function of adipose tissue: focus on persistent organic pollutants. *Environmental Health Perspectives 121*, 162-169.

Laaksonen D. E., Niskanen L., Punnonen K., Nyyssönen K., Tuomainen T., Valkonen V., & Salonen J. T. (2005). The metabolic syndrome and smoking in relation to hypogonadism in middle-aged men: a prospective cohort study. *Journal of Clinical Endocrinology & Metabolism 90*, 712-719.

Laaksonen D. E., Niskanen L., Punnonen K., Nyyssönen K., Tuomainen T., Valkonen V., Salonen R., & Salonen J. T. (2004). Testosterone and sex hormone–binding globulin predict the metabolic syndrome and diabetes in middle-aged men. *Diabetes Care 27*, 1036-1041.

Laaksonen D. E., Niskanen L., Punnonen K., Nyyssonen K., Tuomainen T. P., Salonen R., Rauramaa R., & Salonen J. T. (2003). Sex hormones, inflammation and the metabolic syndrome: a population-based study. *European Journal of Endocrinology / European Federation of Endocrine Societies 149*, 601-608.

Lago F., Dieguez C., Gómez-Reino J., & Gualillo O. (2007). The emerging role of adipokines as mediators of inflammation and immune responses. *Cytokine & Growth Factor Reviews 18*, 313-325.

Lagoa C. E., Vodovotz Y., Stolz D. B., Lhuillier F., McCloskey C., Gallo D., Yang R., Ustinova E., Fink M. P., & Billiar T. R. (2005). The role of hepatic type 1 plasminogen activator inhibitor (PAI-1) during murine hemorrhagic shock. *Hepatology 42*, 390-399.

Lagos K., Filippatos T., Tsimihodimos V., Gazi I., Rizos C., Tselepis A., Mikhailidis D., & Elisaf M. S. (2009). Alterations in the high density lipoprotein phenotype and HDL-associated enzymes in subjects with metabolic syndrome. *Lipids 44*, 9-16.

Lai J. J., Lai K. P., Chuang K. H., Chang P., Yu I. C., Lin W. J., & Chang C. (2009). Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression. *The Journal of Clinical Investigation 119*, 3739-3751.

Lamarche B., Moorjani S., Cantin B., Dagenais G. R., Lupien P. J., & Després J. (1997). Associations of HDL2 and HDL3 Subfractions with ischemic heart disease in Men Prospective Results From the Québec Cardiovascular Study. *Arteriosclerosis, Thrombosis, and Vascular Biology 17*, 1098-1105.

Lamarche B., Moorjani S., Lupien P. J., Cantin B., Bernard P., Dagenais G. R., & Despre J. (1996). Apolipoprotein AI and B Levels and the Risk of Ischemic Heart Disease During a Five-Year Follow-up of Men in the Que′ bec Cardiovascular Study. *Circulation 94*, 273-278.

Landsman L., Bar-On L., Zernecke A., Kim K., Krauthgamer R., Shagdarsuren E., Lira S. A., Weissman I. L., Weber C., & Jung S. (2009). CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood 113*, 963-972.

Lanfranco F., Zitzmann M., Simoni M., & Nieschlag E. (2004). Serum adiponectin levels in hypogonadal males: influence of testosterone replacement therapy. *Clinical Endocrinology 60*, 500-507.

Lapauw B., T'Sjoen G., Mahmoud A., Kaufman J. M., & Ruige J. B. (2009). Short-term aromatase inhibition: effects on glucose metabolism and serum leptin levels in young and elderly men. *European Journal of Endocrinology / European Federation of Endocrine Societies 160*, 397-402.

Lattouf J., Srinivasan R., Pinto P. A., Linehan W. M., & Neckers L. (2006). Mechanisms of disease: the role of heat-shock protein 90 in genitourinary malignancy. *Nature Clinical Practice Urology 3*, 590-601.

Lawn R. M., Wade D. P., Hammer R. E., Chiesa G., Verstuyft J. G., & Rubin E. M. (1992). Atherogenesis in transgenic mice expressing human apolipoprotein (a). *Nature 360*, 670-672.

Le Page C., Genin P., Baines M., & Hiscott J. (1999). Interferon activation and innate immunity. *Reviews in Immunogenetics 2*, 374-386.

Leder B. Z., Rohrer J. L., Rubin S. D., Gallo J., & Longcope C. (2004). Effects of aromatase inhibition in elderly men with low or borderline-low serum testosterone levels. *Journal of Clinical Endocrinology & Metabolism 89*, 1174-1180.

Lee C., Kuo S., Hung Y., Hsieh C., He C., Yang T., Lian W., Chyi-Fan S., & Pei D. (2005). The effect of testosterone supplement on insulin sensitivity, glucose effectiveness, and acute insulin response after glucose load in male type 2 diabetics. *Endocrine Research 31*, 139-148.

Lee E., Vaughan D. E., Parikh S. H., Grodzinsky A. J., Libby P., Lark M. W., & Lee R. T. (1996). Regulation of matrix metalloproteinases and plasminogen activator inhibitor-1 synthesis by plasminogen in cultured human vascular smooth muscle cells. *Circulation Research 78*, 44-49.

Lee H., Shi W., Tontonoz P., Wang S., Subbanagounder G., Hedrick C. C., Hama S., Borromeo C., Evans R. M., & Berliner J. A. (2000). Role for Peroxisome Proliferator-Activated Receptor α in Oxidized Phospholipid–Induced Synthesis of Monocyte Chemotactic Protein-1 and Interleukin-8 by Endothelial Cells. *Circulation Research 87*, 516-521.

Lee J. K., Bettencourt R., Brenner D., Le T., Barrett-Connor E., & Loomba R. (2012). Association between serum interleukin-6 concentrations and mortality in older adults: the Rancho Bernardo study. *PloS One 7*, e34218.

Lee J. S., Pinnamaneni S. K., Eo S. J., Cho I. H., Pyo J. H., Kim C. K., Sinclair A. J., Febbraio M. A., & Watt M. J. (2006). Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. *Journal of Applied Physiology 100*, 1467-1474.

Lee C. H., Olson P., Hevener A., Mehl I., Chong L. W., Olefsky J. M., Gonzalez F. J., Ham J., Kang H., Peters J. M., & Evans R. M. (2006). PPARdelta regulates glucose metabolism and insulin sensitivity. *Proceedings of the National Academy of Sciences of the United States of America 103*, 3444-3449.

Lefebvre P., Chinetti G., Fruchart J., & Staels B. (2006). Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. *Journal of Clinical Investigation 116*, 571-580.

Lesnik P., Haskell C. A., & Charo I. F. (2003). Decreased atherosclerosis in CX3CR1–/–mice reveals a role for fractalkine in atherogenesis. *Journal of Clinical Investigation 111*, 333-340.

Ley S. H., Harris S. B., Connelly P. W., Mamakeesick M., Gittelsohn J., Wolever T. M., Hegele R. A., Zinman B., & Hanley A. J. (2010). Association of apolipoprotein B with incident type 2 diabetes in an aboriginal Canadian population. *Clinical Chemistry 56*, 666-670.

Ley K., Miller Y. I., & Hedrick C. C. (2011). Monocyte and macrophage dynamics during atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology 31*, 1506-1516.

Li A. C., Binder C. J., Gutierrez A., Brown K. K., Plotkin C. R., Pattison J. W., Valledor A. F., Davis R. A., Willson T. M., & Witztum J. L. (2004). Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARα, β/δ, and γ. *Journal of Clinical Investigation 114*, 1564-1576.

Li R., Lu W., Jia J., Zhang S., Shi L., Li Y., Yang Q., & Kan H. (2008). Relationships between indices of obesity and its cardiovascular comorbidities in a Chinese population. *Circulation Journal: Official Journal of the Japanese Circulation Society 72*, 973-978.

Li X., Zhao S., Zhang X., Liu L., Gao M., & Zhou Q. (2000). Protective effect of high density lipoprotein on endothelium-dependent vasodilatation. *International Journal of Cardiology 73*, 231-236.

Li Z., Danis V., & Brooks P. (1993). Effect of gonadal steroids on the production of IL-1 and IL-6 by blood mononuclear cells in vitro. *Clinical and Experimental Rheumatology 11*, 157-162.

Libby P. (2009). Molecular and cellular mechanisms of the thrombotic complications of atherosclerosis. *Journal of Lipid Research 50*, S352-S357.

Libby P. (2008). The molecular mechanisms of the thrombotic complications of atherosclerosis. *Journal of Internal Medicine 263*, 517-527.

Libby P., Okamoto Y., Rocha V. Z., & Folco E. (2010). Inflammation in atherosclerosis. *Circ J 74*, 213-220.

Libby P., Ridker P. M., & Hansson G. K. (2011). Progress and challenges in translating the biology of atherosclerosis. *Nature 473*, 317-325.

Libby P., Ridker P. M., & Hansson G. K. (2009). Inflammation in AtherosclerosisFrom Pathophysiology to Practice. *Journal of the American College of Cardiology 54*, 2129-2138.

Libby P., Ridker P. M., & Maseri A. (2002). Inflammation and atherosclerosis. *Circulation 105*, 1135-1143.

Lihn A. S., Bruun J. M., He G., Pedersen S. B., Jensen P. F., & Richelsen B. (2004). Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Molecular and Cellular Endocrinology 219*, 9-15.

Linton M. F., Atkinson J. B., & Fazio S. (1995). Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science-New York then Washington-* , 1034-1034.

Linton M. F. & Fazio S. (2003). Macrophages, inflammation, and atherosclerosis. *International Journal of Obesity 27*, S35-S40.

Linton M., Farese Jr R. V., Chiesa G., Grass D. S., Chin P., Hammer R., Hobbs H., & Young S. (1993). Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein (a). *Journal of Clinical Investigation 92*, 3029.

Littleton-Kearney M. & Hurn P. D. (2004). Testosterone as a modulator of vascular behavior. *Biological Research for Nursing 5*, 276-285.

Liu A. C., Lawn R., Verstuyft J., & Rubin E. (1994). Human apolipoprotein AI prevents atherosclerosis associated with apolipoprotein [a] in transgenic mice. *Journal of Lipid Research 35*, 2263-2267.

Liu H. & Jiang D. (2011). Fractalkine/CX3CR1 and atherosclerosis. *Clinica Chimica Acta 412*, 1180-1186.

Liu P., Yen-Rei A. Y., Spencer J. A., Johnson A. E., Vallanat C. T., Fong A. M., Patterson C., & Patel D. D. (2008). CX3CR1 deficiency impairs dendritic cell accumulation in arterial intima and reduces atherosclerotic burden. *Arteriosclerosis, Thrombosis, and Vascular Biology 28*, 243-250.

Liu Y. & Miller A. R. (2005). Ligands to peroxisome proliferator-activated receptors as therapeutic options for metabolic syndrome. *Drug Discovery Today: Therapeutic Strategies 2*, 165-169.

Liva S. M. & Voskuhl R. R. (2001). Testosterone acts directly on CD4 T lymphocytes to increase IL-10 production. *The Journal of Immunology 167*, 2060-2067.

Livak, K.J., & T.D. Schmittgen. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*. 25:402-408.

Livak K. J. & Schmittgen T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2< sup>− ΔΔCT</sup> Method. *Methods 25*, 402-408.

Lloyd-Jones D., Adams R. J., Brown T. M., Carnethon M., Dai S., De Simone G., Ferguson T. B., Ford E., Furie K., Gillespie C., Go A., Greenlund K., Haase N., Hailpern S., Ho P. M., Howard V., Kissela B., Kittner S., Lackland D., Lisabeth L., Marelli A., McDermott M. M., Meigs J., Mozaffarian D., Mussolino M., Nichol G., Roger V. L., Rosamond W., Sacco R., Sorlie P., Stafford R., Thom T., Wasserthiel-Smoller S., Wong N. D., & Wylie-Rosett J. (2010). Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation 121*, 948-954.

Loffreda S., Yang S., Lin H., Karp C., Brengman M., Wang D., Klein A., Bulkley G., Bao C., & Noble P. (1998). Leptin regulates proinflammatory immune responses. *The FASEB Journal 12*, 57-65.

Lowe G. D. (2001). The relationship between infection, inflammation, and cardiovascular disease: an overview. *Annals of Periodontology 6*, 1-8.

Lowe G., Woodward M., Hillis G., Rumley A., Li Q., Harrap S., Marre M., Hamet P., Patel A., Poulter N., & Chalmers J. (2014). Circulating inflammatory markers and the risk of vascular complications and mortality in people with type 2 diabetes and cardiovascular disease or risk factors: the ADVANCE study. *Diabetes 63*, 1115-1123.

Luc G., Bard J., Arveiler D., Ferrieres J., Evans A., Amouyel P., Fruchart J., & Ducimetiere P. (2002). Lipoprotein (a) as a predictor of coronary heart disease: the PRIME Study. *Atherosclerosis 163*, 377-384.

Lucas A. D., Bursill C., Guzik T. J., Sadowski J., Channon K. M., & Greaves D. R. (2003). Smooth muscle cells in human atherosclerotic plaques express the fractalkine receptor CX3CR1 and undergo chemotaxis to the CX3C chemokine fractalkine (CX3CL1). *Circulation 108*, 2498-2504.

Lucas A. D. & Greaves D. R. (2001). Atherosclerosis: role of chemokines and macrophages. *Expert Reviews in Molecular Medicine 2*, 1-18.

Ludwig A., Berkhout T., Moores K., Groot P., & Chapman G. (2002). Fractalkine is expressed by smooth muscle cells in response to IFN-γ and TNF-α and is modulated by metalloproteinase activity. *The Journal of Immunology 168*, 604-612.

Ludwig A. & Weber C. (2007). Transmembrane chemokines: Versatilespecial agents' in vascular inflammation. *Thrombosis and Haemostasis-Stuttgart-* 97, 694.

Lumeng C. N., Bodzin J. L., & Saltiel A. R. (2007). Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of Clinical Investigation 117*, 175-184.

Lusis AJ. (2000). Atherosclerosis . *Nature* 407, 233-241.

Luukkaa V., Pesonen U., Huhtaniemi I., Lehtonen A., Tilvis R., Tuomilehto J., Koulu M., & Huupponen R. (1998). Inverse correlation between serum testosterone and leptin in men. *Journal of Clinical Endocrinology & Metabolism 83*, 3243-3246.

Hromadova, T. Hacik ,and I. Riecansk. (1985). Concentration of lipid, Apo protein -B and testosterone in patients with coronarographic finding. *Klin Wochenschr 63*, 1071-1074.

Ma R. C. & Tong P. C. (2010). Testosterone levels and cardiovascular disease. *Heart 96*, 1787-1788.

MacDonald A., Herbison G., Showell M., & Farquhar C. (2010). The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Human Reproduction Update 16*, 293-311.

Mack M., Cihak J., Simonis C., Luckow B., Proudfoot A. E., Plachy J., Bruhl H., Frink M., Anders H. J., Vielhauer V., Pfirstinger J., Stangassinger M., & Schlondorff D. (2001). Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *Journal of Immunology (Baltimore, Md.: 1950) 166*, 4697-4704.

Madjid M., Ali M., & Willerson J. T. (2010). Lipoprotein-associated phospholipase A2 as a novel risk marker for cardiovascular disease: a systematic review of the literature. *Texas Heart Institute Journal 37*, 25.

Maeda S., Nakanishi S., Yoneda M., Awaya T., Yamane K., Hirano T., & Kohno N. (2011). Associations between small dense LDL, HDL subfractions (HDL2, HDL3) and risk of atherosclerosis in Japanese-Americans. *Journal of Atherosclerosis and Thrombosis* .

Maegdefessel L., Schlitt A., Pippig S., Schwaab B., Fingscheidt K., Raaz U., Buerke M., & Loppnow H. (2009). Patients with insulin-dependent diabetes or coronary heart disease following rehabilitation express serum fractalkine levels similar to those in healthy control subjects. *Vascular Health and Risk Management 5*, 849.

Maggio M., Basaria S., Ble A., Lauretani F., Bandinelli S., Ceda G. P., Valenti G., Ling S. M., & Ferrucci L. (2006). Correlation between testosterone and the inflammatory marker soluble interleukin-6 receptor in older men. *Journal of Clinical Endocrinology & Metabolism 91*, 345-347.

Maggio M., Ceda G. P., Lauretani F., Bandinelli S., Metter E. J., Artoni A., Gatti E., Ruggiero C., Guralnik J. M., & Valenti G. (2009). Estradiol and inflammatory markers in older men. *Journal of Clinical Endocrinology & Metabolism 94*, 518-522.

Mahley R. W., Huang Y., & Weisgraber K. H. (2006). Putting cholesterol in its place: apoE and reverse cholesterol transport. *The Journal of Clinical Investigation 116*, 1226-1229.

Makki K., Froguel P., & Wolowczuk I. (2013). Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN Inflammation 2013.*

Malikantiansen, T., Richelsen, B., and Bruun, J. (2005). Monocyte chemoattractant protein-I is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes, 29*, 146.

Malkin C. J., Pugh P. J., Jones R. D., Kapoor D., Channer K. S., & Jones T. H. (2004a). The effect of testosterone replacement on endogenous inflammatory cytokines and lipid profiles in hypogonadal men. *Journal of Clinical Endocrinology & Metabolism 89*, 3313-3318.

Malkin C. J., Pugh P. J., Morris P. D., Kerry K. E., Jones R. D., Jones T. H., & Channer K. S. (2004b). Testosterone replacement in hypogonadal men with angina improves ischaemic threshold and quality of life. Heart (British Cardiac Society) 90, 871-876.

Malkin C. J., Pugh P. J., Morris P. D., Asif S., Jones T. H., & Channer K. S. (2010). Low serum testosterone and increased mortality in men with coronary heart disease. *Heart (British Cardiac Society) 96*, 1821-1825.

Malkin C. J., Pugh P. J., Jones T. H., & Channer K. S. (2003). Testosterone for secondary prevention in men with ischaemic heart disease? *QJM : Monthly Journal of the Association of Physicians 96*, 521-529.

Mallat Z. & Tedgui A. (2001). Current perspective on the role of apoptosis in atherothrombotic disease. *Circulation Research 88*, 998-1003.

Mallat Z. & Tedgui A. (2000). Apoptosis in the vasculature: mechanisms and functional importance. *British Journal of Pharmacology 130*, 947-962.

Mallat Z., Heymes C., Ohan J., Faggin E., Leseche G., & Tedgui A. (1999). Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. *Arteriosclerosis,* 19:611-6.

Manco M, Fernandez-Real JM, Equitani F. (2007). Effect of massive weight loss on inflammatory adipocytokines and the innate immune system in morbidly obese women. J *Clin Endocrinol Metab*; 92:483–90.*Thrombosis, and Vascular Biology 19*, 611-616.

Maneschi E., Morelli A., Filippi S., Cellai I., Comeglio P., Mazzanti B., Mello T., Calcagno A., Sarchielli E., Vignozzi L., Saad F., Vettor R., Vannelli G. B., & Maggi M. (2012). Testosterone treatment improves metabolic syndrome-induced adipose tissue derangements. *The Journal of Endocrinology 215*, 347-362.

Manigrasso M., Ferroni P., Santilli F., Taraborelli T., Guagnano M., Michetti N., & Davi G. (2005). Association between circulating adiponectin and interleukin-10 levels

in android obesity: effects of weight loss. *Journal of Clinical Endocrinology & Metabolism 90*, 5876-5879.

Manolopoulos K., Karpe F., & Frayn K. (2010). Gluteofemoral body fat as a determinant of metabolic health. *International Journal of Obesity 34*, 949-959.

Mantovani A., Bonecchi R., & Locati M. (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature Reviews Immunology 6*, 907-918.

Marcil M., O'Connell B., Krimbou L., & Genest Jr J. (2004). High-density lipoproteins: multifunctional vanguards of the cardiovascular system. *Expert Review of Cardiovascular Therapy 2*, 417-430.

Mårin P., Holmäng S., Jönsson L., Sjöström L., Kvist H., Holm G., Lindstedt G., & Björntorp P. (1992a). The effects of testosterone treatment on body composition and metabolism in middle-aged obese men. *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity 16*, 991-997.

Mårin P., Krotkiewski M., & Björntorp P. (1992b). Androgen treatment of middle-aged, obese men: effects on metabolism, muscle and adipose tissues. *The European Journal of Medicine 1*, 329-336.

Mårin P., Oden B., & Björntorp P. (1995). Assimilation and mobilization of triglycerides in subcutaneous abdominal and femoral adipose tissue in vivo in men: effects of androgens. *Journal of Clinical Endocrinology & Metabolism 80*, 239-243.

Mårin P., Holmtäng S., Gustafsson C., Elander A., Eldh J., Sjöström L., Holm G., & Björntorp P. (1993). Androgen treatment of abdominally obese men. *Obesity Research 1*, 245-251.

Martin S. S., Qasim A. N., Mehta N. N., Wolfe M., Terembula K., Schwartz S., Iqbal N., Schutta M., Bagheri R., & Reilly M. P. (2009). Apolipoprotein B but not LDL cholesterol is associated with coronary artery calcification in type 2 diabetic whites. *Diabetes 58*, 1887-1892.

Martin S. S., Qasim A., & Reilly M. P. (2008). Leptin ResistanceA Possible Interface of Inflammation and Metabolism in Obesity-Related Cardiovascular Disease. *Journal of the American College of Cardiology 52*, 1201-1210.

Martinovic I., Abegunewardene N., Seul M., Vosseler M., Horstick G., Buerke M., Darius H., & Lindemann S. (2005). Elevated monocyte chemoattractant protein-1 serum levels in patients at risk for coronary artery disease. *Circulation Journal : Official Journal of the Japanese Circulation Society 69*, 1484-1489.

Mas M. (2009). A close look at the endothelium: its role in the regulation of vasomotor tone. *European Urology Supplements 8*, 48-57.

Matsuki T., Horai R., Sudo K., & Iwakura Y. (2003). IL-1 plays an important role in lipid metabolism by regulating insulin levels under physiological conditions. *The Journal of Experimental Medicine 198*, 877-888.

Matsuzawa Y. (2005). Adiponectin: identification, physiology and clinical relevance in metabolic and vascular disease. *Atherosclerosis Supplements 6*, 7-14.

Mayes J. & Watson G. (2004). Direct effects of sex steroid hormones on adipose tissues and obesity. *Obesity Reviews 5*, 197-216.

McDermott D. H., Halcox J. P., Schenke W. H., Waclawiw M. A., Merrell M. N., Epstein N., Quyyumi A. A., & Murphy P. M. (2001). Association between polymorphism in the chemokine receptor CX3CR1 and coronary vascular endothelial dysfunction and atherosclerosis. *Circulation Research 89*, 401-407.

McDermott D. H., Yang Q., Kathiresan S., Cupples L. A., Massaro J. M., Keaney J. F., Larson M. G., Vasan R. S., Hirschhorn J. N., & O'Donnell C. J. (2005). CCL2 polymorphisms are associated with serum monocyte chemoattractant protein-1 levels and myocardial infarction in the Framingham Heart Study. *Circulation 112*, 1113-1120.

McGrath K. C., McRobb L. S., & Heather A. K. (2008). Androgen therapy and atherosclerotic cardiovascular disease. *Vascular Health and Risk Management 4*, 11-21.

McNamara J., Campos H., Ordovas J., Peterson J., Wilson P., & Schaefer E. (1987). Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. results from the framingham offspring study. *Arteriosclerosis, Thrombosis, and Vascular Biology 7*, 483-490.

McRobb L., Handelsman D., & Heather A. (2009). Androgen-induced progression of arterial calcification in apolipoprotein E-null mice is uncoupled from plaque growth and lipid levels. *Endocrinology 150*, 841-848.

Meir K. S. & Leitersdorf E. (2004). Atherosclerosis in the Apolipoprotein E–Deficient Mouse A Decade of Progress. *Arteriosclerosis, Thrombosis, and Vascular Biology 24*, 1006-1014.

Melendez-Ramirez L. Y., Richards R. J., & Cefalu W. T. (2010). Complications of type 1 diabetes. *Endocrinology & Metabolism Clinics of North America 39*, 625-640.

Mencin A., Kluwe J., & Schwabe R. F. (2009). Toll-like receptors as targets in chronic liver diseases. *Gut 58*, 704-720.

Mendis, S., Puska, P., & Norrving, B. (2011). *Global atlas on cardiovascular disease prevention and control.* : World Health Organization.

Metcalfe P. D., Leslie J. A., Campbell M. T., Meldrum D. R., Hile K. L., & Meldrum K. K. (2008). Testosterone exacerbates obstructive renal injury by stimulating TNF-α production and increasing proapoptotic and profibrotic signaling. *American Journal of Physiology-Endocrinology and Metabolism 294*, E435-E443.

Meyers C. D. & Kashyap M. L. (2005). Pharmacologic augmentation of high-density lipoproteins: mechanisms of currently available and emerging therapies. *Current Opinion in Cardiology 20*, 307-312.

Michalik L., Auwerx J., Berger J. P., Chatterjee V. K., Glass C. K., Gonzalez F. J., Grimaldi P. A., Kadowaki T., Lazar M. A., & O'rahilly S. (2006). International Union

of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacological Reviews 58*, 726-741.

Miller M., Seidler A., Moalemi A., & Pearson T. A. (1998). Normal Triglyceride Levels and Coronary Artery Disease Events: The Baltimore Coronary Observational Long-Term Study 1. *Journal of the American College of Cardiology 31*, 1252-1257.

Miller N., Hammett F., Saltissi S., Rao S., Van Zeller H., Coltart J., & Lewis B. (1981). Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins. *British Medical Journal (Clinical Research Ed.) 282*, 1741.

Miller N., Hammett F., Saltissi, S. Rao S., van Zeller H., Coltart J., & Lewis, B (1981). Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins. *Br Med J 282*, 1741-1744.

Mineo C., Deguchi H., Griffin J. H., & Shaul P. W. (2006). Endothelial and antithrombotic actions of HDL. *Circulation Research 98*, 1352-1364.

Miyata M. & Smith J. D. (1996). Apolipoprotein E allele–specific antioxidant activity and effects on cytotoxicity by oxidative insults and β–amyloid peptides. *Nature Genetics 14*, 55-61.

Miyazaki T., Shimada K., Mokuno H., & Daida H. (2003). Adipocyte derived plasma protein, adiponectin, is associated with smoking status in patients with coronary artery disease. *Heart (British Cardiac Society) 89*, 663.

Moatti D., Faure S., Fumeron F., Amara M. E. W., Seknadji P., McDermott D. H., Debré P., Aumont M. C., Murphy P. M., & de Prost D. (2001). Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood 97*, 1925-1928.

Mohamed-Ali V., Pinkney J., & Coppack S. (1998). Adipose tissue as an endocrine and paracrine organ. *International Journal of Obesity 22*, 1145-1158.

Mohan Kumar T., Monteiro F. N., Bhagavath P., & Bakkannavar S. M. (2009). Early adipocere formation: a case report and review of literature. *Journal of Forensic and Legal Medicine 16*, 475-477.

Mohasseb M., Nour H., Elsawaf A., & Khalil G. I. (2013). Relationship between testosterone, sex hormone binding globulin, angiopoietin related growth factor and insulin resistance in normal weight and obese men. *Alexandria Journal of Medicine* .

Mohr B. A., Bhasin S., Link C. L., O'Donnell A. B., & McKinlay J. B. (2006). The effect of changes in adiposity on testosterone levels in older men: longitudinal results from the Massachusetts Male Aging Study. *European Journal of Endocrinology / European Federation of Endocrine Societies 155*, 443-452.

Moller D. & Berger J. (2003). Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *International Journal of Obesity 27*, S17-S21.

Monaco C. & Paleolog E. (2004). Nuclear factor κB: a potential therapeutic target in atherosclerosis and thrombosis. *Cardiovascular Research 61*, 671-682.

Moncada S. & Higgs A. (1993). The L-arginine-nitric oxide pathway. *The New England Journal of Medicine 329*, 2002-2012.

Monjo M, Pujol E, Roca P. (2005). Alpha 2- to beta 3-Adrenoceptor switch in 3T3-L1 preadipocytes and adipocytes: modulation by testosterone, 17beta-estradiol, and progesterone. Am J Physiol Endocrinol Metab 289, E145-50.

Monks D. A., Johansen J. A., Mo K., Rao P., Eagleson B., Yu Z., Lieberman A. P., Breedlove S. M., & Jordan C. L. (2007). Overexpression of wild-type androgen receptor in muscle recapitulates polyglutamine disease. *Proceedings of the National Academy of Sciences 104*, 18259-18264.

Montecucco F. & Mach F. (2009). Common inflammatory mediators orchestrate pathophysiological processes in rheumatoid arthritis and atherosclerosis. *Rheumatology 48*, 11-22.

Monteiro R. & Azevedo I. (2010). Chronic inflammation in obesity and the metabolic syndrome. *Mediators of Inflammation 2010.*

Mooradian A. D. (2009). Dyslipidemia in type 2 diabetes mellitus. *Nature Clinical Practice Endocrinology & Metabolism 5*, 150-159.

Mor G., Eliza M., Song J., Wiita B., Chen S., & Naftolin F. (2001). 17α-Methyl testosterone is a competitive inhibitor of aromatase activity in Jar choriocarcinoma cells and macrophage-like THP-1 cells in culture. *The Journal of Steroid Biochemistry and Molecular Biology 79*, 239-246.

Mor G., Yue W., Santen R. J., Gutierrez L., Eliza M., Berstein L. M., Harada N., Wang J., Lysiak J., & Diano S. (1998). Macrophages, estrogen and the microenvironment of breast cancer. *The Journal of Steroid Biochemistry and Molecular Biology 67*, 403-411.

Mora S. (2009). Advanced lipoprotein testing and subfractionation are not (yet) ready for routine clinical use. *Circulation 119*, 2396-2404.

Morange P. E., Alessi M. C., Verdier M., Casanova D., Magalon G., & Juhan-Vague I. (1999). PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 1361-1365.

Morange P. E., Lijnen H. R., Alessi M. C., Kopp F., Collen D., & Juhan-Vague I. (2000). Influence of PAI-1 on adipose tissue growth and metabolic parameters in a murine model of diet-induced obesity. *Arteriosclerosis, Thrombosis, and Vascular Biology 20*, 1150-1154.

Morimoto S., Mendoza-Rodriguez C., Hiriart M., Larrieta M., Vital P., & Cerbon M. (2005). Protective effect of testosterone on early apoptotic damage induced by streptozotocin in rat pancreas. *Journal of Endocrinology 187*, 217-224.

Morinaga H., Talukdar S., Bae E. J., & Olefsky J. M. (2012). Increased macrophage migration into adipose tissue in obese mice. *Diabetes 61*, 346-354.

Moriyama K., Negami M., & Takahashi E. (2014). HDL2-cholesterol/HDL3-cholesterol ratio was associated with insulin resistance, high-molecular-weight adiponectin, and components for metabolic syndrome in Japanese. *Diabetes Research and Clinical Practice* .

Morris D. L., Oatmen K. E., Wang T., DelProposto J. L., & Lumeng C. N. (2012). CX3CR1 Deficiency Does Not Influence Trafficking of Adipose Tissue Macrophages in Mice With Diet-Induced Obesity. *Obesity 20*, 1189-1199.

Morton G. J. & Schwartz M. W. (2011). Leptin and the central nervous system control of glucose metabolism. *Physiological Reviews 91*, 389-411.

Moschen A. R., Molnar C., Enrich B., Geiger S., Ebenbichler C. F., & Tilg H. (2011). Adipose and liver expression of interleukin (IL)-1 family members in morbid obesity and effects of weight loss. *Molecular Medicine (Cambridge, Mass.) 17*, 840-845.

Movérare-Skrtic S., Venken K., Andersson N., Lindberg M. K., Svensson J., Swanson C., Vanderschueren D., Oscarsson J., Gustafsson J., & Ohlsson C. (2006). Dihydrotestosterone treatment results in obesity and altered lipid metabolism in orchidectomized mice. *Obesity 14*, 662-672.

Mudali S. & Dobs A. S. (2004). Effects of testosterone on body composition of the aging male. *Mechanisms of Ageing and Development 125*, 297-304.

Muller M., Grobbee D. E., den Tonkelaar I., Lamberts S. W., & van der Schouw, Yvonne T (2005). Endogenous sex hormones and metabolic syndrome in aging men. *Journal of Clinical Endocrinology & Metabolism 90*, 2618-2623.

Muller M., van den Beld, Annewieke W, Bots M. L., Grobbee D. E., Lamberts S. W., & van der Schouw, Yvonne T (2004). Endogenous sex hormones and progression of carotid atherosclerosis in elderly men. *Circulation 109*, 2074-2079.

Muller M., van der Schouw, Yvonne T, Thijssen J. H., & Grobbee D. E. (2003). Endogenous sex hormones and cardiovascular disease in men. *Journal of Clinical Endocrinology & Metabolism 88*, 5076-5086.

Muraleedharan V. & Jones T. H. (2010). Review: Testosterone and the metabolic syndrome. *Therapeutic Advances in Endocrinology and Metabolism 1*, 207-223.

Musliner T. & Krauss R. (1987). Lipoprotein subspecies and risk of coronary disease. *Clinical Chemistry 34*, B78-83.

Muth N. D., Laughlin G. A., von Mühlen D., Smith S. C., & Barrett-Connor E. (2010). High-density lipoprotein subclasses are a potential intermediary between alcohol intake and reduced risk of cardiovascular disease: the Rancho Bernardo Study. *British Journal of Nutrition 104*, 1034-1042.

Myers Jr M. G., Leibel R. L., Seeley R. J., & Schwartz M. W. (2010). Obesity and leptin resistance: distinguishing cause from effect. *Trends in Endocrinology & Metabolism 21*, 643-651.

Naccache P. H., Molski T., Borgeat P., White J., & Sha'Afi R. (1985). Phorbol esters inhibit the fMet-Leu-Phe-and leukotriene B4-stimulated calcium mobilization and enzyme secretion in rabbit neutrophils. *Journal of Biological Chemistry 260*, 2125-2131.

Naccache P. H., Molski T. F., Borgeat P., & Sha'afi R. I. (1984). Association of leukotriene B< sub> 4</sub> with the cytoskeleton of rabbit neutrophils, effect of chemotactic factor and phorbol esters. *Biochemical and Biophysical Research Communications 124*, 963-969.

Nagasawa T., Inada Y., Nakano S., Tamura T., Takahashi T., Maruyama K., Yamazaki Y., Kuroda J., & Shibata N. (2006). Effects of bezafibrate, PPAR pan-agonist, and GW501516, PPARδ agonist, on development of steatohepatitis in mice fed a methionine-and choline-deficient diet. *European Journal of Pharmacology 536*, 182-191.

Nahrendorf M. & Swirski F. K. (2013). Monocyte and macrophage heterogeneity in the heart. *Circulation Research 112*, 1624-1633.

Nakashima Y., Plump A. S., Raines E. W., Breslow J. L., & Ross R. (1994). ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arteriosclerosis, Thrombosis, and Vascular Biology 14*, 133-140.

Nakashima Y., Raines E. W., Plump A. S., Breslow J. L., & Ross R. (1998). Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arteriosclerosis, Thrombosis, and Vascular Biology 18*, 842-851.

Natarajan P., Ray K. K., & Cannon C. P. (2010). High-Density Lipoprotein and Coronary Heart DiseaseCurrent and Future Therapies. *Journal of the American College of Cardiology 55*, 1283-1299.

Nathan L., Shi W., Dinh H., Mukherjee T. K., Wang X., Lusis A. J., & Chaudhuri G. (2001). Testosterone inhibits early atherogenesis by conversion to estradiol: critical role of aromatase. *Proceedings of the National Academy of Sciences of the United States of America 98*, 3589-3593.

Navab M., Imes S., Hama S., Hough G., Ross L., Bork R., Valente A., Berliner J., Drinkwater D., & Laks H. (1991). Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *Journal of Clinical Investigation 88*, 2039.

Navab M., Reddy S. T., Van Lenten B. J., & Fogelman A. M. (2011). HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms. *Nature Reviews Cardiology 8*, 222-232.

Nelken N., Coughlin S., Gordon D., & Wilcox J. (1991). Monocyte chemoattractant protein-1 in human atheromatous plaques. *Journal of Clinical Investigation 88*, 1121.

Nelson P. J. & Krensky A. M. (2001). Chemokines, chemokine receptors, and allograft rejection. *Immunity 14*, 377-386.

Nettleship J., Jones R., Channer K., & Jones T. (2008). Testosterone and coronary artery disease.

Nettleship J., Pugh P., Channer K., Jones T., & Jones R. (2007a). Inverse relationship between serum levels of interleukin-1β and testosterone in men with stable coronary artery disease. *Hormone and Metabolic Research 39*, 366-371.

Nettleship J. E., Jones T. H., Channer K. S., & Jones R. D. (2007b). Physiological testosterone replacement therapy attenuates fatty streak formation and improves high-density lipoprotein cholesterol in the Tfm mouse an effect that is independent of the classic androgen receptor. *Circulation 116*, 2427-2434.

Ng M. K., Liu P. Y., Williams A. J., Nakhla S., Ly L. P., Handelsman D. J., & Celermajer D. S. (2002). Prospective study of effect of androgens on serum inflammatory markers in men. *Arteriosclerosis, Thrombosis, and Vascular Biology 22*, 1136-1141.

Ng M. K., Quinn C. M., McCrohon J. A., Nakhla S., Jessup W., Handelsman D. J., Celermajer D. S., & Death A. K. (2003). Androgens up-regulate atherosclerosis-related genes in macrophages from males but not femalesmolecular insights into gender differences in atherosclerosis. *Journal of the American College of Cardiology 42*, 1306-1313.

Nieschlag E Z. M. (2004). Pharmacogenetics of testosterone action. *The 4th World Congress on the Aging Male* .

Nieschlag, E., Behre, H. M., & Nieschlag, S. (2012). *Testosterone: action, deficiency, substitution.* : Cambridge University Press.

Nieschlag E., Behre H. M., Bouchard P., Corrales J. J., Jones T. H., Stalla G. K., Webb S. M., & Wu F. C. (2004). Testosterone replacement therapy: current trends and future directions. *Human Reproduction Update 10*, 409-419.

Niessner A., Marculescu R., Haschemi A., Endler G., Zorn G., Weyand C. M., Maurer G., Mannhalter C., Wojta J., & Wagner O. (2005). Opposite effects of CX3CRI receptor polymorphisms V249I and T280M on the development of acute coronary syndrome: A possible implication of fractalkine in inflammatory activation. *Thrombosis and Haemostasis 93*, 949-954.

Nikolaenko L., Jia Y., Wang C., Diaz-Arjonilla M., Yee JK., French SW., Liu PY., Laurel S., Chong C., & Lee K. (2014). Testosterone Replacement Ameliorates Nonalcoholic Fatty Liver Disease in Castrated Male Rats. *Endocrinology*, 2013, 155, 2, 417-428.

Nikoulin I. R. & Curtiss L. K. (1998). An apolipoprotein E synthetic peptide targets to lipoproteins in plasma and mediates both cellular lipoprotein interactions in vitro and acute clearance of cholesterol-rich lipoproteins in vivo. *Journal of Clinical Investigation 101*, 223.

Nishizawa H., Shimomura I., Kishida K., Maeda N., Kuriyama H., Nagaretani H., Matsuda M., Kondo H., Furuyama N., Kihara S., Nakamura T., Tochino Y., Funahashi

T., & Matsuzawa Y. (2002). Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes 51*, 2734-2741.

Norata G. D., Tibolla G., Seccomandi P. M., Poletti A., & Catapano A. L. (2006). Dihydrotestosterone decreases tumor necrosis factor-α and lipopolysaccharide-induced inflammatory response in human endothelial cells. *Journal of Clinical Endocrinology & Metabolism 91*, 546-554.

Norata G. D., Cattaneo P., Poletti A., & Catapano A. L. (2010). The androgen derivative 5α-androstane-3β, 17β-diol inhibits tumor necrosis factor α and lipopolysaccharide induced inflammatory response in human endothelial cells and in mice aorta. *Atherosclerosis 212*, 100-106.

Nordestgaard B. G., Chapman M. J., Ray K., Boren J., Andreotti F., Watts G. F., Ginsberg H., Amarenco P., Catapano A., Descamps O. S., Fisher E., Kovanen P. T., Kuivenhoven J. A., Lesnik P., Masana L., Reiner Z., Taskinen M. R., Tokgozoglu L., Tybjaerg-Hansen A., & European Atherosclerosis Society Consensus Panel (2010). Lipoprotein(a) as a cardiovascular risk factor: current status. *European Heart Journal 31*, 2844-2853.

Nov O., Shapiro H., Ovadia H., Tarnovscki T., Dvir I., Shemesh E., Kovsan J., Shelef I., Carmi Y., & Voronov E. (2013). Interleukin-1β regulates fat-liver crosstalk in obesity by auto-paracrine modulation of adipose tissue inflammation and expandability. *PloS One 8*, e53626.

Ntambi J. M. & Young-Cheul K. (2000). Adipocyte differentiation and gene expression. *The Journal of Nutrition 130*, 3122S-3126S.

Nussey, S. S. & Whitehead, S. A. (2013). *Endocrinology: an integrated approach.* : CRC Press.

O'Brien T., Nguyen T., Hallaway B., Hodge D., Bailey K., Holmes D., & Kottke B. (1995). The role of lipoprotein AI and lipoprotein AI/A-II in predicting coronary artery disease. *Arteriosclerosis, Thrombosis, and Vascular Biology 15*, 228-231.

O'Connell B. J. & Genest J. (2001). High-density lipoproteins and endothelial function. *Circulation 104*, 1978-1983.

O'Connor J. C., Sherry C. L., Guest C. B., & Freund G. G. (2007). Type 2 diabetes impairs insulin receptor substrate-2-mediated phosphatidylinositol 3-kinase activity in primary macrophages to induce a state of cytokine resistance to IL-4 in association with overexpression of suppressor of cytokine signaling-3. *Journal of Immunology (Baltimore, Md.: 1950) 178*, 6886-6893.

Ogden C. L., Carroll M. D., Kit B. K., & Flegal K. M. (2014). Prevalence of childhood and adult obesity in the United States, 2011-2012. *Jama 311*, 806-814.

Oh J. Y., Barrett-Connor E., Wedick N. M., Wingard D. L., & Rancho Bernardo Study (2002). Endogenous sex hormones and the development of type 2 diabetes in older men and women: the Rancho Bernardo study. *Diabetes Care 25*, 55-60.

Oh Y., Morinaga H., Talukdar S., Bae J., & Olefsky J. (2012). Increased macrophage migration into adipose tissue in obese mice. Diabetes, 61; 346-54.

Ohl F., Jung M., Xu C., Stephan C., Rabien A., Burkhardt M., Nitsche A., Kristiansen G., Loening S. A., & Radonić A. (2005). Gene expression studies in prostate cancer tissue: which reference gene should be selected for normalization? *Journal of Molecular Medicine 83*, 1014-1024.

Ohta H., Wada H., Niwa T., Kirii H., Iwamoto N., Fujii H., Saito K., Sekikawa K., & Seishima M. (2005). Disruption of tumor necrosis factor-α gene diminishes the development of atherosclerosis in ApoE-deficient mice. *Atherosclerosis 180*, 11-17.

Okamoto Y., Folco E. J., Minami M., Wara A., Feinberg M. W., Sukhova G. K., Colvin R. A., Kihara S., Funahashi T., & Luster A. D. (2008). Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis. *Circulation Research 102*, 218-225.

Okumoto S., Taniguchi Y., Nakashima A., Masaki T., Ito T., Ogawa T., Takasugi N., Kohno N., & Yorioka N. (2009). C-C Chemokine Receptor 2 Expression by Circulating Monocytes Influences Atherosclerosis in Patients on Chronic Hemodialysis. *Therapeutic Apheresis and Dialysis 13*, 205-212.

Olefsky J. M. & Glass C. K. (2010). Macrophages, inflammation, and insulin resistance. *Annual Review of Physiology 72*, 219-246.

Olga Gruzdeva, Evgenya Uchasova, Yulia Dyleva, Ekaterina Belik, Ekaterina Shurygina, Olga Barbarash (2013). Plasminogen activator inhibitor-, free fatty acids, and insulin resistance in patients with myocardial infarction.Diabetes, Metabolic Syndrome and Obesity. *Targets and Therapy 6*, 293-301.

Oliver W. R., Shenk J. L., Snaith M. R., Russell C. S., Plunket K. D., Bodkin N. L., Lewis M. C., Winegar D. A., Sznaidman M. L., & Lambert M. H. (2001). A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences 98*, 5306-5311.

Ollivier V., Faure S., Tarantino N., Chollet-Martin S., Deterre P., Combadière C., & de Prost D. (2003). Fractalkine/CX3CL1 production by human aortic smooth muscle cells impairs monocyte procoagulant and inflammatory responses. *Cytokine 21*, 303-311.

Olofsson S. & Boren J. (2005). Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. *Journal of Internal Medicine 258*, 395-410.

Olofsson S. O., Wiklund O., & Boren J. (2007). Apolipoproteins A-I and B: biosynthesis, role in the development of atherosclerosis and targets for intervention against cardiovascular disease. *Vascular Health and Risk Management 3*, 491-502.

Olsen N. J. & Kovacs W. J. (1995). Testosterone treatment of systemic lupus erythematosus in a patient with Klinefelter's syndrome. *The American Journal of the Medical Sciences 310*, 158-160.

Omenn G. S., Beresford S. A., & Motulsky A. G. (1998). Preventing Coronary Heart Disease B Vitamins and Homocysteine. *Circulation 97*, 421-424.

Onat A., Can G., G Hergenç M. Y., & A Karabulut S. A. (2007). Serum apolipoprotein B predicts dyslipidemia, metabolic syndrome and, in women, hypertension and diabetes, independent of markers of central obesity and inflammation. *International Journal of Obesity 31*, 1119-1125.

Onat A., Uğur M., Can G., Yüksel H., & Hergenç G. (2010). Visceral adipose tissue and body fat mass: predictive values for and role of gender in cardiometabolic risk among Turks. *Nutrition 26*, 382-389.

Opalek J. M., Ali N. A., Lobb J. M., Hunter M. G., & Marsh C. B. (2007). Alveolar macrophages lack CCR2 expression and do not migrate to CCL2. *J Inflamm (Lond) 4*, 19.

Orekhov A., Sobenin I., & Smirnov A. (2009). P1136 sex hormones decrease intracellular cholesterol accumulation caused by modified lipoprotein. *Atherosclerosis Supplements 10*, e1162.

Oslund L. J. P., Hedrick C. C., Olvera T., Hagenbaugh A., Territo M., Berliner J. A., & Fyfe A. I. (1999). Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 2847-2853.

Ota T. (2013). Chemokine Systems Link Obesity to Insulin Resistance. *Diabetes & Metabolism Journal 37*, 165-172.

Ouchi N., Kihara S., Arita Y., Maeda K., Kuriyama H., Okamoto Y., Hotta K., Nishida M., Takahashi M., & Nakamura T. (1999). Novel modulator for endothelial adhesion molecules adipocyte-derived plasma protein adiponectin. *Circulation 100*, 2473-2476.

Ouchi N., Kihara S., Arita Y., Nishida M., Matsuyama A., Okamoto Y., Ishigami M., Kuriyama H., Kishida K., & Nishizawa H. (2001). Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation 103*, 1057-1063.

Ouchi N., Kihara S., Arita Y., Okamoto Y., Maeda K., Kuriyama H., Hotta K., Nishida M., Takahashi M., & Muraguchi M. (2000). Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-κB signaling through a cAMP-dependent pathway. *Circulation 102*, 1296-1301.

Ouchi N., Parker J. L., Lugus J. J., & Walsh K. (2011). Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology 11*, 85-97

Ouyang W., Rutz S., Crellin N. K., Valdez P. A., & Hymowitz S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology 29*, 71-109.

Ozata M., Yildirimkaya M., Bulur M., Yilmaz K., Bolu E., Corakci A., & Gundogan M. (1996). Effects of gonadotropin and testosterone treatments on Lipoprotein (a), high density lipoprotein particles, and other lipoprotein levels in male hypogonadism. *Journal of Clinical Endocrinology & Metabolism 81*, 3372-3378.

Ozata M., Oktenli C., Bingol N., & Ozdemir I. C. (2001). The effects of metformin and diet on plasma testosterone and leptin levels in obese men. *Obesity Research 9*, 662-667.

Page S. T., Amory J. K., Bowman F. D., Anawalt B. D., Matsumoto A. M., Bremner W. J., & Tenover J. L. (2005a). Exogenous testosterone (T) alone or with finasteride increases physical performance, grip strength, and lean body mass in older men with low serum T. *Journal of Clinical Endocrinology & Metabolism 90*, 1502-1510.

Page S. T., Herbst K. L., Amory J. K., Coviello A. D., Anawalt B. D., Matsumoto A. M., & Bremner W. J. (2005b). Testosterone administration suppresses adiponectin levels in men. *Journal of Andrology 26*, 85-92.

Pai J. K., Pischon T., Ma J., Manson J. E., Hankinson S. E., Joshipura K., Curhan G. C., Rifai N., Cannuscio C. C., & Stampfer M. J. (2004). Inflammatory markers and the risk of coronary heart disease in men and women. *New England Journal of Medicine 351*, 2599-2610.

Panee J. (2012). Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. *Cytokine 60*, 1-12.

Papanicolaou D. A. (2000). Interleukin-6: the endocrine cytokine. *Journal of Clinical Endocrinology & Metabolism 85*, 1331-1333.

Papanicolaou D. A., Wilder R. L., Manolagas S. C., & Chrousos G. P. (1998). The pathophysiologic roles of interleukin-6 in human disease. *Annals of Internal Medicine 128*, 127-137.

Parish S., Peto R., Palmer A., Clarke R., Lewington S., Offer A., Whitlock G., Clark S., Youngman L., Sleight P., Collins R., & International studies of infarct survival collaborators (2009). The joint effects of apolipoprotein B, apolipoprotein A1, LDL cholesterol, and HDL cholesterol on risk: 3510 cases of acute myocardial infarction and 9805 controls. *European Heart Journal 30*, 2137-2146.

Park K., Park K. S., Kim M., Kim H., Suh Y., Ahn J. D., Park K., Chang Y., & Lee I. (2004). Relationship between serum adiponectin and leptin concentrations and body fat distribution. *Diabetes Research and Clinical Practice 63*, 135-142.

Parkar M., Tabona P., Newman H., & Olsen I. (1998). IL-6 expression by oral fibroblasts is regulated by androgen. *Cytokine 10*, 613-619.

Parthasarathy C., Renuka V. N., & Balasubramanian K. (2009). Sex steroids enhance insulin receptors and glucose oxidation in Chang liver cells. *Clinica Chimica Acta 399*, 49-53.

Pasarica M., Sereda O. R., Redman L. M., Albarado D. C., Hymel D. T., Roan L. E., Rood J. C., Burk D. H., & Smith S. R. (2009). Reduced adipose tissue oxygenation in human obesity evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes 58*, 718-725.

Pease J. (2006). Tails of the unexpected–an atypical receptor for the chemokine RANTES/CCL5 expressed in brain. *British Journal of Pharmacology 149*, 460-462.

Pedro V., Carlota G., & Miguel S. Omega 3 fatty acids induce a marked reduction of apolipoprotein B48 when added to fluvastatin in patients with type 2 diabetes and mixed hyperlipidemia: a preliminary report.

Peeters W., Hellings W., De Kleijn D., de Vries J., Moll F., Vink A., & Pasterkamp G. (2009). Carotid atherosclerotic plaques stabilize after stroke insights into the natural process of atherosclerotic plaque stabilization. *Arteriosclerosis, Thrombosis, and Vascular Biology 29*, 128-133.

Pennline K. J., Roque-Gaffney E., & Monahan M. (1994). Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clinical Immunology and Immunopathology 71*, 169-175.

Pergola G. d. (2000). The adipose tissue metabolism: role of testosterone and dehydroepiandrosterone. *International Journal of Obesity 24*, S59-S63.

Peters J. M., Lee S. S., Li W., Ward J. M., Gavrilova O., Everett C., Reitman M. L., Hudson L. D., & Gonzalez F. J. (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β (δ). *Molecular and Cellular Biology 20*, 5119-5128.

Pfaffl M. W., Horgan G. W., & Dempfle L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research 30*, e36.

Pfaffl M. W. (2006). Relative quantification. *Real-Time PCR* , 63-82.

Phillips G. B., Pinkernell B. H., & Jing T. (1994). The association of hypotestosteronemia with coronary artery disease in men. *Arteriosclerosis, Thrombosis, and Vascular Biology 14*, 701-706.

Pickersgill L., Litherland G. J., Greenberg A. S., Walker M., & Yeaman S. J. (2007). Key role for ceramides in mediating insulin resistance in human muscle cells. *Journal of Biological Chemistry 282*, 12583-12589.

Pickup J. & Crook M. (1998). Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia 41*, 1241-1248.

Piedrahita J. A., Zhang S. H., Hagaman J. R., Oliver P. M., & Maeda N. (1992). Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proceedings of the National Academy of Sciences 89*, 4471-4475.

Pirillo A., Norata G. D., & Catapano A. L. (2013). LOX-1, OxLDL, and atherosclerosis. *Mediators of Inflammation 2013*.

Pischon N., Heng N., Bernimoulin J., Kleber B., Willich S., & Pischon T. (2007). Obesity, inflammation, and periodontal disease. *Journal of Dental Research 86*, 400-409.

Pischon T., Girman C. J., Rifai N., Hotamisligil G. S., & Rimm E. B. (2005). Association between dietary factors and plasma adiponectin concentrations in men. *The American Journal of Clinical Nutrition 81*, 780-786.

Pittas A. G., Joseph N. A., & Greenberg A. S. (2004). Adipocytokines and insulin resistance. *Journal of Clinical Endocrinology & Metabolism 89*, 447-452.

Plaisance E. P., Grandjean P. W., Judd R. L., Jones K. W., & Taylor J. K. (2009). The influence of sex, body composition, and nonesterified fatty acids on serum adipokine concentrations. *Metabolism 58*, 1557-1563.

Plump A. S., Smith J. D., Hayek T., Aalto-Setälä K., Walsh A., Verstuyft J. G., Rubin E. M., & Breslow J. L. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell 71*, 343-353.

Poirier P., Giles T. D., Bray G. A., Hong Y., Stern J. S., Pi-Sunyer F. X., & Eckel R. H. (2006). Obesity and cardiovascular disease pathophysiology, evaluation, and effect of weight loss. *Arteriosclerosis, Thrombosis, and Vascular Biology 26*, 968-976.

Polyak A., Ferenczi S., Denes A., Winkler Z., Kriszt R., Pinter-Kubler B., & Kovacs K. J. (2014). The fractalkine/Cx3CR1 system is implicated in the development of metabolic visceral adipose tissue inflammation in obesity. *Brain, Behavior, and Immunity 38*, 25-35.

Poole J. & Florey H. (1958). Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits. *The Journal of Pathology and Bacteriology 75*, 245-251.

Pradhan A. (2007). Obesity, metabolic syndrome, and type 2 diabetes: inflammatory basis of glucose metabolic disorders. *Nutrition Reviews 65*, S152-S156.

Pratley R. E. (2005). The evolving role of inflammation in obesity and the metabolic syndrome. *Current Diabetes Reports 5*, 70-75.

Pratt W. B. & Toft D. O. (1997). Steroid receptor interactions with heat shock protein and immunophilin chaperones 1. *Endocrine Reviews 18*, 306-360.

Pugh P. J., Channer K. S., Parry H., Downes T., & Hugh Jones T. (2002). Bio-available testosterone levels fall acutely following myocardial infarction in men: association with fibrinolytic factors. *Endocrine Research 28*, 161-173.

Puren A. J., Fantuzzi G., Gu Y., Su M., & Dinarello C. A. (1998). Interleukin-18 (IFNgamma-inducing factor) induces IL-8 and IL-1beta via TNFalpha production from non-CD14 human blood mononuclear cells. *Journal of Clinical Investigation 101*, 711.

Qatanani M., & Lazar M. A. (2007). Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes & Development 21*, 1443-1455.

Qiang W. Xiao L. (2003). Effects of serum testosterone level on plasma lipid and apolipoprotein spectrum in orchiectomized rabbits. *Chinese Journal of Applied Physiology 3*, 295-7

Qiu H., Gabrielsen A., Agardh H. E., Wan M., Wetterholm A., Wong C., Hedin U., Swedenborg J., Hansson G. K., & Samuelsson B. (2006). Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. *Proceedings of the National Academy of Sciences 103*, 8161-8166.

Qiu Y., Yanase T., Hu H., Tanaka T., Nishi Y., Liu M., Sueishi K., Sawamura T., & Nawata H. (2010). Dihydrotestosterone suppresses foam cell formation and attenuates atherosclerosis development. *Endocrinology 151*, 3307-3316.

Rabinovitch A., Suarez-Pinzon W., Strynadka K., Ju Q., Edelstein D., Brownlee M., Korbutt G. S., & Rajotte R. V. (1999). Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. *Diabetes 48*, 1223-1229.

Rader D. J., Cain W., Zech L. A., Usher D., & Brewer Jr H. B. (1993). Variation in lipoprotein (a) concentrations among individuals with the same apolipoprotein (a) isoform is determined by the rate of lipoprotein (a) production. *Journal of Clinical Investigation 91*, 443.

Rader D., Mann W., Cain W., Kraft H., Usher D., Zech L., Hoeg J., Davignon J., Lupien P., & Grossman M. (1995). The low density lipoprotein receptor is not required for normal catabolism of Lp (a) in humans. *Journal of Clinical Investigation 95*, 1403.

Rai A. K., Srivastava N. S., Maurizio A., & Renee L. (1997). Estrogen up-regulates apolipoprotein E (ApoE) gene expression by increasing ApoE mRNA in the translating pool via the estrogen receptor a-mediated pathway. *The Journal of Biological Chemistry 272*, 33360-33366.

Rainwater D. L., McMahan C. A., Malcom G. T., Scheer W. D., Roheim P. S., McGill H. C., & Strong J. P. (1999). Lipid and Apolipoprotein Predictors of Atherosclerosis in Youth Apolipoprotein Concentrations Do Not Materially Improve Prediction of Arterial Lesions in PDAY Subjects. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 753-761.

Rajala M. W. & Scherer P. E. (2003). Minireview: the adipocyte—at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology 144*, 3765-3773.

Ramirez M. E., McMurry M. P., Wiebke G. A., Felten K. J., Ren K., Meikle A. W., & Iverius P. (1997). Evidence for sex steroid inhibition of lipoprotein lipase in men: comparison of abdominal and femoral adipose tissue. *Metabolism 46*, 179-185.

Rana J. S., Arsenault B. J., Després J., Côté M., Talmud P. J., Ninio E., Jukema J. W., Wareham N. J., Kastelein J. J., & Khaw K. (2011). Inflammatory biomarkers, physical activity, waist circumference, and risk of future coronary heart disease in healthy men and women. *European Heart Journal 32*, 336-344.

Randolph G. J. & Furie M. B. (1996). Mononuclear phagocytes egress from an in vitro model of the vascular wall by migrating across endothelium in the basal to apical direction: role of intercellular adhesion molecule 1 and the CD11/CD18 integrins. *The Journal of Experimental Medicine 183*, 451-462.

Rasul S., Ilhan A., Reiter M. H., Baumgartner-Parzer S., & Kautzky-Willer A. (2011). Relations of adiponectin to levels of metabolic parameters and sexual hormones in elderly type 2 diabetic patients. *Gender Medicine 8*, 93-102.

Rasouli N. & Kern P. A. (2008). Adipocytokines and the metabolic complications of obesity. *The Journal of Clinical Endocrinology & Metabolism 93*, s64-s73

Rath M., Niendorf A., Reblin T., Dietel M., Krebber H. J., & Beisiegel U. (1989). Detection and quantification of lipoprotein (a) in the arterial wall of 107 coronary bypass patients. *Arteriosclerosis, Thrombosis, and Vascular Biology 9*, 579-592.

Rayner K., Van Eersel S., Groot P. H., & Reape T. J. (2000). Localisation of mRNA for JE/MCP-1 and its receptor CCR2 in atherosclerotic lesions of the ApoE knockout mouse. *Journal of Vascular Research 37*, 93-102.

Rebuffe-Scrive M., Mårin P., & Björntorp P. (1991). Effect of testosterone on abdominal adipose tissue in men. *International Journal of Obesity 15*, 791-795.

Reddick R. L., Zhang S. H., & Maeda N. (1994). Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. *Arteriosclerosis, Thrombosis, and Vascular Biology 14*, 141-147.

Reddy J. & Ohno S. (1981). Testicular feminization of the mouse: paucity of peroxisomes in Leydig cell of the testis. *The American Journal of Pathology 103*, 96.

Reggie Johal (2012). Testosterone, leptin, body fat levels and how they correlate *Http://www.Predatornutrition.com/blog/2012/03/15/testosterone-Leptin-Body-Fat-Levels-and-how-they-Correlate/* .

Rentsch J. & Chiesi M. (1996). Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Letters 379*, 55-59.

Reyes L., Davidson M. K., Thomas L. C., & Davis J. K. (1999). Effects of Mycoplasma fermentansincognitus on Differentiation of THP-1 Cells. *Infection and Immunity 67*, 3188-3192.

Rice D., Brannigan R. E., Campbell R. K., Fine S., Jack L., Nelson J. B., & Regan-Klich J. (2008). Men's Health, Low Testosterone, and Diabetes Individualized Treatment and a Multidisciplinary Approach. *The Diabetes Educator 34*, 97S-112S.

Ricote M., Huang J. T., Welch J. S., & Glass C. K. (1999). The peroxisome proliferator-activated receptor (PPARgamma) as a regulator of monocyte/macrophage function. *Journal of Leukocyte Biology 66*, 733-739.

Rizk A., Robertson J., & Raber J. (2005). Behavioral performance of< i> tfm</i> mice supports the beneficial role of androgen receptors in spatial learning and memory. *Brain Research 1034*, 132-138.

Robaye B., Mosselmans R., Fiers W., Dumont J. E., & Galand P. (1991). Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. *The American Journal of Pathology 138*, 447-453.

Rocha V. Z. & Folco E. J. (2011). Inflammatory concepts of obesity. *International Journal of Inflammation 2011.*

Romagnani S. (2002). Cytokines and chemoattractants in allergic inflammation. *Molecular Immunology 38*, 881-885.

Rommerts, F. F. (2004). Testosterone: an overview of biosynthesis, transport, metabolism and nongenomic actions. In Anonymous *Testosterone* (pp. 1-31). : Springer.

Roque M., Kim W. J., Gazdoin M., Malik A., Reis E. D., Fallon J. T., Badimon J. J., Charo I. F., & Taubman M. B. (2002). CCR2 deficiency decreases intimal hyperplasia after arterial injury. *Arteriosclerosis, Thrombosis, and Vascular Biology 22*, 554-559.

Rosano G. M., Leonardo F., Pagnotta P., Pelliccia F., Panina G., Cerquetani E., della Monica P. L., Bonfigli B., Volpe M., & Chierchia S. L. (1999). Acute anti-ischemic effect of testosterone in men with coronary artery disease. *Circulation 99*, 1666-1670.

Rosen E. D. & Spiegelman B. M. (2014). What We Talk About When We Talk About Fat. *Cell 156*, 20-44.

Rosenfeld M. E., Butler S., Ord V. A., Lipton B. A., Dyer C. A., Curtiss L. K., Palinski W., & Witztum J. L. (1993). Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions. *Arteriosclerosis, Thrombosis, and Vascular Biology 13*, 1382-1389.

Rosengren A., Wilhelmsen L., Eriksson E., Risberg B., & Wedel H. (1990). Lipoprotein (a) and coronary heart disease: a prospective case-control study in a general population sample of middle aged men. *BMJ: British Medical Journal 301*, 1248.

Rosner W., Hryb D. J., Khan M. S., Nakhla A. M., & Romas N. A. (1992). Sex Hormone-Binding Globulin Binding to Cell Membranes and Generation of a Second Messenger. *Journal of Andrology 13*, 101-106.

Rothenbacher D., Brenner H., März W., & Koenig W. (2005). Adiponectin, risk of coronary heart disease and correlations with cardiovascular risk markers. *European Heart Journal 26*, 1640-1646.

Rubinow K. B., Vaisar T., Tang C., Matsumoto A. M., Heinecke J. W., & Page S. T. (2012). Testosterone replacement in hypogonadal men alters the HDL proteome but not HDL cholesterol efflux capacity. *Journal of Lipid Research 53*, 1376-1383.

Russo I. (2012). The prothrombotic tendency in metabolic syndrome: focus on the potential mechanisms involved in impaired haemostasis and fibrinolytic balance. *Scientifica 2012.*

Ryo M., Nakamura T., Kihara S., Kumada M., Shibazaki S., Takahashi M., Nagai M., Matsuzawa Y., & Funahashi T. (2004). Adiponectin as a biomarker of the metabolic syndrome. *Circulation Journal: Official Journal of the Japanese Circulation Society 68*, 975-981.

Saad F. (2009). The role of testosterone in type 2 diabetes and metabolic syndrome in men. *Arquivos Brasileiros De Endocrinologia & Metabologia 53*, 901-907.

Saad F. & Gooren L. (2009). The role of testosterone in the metabolic syndrome: a review. *The Journal of Steroid Biochemistry and Molecular Biology 114*, 40-43.

Saad F., Gooren L. J., Haider A., & Yassin A. (2008). A dose-response study of testosterone on sexual dysfunction and features of the metabolic syndrome using testosterone gel and parenteral testosterone undecanoate. *Journal of Andrology 29*, 102-105.

Saad F., Gooren L., Haider A., & Yassin A. (2007). An exploratory study of the effects of 12 month administration of the novel long-acting testosterone undecanoate on measures of sexual function and the metabolic syndrome. *Systems Biology in Reproductive Medicine 53*, 353-357.

Saad M. F., Damani S., Gingerich R. L., Riad-Gabriel M. G., Khan A., Boyadjian R., Jinagouda S. D., El-Tawil K., Rude R. K., & Kamdar V. (1997). Sexual dimorphism in plasma leptin concentration. *Journal of Clinical Endocrinology & Metabolism 82*, 579-584.

Saad F., Aversa A., Isidori A. M., & Gooren L. J. (2012). Testosterone as potential effective therapy in treatment of obesity in men with testosterone deficiency: a review. *Current Diabetes Reviews 8*, 131-143.

Saba A. & Oridupa O. (2012). Lipoproteins and Cardiovascular Diseases.

Sacks F. M., Gerhard M., & Walsh B. W. (1995). Sex hormones, lipoproteins, and vascular reactivity. *Current Opinion in Lipidology 6*, 161-166.

Saederup N., Chan L., Lira S. A., & Charo I. F. (2008). Fractalkine deficiency markedly reduces macrophage accumulation and atherosclerotic lesion formation in CCR2−/− Mice evidence for independent chemokine functions in atherogenesis. *Circulation 117*, 1642-1648.

Saigal C. S., Gore J. L., Krupski T. L., Hanley J., Schonlau M., & Litwin M. S. (2007). Androgen deprivation therapy increases cardiovascular morbidity in men with prostate cancer. *Cancer 110*, 1493-1500.

Sakkinen P. A., Wahl P., Cushman M., Lewis M. R., & Tracy R. P. (2000). Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. *American Journal of Epidemiology 152*, 897-907.

Saku K., Ahmad M., Glas-Greenwalt P., & Kashyap M. L. (1985). Activation of fibrinolysis by apolipoproteins of high density lipoproteins in man. *Thrombosis Research 39*, 1-8.

Sakurai T., Ogasawara J., Kizaki T., Sato S., Ishibashi Y., Takahashi M., Kobayashi O., Oh-ishi S., Nagasawa J., & Takahashi K. (2013). The effects of exercise training on obesity-induced dysregulated expression of adipokines in white adipose tissue. *International Journal of Endocrinology 2013*.

Salonen J. T., Salonen R., Seppänen K., Rauramaa R., & Tuomilehto J. (1991). HDL, HDL2, and HDL3 subfractions, and the risk of acute myocardial infarction. A prospective population study in eastern Finnish men. *Circulation 84*, 129-139.

Sandler S., Bendtzen K., Eizirik D. L., & Welsh M. (1990). Interleukin-6 affects insulin secretion and glucose metabolism of rat pancreatic islets in vitro. *Endocrinology 126*, 1288-1294.

Santos A., Lopes C., Guimaraes J., & Barros H. (2005). Central obesity as a major determinant of increased high-sensitivity C-reactive protein in metabolic syndrome. *International Journal of Obesity 29*, 1452-1456.

Sartipy P. & Loskutoff D. J. (2003). Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences 100*, 7265-7270.

Sato T., Matsumoto T., Yamada T., Watanabe T., Kawano H., & Kato S. (2003). Late onset of obesity in male androgen receptor-deficient (AR KO) mice. *Biochemical and Biophysical Research Communications 300*, 167-171.

Satterfield M. C. & Wu G. (2011). Brown adipose tissue growth and development: significance and nutritional regulation. *Frontiers in Bioscience (Landmark Edition) 16*, 1589-1608.

Satterthwaite G., Francis S. E., Suvarna K., Blakemore S., Ward C., Wallace D., Braddock M., & Crossman D. (2005). Differential gene expression in coronary arteries from patients presenting with ischemic heart disease: further evidence for the inflammatory basis of atherosclerosis. *American Heart Journal 150*, 488-499.

Saunders, N. A. & Lee, M. A. (2013). *Real-time PCR: Advanced Technologies and Applications.* : Horizon Scientific Press.

Scarpelli D., Cardellini M., Andreozzi F., Laratta E., Hribal M. L., Marini M. A., Tassi V., Lauro R., Perticone F., & Sesti G. (2006). Variants of the interleukin-10 promoter gene are associated with obesity and insulin resistance but not type 2 diabetes in Caucasian Italian subjects. *Diabetes 55*, 1529-1533.

Schaefer e. J., Foster D. M., Zech L. A., Lindgren F. T., Brewer H. B., & Levy r. I. (1983). The effects of estrogen administration on plasma lipoprotein metabolism in premenopausal females. *Journal of Clinical Endocrinology & Metabolism 57*, 262-267.

Schäfer A., Schulz C., Fraccarollo D., Tas P., Leutke M., Eigenthaler M., Seidl S., Heider P., Ertl G., & Massberg S. (2007). The CX3C chemokine fractalkine induces vascular dysfunction by generation of superoxide anions. *Arteriosclerosis, Thrombosis, and Vascular Biology 27*, 55-62.

Schenk S., Saberi M., & Olefsky J. M. (2008). Insulin sensitivity: modulation by nutrients and inflammation. *The Journal of Clinical Investigation 118*, 2992-3002.

Schieffer B., Selle T., Hilfiker A., Hilfiker-Kleiner D., Grote K., Tietge U. J., Trautwein C., Luchtefeld M., Schmittkamp C., Heeneman S., Daemen M. J., & Drexler H. (2004). Impact of interleukin-6 on plaque development and morphology in experimental atherosclerosis. *Circulation 110*, 3493-3500.

Schling P. & Löffler G. (2002). Cross talk between adipose tissue cells: impact on pathophysiology. *Physiology 17*, 99-104.

Schmittgen T. D. & Livak K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols 3*, 1101-1108.

Schober A., Zernecke A., Liehn E. A., von Hundelshausen P., Knarren S., Kuziel W. A., & Weber C. (2004). Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets. *Circulation Research 95*, 1125-1133.

Schreiner P. J., Morrisett J. D., Sharrett A. R., Patsch W., Tyroler H. A., Wu K., & Heiss G. (1993). Lipoprotein [a] as a risk factor for preclinical atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 13*, 826-833.

Schulz C., Schäfer A., Stolla M., Kerstan S., Lorenz M., von Brühl M., Schiemann M., Bauersachs J., Gloe T., & Busch D. H. (2007). Chemokine Fractalkine Mediates Leukocyte Recruitment to Inflammatory Endothelial Cells in Flowing Whole Blood A Critical Role for P-Selectin Expressed on Activated Platelets. *Circulation 116*, 764-773.

Schulze M. B., Shai I., Rimm E. B., Li T., Rifai N., & Hu F. B. (2005). Adiponectin and future coronary heart disease events among men with type 2 diabetes. *Diabetes 54*, 534-539.

Schwarcz M. D. & Frishman W. H. (2010). Testosterone and coronary artery disease. *Cardiology in Review 18*, 251-257.

See R., Abdullah S. M., McGuire D. K., Khera A., Patel M. J., Lindsey J. B., Grundy S. M., & De Lemos J. A. (2007). The association of differing measures of overweight and obesity with prevalent atherosclerosisthe dallas heart study. *Journal of the American College of Cardiology 50*, 752-759.

Seed M. (1991). Sex hormones, lipoproteins, and cardiovascular risk. *Atherosclerosis 90*, 1-7.

Seidell J. C., Björntorp P., Sjöström L., Kvist H., & Sannerstedt R. (1990). Visceral fat accumulation in men is positively associated with insulin, glucose, and C-peptide levels, but negatively with testosterone levels. *Metabolism 39*, 897-901.

Seidell J. & Bouchard C. (1997). Visceral fat in relation to health: is it a major culprit or simply an innocent bystander? *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity 21*, 626.

Seishima M., Bisgaier C., Davies S., & Glickman R. M. (1991). Regulation of hepatic apolipoprotein synthesis in the 17 alpha-ethinyl estradiol-treated rat. *Journal of Lipid Research 32*, 941-951.

Sell H. & Eckel J. (2009). Chemotactic cytokines, obesity and type 2 diabetes: in vivo and in vitro evidence for a possible causal correlation? *Diabetes 5*, 6.

Seman L. J., DeLuca C., Jenner J. L., Cupples L. A., McNamara J. R., Wilson P. W., Castelli W. P., Ordovas J. M., & Schaefer E. J. (1999). Lipoprotein (a)-cholesterol and coronary heart disease in the Framingham Heart Study. *Clinical Chemistry 45*, 1039-1046.

Sewter C. P., Digby J. E., Blows F., Prins J., & O'Rahilly S. (1999). Regulation of tumour necrosis factor-alpha release from human adipose tissue in vitro. *The Journal of Endocrinology 163*, 33-38.

Shah PK (2011). Atherosclerosis: targeting endogenous apo A-I-a new approach for raising HDL. *Nature Reviews. Cardiology 8*, 187-188.

Shah R., Hinkle C. C., Ferguson J. F., Mehta N. N., Li M., Qu L., Lu Y., Putt M. E., Ahima R. S., & Reilly M. P. (2011). Fractalkine is a novel human adipochemokine associated with type 2 diabetes. *Diabetes 60*, 1512-1518.

Shah R., Lu Y., Hinkle C. C., McGillicuddy F. C., Kim R., Hannenhalli S., Cappola T. P., Heffron S., Wang X., & Mehta N. N. (2009). Gene profiling of human adipose tissue during evoked inflammation in vivo. *Diabetes 58*, 2211-2219.

Shaul M. E., Bennett G., Strissel K. J., Greenberg A. S., & Obin M. S. (2010). Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. *Diabetes 59*, 1171-1181.

Shi H., Kokoeva M. V., Inouye K., Tzameli I., Yin H., & Flier J. S. (2006). TLR4 links innate immunity and fatty acid–induced insulin resistance. *Journal of Clinical Investigation 116*, 3015-3025.

Shi H., Tzameli I., Bjørbæk C., & Flier J. S. (2004). Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling. *Journal of Biological Chemistry 279*, 34733-34740.

Shimano H., Ohsuga J., Shimada M., Namba Y., Gotoda T., Harada K., Katsuki M., Yazaki Y., & Yamada N. (1995). Inhibition of diet-induced atheroma formation in transgenic mice expressing apolipoprotein E in the arterial wall. *Journal of Clinical Investigation 95*, 469.

Shimomura I., Funahasm T., Takahashi M., Maeda K., Kotani K., Nakamura T., Yamashita S., Miura M., Fukuda Y., & Takemura K. (1996). Enhanced expression of PAI–1 in visceral fat: Possible contributor to vascular disease in obeisty. *Nature Medicine 2*, 800-803.

Shoelson S. E., Herrero L., & Naaz A. (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology 132*, 2169-2180.

Shoelson S. E., Lee J., & Goldfine A. B. (2006). Inflammation and insulin resistance. *Journal of Clinical Investigation 116*, 1793-1801.

Shulman A. I. & Mangelsdorf D. J. (2005). Retinoid X receptor heterodimers in the metabolic syndrome. *New England Journal of Medicine 353*, 604-615.

Siasos G., Tousoulis D., Kollia C., Oikonomou E., Siasou Z., Stefanadis C., & G Papavassiliou A. (2012). Adiponectin and cardiovascular disease: mechanisms and new therapeutic approaches. *Current Medicinal Chemistry 19*, 1193-1209.

Sica A., Saccani A., Borsatti A., Power C. A., Wells T. N., Luini W., Polentarutti N., Sozzani S., & Mantovani A. (1997). Bacterial lipopolysaccharide rapidly inhibits

expression of C–C chemokine receptors in human monocytes. *The Journal of Experimental Medicine 185*, 969-974.

Sierra-Honigmann M. R., Nath A. K., Murakami C., García-Cardeña G., Papapetropoulos A., Sessa W. C., Madge L. A., Schechner J. S., Schwabb M. B., & Polverini P. J. (1998). Biological action of leptin as an angiogenic factor. *Science 281*, 1683-1686.

Sigurdsson G., Baldursdottir A., Sigvaldason H., Agnarsson U., Thorgeirsson G., & Sigfusson N. (1992). Predictive value of apolipoproteins in a prospective survey of coronary artery disease in men. *The American Journal of Cardiology 69*, 1251-1254.

Sih R., Morley J. E., Kaiser F. E., Perry III H. M., Patrick P., & Ross C. (1997). Testosterone replacement in older hypogonadal men: a 12-month randomized controlled trial. *The Journal of Clinical Endocrinology & Metabolism 82*, 1661-1667.

Sih R., Morley J. E., Kaiser F. E., Perry H. M., Patrick P., & Ross C. (1997). Testosterone replacement in older hypogonadal men: a 12-month randomized controlled trial. *Journal of Clinical Endocrinology & Metabolism 82*, 1661-1667.

Silver N., Cotroneo E., Proctor G., Osailan S., Paterson K. L., & Carpenter G. H. (2008). Selection of housekeeping genes for gene expression studies in the adult rat submandibular gland under normal, inflamed, atrophic and regenerative states. *BMC Molecular Biology 9*, 64.

Silverman D. I., Ginsburg G. S., & Pasternak R. C. (1993). High-density lipoprotein subfractions. *The American Journal of Medicine 94*, 636-645.

Simon D., Charles M., Lahlou N., Nahoul K., Oppert J., Gouault-Heilmann M., Lemort N., Thibult N., Joubert E., & Balkau B. (2001). Androgen Therapy Improves Insulin Sensitivity and Decreases Leptin Level in Healthy Adult Men With Low Plasma Total Testosterone A 3-month randomized placebo-controlled trial. *Diabetes Care 24*, 2149-2151.

Simon D., Charles M., Nahoul K., Orssaud G., Kremski J., Hully V., Joubert E., Papoz L., & Eschwege E. (1997). Association between plasma total testosterone and cardiovascular risk factors in healthy adult men: The Telecom Study. *Journal of Clinical Endocrinology & Metabolism 82*, 682-685.

Singh A. B., Hsia S., Alaupovic P., Sinha-Hikim I., Woodhouse L., Buchanan T. A., Shen R., Bross R., Berman N., & Bhasin S. (2002). The effects of varying doses of T on insulin sensitivity, plasma lipids, apolipoproteins, and C-reactive protein in healthy young men. *Journal of Clinical Endocrinology & Metabolism 87*, 136-143.

Singh M., Mastana S. S., & Singh P. P. (2002). Genetic variation of apolipoproteins in North Indians. *Human Biology 74*, 673-682.

Singh R., Artaza J. N., Taylor W. E., Braga M., Yuan X., Gonzalez-Cadavid N. F., & Bhasin S. (2006). Testosterone inhibits adipogenic differentiation in 3T3-L1 cells: nuclear translocation of androgen receptor complex with β-catenin and T-cell factor 4 may bypass canonical Wnt signaling to down-regulate adipogenic transcription factors. *Endocrinology 147*, 141-154.

Singh R., Artaza J. N., Taylor W. E., Gonzalez-Cadavid N. F., & Bhasin S. (2003). Androgens stimulate myogenic differentiation and inhibit adipogenesis in C3H 10T1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology 144*, 5081-5088.

Singh R. & Sobhia M. E. (2013). Structure prediction and molecular dynamics simulations of a G-protein coupled receptor: human CCR2 receptor. *Journal of Biomolecular Structure and Dynamics 31*, 694-715.

Sirois-Gagnon D., Chamberland A., Perron S., Brisson D., Gaudet D., & Laprise C. (2011). Association of common polymorphisms in the fractalkine receptor (CX3CR1) with obesity. *Obesity 19*, 222-227.

Skurk T. & Hauner H. (2004). Obesity and impaired fibrinolysis: role of adipose production of plasminogen activator inhibitor-1. *International Journal of Obesity 28*, 1357-1364.

Skurk T., Kolb H., Müller-Scholze S., Röhrig K., Hauner H., & Herder C. (2005). The proatherogenic cytokine interleukin-18 is secreted by human adipocytes. *European Journal of Endocrinology 152*, 863-868.

Smith C. D., Uhing R., & Snyderman R. (1987). Nucleotide regulatory protein-mediated activation of phospholipase C in human polymorphonuclear leukocytes is disrupted by phorbol esters. *Journal of Biological Chemistry 262*, 6121-6127.

Smith G. D., Ben-Shlomo Y., Beswick A., Yarnell J., Lightman S., & Elwood P. (2005). Cortisol, testosterone, and coronary heart disease prospective evidence from the Caerphilly Study. *Circulation 112*, 332-340.

Smith J. D., Trogan E., Ginsberg M., Grigaux C., Tian J., & Miyata M. (1995). Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proceedings of the National Academy of Sciences 92*, 8264-8268.

Smith A. M., English K. M., Malkin C. J., Jones R. D., Jones T. H., & Channer K. S. (2005). Testosterone does not adversely affect fibrinogen or tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) levels in 46 men with chronic stable angina. *European Journal of Endocrinology / European Federation of Endocrine Societies 152*, 285-291.

Smuts C. M., Weich H. F., Weight M. J., Faber M., Kruger M., Lombard C. J., & Benadé A. S. (1994). Free cholesterol concentrations in the high-density lipoprotein subfraction-3 as a risk indicator in patients with angiographically documented coronary artery disease. *Coronary Artery Disease 5*, 331-338.

Sniderman A. D., Scantlebury T., & Cianflone K. (2001). Hypertriglyceridemic hyperapob: the unappreciated atherogenic dyslipoproteinemia in type 2 diabetes mellitus. *Annals of Internal Medicine 135*, 447-459.

Snyder P. J., Peachey H., Berlin J. A., Hannoush P., Haddad G., Dlewati A., Santanna J., Loh L., Lenrow D. A., & Holmes J. H. (2000). Effects of testosterone replacement in hypogonadal men. *Journal of Clinical Endocrinology & Metabolism 85*, 2670-2677.

Snyder P. J., Peachey H., Berlin J. A., Rader D., Usher D., Loh L., Hannoush P., Dlewati A., Holmes J. H., & Santanna J. (2001). Effect of transdermal testosterone treatment on serum lipid and apolipoprotein levels in men more than 65 years of age. *The American Journal of Medicine 111*, 255-260.

Snyder P. J., Peachey H., Hannoush P., Berlin J. A., Loh L., Lenrow D. A., Holmes J. H., Dlewati A., Santanna J., & Rosen C. J. (1999). Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age. *Journal of Clinical Endocrinology & Metabolism 84*, 2647-2653.

Sofi F., Marcucci R., Abbate R., Gensini G. F., & Prisco D. (2007). Lipoprotein (a) and venous thromboembolism in adults: a meta-analysis. *The American Journal of Medicine 120*, 728-733.

Sotiropoulos A., Papapanagiotou A., Souglis A., Giosos G., Kotsis G., & Bogdanis G. C. (2008). Changes in hormonal and lipid profile after a soccer match in male amateur players. *Serbian Journal of Sports Sciences 2*, 31-36.

Spagnoli L. G., Bonanno E., Sangiorgi G., & Mauriello A. (2007). Role of inflammation in atherosclerosis. *Journal of Nuclear Medicine 48*, 1800-1815.

Spinedi E., Suescun M., Hadid R., Daneva T., & Gaillard R. (1992). Effects of gonadectomy and sex hormone therapy on the endotoxin-stimulated hypothalamo-pituitary-adrenal axis: evidence for a neuroendocrine-immunological sexual dimorphism. *Endocrinology 131*, 2430-2436.

Srivastava R, Srivastava N, Averna M, Lin RC, Korach KS, Lubahn DB, Schonfeld G. (1997). Estrogen Upregulates Apolipoprotein E (ApoE) Gene Expression by Increasing ApoE mRNA in the Translating Pool via the Estrogen Receptor a-Mediated Pathway. J Biol Chem 272, 33360.

Srivastava R., Baumann d., & Schonfeld G. (1993). In vivo regulation of low-density lipoprotein receptors by estrogen differs at the post-transcriptional level in rat and mouse. *European Journal of Biochemistry 216*, 527-538.

Stampfer M. J., Sacks F. M., Salvini S., Willett W. C., & Hennekens C. H. (1991). A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. *New England Journal of Medicine 325*, 373-381.

Stanhope, K., Graham, J., Sinha, M., & Peter, H. (2002). Low circulating adiponectin levels and reduced adipocyte adiponectin production in obese, insulin-resistant Sprague-Dawley rats. *Diabetes,* A404-A404).

Stannard A. K., Riddell D. R., Sacre S. M., Tagalakis A. D., Langer C., von Eckardstein A., Cullen P., Athanasopoulos T., Dickson G., & Owen J. S. (2001). Cell-derived apolipoprotein E (ApoE) particles inhibit vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells. *Journal of Biological Chemistry 276*, 46011-46016.

Stanworth R. & Jones T. (2008). Testosterone in obesity, metabolic syndrome and type 2 diabetes.

Stanworth R., Kapoor D., Channer K., & Jones T. (2008). Androgen receptor CAG repeat polymorphism is associated with serum testosterone levels, obesity and serum leptin in men with type 2 diabetes. *European Journal of Endocrinology 159*, 739-746.

Stenmark K. R., Yeager M. E., El Kasmi K. C., Nozik-Grayck E., Gerasimovskaya E. V., Li M., Riddle S. R., & Frid M. G. (2013). The adventitia: essential regulator of vascular wall structure and function. *Annual Review of Physiology 75*, 23-47.

Steppan C. M., Bailey S. T., Bhat S., Brown E. J., Banerjee R. R., Wright C. M., Patel H. R., Ahima R. S., & Lazar M. A. (2001). The hormone resistin links obesity to diabetes. *Nature 409*, 307-312.

Stevenson S. C., Marshall-Neff J., Teng B., Lee C. B., Roy S., & McClelland A. (1995). Phenotypic correction of hypercholesterolemia in apoE-deficient mice by adenovirus-mediated in vivo gene transfer. *Arteriosclerosis, Thrombosis, and Vascular Biology 15*, 479-484.

Stienstra R., Duval C., Müller M., & Kersten S. (2006). PPARs, obesity, and inflammation. *PPAR Research 2007.*

Stoneman V. E. & Bennett M. R. (2009). Role of Fas/Fas-L in vascular cell apoptosis. *Journal of Cardiovascular Pharmacology 53*, 100-108.

Stoner L., Lucero A. A., Palmer B. R., Jones L. M., Young J. M., & Faulkner J. (2013). Inflammatory biomarkers for predicting cardiovascular disease. *Clinical Biochemistry 46*, 1353-1371.

Straus D. S. & Glass C. K. (2007). Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. *Trends in Immunology 28*, 551-558.

Strazzullo P., Barbato A., Galletti F., Barba G., Siani A., Iacone R., D'Elia L., Russo O., Versiero M., & Farinaro E. (2006). Abnormalities of renal sodium handling in the metabolic syndrome. Results of the Olivetti Heart Study. *Journal of Hypertension 24*, 1633-1639.

Su L., Chang C., Wu Y., Chen K., Lin C., Liang S., Lin C., Whang-Peng J., Hsu S., & Chen C. (2007). Selection of DDX5 as a novel internal control for Q-RT-PCR from microarray data using a block bootstrap re-sampling scheme. *BMC Genomics 8*, 140.

Su C. L., Huang H. Y., Shen Z. Q., & Lin J. F. (2009). The effects of testosterone on the expression of inflammatory factors in 3T3-L1 adipocytes and its mechanism. *Zhonghua Yi Xue Za Zhi 89*, 1493-1497.

Su C. L., Huang H. Y., Shen Z. Q., & Lin J. F. (2009). The effects of testosterone on the expression of inflammatory factors in 3T3-L1 adipocytes and its mechanism. *Zhonghua Yi Xue Za Zhi 89*, 1493-1497.

Sucharda P. (2010). Obesity and atherosclerosis--what's the link? *Vnitrni Lekarstvi 56*, 289-291.

Sudhir K. (2005). Lipoprotein-associated phospholipase A2, a novel inflammatory biomarker and independent risk predictor for cardiovascular disease. *Journal of Clinical Endocrinology & Metabolism 90*, 3100-3105.

Suganami T. & Ogawa Y. (2010). Adipose tissue macrophages: their role in adipose tissue remodeling. *Journal of Leukocyte Biology 88*, 33-39.

Sung M. J., Kim D. H., Davaatseren M., Hur H. J., Kim W., Jung Y. J., Park S. K., & Kwon D. Y. (2010). Genistein suppression of TNF-alpha-induced fractalkine expression in endothelial cells. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology 26*, 431-440.

Superko H. R., Pendyala L., Williams P. T., Momary K. M., King III S. B., & Garrett B. C. (2012). High-density lipoprotein subclasses and their relationship to cardiovascular disease. *Journal of Clinical Lipidology 6*, 496-523.

Surampudi P. N., Wang C., & Swerdloff R. (2012). Hypogonadism in the aging male diagnosis, potential benefits, and risks of testosterone replacement therapy. *International Journal of Endocrinology 2012*.

Suriyaphol P., Fenske D., Zähringer U., Han S., Bhakdi S., & Husmann M. (2002). Enzymatically Modified Nonoxidized Low-Density Lipoprotein Induces Interleukin-8 in Human Endothelial Cells Role of Free Fatty Acids. *Circulation 106*, 2581-2587.

Suzuki M. & O'Neal R. (1967). Circulating lipophages, serum lipids, and atherosclerosis in rats. *Archives of Pathology 83*, 169.

Swaminathan S. & Shah S. V. (2011). Novel inflammatory mechanisms of accelerated atherosclerosis in kidney disease. *Kidney International 80*, 453-463.

Swarbrick M. M. & Havel P. J. (2008). Physiological, pharmacological, and nutritional regulation of circulating adiponectin concentrations in humans. *Metabolic Syndrome and Related Disorders 6*, 87-102.

Sweetnam P. M., Bolton C. H., Yarnell J., Bainton D., Baker I. A., Elwood P. C., & Miller N. E. (1994). Associations of the HDL2 and HDL3 cholesterol subfractions with the development of ischemic heart disease in British men. The Caerphilly and Speedwell Collaborative Heart Disease Studies. *Circulation 90*, 769-774.

Symonds M. E. (2013). Brown adipose tissue growth and development. *Scientifica 2013*.

Syvänne M., Ahola M., Lahdenperä S., Kahri J., Kuusi T., Virtanen K. S., & Taskinen M. (1995). High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *Journal of Lipid Research 36*, 573-582.

Szalai C., Duba J., Prohászka Z., Kalina Á., Szabó T., Nagy B., Horváth L., & Császár A. (2001). Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp (a) and MCP-1− 2518 G/G genotype in CAD patients. *Atherosclerosis 158*, 233-239.

Szanto A. & Nagy L. (2008). The many faces of PPAR< i> γ</i>: Anti-inflammatory by any means? *Immunobiology 213*, 789-803.

Szodoray P., Timar O., Veres K., Der H., Szomjak E., Lakos G., Aleksza M., Nakken B., Szegedi G., & Soltesz P. (2006). TH1/TH2 imbalance, measured by circulating and intracytoplasmic inflammatory cytokines–immunological alterations in acute coronary syndrome and stable coronary artery disease. *Scandinavian Journal of Immunology 64*, 336-344.

T'Sjoen G. G., Giagulli V. A., Delva H., Crabbe P., De Bacquer D., & Kaufman J. (2005). Comparative assessment in young and elderly men of the gonadotropin response to aromatase inhibition. *Journal of Clinical Endocrinology & Metabolism 90*, 5717-5722.

Tabas I. (2005). Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis the importance of lesion stage and phagocytic efficiency. *Arteriosclerosis, Thrombosis, and Vascular Biology 25*, 2255-2264.

Tabas I., Williams K. J., & Borén J. (2007). Subendothelial lipoprotein retention as the initiating process in atherosclerosis update and therapeutic implications. *Circulation 116*, 1832-1844.

Tabas I. & Glass C. K. (2013). Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science (New York, N.Y.) 339*, 166-172.

Tacke F., Alvarez D., Kaplan T. J., Jakubzick C., Spanbroek R., Llodra J., Garin A., Liu J., Mack M., van Rooijen N., Lira S. A., Habenicht A. J., & Randolph G. J. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *The Journal of Clinical Investigation 117*, 185-194.

Takahashi K., Naito M., & Takeya M. (1996). Development and heterogeneity of macrophages and their related cells through their differentiation pathways. *Pathology International 46*, 473-485.

Talayero B. G. & Sacks F. M. (2011). The role of triglycerides in atherosclerosis. *Current Cardiology Reports 13*, 544-552.

Tam S., Archer T. K., & Deeley R. G. (1986). Biphasic effects of estrogen on apolipoprotein synthesis in human hepatoma cells: mechanism of antagonism by testosterone. *Proceedings of the National Academy of Sciences 83*, 3111-3115.

Tan K., Shiu S., Pang R., & Kung A. (1998). Effects of testosterone replacement on HDL subfractions and apolipoprotein A-I containing lipoproteins. *Clinical Endocrinology 48*, 187-194.

Tan B., Chen J., Digby J., Keay S., Kennedy C., & Randeva H. S. (2006). Upregulation of adiponectin receptor 1 and 2 mRNA and protein in adipose tissue and adipocytes in insulin-resistant women with polycystic ovary syndrome. *Diabetologia 49*, 2723-2728.

Tanaka T., Yamamoto J., Iwasaki S., Asaba H., Hamura H., Ikeda Y., Watanabe M., Magoori K., Ioka R. X., Tachibana K., Watanabe Y., Uchiyama Y., Sumi K., Iguchi H., Ito S., Doi T., Hamakubo T., Naito M., Auwerx J., Yanagisawa M., Kodama T., & Sakai J. (2003). Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome.

Proceedings of the National Academy of Sciences of the United States of America 100, 15924-15929.

Tang H., Sebastian B. M., Axhemi A., Chen X., Hillian A. D., Jacobsen D. W., & Nagy L. E. (2012). Ethanol-Induced Oxidative Stress via the CYP2E1 Pathway Disrupts Adiponectin Secretion from Adipocytes. Alcoholism: Clinical and Experimental Research 36, 214-222.

Tang J., Srivastava R., Krul E. S., Baumann D., Pfleger B., Kitchens R., & Schonfeld G. (1991). In vivo regulation of apolipoprotein AI gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice. *Journal of Lipid Research 32*, 1571-1585.

Tang W., Zeve D., Suh J. M., Bosnakovski D., Kyba M., Hammer R. E., Tallquist M. D., & Graff J. M. (2008). White fat progenitor cells reside in the adipose vasculature. *Science (New York, N.Y.) 322*, 583-586.

Tangirala R. K., Murao K., & Quehenberger O. (1997). Regulation of expression of the human monocyte chemotactic protein-1 receptor (hCCR2) by cytokines. *Journal of Biological Chemistry 272*, 8050-8056.

Tao B. J., YANG, Y. W., Yu-li Z, & Kun Y. (2011). Effect of sex hormones on secretions of leptin and adiponectin by adipocytes . Chinese Journal of Endocrinology and Metabolism 27, 1009-1012.

Tardif J. (2009). Heart rate and atherosclerosis. *European Heart Journal Supplements 11*, D8-D12.

Taslipinar A., Sonmez Y. A., Bolu E., Tapan S., Uckaya G., Aydogdu A., & Kutlu M. (2010). The effect of testesterone replacement therapy on HDL subfraction levels in hypogonadotropic hypogonadism.

Tataranni P. (2002). Pathophysiology of obesity-induced insulin resistance and type 2 diabetes mellitus. *European Review for Medical and Pharmacological Sciences 6*, 27-32.

Tateya S., Tamori Y., Kawaguchi T., Kanda H., & Kasuga M. (2010). An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology 151*, 971-979.

Taube A., Schlich R., Sell H., Eckardt K., & Eckel J. (2012). Inflammation and metabolic dysfunction: links to cardiovascular diseases. *American Journal of Physiology-Heart and Circulatory Physiology 302*, H2148-H2165.

Tchernof A., Labrie F., Bélanger A., Prud'homme D., Bouchard C., Tremblay A., Nadeau A., & Després J. (1997). Relationships between endogenous steroid hormone, sex hormone-binding globulin and lipoprotein levels in men: contribution of visceral obesity, insulin levels and other metabolic variables. *Atherosclerosis 133*, 235-244.

Tedgui A. & Mallat Z. (2006). Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological Reviews 86*, 515-581.

Teupser D., Pavlides S., Tan M., Gutierrez-Ramos J., Kolbeck R., & Breslow J. L. (2004). Major reduction of atherosclerosis in fractalkine (CX3CL1)-deficient mice is at the brachiocephalic artery, not the aortic root. *Proceedings of the National Academy of Sciences of the United States of America 101*, 17795-17800.

Thalmann, S., Juge-Aubry, C. E., & Meier, C. A. (2008). Explant cultures of white adipose tissue. In Anonymous *Adipose Tissue Protocols* (pp. 195-199). : Springer.

Thamer C., Haap M., Fritsche A., Haering H., & Stumvoll M. (2004). Relationship between moderate alcohol consumption and adiponectin and insulin sensitivity in a large heterogeneous population. *Diabetes Care 27*, 1240.

Thompson P. D., Cullinane E. M., Sady S. P., Chenevert C., Saritelli A. L., Sady M. A., & Herbert P. N. (1989). Contrasting effects of testosterone and stanozolol on serum lipoprotein levels. *Jama 261*, 1165-1168.

Thorand, B., Lowel, H., Schneider, A., Kolb, H.,Meisinger, C., Frohlich, M.,and Koenig, W. 2003 (2003). C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: results from the Schwarcz and Frishman Augsburg cohort study 1984–1998. *Arch Intern Med 163*, 93-99.

Toda T., Toda Y., Cho B., & Kummerow F. A. (1984). Ultrastructural changes in the comb and aorta of chicks fed excess testosterone. *Atherosclerosis 51*, 47-57.

Tontonoz P., Nagy L., Alvarez J. G., Thomazy V. A., & Evans R. M. (1998). PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell 93*, 241-252.

Toth L. R., Smith T. J., Jones C., de Silva H. V., Smithies O., & Maeda N. (1996). Two distinct apolipoprotein B alleles in mice generated by a single 'in-out'targeting. *Gene 178*, 161-168.

Traish A. M., Abdou R., & Kypreos K. E. (2009). Androgen deficiency and atherosclerosis: The lipid link. *Vascular Pharmacology 51*, 303-313.

Traish A. M. & Kypreos K. E. (2011). Testosterone and cardiovascular disease: an old idea with modern clinical implications. *Atherosclerosis 214*, 244-248.

Trayhurn P. (2005). Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiologica Scandinavica 184*, 285-293.

Trayhurn P. & Beattie J. H. (2001). Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proceedings of the Nutrition Society 60*, 329-339.

Trayhurn P. & Wood I. (2005). Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochemical Society Transactions 33*, 1078-1081.

Trayhurn P. & Wood I. S. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition 92*, 347-355.

Tripathy D., Shah P., Lakshmy R., & Reddy K. (1998). Effect of testosterone replacement on whole body glucose utilisation and other cardiovascular risk factors in males with idiopathic hypogonadotrophic hypogonadism. *Hormone and Metabolic Research 30*, 642-645.

Tsai E. C., Boyko E. J., Leonetti D. L., & Fujimoto W. Y. (2000). Low serum testosterone level as a predictor of increased visceral fat in Japanese-American men. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity 24*, 485-491

Tsigos C., Papanicolaou D. A., Kyrou I., Defensor R., Mitsiadis C. S., & Chrousos G. P. (1997). Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. *Journal of Clinical Endocrinology & Metabolism 82*, 4167-4170.

Tsimikas S., Kiechl S., Willeit J., Mayr M., Miller E. R., Kronenberg F., Xu Q., Bergmark C., Weger S., & Oberhollenzer F. (2006). Oxidized phospholipids predict the presence and progression of carotid and femoral atherosclerosis and symptomatic cardiovascular diseasefive-year prospective results from the bruneck study. *Journal of the American College of Cardiology 47*, 2219-2228.

Tsimikas S. & Witztum J. L. (2008). The role of oxidized phospholipids in mediating lipoprotein (a) atherogenicity. *Current Opinion in Lipidology 19*, 369-377.

Tsukamoto K., Tangirala R., Chun S. H., Puré E., & Rader D. J. (1999). Rapid regression of atherosclerosis induced by liver-directed gene transfer of ApoE in ApoE-deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 2162-2170.

Ueshiba H. (2013). Testosterone treatment improves insulin resistance in japanese male metabolic syndrome. *J Steroids Horm Sci 4*, 2.

Umehara H., Bloom E. T., Okazaki T., Nagano Y., Yoshie O., & Imai T. (2004). Fractalkine in vascular biology from basic research to clinical disease. *Arteriosclerosis, Thrombosis, and Vascular Biology 24*, 34-40.

Umehara H., Bloom E., Okazaki T., Domae N., & Imai T. (2001). Fractalkine and vascular injury. *Trends in Immunology 22*, 602-607.

Umehara H., Goda S., Imai T., Nagano Y., Minami Y., Tanaka Y., Okazaki T., Bloom E. T., & Domae N. (2001). Fractalkine, a CX3C-chemokine, functions predominantly as an adhesion molecule in monocytic cell line THP-1. *Immunology and Cell Biology 79*, 298-302.

Upadhya S., Mooteri S., Peckham N., & Pai R. G. (2004). Atherogenic effect of interleukin-2 and antiatherogenic effect of interleukin-2 antibody in apo-E-deficient mice. *Angiology 55*, 289-294.

Uyanik B. S., Ari Z., Gumus B., Yigitoglu M. R., & Arslan T. (1997). Beneficial effects of testosterone undecanoate on the lipoprotein profiles in healthy elderly men. A placebo controlled study. *Japanese Heart Journal 38*, 73-82.

Valente V., Teixeira S. A., Neder L., Okamoto O. K., Oba-Shinjo S. M., Marie S. K., Scrideli C. A., Paçó-Larson M. L., & Carlotti C. G. (2009). Selection of suitable

housekeeping genes for expression analysis in glioblastoma using quantitative RT-PCR. *BMC Molecular Biology 10*, 17.

Van Anders S. M. & Hampson E. (2005). Waist-to-hip ratio is positively associated with bioavailable testosterone but negatively associated with sexual desire in healthy premenopausal women. *Psychosomatic Medicine 67*, 246-250.

Van den Beld A., Bots M., Janssen J., Pols H., Lamberts S., & Grobbee D. (2003). Endogenous hormones and carotid atherosclerosis in elderly men. *American Journal of Epidemiology 157*, 25-31.

Van der Poll T., Levi M., Büller H., Van Deventer S., De Boer J., Hack C., & Ten Cate J. (1991). Fibrinolytic response to tumor necrosis factor in healthy subjects. *The Journal of Experimental Medicine 174*, 729-732.

Van Dielen F., Van't Veer C., Schols A., Soeters P., Buurman W., & Greve J. (2001). Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity 25*, 1759.

Van Eck M., Herijgers N., Yates J., Pearce N. J., Hoogerbrugge P. M., Groot P. H., & Van Berkel T. J. (1997). Bone Marrow Transplantation in Apolipoprotein E–Deficient Mice Effect of ApoE Gene Dosage on Serum Lipid Concentrations,(β) VLDL Catabolism, and Atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology 17*, 3117-3126.

Van Gaal L. F., Mertens I. L., & Christophe E. (2006). Mechanisms linking obesity with cardiovascular disease. *Nature 444*, 875-880.

Van Pottelbergh I., Braeckman L., De Bacquer D., De Backer G., & Kaufman J. (2003). Differential contribution of testosterone and estradiol in the determination of cholesterol and lipoprotein profile in healthy middle-aged men. *Atherosclerosis 166*, 95-102.

Van Stijn C. M., Kim J., Barish G. D., Tietge U. J., & Tangirala R. K. (2014). Adiponectin Expression Protects against Angiotensin II-Mediated Inflammation and Accelerated Atherosclerosis. *PloS One 9*, e86404.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., & Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology 3*, research0034.

Vázquez-Vela M. E. F., Torres N., & Tovar A. R. (2008). White adipose tissue as endocrine organ and its role in obesity. *Archives of Medical Research 39*, 715-728.

Veillard N. R., Braunersreuther V., Arnaud C., Burger F., Pelli G., Steffens S., & Mach F. (2006). Simvastatin modulates chemokine and chemokine receptor expression by geranylgeranyl isoprenoid pathway in human endothelial cells and macrophages. *Atherosclerosis 188*, 51-58.

Veldhuis J. D., Keenan D. M., Liu P. Y., Iranmanesh A., Takahashi P. Y., & Nehra A. X. (2009). The aging male hypothalamic–pituitary–gonadal axis: Pulsatility and feedback. *Molecular and Cellular Endocrinology 299*, 14-22.

Vgontzas A. N. (2008). Does obesity play a major role in the pathogenesis of sleep apnoea and its associated manifestations via inflammation, visceral adiposity, and insulin resistance? *Archives of Physiology and Biochemistry 114*, 211-223.

Vidal H., Auboeuf D., De Vos P., Staels B., Riou J. P., Auwerx J., & Laville M. (1996). The expression of ob gene is not acutely regulated by insulin and fasting in human abdominal subcutaneous adipose tissue. *Journal of Clinical Investigation 98*, 251.

Vigen R., O'Donnell C. I., Barón A. E., Grunwald G. K., Maddox T. M., Bradley S. M., Barqawi A., Woning G., Wierman M. E., & Plomondon M. E. (2013). Association of Testosterone Therapy With Mortality, Myocardial Infarction, and Stroke in Men With Low Testosterone LevelsTestosterone Therapy and Mortality, MI, and StrokeTestosterone Therapy and Mortality, MI, and Stroke. *Jama 310*, 1829-1836.

Vodo S., Bechi N., Petroni A., Muscoli C., & Aloisi A. M. (2013). Testosterone-induced effects on lipids and inflammation. *Mediators of Inflammation 2013*.

Von Dehn G., von Dehn O., Völker W., Langer C., Weinbauer G., Behre H., Nieschlag E., Assmann G., & von Eckardstein A. (2001). Atherosclerosis in apolipoprotein E-deficient mice is decreased by the suppression of endogenous sex hormones. *Hormone and Metabolic Research 33*, 110-114.

Von Eckardstein A., Kliesch S., Nieschlag E., Chirazi A., Assmann G., & Behre H. M. (1997). Suppression of endogenous testosterone in young men increases serum levels of high density lipoprotein subclass lipoprotein AI and lipoprotein (a). *Journal of Clinical Endocrinology & Metabolism 82*, 3367-3372.

Von Eynatten M., Hamann A., Twardella D., Nawroth P. P., Brenner H., & Rothenbacher D. (2006). Relationship of adiponectin with markers of systemic inflammation, atherogenic dyslipidemia, and heart failure in patients with coronary heart disease. *Clinical Chemistry 52*, 853-859.

Wagenknecht L. E., Langefeld C. D., Scherzinger A. L., Norris J. M., Haffner S. M., Saad M. F., & Bergman R. N. (2003). Insulin Sensitivity, Insulin Secretion, and Abdominal Fat The Insulin Resistance Atherosclerosis Study (IRAS) Family Study. *Diabetes 52*, 2490-2496.

Wagner A. M., Perez A., Zapico E., & Ordonez-Llanos J. (2003). Non-HDL cholesterol and apolipoprotein B in the dyslipidemic classification of type 2 diabetic patients. *Diabetes Care 26*, 2048-2051.

Walldius G. & Jungner I. (2004). Apolipoprotein B and apolipoprotein A-I: risk indicators of coronary heart disease and targets for lipid-modifying therapy. *Journal of Internal Medicine 255*, 188-205.

Walldius G., Jungner I., Holme I., Aastveit A. H., Kolar W., & Steiner E. (2001). High apolipoprotein B, low apolipoprotein AI, and improvement in the prediction of fatal

myocardial infarction (AMORIS study): a prospective study. *The Lancet 358*, 2026-2033.

Wallenius V., Wallenius K., Ahrén B., Rudling M., Carlsten H., Dickson S. L., Ohlsson C., & Jansson J. (2002). Interleukin-6-deficient mice develop mature-onset obesity. *Nature Medicine 8*, 75-79.

Walpola P. L., Gotlieb A. I., Cybulsky M. I., & Langille B. L. (1995). Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress. *Arteriosclerosis, Thrombosis, and Vascular Biology 15*, 2-10.

Walsh B. W., Schiff I., Rosner B., Greenberg L., Ravnikar V., & Sacks F. M. (1991). Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *New England Journal of Medicine 325*, 1196-1204.

Walsh B., Li H., & Sacks F. (1994). Effects of postmenopausal hormone replacement with oral and transdermal estrogen on high density lipoprotein metabolism. *Journal of Lipid Research 35*, 2083-2093.

Wang C., Jackson G., Jones T. H., Matsumoto A. M., Nehra A., Perelman M. A., Swerdloff R. S., Traish A., Zitzmann M., & Cunningham G. (2011). Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes. *Diabetes Care 34*, 1669-1675.

Wang C., Nieschlag E., Swerdloff R., Behre H., Hellstrom W. J., Gooren L. J., Kaufman J. M., Legros J., Lunenfeld B., & Morales A. (2009). ISA, ISSAM, EAU, EAA and ASA recommendations: investigation, treatment and monitoring of late-onset hypogonadism in males. *International Journal of Impotence Research 21*, 1-8.

Wang N., Wang W., Breslow J. L., & Tall A. R. (1996). Scavenger Receptor BI (SR-BI) Is Up-regulated in Adrenal Gland in Apolipoprotein AI and Hepatic Lipase Knock-out Mice as a Response to Depletion of Cholesterol Stores *in vivo* evidences that sr-bi is a functional high density lipoprotein receptor under feedback controL. *Journal of Biological Chemistry 271*, 21001-21004.

Wang S. S., Hung Y., Wen W., Lin K., & Chen G. (2011). Exploring the inhibitory activity of short-chain phospholipids against amyloid fibrillogenesis of hen egg-white lysozyme. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids 1811*, 301-313.

Wang, Y., Wang, L., Zhao, J., Qiao, Z (2005). Estrogen, but not testosterone down-regulates cytokine production in nicotine-mediated murine macrophage. *Methods Find Exp Clin Pharmacol 27*, 311.

Wang Y., Zhu W., & Levy D. E. (2006). Nuclear and cytoplasmic mRNA quantification by SYBR green based real-time RT-PCR. *Methods 39*, 356-362.

Wang Y., Lee C., Tiep S., Yu R. T., Ham J., Kang H., & Evans R. M. (2003). Peroxisome-proliferator-activated receptor δ activates fat metabolism to prevent obesity. *Cell 113*, 159-170.

Wang Y., Lam K., Yau M., & Xu A. (2008). Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem.J 409*, 623-633.

Wang C., Jackson G., Jones T. H., Matsumoto A. M., Nehra A., Perelman M. A., Swerdloff R. S., Traish A., Zitzmann M., & Cunningham G. (2011). Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes. *Diabetes Care 34*, 1669-1675.

Wang N., Yvan-Charvet L., Lutjohann D., Mulder M., Vanmierlo T., Kim T. W., & Tall A. R. (2008). ATP-binding cassette transporters G1 and G4 mediate cholesterol and desmosterol efflux to HDL and regulate sterol accumulation in the brain. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 22*, 1073-1082.

Wang X., Bao W., Liu J., Ouyang Y. Y., Wang D., Rong S., Xiao X., Shan Z. L., Zhang Y., Yao P., & Liu L. G. (2013). Inflammatory markers and risk of type 2 diabetes: a systematic review and meta-analysis. *Diabetes Care 36*, 166-175.

Wataru N. (1990). C-reactive protein in rat: in development, pregnancy and effect of sex hormones. *Comparative Biochemistry and Physiology Part A: Physiology 96*, 489-493.

Webb C. M., Adamson D. L., de Zeigler D., & Collins P. (1999). Effect of acute testosterone on myocardial ischemia in men with coronary artery disease. *The American Journal of Cardiology 83*, 437-439.

Weber C. (2003). Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules. *Journal of Molecular Medicine 81*, 4-19.

Weber C., Schober A., & Zernecke A. (2004). Chemokines key regulators of mononuclear cell recruitment in atherosclerotic vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology 24*, 1997-2008.

Weber C., Zernecke A., & Libby P. (2008). The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nature Reviews Immunology 8*, 802-815.

Weber K. S., Nelson P. J., Grone H. J., & Weber C. (1999). Expression of CCR2 by endothelial cells : implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium. *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 2085-2093.

Wehling M. (1997). Specific, nongenomic actions of steroid hormones. *Annual Review of Physiology 59*, 365-393.

Weisberg S. P., Hunter D., Huber R., Lemieux J., Slaymaker S., Vaddi K., Charo I., Leibel R. L., & Ferrante Jr A. W. (2006). CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *Journal of Clinical Investigation 116*, 115-124.

Weisberg S. P., McCann D., Desai M., Rosenbaum M., Leibel R. L., & Ferrante A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation 112*, 1796-1808.

Weiss R., Dziura J., Burgert T. S., Tamborlane W. V., Taksali S. E., Yeckel C. W., Allen K., Lopes M., Savoye M., & Morrison J. (2004). Obesity and the metabolic syndrome in children and adolescents. *New England Journal of Medicine 350*, 2362-2374.

Wellen K. E. & Hotamisligil G. S. (2005). Inflammation, stress, and diabetes. *Journal of Clinical Investigation 115*, 1111-1119.

Wellen K. E. & Hotamisligil G. S. (2003). Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation 112*, 1785-1788.

Westerbacka J., Kolak M., Kiviluoto T., Arkkila P., Sirén J., Hamsten A., Fisher R. M., & Yki-Järvinen H. (2007). Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes 56*, 2759-2765.

Wexler D. J., Hu F. B., Manson J. E., Rifai N., & Meigs J. B. (2005). Mediating effects of inflammatory biomarkers on insulin resistance associated with obesity. *Obesity Research 13*, 1772-1783.

Weyer C., Funahashi T., Tanaka S., Hotta K., Matsuzawa Y., Pratley R. E., & Tataranni P. A. (2001). Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *Journal of Clinical Endocrinology & Metabolism 86*, 1930-1935.

White G. E., Tan T. C., John A. E., Whatling C., McPheat W. L., & Greaves D. R. (2010). Fractalkine has anti-apoptotic and proliferative effects on human vascular smooth muscle cells via epidermal growth factor receptor signalling. *Cardiovascular Research 85*, 825-835.

Whitsel E. A., Boyko E. J., Matsumoto A. M., Anawalt B. D., & Siscovick D. S. (2001). Intramuscular testosterone esters and plasma lipids in hypogonadal men: a meta-analysis. *The American Journal of Medicine 111*, 261-269.

Wiesbauer F., Kaun C., Zorn G., Maurer G., Huber K., & Wojta J. (2002). HMG CoA reductase inhibitors affect the fibrinolytic system of human vascular cells in vitro: a comparative study using different statins. *British Journal of Pharmacology 135*, 284-292.

Williams P. T. (2012). Fifty-three year follow-up of coronary heart disease versus HDL2 and other lipoproteins in Gofman's Livermore Cohort. *Journal of Lipid Research* 53, 266-272.

Williams K. J. & Tabas I. (1998). The response-to-retention hypothesis of atherogenesis reinforced. *Current Opinion in Lipidology 9*, 471-474.

Wilson M. E., Westberry J. M., & Trout A. L. (2011). Estrogen receptor-alpha gene expression in the cortex: sex differences during development and in adulthood. *Hormones and Behavior* 59, 353-357.

Winegar D. A., Brown P. J., Wilkison W. O., Lewis M. C., Ott R. J., Tong W., Brown H. R., Lehmann J. M., Kliewer S. A., & Plunket K. D. (2001). Effects of fenofibrate on lipid parameters in obese rhesus monkeys. *Journal of Lipid Research 42*, 1543-1551.

Witkowska-Zimny M. & Walenko K. (2011). Stem cells from adipose tissue. *Cellular & Molecular Biology Letters 16*, 236-257.

Wittert G. A., Chapman I. M., Haren M. T., Mackintosh S., Coates P., & Morley J. E. (2003). Oral testosterone supplementation increases muscle and decreases fat mass in healthy elderly males with low–normal gonadal status. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 58*, M618-M625.

Witztum J. L. & Steinberg D. (1991). Role of oxidized low density lipoprotein in atherogenesis. *Journal of Clinical Investigation 88*, 1785.

Wolk R., Berger P., Lennon R. J., Brilakis E. S., Johnson B. D., & Somers V. K. (2004). Plasma leptin and prognosis in patients with established coronary atherosclerosis. *Journal of the American College of Cardiology 44*, 1819-1824.

Wolowczuk I., Verwaerde C., Viltart O., Delanoye A., Delacre M., Pot B., & Grangette C. (2008). Feeding our immune system: impact on metabolism. *Clinical and Developmental Immunology 2008*.

Wong B. W., Wong D., & McManus B. M. (2002). Characterization of fractalkine (CX3CL1) and CX3CR1 in human coronary arteries with native atherosclerosis, diabetes mellitus, and transplant vascular disease. *Cardiovascular Pathology 11*, 332-338.

Wong M. L. & Medrano J. F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques 39*, 75.

Wong N., Malik S., & Kashyap M. (2005). Dyslipidemia. *Preventive Cardiology.ND Wong, HR Black, JM Gardin, Editors.McGraw Hill Co., Inc., New York*, 183-211.

Wood I. S., Wang B., & Trayhurn P. (2009). IL-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochemical and Biophysical Research Communications 384*, 105-109.

Wright S. D. & Silverstein S. C. (1982). Tumor-promoting phorbol esters stimulate C3b and C3b'receptor-mediated phagocytosis in cultured human monocytes. *The Journal of Experimental Medicine 156*, 1149-1164.

Wu F. C. & von Eckardstein A. (2003). Androgens and coronary artery disease. *Endocrine Reviews 24*, 183-217.

Wu K.. & Huan Y. (2007). Diabetic atherosclerosis mouse models. *Atherosclerosis 191*, 241-249.

Wu M., Yang S., Elliott M. H., Fu D., Wilson K., Zhang J., Du M., Chen J., & Lyons T. (2012). Oxidative and endoplasmic reticulum stresses mediate apoptosis induced by modified LDL in human retinal Müller cells. *Investigative Ophthalmology & Visual Science 53*, 4595-4604.

Xaus J., Comalada M., Valledor A. F., Lloberas J., López-Soriano F., Argilés J. M., Bogdan C., & Celada A. (2000). LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-α. *Blood 95*, 3823-3831.

Xu A., Chan K. W., Hoo R. L., Wang Y., Tan K. C., Zhang J., Chen B., Lam M. C., Tse C., & Cooper G. J. (2005). Testosterone selectively reduces the high molecular weight form of adiponectin by inhibiting its secretion from adipocytes. *Journal of Biological Chemistry 280*, 18073-18080.

Xu H., Barnes G. T., Yang Q., Tan G., Yang D., Chou C. J., Sole J., Nichols A., Ross J. S., & Tartaglia L. A. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *Journal of Clinical Investigation 112*, 1821-1830.

Xu X., De pergola G., & Björntorp P. (1990). The effects of androgens on the regulation of lipolysis in adipose precursor cells. *Endocrinology 126*, 1229-1234.

Yamauchi T., Kamon J., Minokoshi Y. a., Ito Y., Waki H., Uchida S., Yamashita S., Noda M., Kita S., & Ueki K. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine 8*, 1288-1295.

Yamauchi T., Kamon J., Waki H., Terauchi Y., Kubota N., Hara K., Mori Y., Ide T., Murakami K., & Tsuboyama-Kasaoka N. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine 7*, 941-946.

Yamauchi T., Kamon J., Ito Y., Tsuchida A., Yokomizo T., Kita S., Sugiyama T., Miyagishi M., Hara K., & Tsunoda M. (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature 423*, 762-769.

Yang, K. (2008). *Adipose tissue protocols. Biology 456,* 1-22.

Yang M., Leake D., & Rice-Evans C. (1996). Non-oxidative modification of native low-density lipoprotein by oxidized low-density lipoprotein. *Biochem.J 316*, 377-380.

Yang Q., Graham T. E., Mody N., Preitner F., Peroni O. D., Zabolotny J. M., Kotani K., Quadro L., & Kahn B. B. (2005). Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature 436*, 356-362.

Yao L., Herlea-Pana O., Heuser-Baker J., Chen Y., & Barlic-Dicen J. (2014). Roles of the chemokine system in development of obesity, insulin resistance, and cardiovascular disease. *Journal of Immunology Research 2014.*

Yatagai T., Nagasaka S., Taniguchi A., Fukushima M., Nakamura T., Kuroe A., Nakai Y., & Ishibashi S. (2003). Hypoadiponectinemia is associated with visceral fat

accumulation and insulin resistance in Japanese men with type 2 diabetes mellitus. *Metabolism 52*, 1274-1278.

Yildiz B. O. & Haznedaroglu I. C. (2006). Rethinking leptin and insulin action: therapeutic opportunities for diabetes. *The International Journal of Biochemistry & Cell Biology 38*, 820-830.

Yin K., Liao D., & Tang C. (2010). ATP-binding membrane cassette transporter A1 (ABCA1): a possible link between inflammation and reverse cholesterol transport. *Molecular Medicine 16*, 438.

Ylä-Herttuala S., Lipton B. A., Rosenfeld M. E., Särkioja T., Yoshimura T., Leonard E. J., Witztum J. L., & Steinberg D. (1991). Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proceedings of the National Academy of Sciences 88*, 5252-5256.

Yu C., Chen Y., Cline G. W., Zhang D., Zong H., Wang Y., Bergeron R., Kim J. K., Cushman S. W., & Cooney G. J. (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *Journal of Biological Chemistry 277*, 50230-50236.

Yu X., Dluz S., Graves D. T., Zhang L., Antoniades H. N., Hollander W., Prusty S., Valente A. J., Schwartz C. J., & Sonenshein G. E. (1992). Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proceedings of the National Academy of Sciences 89*, 6953-6957.

Yudkin J. S., Kumari M., Humphries S. E., & Mohamed-Ali V. (2000). Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis 148*, 209-214.

Yudkin J. S., Stehouwer C., Emeis J., & Coppack S. (1999). C-Reactive Protein in Healthy Subjects: Associations With Obesity, Insulin Resistance, and Endothelial Dysfunction A Potential Role for Cytokines Originating From Adipose Tissue? *Arteriosclerosis, Thrombosis, and Vascular Biology 19*, 972-978.

Yun-mei Y., Xue-ying L., Wei-dong H., Zhe-rong X., & Ling-jiao W. (2005). Study of androgen and atherosclerosis in old-age male. *Journal of Zhejiang University Science B 6*, 931-935.

Yusuf S., Hawken S., Ôunpuu S., Bautista L., Franzosi M. G., Commerford P., Lang C. C., Rumboldt Z., Onen C. L., & Lisheng L. (2005). Obesity and the risk of myocardial infarction in 27 000 participants from 52 countries: a case-control study. *The Lancet 366*, 1640-1649.

Zancanaro C., Carnielli V. P., Moretti C., Benati D., & Gamba P. (1995). An ultrastructural study of brown adipose tissue in pre-term human new-borns. *Tissue and Cell 27*, 339-348.

Zari Dastani Carole Dangoisse,Betsie Boucher (2006). A novel nonsense apolipoprotein A-I mutation (apoA IE136X) causes low HDL cholesterol in French Canadians . *Atherosclerosis 185*, 127-136.

Zebisch K., Voigt V., Wabitsch M., & Brandsch M. (2012). Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry 425*, 88-90.

Zeng L., Geng Y., Tretiakova M., Yu X., Sicinski P., & Kroll T. G. (2008). Peroxisome Proliferator-Activated Receptor-δ Induces Cell Proliferation by a Cyclin E1–Dependent Mechanism and Is Up-regulated in Thyroid Tumors. *Cancer Research 68*, 6578-6586.

Zernecke A., Shagdarsuren E., & Weber C. (2008). Chemokines in atherosclerosis an update. *Arteriosclerosis, Thrombosis, and Vascular Biology 28*, 1897-1908.

Zernecke A. & Weber C. (2010). Chemokines in the vascular inflammatory response of atherosclerosis. *Cardiovascular Research 86*, 192-201.

Zernecke A., Weber K. S., Erwig L. P., Kluth D. C., Schröppel B., Rees A. J., & Weber C. (2001). Combinatorial model of chemokine involvement in glomerular monocyte recruitment: role of CXC chemokine receptor 2 in infiltration during nephrotoxic nephritis. *The Journal of Immunology 166*, 5755-5762.

Zeyda M., Gollinger K., Kriehuber E., Kiefer F., Neuhofer A., & Stulnig T. (2010). Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. *International Journal of Obesity 34*, 1684-1694.

Zeyda M. & Stulnig T. M. (2007). Adipose tissue macrophages. *Immunology Letters 112*, 61-67.

Zgliczynski S., Ossowski M., Slowinska-Srzednicka J., Brzezinska A., Zgliczynski W., Soszynski P., Chotkowska E., Srzednicki M., & Sadowski Z. (1996). Effect of testosterone replacement therapy on lipids and lipoproteins in hypogonadal and elderly men. *Atherosclerosis 121*, 35-43.

Zhang L., Kamanna V. S., Ganji S. H., Xiong X., & Kashyap M. L. (2010). Pioglitazone increases apolipoprotein AI production by directly enhancing PPRE-dependent transcription in HepG2 cells. *Journal of Lipid Research 51*, 2211-2222.

Zhang S. H., Reddick R. L., Piedrahita J. A., & Maeda N. (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science 258*, 468-471.

Zhang Y., Proenca R., Maffei M., Barone M., Leopold L., & Friedman J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature 372*, 425-432.

Zhu Y. & Hui D. Y. (2003). Apolipoprotein E binding to low density lipoprotein receptor-related protein-1 inhibits cell migration via activation of cAMP-dependent protein kinase A. *Journal of Biological Chemistry 278*, 36257-36263.

Zietz B., Cuk A., Hügl S., Büttner R., Straub R. H., Bauer B., Daffner P., Schölmerich J., & Palitzsch K. (2000). Association of increased C-peptide serum levels and testosterone in type 2 diabetes. *European Journal of Internal Medicine 11*, 322-328.

Zitzmann M. (2009). Pharmacogenetics of testosterone replacement therapy. *Pharmacogenomics 10*, 1341-1349.

Zmuda J. M., Cauley J. A., Kriska A., Glynn N. W., Gutai J. P., & Kuller L. H. (1997). Longitudinal relation between endogenous testosterone and cardiovascular disease risk factors in middle-aged men a 13-year follow-up of former multiple risk factor intervention trial participants. *American Journal of Epidemiology 146*, 609-617.