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CARDIOVASCULAR RISK IN NON-DIABETES MELLITUS INSULIN RESISTANT STATES

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DECLARATION

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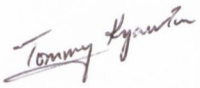
Signed: 

Dr. Tommy Kyaw Tun

SUMMARY

This thesis explores the various cardiovascular risks that occur in insulin resistant states apart from type 2 diabetes mellitus (T2DM). Various cardiovascular risk markers and metabolic derangements related to cardiovascular risk are investigated in two highly prevalent insulin resistant conditions: polycystic ovary syndrome (PCOS) and the metabolic syndrome (MetS).

PCOS is the commonest endocrinopathy in women of reproductive age. The three core features of PCOS include oligomenorrhoea, hyperandrogenism and polycystic ovaries (PCO). Insulin resistance (IR) is thought to play a key role in the pathogenesis of the syndrome. Women with PCOS have a higher prevalence of impaired glucose tolerance (IGT), T2DM, hypertension, dyslipidaemia and central obesity. Whilst a lot of work has already been done to investigate these abnormalities in PCOS, more recent research has raised the possibility of the importance of non-classical cardiovascular risk markers such as postprandial hypertriglyceridaemia and lipoproteins, different oligomers of adiponectin and newer inflammatory biomarkers. The first three studies of this thesis explore these.

The first study evaluates postprandial metabolism and explores the relationship between hypertriglyceridaemia and IR, pancreatic beta-**islet cell (β**-cell) function and androgens. In obese women with PCOS, postprandial hypertriglyceridaemia strongly correlated with IR, more so than hyperandrogenaemia. Stepwise multiple regression analyses revealed that IR and area under the curve (AUC) of apolipoprotein (apo) B_{48} were predictors of postprandial triglycerides. This suggests that postprandial apoB_{48}-containing chylomicron particles probably play an important role in contributing towards postprandial hypertriglyceridaemia. Postprandial insulin levels were also higher even after adjusting for body mass index (BMI) and IR, suggesting a compensatory insulin hypersecretion to counter hyperglycaemia.

In the second study, different oligomeric forms of adiponectin were investigated in PCOS women in relation to IR, β-cell function and androgens. This study found that insulin sensitivity was best predicted by PCOS status, BMI, and medium-molecular weight (MMW) adiponectin levels in pre-menopausal women. MMW adiponectin was also the strongest predictor of disposition index (DI) – a marker for future risk of development of T2DM. The novel association between MMW adiponectin and both insulin sensitivity and DI is one that has not been previously described.

In the third study, oxidative stress, traditional and novel cardiovascular risk biomarkers were quantified in obese and lean PCOS women and BMI-matched control subjects. This is to further help clarify whether the any differences in oxidation susceptibility and biomarkers are due to obesity or whether they are due to PCOS status and hyperandrogenaemia. Results suggest that obesity and PCOS together contributes to oxidative stress, and that obesity rather than PCOS status is the main determinant of elevated levels of inflammatory markers in women with PCOS.

Finally, to investigate lipoprotein sizes in a large cohort of insulin resistant subjects, a study was performed to analyse low-density lipoprotein (LDL) and HDL particle sizes using the Quantimetrix Lipoprint™ gel electrophoresis system in MetS subjects. The proportion of small dense LDL (sdLDL) particle and small HDL were assessed pre- and post-dietary intervention. Whilst a 12-week dietary intervention had little impact on lipids and lipoprotein subfractions, the study confirmed that sdLDL and small HDL were strongly associated with the presence of hypertriglyceridaemia.

In summary, results of these studies provide further understanding of how IR individuals, even without diabetes are at risk of cardiovascular disease. Obesity appears to be a major factor contributing towards many of the perturbations found in IR subjects. Some of the findings may help risk stratification and guide treatments with early primary prevention therapy.

DEDICATED TO GOD AND TO MY FAMILY

"By three methods we may learn wisdom: First, by reflection, which is noblest; second, by imitation, which is easiest; and third by experience, which is the bitterest."

Confucius

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First and foremost, I would like to thank God who has given me the strength, patience and endurance to complete this work. He gently guided me all the way from beginning to end, even though many times I deviated from the path... And although it has not been easy, by His grace, He has orchestrated the following people in to my life to help me through this challenging and memorable journey.

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PUBLICATIONS AND PRESENTATIONS

* Joint first authorship

Published articles

1) Blair SA*, Kyaw Tun T*, Young IS, Phelan NA, Gibney J, McEneny J

   Oxidative Stress and Inflammation in Lean and Obese Subjects with PCOS

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1) Blair SA, Kyaw Tun T, Young IS, Phelan NA, Gibney J, McEneny J

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2) Blair SA, Young IS, Kyaw Tun T, Phelan NA, Gibney J, McEneny J

   Classic and novel cardiovascular risk markers associated with polycystic ovary syndrome

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3) Kyaw Tun T, **O'Connor A, Phelan NA, Correia N, Roche HM,** Boran G, Gibney J

Oligomeric forms of adiponectin are associated with insulin sensitivity and beta cell function in premenopausal women with and without polycystic ovary syndrome

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4) Kyaw Tun T, **O'Connor A, Phelan NA, Correia N, Roche HM,** Boran G, Gibney J

Insulin sensitivity, insulin secretion and glucose effectiveness in women with Polycystic Ovary Syndrome

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Presented as an Oral Communication OC6 at the Irish Endocrine Society 2007

5) Kyaw Tun T, **O'Connor A, Phelan NA, Correia N, Roche HM,** Boran G, Gibney J

Fasting and Postprandial hypertriglyceridaemia is explained by Insulin Resistance in Polycystic Ovary Syndrome

Irish J Med Sci 2006; **175**(4) S2:15 – Oral Communication OC3

Presented as an Oral Communication OC3 at the Irish Endocrine Society 2006

ABBREVIATIONS

11β-HSD1	11-beta-hydroxysteroid dehydrogenase-1
αLA	α-linoleic acid
ΔA4	androstenedione
Acrp30	adipocytes complement related protein of 30-kDa
ADSF	adipose tissue secretory factor
AgRP	agout-related peptide
AIR_G	acute insulin response to glucose
AMPK	adenosine monophosphate-activated protein kinase
apM1	adipose most abundant gene transcript 1
apoA-I	apolipoprotein A-I
apoA-II	apolipoprotein A-II
apoA-IV	apolipoprotein A-IV
apoB	apolipoprotein B
$apoB_{48}$	apolipoprotein B_{48}
$apoB_{100}$	apolipoprotein B_{100}
apoC-I	apolipoprotein C-I
apoC-II	apolipoprotein C-II
apoC-III	apolipoprotein C-III
AT	adipose tissue

AUC	area under the curve
β-cell	pancreatic beta-cell
BAT	brown adipose tissue
BMI	body mass index
BP	blood pressure
CART	cocaine and amphetamine-regulated transcript
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CM	chylomicron
CNS	central nervous system
CR	chylomicron remnants
CRP	C-reactive protein
DHA	docosahexaenoic acid
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulphate
DI	disposition index
DM	diabetes mellitus
EC	endothelial cells
EGF	epidermal growth factor
EPA	eicosapentaenoic acid
FA	fatty acid

FAI	free androgen index
FFA	free fatty acid
FIZZ	found in inflammatory zone
FSH	follicle stimulating hormone
fsIVGTT	frequently sampled intravenous glucose tolerance test
fT4	free thyroxine
GBP-28	gelatine binding protein of 28kDa
gluc	glucose
GLUT4	glucose transporter type 4
GTP	guanosine triphosphate
HC	hip circumference
HDL	high-density lipoprotein
HDL-chol	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus
HL	hepatic lipase
HMW	high-molecular weight
$HOMA_{IR}$	homeostatic model assessment of insulin resistance
hsCRP	highly sensitive C-reactive protein
HSFA	high saturated fatty acid
iAUC	incremental area under the curve
IDL	intermediate-density lipoprotein

IFN-γ	interferon-γ
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL	interleukin
IL-1	interleukin-1
IL-6	interleukin-6
IMT	intima-media thickness
ins	insulin
IR	insulin resistance / insulin resistant
IRS-1	insulin receptor substrate 1
JAK	Janus kinase
JAK2	Janus kinase 2
kDa	kilo Dalton
LCAT	lecithin cholesterol acyltransferase
LDL	low-density lipoprotein
LDL-chol	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
LFHCC	low-fat high complex carbohydrate
LH	luteinising hormone
LMW	low-molecular weight
Lp(a)	lipoprotein(a)

LPL	lipoprotein lipase
MCP-1	monocyte chemotactic protein-1
MetS	metabolic syndrome
MMW	medium-molecular weight
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MUFA	mono-unsaturated fatty acid
n-3 PUFA	n-3 polyunsaturated fatty acid
n-6 PUFA	n-6 polyunsaturated fatty acid
NEFA	non-esterified fatty acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
oxLDL	oxidised low-density lipoprotein
PAI-1	plasminogen activator inhibitor-1
PCOS	polycystic ovary syndrome
PDGF	platelet derived growth factor
POMC	pro-opiomelanocortin
PPAR	peroxisome proliferator-activated receptor
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma

PRL	prolactin
PUFA	polyunsaturated fatty acid
RELM	resistin-like molecule
SAA	serum amyloid A
SAP	serum amyloid P
sdLDL	small dense low-density lipoprotein
SFA	saturated fatty acid
S_G	insulin sensitivity to glucose
SHBG	sex hormone binding globulin
S_I	insulin sensitivity
STAT	signal transducers activators of transcription
sICAM-1	soluble intercellular adhesion molecule-1
sTNF-α	soluble tumour necrosis factor alpha
sVCAM-1	soluble vascular cell adhesion molecule-1
T2DM	type 2 diabetes mellitus
TAC	total anti-oxidation capacity
TAG	triacylglycerol
TG	triglycerides
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α

TNFR	tumour necrosis factor receptor
tPA	tissue plasminogen activator
TOS	total oxidant status
TSH	thyroid stimulating hormone
TZD	thiazolidinedione
VLDL	very low-density lipoprotein
WAT	white adipose tissue
WC	waist circumference
WHR	waist-to-hip ratio

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Chapter 1

Introduction

"Learn not only to find what you like, but to like what you find."

Anthony J. D'Angelo

1.1 CARDIOVASCULAR DISEASE & ATHEROSCLEROSIS

1.1.1 Introduction

Cardiovascular disease (CVD) is the class of diseases which affect the heart and blood vessels (arteries and veins). Whilst the term can be used to refer to any condition affecting the heart or blood vessels, it usually is specific to diseases affecting organs as a result of atherosclerosis in the large and medium sized arteries. Depending on the distribution and extent of atherosclerotic plaques, different clinical manifestations ensue e.g. angina pectoris and myocardial infarction if the coronary arteries are affected; transient ischaemic attack and stroke if the cerebral arteries are affected; and intermittent claudication and toe/foot amputations if the peripheral arteries are affected.

Atherosclerosis commences even in childhood/adolescence – "fatty streaks", the earliest lesions of atherosclerosis – can be found in children as young as 10 years of age (McGill 1984). But clinically relevant disease has traditionally only manifested much later in life, typically after about 50 years of age. Recent advances in the understanding and therapeutic options for this disease has led to a steady gentle decline of CVD deaths over the last 20-30 years in certain Western countries (Kesteloot, Sans et al. 2006). However, with the increasing prevalence of well defined cardiovascular risk factors such as obesity, T2DM and smoking, this decline is set to reduce or even reverse, especially in young adults (Capewell, Ford et al.; O'Flaherty, Ford et al. 2008). The prevalence of CVD is also set to rise especially in the developing and lower- to middle-income countries as they go through economic, social, and cultural changes (Levenson, Skerrett et al. 2002). In fact, such is the increase in the global rate of CVD mortality that it has now become the leading cause of death worldwide, superseding infectious diseases (Mathers, Boerma et al. 2009).

Whilst traditional modifiable risk factors of CVD such as obesity, hypertension, hypercholesterolaemia and smoking have long been routinely addressed in clinical practice, there are other factors which are now generally accepted as risks for CVD but their roles and mechanisms are much less understood and are still mainly studied only in clinical or laboratory research settings. This thesis explores the roles of adipokines, classical and novel cardiovascular risk markers, postprandial dyslipidaemia and qualitative lipid abnormalities in subjects with IR but without diabetes, with a view of possibly introducing them for future use in clinical settings.

1.1.2 Pathophysiology of atherosclerosis

Atherosclerosis is a disease characterised by chronic immunoinflammation and fibroproliferation driven by abnormal lipid metabolism (Glass and Witztum 2001; Libby 2002; Hansson 2005). Endothelial or intimal injury is thought to result from smoking, hypertension, obesity, diabetes mellitus and hypercholesterolaemia. This results in platelet aggregation and the subsequent release of proliferative agents such as epidermal growth factor (EGF), platelet derived growth factor (PDGF) and tumour necrosis factor (TNF), all of which promote vascular smooth muscle proliferation (Weber 2005). The smooth muscle cells accumulate lipid and stimulate the production of the extracellular matrix which acts like a mesh trapping migrating lipid-laden cells. Chemotactic factors such as monocyte chemotactic protein-1 (MCP-1), transforming growth factor-β (TGF-β) and various interleukins (ILs) are also released and mediate the migration of monocytes and lymphocytes to the point of inflammation where they are localised in the subendothelial space and migrate further into the intima of the wall as the process continues (Quehenberger 2005). Monocytes become macrophages and further accumulate lipid to become foam cells (de Villiers and Smart 1999; Mestas and Ley 2008). During this process the cells stop expressing low-density lipoprotein receptors (LDLRs). Instead, scavenger receptors are expressed (de Villiers and Smart 1999). LDL is the major cholesterol carrying lipoprotein in the circulation and enters cells along with other lipoproteins through this and other receptors. The change in receptor expression results in the preferential

uptake of modified forms of LDL such as oxidised LDL (oxLDL). Modified LDL (Shaikh, Martini et al. 1988) and other lipoproteins (Mamo, Proctor et al. 1998) have been identified in atherosclerotic plaques. As the plaque advances, a dense fibrous cap of connective tissue develops, covering the core of lipid and necrotic debris. Thrombus formation may occur at points of lesion eruption. Advanced lesions can cause stenosis of the arteries, and further rupture and haemorrhage into the plaque can cause complete occlusion. Occasionally, parts of the unstable plaque can dislodge and becomes an embolus which occludes a narrower part of the vessel at a more distal site.

1.1.3 Risk factors of atherosclerosis

Multiple risk factors for the development of atherosclerosis have been identified. Non-modifiable risk factors include age, family history of diseases caused by atherosclerosis and gender. Modifiable risk factors include lifestyle factors such as smoking, high dietary intake of saturated fat and cholesterol, sedentary lifestyle and stress. Other modifiable risk factors amenable to lifestyle and pharmacotherapy include hypertension, dyslipidaemia, diabetes mellitus and obesity. Attention to the above factors is important in the management of patients with increased cardiovascular risk. However, a substantial proportion of individuals without any of the above risk factors are thought to be at increased risk of CVD. Conventional risk factors are not able to fully account for the risk of CVD (Wilson, D'Agostino et al. 1998; D'Agostino, Grundy et al. 2001; Golden, Folsom et al. 2002) and it is thought that IR may be the missing factor.

1.1.4 Risk Markers of Cardiovascular Disease in Insulin Resistant States

There are many other factors which are thought to be associated with, or even directly implicated in increasing cardiovascular risk. These include inflammatory cytokines, adipokines, postprandial lipoproteins and qualitative lipid abnormalities which are thought to either be implicated in the pathogenesis of atherosclerosis, or at

least be a marker of it. These potentially serve as useful cardiovascular risk markers in those who may not have clinically overt diabetes, but are insulin resistant individuals who are at risk of developing diabetes and CVD.

1.1.5 Adipokines and Cardiovascular Disease

1.1.5.1 Introduction

Adipose tissue (AT) is a loose connective tissue that consists of a mass of adipocytes (fat cells) loosely held together by collagen fibres (Trayhurn 2007). Even in normal, lean individuals, AT makes up a substantial proportion of total tissue mass. Typical fat mass for a male is between 9-18% body weight, and for a female between 14-28% body weight (Hausman, DiGirolamo et al. 2001). However, in obese individuals, up to 50% of total body tissue mass may be due to white fat in subjects with a body mass index (BMI) of 30kg/m^2 (Trayhurn and Bing 2006).

AT can be classified in several ways, either as white adipose tissue (WAT) or brown adipose tissue (BAT), as subcutaneous or visceral fat, as android or gynoid fat and as hypertrophic or hyperplastic fat. WAT, which is white or yellow in colour, contains few nerves and blood vessels and at a histological level, appears to have a simple structure consisting mainly of mature adipocytes filled with a large single droplet of triacylglycerol (TAG) that is coated by the peripilin phospholipoproteins (Granneman and Moore 2008). This apparent simplicity of the WAT partly explains why it is only recently that the complexity of the tissue has become recognised (Trayhurn and Bing 2006). However adipocytes comprise only 30-60% of WAT. Several other cell types including pericytes, white and brown preadipocytes, fibroblasts, mast cells, lymphocytes, vascular cells, dendritic cells and macrophages are present also.

Adipocytes are insulin-sensitive cells that take up glucose and store energy in the form of TAG, which is subsequently released as NEFA and glycerol in times of energy need (Lyon, Law et al. 2003). In addition to their storage function, adipocytes also have an endocrine function, secreting adipocytokines, or adipokines for short (Kanda, Tateya et al. 2006).

The understanding of cytokines is important in order to appreciate the pathophysiology of how inflammation contributes to atherosclerosis. Cytokines are humoral immunoregulatory proteins and glycoproteins which have the ability to regulate local and/or systemic inflammation, immunity, angiogenesis, blood pressure, appetite and energy balance, insulin sensitivity, lipid metabolism and homeostasis (Lago, Dieguez et al. 2007). They differ from classical hormones in that they are produced by a number of tissue or cell types rather than by specialized glands. Some cytokines are small soluble polypeptides that function as intercellular signal mediators acting in an autocrine, paracrine or even endocrine manner (Sano, Fukuda et al. 2000). At concentrations as low as 10^{-10} to 10^{-15} mmol/L these molecules have the ability to bind to specific receptors and induce downstream signalling events (Schindler, Senf et al. 2002). Cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are pro-inflammatory and can induce the hepatic acute phase response, which consists of local and systemic reactions and is accompanied by upregulated and downregulated synthesis and/or activation of liver-enriched transcription factors (Rai, Lee et al. 1998). Excessive production of cytokines can lead to tissue damage, and hence their production is meticulously controlled in healthy individuals. Cytokine profiles of healthy individuals differ from those mounting an immune / inflammatory response. There also appears to be a hierarchy of cytokines, with cytokines such as TNFα being able to orchestrate the synthesis, secretion and activity of other cytokines (Coppack 2001).

1.1.5.2 Leptin

The key development that established WAT as an endocrine organ was the discovery of leptin in 1994, with its wide range of biological functions (Trayhurn and Wood 2004). Leptin signals the status of energy stores and its secretion can reduce appetite and increase energy expenditure. Leptin is a 167 amino acid peptide hormone that is synthesized and released from fat cells in response to changes in body fat. It is encoded by the gene obese (*ob*), the murine homologue of the human gene LEP (Zhang, Proenca et al. 1994). Structurally, it belongs to the class I cytokine superfamily, consisting of four α-helices and 2 short β-strands, with an intra-chain disulfide bond necessary for its biological activity (Zhang, Basinski et al. 1997). It circulates partially bound to plasma proteins and enters the central nervous system (CNS) by diffusion through capillary junctions in the median eminence and by receptor transport in the choroid plexus (Ronti, Lupattelli et al. 2006). Plasma levels correlate with WAT mass (Peelman, Waelput et al. 2004). In lean subjects, the majority of leptin circulates in the bound form whereas in obese subjects, the majority of leptin is present in the free-form (Sinha and Caro 1998).

The discovery of leptin led to increased understanding of the pathophysiology of human obesity. The word leptin comes from the Greek word *leptos* meaning thin, as it a key molecule in the regulation of body weight and energy balance. The primary role of leptin is to provide the CNS with information regarding body energy balance, controlling appetite and food intake to maintain a stable body weight (Ronti, Lupattelli et al. 2006). Leptin expression and serum levels increase after food intake, causing satiety. In contrast, leptin expression is rapidly suppressed with food restriction, faster than rates of reduction of fat mass and adipocyte size (Peelman, Waelput et al. 2004). Leptin levels can vary between 30% of baseline levels after 24 hours of fasting, to 150% of basal levels after over 12 hours of overfeeding (Sinha and Caro 1998).

Leptin exerts its effects by binding to its receptors, coded by the LEPR gene (Bjorbaek, Elmquist et al. 1998). 6 isoforms of the receptor exist (a-f), all containing an extracellular leptin-binding domain. However only the b isoform, being the longest form, contains a full length intracellular domain required for cell signalling (Friedman and Halaas 1998). LEPRb belongs to the gp130 family of cytokine receptors (Baumann, Morella et al. 1996). Its deficiency results in profound leptin resistance and morbid obesity in animals (Baumann, Morella et al. 1996; Chen, Charlat et al. 1996). Binding of leptin to its receptor causes LEPRb dimerisation, Janus Kinase 2 (JAK2) activation and autophosphorylation and subsequent downstream signalling (Fruhbeck 2006).

Leptin's main site of action is the hypothalamus (Flier 1998) where it decreases the desire for food intake and increases energy consumption by acting on the hypothalamic cell populations (Chan, Heist et al. 2003). Leptin activates anorexigenic neurons secreting cocaine and amphetamine-regulated transcript (CART) (Elias, Lee et al. 1998) and pro-opiomelanocortin (POMC) (Millington 2007), and inhibition of the orexigenic neurons secreting neuropeptide Y (NPY)/agouti-related peptide (AgRP) (van den Top, Lee et al. 2004) and orexin (Lopez, Seoane et al. 2000). In addition, leptin levels correlate negatively with glucocorticoids (Zakrzewska, Cusin et al. 1997) yet, correlate positively with insulin concentrations (Boden, Chen et al. 1997). It is known that leptin synthesis is regulated by food intake and eating-related hormones, but synthesis also depends on energy status, sex hormones (inhibited by testosterone and increased by ovarian sex steroids) and cytokines (Sarraf, Frederich et al. 1997). Interestingly, age, basal glucose levels and ethnicity do not influence circulating leptin levels (Sinha and Caro 1998).

1.1.5.3 Resistin

Resistin is a newer adipokine that belongs to the class of cysteine-rich, C-terminal proteins that is induced during adipogenesis. Originally named for its resistance to

insulin, resistin belongs to the resistin-like molecules (RELM) family or found in inflammatory zone (FIZZ) proteins (Holcomb, Kabakoff et al. 2000). Resistin is also known as adipose tissue secretory factor (ADSF) (Kim, Lee et al. 2001). This 114 amino acid peptide was discovered by Steppan & Lazar (Steppan and Lazar 2002) when identifying potential targets of the thiazolidinedione (TZD) class of insulin sensitizers in 3T3-L1 adipocytes. Messenger ribonucleic acid (mRNA) levels of resistin were markedly increased during 3T3-L1 and primary preadipocytes differentiation into adipocytes, although resistin itself has an inhibitory effect on adipose conversion. Thus it may be a negative feedback regulator restricting AT formation (Kim, Lee et al. 2001).

Resistin is encoded by the *Retn* gene and is secreted as a disulfide-linked dimmer. In mice, the *Retn* gene is expressed almost exclusively in WAT and the protein is detectable in adipocytes and in the circulation (Steppan, Bailey et al. 2001). Unlike murine resistin, human resistin is expressed at low levels in adipocytes but is readily detectable in mononuclear blood cells (Savage, Sewter et al. 2001; Banerjee and Lazar 2003). Expression of resistin from human monocytes is correlated with cytokines such as C-reactive protein (CRP) (Kunnari, Ukkola et al. 2006). Recombinant resistin upregulates cytokines and expression of cellular adhesion molecules expression on human endothelial cells (EC) (Verma, Li et al. 2003) and therefore may be implicated in atherosclerosis.

Resistin appears to be an important regulator of glucose homeostasis, adipogenesis, adipocyte differentiation and inflammation. Administration of recombinant resistin to otherwise normal mice caused IR, whereas administration of an anti-resistin antibody increased insulin sensitivity of obese and IR animals (Steppan, Bailey et al. 2001). However, the role of resistin in human obesity remains controversial. Previous resistin mRNA expression studies have produced conflicting reports about the levels of expression of this gene in human AT (Janke, Engeli et al. 2002; McTernan, McTernan et al. 2002). Higher resistin levels have been found in T2DM subjects

compared to non-diabetic controls (Azuma, Katsukawa et al. 2003) although it is not clear whether it is related to IR in all instances (Vozarova de Courten, Degawa-Yamauchi et al. 2004; Hasegawa, Ohta et al. 2005).

1.1.5.4 Adiponectin

Adiponectin was independently characterised by four groups using different methods between 1995 and 1996, hence its alternative names: apM1 (adipose most abundant gene transcript 1) (Maeda, Okubo et al. 1996), Acrp30 (adipocytes complement related protein of 30-kDa) (Scherer, Williams et al. 1995), GBP-28 (gelatine binding protein of 28kDa) (Nakano, Tobe et al. 1996) and AdipoQ (Hu, Liang et al. 1996). It is the product of the AD/POQ gene located on chromosome 3q27, a locus for diabetes and MetS susceptibility (Mori, Otabe et al. 2002). Initially it was thought to be exclusively secreted from the AT (Scherer, Williams et al. 1995). However there is now evidence that it is also expressed from adipocytes of bone marrow (Yokota, Meka et al. 2002), osteoblasts (Berner, Lyngstadaas et al. 2004) and cardiomyocytes (Pineiro, Iglesias et al. 2005).

Adiponectin is a 244 amino acid peptide. It has a collagen-like domain at its N-terminus and a globular domain at its carboxyl-terminus, which is structural homology with collagens VIII and X and complement factor C1q. Despite a lack of homology in primary sequence, the tertiary crystal structure of the C-terminal globular domain has striking resemblance to that of TNF-α (Shapiro and Scherer 1998).

Adiponectin is present in full-length multimers, with a wide range of multimer complexes in plasma and combines via its collagen domain to create 3 major oligomeric isoforms: a low-molecular weight (LMW) trimer, medium-molecular weight (MMW) hexamer and high-molecular weight (HMW) isoform of 12-18

subunits (Behre 2007). The HMW species may be the most active form of the protein and may have a more relevant role in IR. A high HMW-to-total adiponectin ratio correlated more significantly with glucose and insulin levels than did total adiponectin levels alone (Lara-Castro, Luo et al. 2006), and it has been proposed that HMW adiponectin is more predictive of IR and MetS (Hara, Horikoshi et al. 2006).

Two adiponectin receptors have been identified (Yamauchi, Kamon et al. 2003). AdipoR1 is found predominantly expressed in the skeletal muscle, while AdipoR2 is found primarily in the liver (Lago, Dieguez et al. 2007). Adiponectin binds to the C-terminal extracellular domain of AdipoR1, whereas the N-terminal cytoplasmic domain interacts with an adaptor protein containing a pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper motif (APPL). Transduction of the adiponectin signal by AdipoR1 and AdipoR2 involves the activation of adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-alpha (PPAR-α). Kadowaki et al. (Kadowaki, Yamauchi et al. 2006) demonstrated that full-length adiponectin activates AMPK in the liver inhibiting gluconeogenesis (Combs, Berg et al. 2001) while globular adiponectin exerts its effects in both muscles and in the liver, resulting in increased fatty acid combustion. Glucose transport is also enhanced by adiponectin in the muscles (Kuoppamaa, Skrobuk et al. 2008). Furthermore, adiponectin also activates PPAR-α, leading to decreased hepatic and muscular TAG content and thus an increase in *in vivo* insulin sensitivity. PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor family. PPAR-α is mainly expressed in the liver, BAT, small intestine, skeletal muscle and heart. The level of PPAR-α in the WAT and in adipocyte cell lines is very low suggesting a limited role for this isoform during adipogenesis (Grimaldi 2001).

Both TNF-α and IL-6 are potent inhibitors of adiponectin expression and secretion from human WAT and cultured adipose cells, suggesting that TNF-α and IL-6 induced IR may rely on an autocrine-paracrine inhibition of adiponectin release (Bruun, Lihn

et al. 2003; Fasshauer, Kralisch et al. 2003). Evidence also suggests that adiponectin regulates TNF-α metabolism, as increased TNF-α has been reported in adiponectin knockout mice (Yamauchi, Kamon et al. 2003).

It is well recognised now that adiponectin is strongly inversely correlated with IR and inflammation (Chandran, Phillips et al. 2003; Diez and Iglesias 2003; Fortuno, Rodriguez et al. 2003). Even before the onset of obesity and T2DM, adiponectin is found to be lower than expected, suggesting that hypoadiponectinaemia may implicated in the pathogenesis of these conditions, at least in primates (Hotta, Funahashi et al. 2001). Administration of adiponectin in IR obese subjects improves metabolic parameters (Chandran, Phillips et al. 2003; Diez and Iglesias 2003). A similar finding has been reported in non-obese IR subjects with lipodystrophy secondary to human immunodeficiency virus (HIV) (Kinlaw and Marsh 2004). On the other hand, improving insulin sensitivity, either by weight reduction or insulin-sensitising drugs increases adiponectin levels (Chandran, Phillips et al. 2003; Diez and Iglesias 2003). Lastly, polymorphisms of adiponectin are associated with IR and obesity (Chandran, Phillips et al. 2003; Diez and Iglesias 2003). It has been suggested that adiponectin appears to have anti-diabetic, anti-inflammatory and anti-atherogenic properties (Kershaw and Flier 2004).

1.1.6 Inflammatory Markers

1.1.6.1 Introduction

Inflammation is part of the non-specific immune response as a result of any bodily injury, be it caused by pathogens, damaged cells or irritants. It is an attempt of a multicellular organism to remove the noxious stimuli and initiate healing. It is now well recognised that atherosclerosis, previously thought to be a bland lipid storage disease, is actually a disease with ongoing low grade inflammation (Libby 2002). As

mentioned above, the pathophysiology of atherosclerosis involves complex processes involving immune cells and inflammation. Various markers of inflammation might serve to be useful markers of cardiovascular risk.

1.1.6.2 Tumour Necrosis Factor-α (TNF-α)

The first molecular link between inflammation and obesity was described by Carswell et al. (Carswell, Old et al. 1975) in 1975. Although originally described as a circulating factor that can cause necrosis of tumours, TNF-α has since been identified as a key regulator of inflammation (Bradley 2008). TNF-α is a 185 amino acid, 26-kDa glycoprotein transmembrane peptide. Soluble TNF-α (sTNF-α) is the form found in the circulation and is the result of cleavage by TNF-α converting enzyme (Moller 2000). Whilst both cell-bound TNFα and sTNF-α can affect biological and metabolic responses (Perez, Albert et al. 1990; Xu, Sethi et al. 1999) TNF-α may mainly mediate paracrine and autocrine signals, whereas sTNF-α mediates endocrine effects (Grell 1995).

TNF-α exerts its effects by binding to two different cell-surface receptors that have been identified, TNF receptor-1 (TNFR-1) and TNF receptor-2 (TNFR-2), which are present on virtually all cells except for erythrocytes. In humans these receptors are also known as gp60/TNFR60 and gp80/TNFR80, respectively and in mice the p55 receptor and p75 receptor, respectively (Coppack 2001). TNFα converting enzyme (TACE/ADAM-17) is a transmembrane metalloproteinase-disintegrin that cleaves the release of TNFR-1 and TNFR-2 from the extracellular domain (Loetscher, Pan et al. 1990) and thus releasing the soluble fraction of these receptors, sTNFR1 and sTNFR2 (Reddy, Slack et al. 2000). Hotamisligil et al. (Hotamisligil, Arner et al. 1997) found that obese females had higher circulating levels and mRNA levels of sTNFR2 compared with lean controls. This study also found that mRNA levels of sTNFR2 were strongly correlated with BMI and degree of hyperinsulinaemia. These results concur with a recent study that found strong correlations between circulating sTNFR1 and

sTNFR2 levels and BMI, total body fat and subcutaneous abdominal fat. However, no significant relationship was found between the TNF-α receptors and insulin levels (Lo, Bernstein et al. 2007).

In AT, TNF-α expression and secretion is primarily from the stromal vascular fraction and to a lesser extent from adipocytes (Fain, Madan et al. 2004). Levels of circulating sTNF-α seem are strongly correlated with BMI and WHR (Hotamisligil, Arner et al. 1995; Zahorska-Markiewicz, Janowska et al. 2000), probably due to increased AT macrophage accumulation in obesity states (Weisberg, McCann et al. 2003; Cinti, Mitchell et al. 2005). TNF-α has potent effects on adipocyte metabolism including inhibition of adipogenesis and fat-producing lipogenic enzymes as well as activation of lipolytic activity (Beutler, Greenwald et al. 1985; Cornelius, Enerback et al. 1988). TNF-α **has been shown to increase** plasma TAG and very low-density lipoprotein (VLDL) as well as lipolysis in rodent and human fat cells. TNF-α also reduces insulin sensitivity by down-regulating expression of the insulin receptor, insulin receptor substrate 1 (IRS-1) and glucose transporter type 4 (GLUT4). It also reduces insulin stimulated receptor tyrosine kinase activity at low concentrations and increases the phosphorylation of IRS-1 serine 307 and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), thus impairing its ability to bind to the insulin receptor and initiate downstream signalling (Hotamisligil, Peraldi et al. 1996).

Under normal healthy circumstances TNF-α circulates in low concentrations, reported to be in the ranges of 10-80pg/mL (Yudkin 2007). However, obese women express 2-fold more TNFR2 mRNA in AT and 6-fold more sTNFR2 in circulation relative to lean controls (Hotamisligil, Arner et al. 1997). Interestingly, concentrations of TNF-α have been reported to decrease with weight-loss and improvements in insulin sensitivity (Bruun, Verdich et al. 2003). More recently, it has been shown that TNF-α is also expressed in skeletal muscle and that levels are 4-fold higher in IR, obese humans than in healthy humans (Ferrier, Nestel et al. 2004). Anti-TNF-α

antibodies improve insulin sensitivity in obese rodents and TNF-α deficient mice are protected from obesity induced IR, which is characterised by a less than normal physiological response to normal hormone levels (Guerre-Millo 2004). Based on these findings anti-inflammatory therapies targeting TNF-α activity have emerged as novel strategies for the treatment of obesity-related diseases.

1.1.6.3 Interleukin-6 (IL-6)

IL-6 is a multi-functional cytokine that regulates immune responses, growth and differentiation of a variety of cells and metabolic processes. First described by Kishimoto et al. (Hirano, Yasukawa et al. 1986), IL-6 has been alternatively named interferon β2, IL-1-inducible protein, hepatocyte-stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor and/or B cell stimulatory factor 2 (Ferguson-Smith, Chen et al. 1988). IL-6 is produced by numerous cell types including fibroblasts, EC, monocytes/macrophages and T-cells (Van Snick 1990). Human IL-6 is a variably glycosylated, 22-27 kDa secreted glycoprotein and it is translated as a 212 amino acid molecule, with a 28 amino acid signal sequence and a 184 amino acid mature segment (Somers, Stahl et al. 1997). IL-6 is often involved in the acute phase response together with IL-1 and TNF-α. IL-6 regulates these cytokines by directly influencing TNF-α and promoting the release of IL-1 from the IL-1 receptor activity.

IL-6 exerts its effects through a cell-surface type 1 cytokine receptor complex consisting of the ligand binding IL-6Rα chain (CD126) and the signal transducing sub-unit (gp130), which is ubiquitously expressed in most tissues. As IL-6 interacts with its receptors it triggers the gp130 and IL-6R protein to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through certain transcription factors; Janus kinases (JAKs) and signal transducers activators of transcription (STAT). Soluble forms of the IL-6 receptor have been identified, but their regulation and role is poorly understood (Fruchart, Staels et al. 2001).

Human AT is the main site for IL-6 secretion accounting for 15-35% of the circulating levels and visceral AT produces two to three times more IL-6 compared with subcutaneous AT (Fried, Bunkin et al. 1998; Aldhahi and Hamdy 2003; Rotter, Nagaev et al. 2003). Circulating IL-6 concentrations of normal healthy individuals tend to approximately 1pg/mL (Yamamura, Yamada et al. 1998) with cyclical variations with the menstrual cycle (Angstwurm, Gartner et al. 1997). Among all the cytokines, IL-6 presents the strongest correlation with IR and T2DM in humans (Pradhan, Manson et al. 2001; Bastard, Maachi et al. 2002). IL-6 levels also increase postprandialy in subcutaneous AT interstitial fluid (Orban, Remaley et al. 1999).

1.1.6.4 C-Reactive Protein (CRP)

CRP is a membrane of the phylogenetically **ancient and highly conserved 'pentraxin'** family of proteins, which also includes serum amyloid P (SAP) component. In man and other animal species, CRP is a major acute phase reactant displaying rapid and pronounced rise of its serum concentration in response to infection or tissue injury (Volanakis 2001). CRP was first discovered many decades ago in 1930. It has a half-life of about 19 hrs and has a Ca^{2+} dependent binding specificity for phosphocholine, a constitution of many bacterial and fungal polysaccharides and for most biological cell membranes (Volanakis and Kaplan 1971). Ligand-complexed CRP is subsequently recognised by C1q of the complement, activating the classical pathway. Thus CRP is an important constituent of the first line of host defence. Furthermore, this protein appears to play a role in the clearance of apoptotic and necrotic host cells, thus contributing to restoration of normal structure and function of injured tissues. Despite these beneficial effects, CRP can become highly elevated in the presence of other inflammatory processes such as infections as well as chronic diseases such as hypertension, obesity, and diabetes (Zwaka, Hombach et al. 2001). Additionally, CRP can also induce the expression of cell adhesion molecules in EC, thereby propagating antigens and stimulating monocyte recruitment (Labarrere and Zaloga 2004).

CRP is primarily synthesised by hepatocytes following stimulation by IL-6 in an acute-phase response (Collins, Saxena et al. 1984). *In vitro* and *in vivo* CRP mRNA transcription is dramatically upregulated by IL-6 and this response is greatly enhanced by IL-1β, glucocorticoids and complement activation products (Toniatti, Arcone et al. 1990; Szalai, van Ginkel et al. 2000). This synergistic phenomenon occurs due to the regulation of CRP synthesis at the transcriptional level by IL-1β. After transcription, CRP mRNA is translated to protomers. In the endoplasmic reticulum five protomers are assembled to one cyclic pentamer, which is either stored or secreted (Hu, Miller et al. 1986). Under physiological circumstances, human CRP is a protein with a medium serum concentration of 0.8mg/L. Circulating plasma CRP concentrations of over 2.0mg/L is associated with increased risk of CVD (Danesh, Wheeler et al. 2004). Several studies have found that MetS subjects have increased concentrations of CRP compared to healthy lean controls, suggesting a relationship between IR and CRP levels (Novo, Corrado et al. 2007; Yoshikane, Yamamoto et al. 2007). In fact, all 5 characteristics of the MetS are associated with increased CRP levels (Ridker, Buring et al. 2003; Nakamura, Ito et al. 2008). It has even been suggested that CRP and LDL-chol should be measured routinely to assess cardiovascular risk (Ridker, Rifai et al. 2002). However, since normal individuals have CRP levels of <1mg/L, assays must have sensitivities below this. Such measurements are known to measure highly sensitive-CRP (hsCRP) being able to detect levels below the sensitivities of CRP assays used in standard clinical practice.

1.1.6.5 Soluble Intercellular Adhesion Molecule-1 (sICAM-1)

ICAM-1, also known as CD54 (Cluster of Differentiation 54) is an 80-110-kD glycoprotein and a member of an immunoglobulin (Ig) supergene family. Both ICAM-1 and VCAM-1 are implicated in the attachment of leukocytes to ECs in response to inflammatory cytokines (Malik, Danesh et al. 2001). ICAM-1 is the most widely expressed cell adhesion molecule and is found on leukocytes, fibroblasts, epithelial and ECs. It is constitutively expressed, but its expression is upregulated in response to lipopolysaccharide (LPS), phorbol esters, pro-inflammatory cytokines and LDL

exposure (Yuan, Verna et al. 2001). Proteolytic cleavage at the cell surface releases the extracellular domain and can be detected in the circulation as soluble forms i.e. sICAM-1.

Studies have shown an increased expression of cellular adhesion molecule in atherosclerotic plaques (Endres, Laufs et al. 1997) and a positive correlation between plasma concentrations of sICAM-1 and risk of coronary artery disease (Ridker, Hennekens et al. 1998). Levels of sICAM-1 are higher in T2DM patients compared to non-diabetic controls (Fasching, Waldhausl et al. 1996). A high-fat diet, now typical of **the "Western diet"** is associated with increased ICAM-1 exposure (Brake, Smith et al. 2006).

1.1.6.6 Soluble Vascular Adhesion Molecule-1 (sVCAM-1)

VCAM-1, also known as CD106, is also a member of the Ig superfamily. It is a glycoprotein that contains 6-7 Ig domains. It is expressed on ECs of large and small vessels following stimulation by TNF-α, IL-1 and IL-4. Apart from ECs, it is also expressed on macrophages, epithelial cells and dendritic cells (DCs). VCAM-1 has been found to bind to its counter-receptor α4β1-integrin, very late antigen 4 (VLA-4) (Elices, Osborn et al. 1990) and participates in the adhesion of circulating leukocytes to ECs allowing subsequent transmigration to the arterial intima (Munro and Cotran 1988). Like ICAM-1, VCAM-1 is also subject to proteolytic cleavage on cell surfaces and is detected in the circulation as sVCAM-1 (Singh, Mason et al. 2005).

Expression of sVCAM-1 is upregulated by the proinflammatory cytokines IL-1 and TNF-α in vascular EC and SMC (Singh, Mason et al. 2005). sVCAM-1 levels are higher in subjects with diabetes compared to non-diabetic subjects (Wautier and Wautier 2001). Moreover, the serum level of sVCAM-1 is increased in hypercholesterolaemic

and hypertensive patients (Parissis, Venetsanou et al. 2001), implicating its role in atherogenesis.

1.1.6.7 Plasminogen Activator Inhibitor-1 (PAI-1)

PAI-1 was first described in the early 1980's as a rapid inhibitor of tissue plasminogen activator (tPA) and that of fibrinolysis in the blood (Wiman, Chmielewska et al. 1984). It is a 50 kDa glycoprotein of a 379 amino acid and belongs to the serine protease inhibitor (serpin) family (Gils and Declerck 2004). A number of cell types produce PAI-1, including EC, hepatocytes, adipocytes and AT stromal cells (Samad and Loskutoff 1996; Bastelica, Morange et al. 2002). Over expression and production of PAI-1 are thought to be related to transforming growth factor beta (TGF-β), TNF and insulin (Samad and Loskutoff 1996; Fujita, Kang et al. 2006), leading to IR and MetS (Alessi and Juhan-Vague 2006). Deletion of TNF receptors significantly reduces PAI-1 mRNA and TNF-neutralising antibodies reduce plasma PAI-1 levels (Samad, Uysal et al. 1999; Pandey, Tuncman et al. 2003). Furthermore, thiazolidinediones inhibit TNF-α induction of PAI-1 independent of peroxisome proliferator-activated receptor gamma (PPARγ) activation (Liu, Hu et al. 2005). That TGF-β is involved in PAI-1 production comes from studies of the $FOXC2^{+/-}$ mice. FOXC2 is implicated in IR, and treatment of FOXC2+/- mice with TGF-β1 treatment lowers PAI-1 (Fujita, Kang et al. 2006). Other factors that contribute to increased PAI-1 levels include glucocorticoids (Halleux, Declerck et al. 1999), probably due to elevated 11-beta-hydroxysteroid dehydrogenase-1 (11β-HSD1) activity (Kotelevtsev, Holmes et al. 1997; Ayachi, Paulmyer-Lacroix et al. 2006) and angiotensin II (Vaughan, Lazos et al. 1995; Skurk, Lee et al. 2001).

Free fatty acids (FFA) and VLDL have also been shown to stimulate PAI-1 synthesis (Stiko-Rahm, Wiman et al. 1990; Nilsson, Banfi et al. 1998; Banfi, Mussoni et al. 1999). However, studies investigating IR on PAI-1 synthesis have shown conflicting results. Cell culture studies suggested that PAI-1 synthesis is stimulated by insulin or

proinsulin (Alessi, Juhan-Vague et al. 1988; Kooistra, Bosma et al. 1989; Schneider, Nordt et al. 1992) but clinical studies in humans have not supported such findings (Grant, Kruithof et al. 1990; Lormeau, Aurousseau et al. 1997).

It has been demonstrated that in women with PCOS, PAI-1 levels are significantly elevated compared to healthy controls (Dhindsa, Bhatia et al. 2004), an association that has been demonstrated in several studies (Sampson, Kong et al. 1996; Orio, Palomba et al. 2004). However increased PAI-1 levels may be due to obesity rather than PCOS *per se* (Atiomo, Fox et al. 2000). Due to inconsistencies regarding the outcomes of these studies, the answer to whether or not PCOS *per se*, has an independent influence on PAI-1 levels remains inconclusive.

1.1.6.8 Myeloperoxidase (MPO)

MPO is a heme protein expressed abundantly in the azurophilic granules of most leukocytes including neutrophils and monocytes (Klebanoff 2005). It is released during inflammation and catalyses the formation of several reactive species including hypochlorous acid and thus has a role in the host defence against microorganism (Klebanoff 2005). However, in recent years, its role in atherosclerosis has also been acknowledged. Many epidemiological studies have shown that higher levels of MPO are associated with increased CVD risk, independent of classical CVD risk factors (Baldus, Heeschen et al. 2003; Exner, Minar et al. 2006; Khan, Kelly et al. 2007). Several studies, typically involving patients with stable CVD have reported no association (Kubala, Lu et al. 2008; Stefanescu, Braun et al. 2008) suggesting that MPO may only be a useful risk marker in subjects at higher risk.

The role of MPO in atherosclerosis is not surprising given the important roles of inflammation and monocytes in the development of CVD. MPO released from leukocytes is initially bound to the vascular endothelium and subsequently

transcytosed to the subendothelial matrix (Baldus, Eiserich et al. 2001). In the subendothelial space, MPO, together with its co-substrate hydrogen peroxide, catalyses various substrates including LDL and HDL. As will be discussed in a later section, oxidised LDL and HDL particles are both implicated in the progression of atherosclerosis. MPO is also deranged in dyslipidaemia (Francisco, Hernandez et al. 2006). Finally, MPO contributes to the development of endothelial dysfunction, by regulating nitric oxide availability, which can lead to decreased vasodilatation (Eiserich, Baldus et al. 2002).

1.1.6.9 Serum Amyloid A (SAA)

Human SAA is a 12.5kDa protein synthesised by the liver (Malle and De Beer 1996). Its serum levels can increase up to 1000- fold 24-36 hours after infection or injury, decline after 4-5 days, and return to baseline after 10-14 days (Gabay and Kushner 1999). The human genome encompasses four SAA genes (SAA1 – SAA4). SAA1 and SAA2 are highly homologous reactants whose concentrations increase with infection, trauma and obesity (Poitou, Viguerie et al. 2005). SAA3 is a pseudogene and SAA4 is a constitutively expressed minor constituent of the non-acute-phase HDL (Kisilevsky and Tam 2002).

It has been hypothesised that SAA is one of the inflammatory molecules in the pathogenesis of CVD (Yang, Lee et al. 2006; Hatanaka, Monteagudo et al. 2007), being able to cause proatherogenic changes to vascular proteoglycans (Wilson, Thompson et al. 2008) and stimulate inflammatory mediators from cultured vascular EC, neutrophils and monocytes (Song, Hsu et al. 2009; Song, Shen et al. 2009). Some of these cells may even account for extrahepatic SAA production, as suggested by detection of SAA mRNA in these cells (Poitou, Divoux et al. 2009). Indeed, studies show that SAA elevation predict cardiovascular events analogously with or even better than CRP by itself (Ridker, Hennekens et al. 2000; Johnson, Kip et al. 2004; Kosuge, Ebina et al. 2007). SAA is also a recognised biomarker of cerebrovascular

disease (Brea, Sobrino et al. 2009) and carotid intima-media thickness (IMT) (Uurtuya, Kotani et al. 2009). Lastly, SAA is associated with IR, MetS and DM (Samaras, Botelho et al. ; Ley, Harris et al. 2008).

1.1.6.10 Neopterin

Neopterin is a pteridine derivative produced by activated macrophages in response to interferon-γ (IFN-γ) (Huber, Batchelor et al. 1984; Murr, Widner et al. 2002). Guanosine triphosphate (GTP) within macrophages is broken down into 7, 8-dihydroneopterin which diffuses into plasma and is oxidised to neopterin by hypochlorous acid released from neutrophils during inflammation (Widner, Mayr et al. 2000). Although its exact function is unknown, several studies suggest that it is a pro-oxidant, enhancing oxidant production and promoting cell death or atherosclerosis (Weiss, Fuchs et al. 1993; Murr, Fuchs et al. 1994). It is raised in various conditions including infection (Fuchs, Hausen et al. 1988), autoimmune disease (Reibnegger, Egg et al. 1986), cancer (Murr, Fuith et al. 1999) and allograft rejection (Margreiter, Fuchs et al. 1983).

Numerous studies now also show that neopterin may be a useful marker for cardiovascular risk stratification as it appears to predict CVD independently of coronary artery disease (Garcia-Moll, Cole et al. 2000; van Haelst, Liem et al. 2003; Avanzas, Arroyo-Espliguero et al. 2004; Avanzas, Arroyo-Espliguero et al. 2005; Ray, Morrow et al. 2007; Kaski, Consuegra-Sanchez et al. 2008). It is interesting to note that neopterin is useful in patients with chronic stable angina (Avanzas, Arroyo-Espliguero et al. 2005) and patients with hypertension without obstructive coronary artery disease (Avanzas, Arroyo-Espliguero et al. 2004). Finally, neopterin levels are also closely associated with obesity status and IR (Ledochowski, Murr et al. 1999).

Table 1-1: Summary of Adipokines & Inflammatory Markers

Adipokine / Inflammatory Marker	Main metabolic functions	Levels in IR / CVD
Leptin	Regulates food intake Levels increase postprandially	Increased levels
Resistin	Adipocyte differentiation/adipogenesis Possibly ↑ IR	Increased levels
Adiponectin	↑ fatty acid oxidation & ↓ gluconeogenesis ↓ IR	Decreased levels
TNF-α	Down regulates IRS-1 & GLUT 4 ↑ IR	Increased levels
IL-6	Directly influences TNF-α And IL-1 release	Increased levels
hs-CRP	Activates classical pathway of complement Induces cell adhesion molecule expression	Increased levels
sICAM-1	Allows leucocytes to attach to Endothelial cells	Increased levels
PAI-1	Rapid inhibitor of tissue plasminogen activator	Increased levels
MPO	Catalyses the formation of Reactive species e.g. hypochlorous acid	Increased levels
SAA	Associated with LCAT	Increased levels
Neopterin	Pro-oxidant, promoting cell death	Increased levels

1.1.7 Lipids and Lipoproteins

1.1.7.1 Introduction

Lipids are by nature hydrophobic, and are not able to circulate in the aqueous plasma. Plasma lipoproteins are a heterogeneous group of water-soluble globular particles that serve to transport hydrophobic lipids in the circulation. They consist of both lipids (cholesterol, triglycerides and phospholipids) and proteins (apolipoproteins) and hence their name – lipoprotein. The hydrophobic components, triglycerides and cholesteryl esters are located within the central core of the lipoprotein body, whereas the amphipathic components, including apolipoproteins, cholesterol and phospholipids are located on the outer surface of the particle as shown in Figure 1-1. The combination of lipid and protein constituents gives rise to five main subclasses of lipoproteins, all of which differ in size, composition, hydrated density, and electrophoretic mobility.

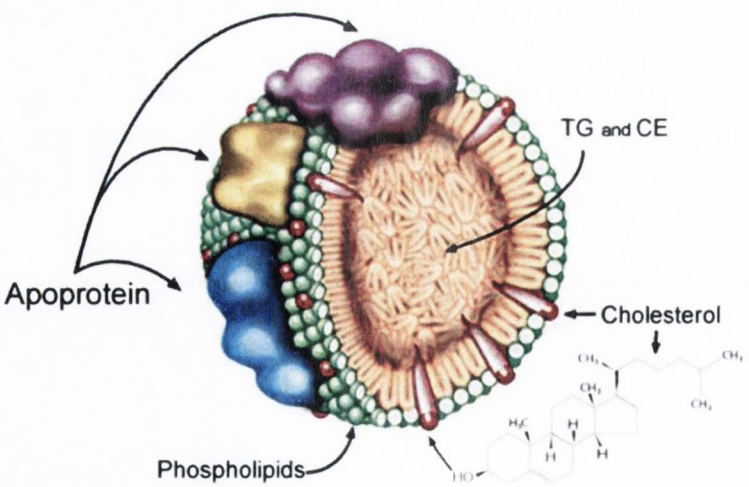

Figure 1-1: The composition of a typical lipoprotein particle. The particle is spherical in shape and is composed of two regions, a hydrophilic layer and a hydrophobic core. Apoproteins intersperse between the layers and add structural integrity. Taken from Peprotech (2009).

Lipoproteins are also readily classified by the apoprotein content. Apolipoproteins contain helical structures interdigitated between non-helical sections and represent the major protein component of lipoprotein particles. They have various functions, including: (i) provide structural support to the lipoprotein, (ii) contribute to the mediation of normal lipoprotein metabolism, (iii) regulate important enzymes involved in lipid metabolism, including hepatic lipase (HL) and lipoprotein lipase (LPL), and (iv) interact with extracellular matrix and cell surface receptors, for example, apoprotein B_{100} ($apoB_{100}$) (Frohlich, Seccombe et al. 1989). A summary of the major apolipoproteins, including their associated lipoproteins and metabolic functions is given in Table 1-2.

Table 1-2: Summary of apoproteins, function and associated lipoproteins.

Apoprotein	Metabolic function	Associated Lipoprotein
A-I	Ligand for HDL Co-factor for LCAT	CM, HDL
A-II	Ligand for HDL Co-factor for LCAT	CM, HDL
A-IV	Ligand for HDL Activates LCAT	CM, HDL
(a)	Provides structural integrity for Lp(a)	Lp(a)
B_{48}	Provides structural integrity for CM	CM
B_{100}	Ligand for LDL receptor Provide structural integrity for VLDL, IDL, LDL	VLDL, IDL, LDL
C-I	Activates LCAT Activates LPL	CM, VLDL, IDL, LDL
C-II	Activates LCAT Activates LPL	CM, VLDL, IDL, LDL
C-III	Inhibits LPL Inhibits HL	CM, VLDL, IDL, LDL
D	Associated with LCAT	HDL
E	Ligand for Apo B/E receptor Ligand for Apo E2 receptor	CM, VLDL, IDL, LDL
F	Lipid transfer-inhibitor protein	HDL
H	Implicated in platelet function	Not associated
J	Implicated in cell membrane protection	HDL

Abbreviations: LCAT – Lecithin cholesterol acyltransferase, CM – chylomicrons, VLDL – very low density lipoprotein, IDL – intermediate density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein, Lp(a) – Lipoprotein(a), LPL – lipoprotein lipase, HL – hepatic lipase

1.1.7.2 Lipoprotein Subclasses

There are five major classifications of plasma lipoproteins, originally classified according to their electrophoretic mobility, but now according to their density as determined by ultracentrifugation. The largest and least dense particles are chylomicrons (chylo = milky fluid, micron = indicates particle size); followed by very low density lipoproteins (VLDL); intermediate density lipoprotein (IDL); low density lipoproteins (LDL); and high density lipoproteins (HDL), the latter being the smallest and most dense of the lipoprotein particles. The lipid component and apoprotein content of these five lipoproteins are continuously interchanging, giving rise to a heterogeneous population of subclasses. Each lipoprotein can therefore be further divided into various subclasses, usually according to size or density. VLDL can be subdivided into subfractions A, B, C and D (McEneny, McMaster et al. 2002). LDL can be subdivided, most commonly into LDL-I, LDL-II and LDL-III (de Graaf, Hendriks et al. 1993). Finally, HDL can be further subdivided into at least HDL_2 and HDL_3, (McPherson, Young et al. 2007), and some have identified 5 subclasses (Wilson, Griffin et al. 1992; Guerin, Lassel et al. 2000). Table 1-3 provides a summary of the five main lipoproteins found *in vivo*, including their density range, particle size, electrophoretic mobility and function.

Table 1-3: The 5 main classes of lipoproteins

Lipoprotein	Density (Range) (g/mL)	Diameter (mean) (nm)	Electrophoretic mobility	Major apoproteins	Protein content (% of dry weight)	PL content (% of dry weight)	TG content (% of dry weight)	Chol content (% of dry weight)	Main function
CM	< 0.950	500	Origin	B48, CI, CII, CIII, E	1 - 2	7	84	7	Transport of exogenous TG from intestine to liver
VLDL	0.950 - 1.006	43	Preβ	B100, CI, CII, CIII, E	10	18	50	22	Transport of endogenous TG
IDL	1.006 - 1.019	27	Preβ-1	B100, CI, CII, CIII, E	18	22	31	29	Endocytosis by liver and conversion to LDL
LDL	1.019 - 1.063	22	β	B100	25	21	4	50	Transport of cholesterol from liver to peripheral tissues
HDL	1.063 - 1.210	8	α	AI, AII, CI, CII, CIII, E	35	29	8	28	Transport of cholesterol from peripheral tissues to liver

Abbreviations: PL – phospholipid, TG – triglyceride, Chol – cholesterol, CM – chylomicrons, VLDL – very low density lipoprotein, IDL – intermediate density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein

1.1.7.3 Normal Lipoprotein Metabolism

Lipoprotein metabolism is a complex process involving lipoprotein receptors, enzymes and lipid transfer proteins. The liver and intestine are the two most important players in lipoprotein metabolism. The intricate metabolism of lipoproteins occurs *via* three separate metabolic pathways: the exogenous pathway, the endogenous pathway and reverse cholesterol transport (Ginsberg 1998).

1.1.7.3.1 Exogenous Pathway

Exogenous lipid metabolism describes the process by which dietary lipids are absorbed and utilised – particularly in skeletal muscle, adipose tissue and the liver. Initially dietary lipids are absorbed by intestinal mucosal cells, or enterocytes, which re-esterify cholesterol and free fatty acids to produce cholesterol ester (CE) and triglyceride. Together with phospholipids and unesterified cholesterol, these lipids are then packaged together with an apolipoprotein B48 (apoB48) as well as some apolipoprotein A-I and A-IV (apoA-I and apoA-IV) to form nascent chylomicrons (Guo, Avramoglu et al. 2005). ApoB48 is a low molecular weight form of apoB100 – it contains only residues 1-2152 i.e. 48% of an apoB100 molecule. Each chylomicron particle only has one apoB48 particle, and therefore they have a high lipid to protein content – and hence the term intestinal-derived triglyceride rich lipoprotein (TRL). They are the least dense of all lipoproteins. These lipid-rich chylomicrons are then secreted into the lymphatic circulation and subsequently enter the circulation *via* the thoracic duct.

Once in the circulation they are rapidly metabolised in to chylomicron remnants. HDL particles exchange their apolipoproteins C-II, C-III and E (apoC-II, apoC-III, apoE) with apolipoprotein A from chylomicrons. Endothelial lipoprotein lipase (LPL) depletes chylomicrons of their TGs breaking the lipids into FFAs and glycerol again. FFAs are then taken up where they may be re-synthesised into triglycerides and stored, or

oxidised to provide energy. The now triglyceride-poor chylomicron particles get progressively smaller in size and also receive CE from mature HDL *via* the action of cholesteryl ester transfer protein (CETP) (de Grooth, Zerba et al. 2004). The presence of apoE on these now cholesterol-enriched remnants helps in their clearance *via* the hepatic apo B/E receptor. The remnants are then rapidly taken up by the liver and removed from the circulation (Ginsberg 1998).

1.1.7.3.2 Endogenous pathway

Endogenous lipid metabolism describes the process by which the hepatically-derived lipids are released into the circulation during periods of fasting. Chylomicrons are only present after ingestion of food and generally become undetectable in the circulation after 12 hours of fasting. During the fasting state, the liver packages triglycerides, cholesterol, CE and phospholipids, together with apoC, apoE and apoB100 into very-low density lipoprotein (VLDL) particles. As a result, large, nascent VLDL particles are produced, and secreted into the bloodstream *via* exocytosis (Gibbons, Wiggins et al. 2004).

Nascent VLDL, like chylomicrons, are also rich in TG – and hence the term hepatic-derived TRL. They undergo a similar metabolic process to chylomicrons, with HDL donating apoC-II, C-III and E (Gibbons, Wiggins et al. 2004). These VLDL particles then bind to the same LPL, where the triglycerides are hydrolysed to FFAs and glycerol. Again, these FFAs are re-synthesised into triglycerides for storage, or undergo beta-oxidation to provide energy. As the tissues absorb these FFAs, the VLDL **particle becomes progressively smaller in size, resulting in the formation of 'VLDL remnants', otherwise known as** intermediate density lipoproteins (IDL). As these VLDL particles become smaller in size, a significant proportion of phospholipids and apo Cs are removed to HDL. IDL can then either be taken up by the liver, *via* the same apo B/E receptor, in the same manner as chylomicrons remnants, or undergo further metabolism by hepatic lipase resulting in triglyceride removal to become a smaller

lipoprotein – low-density lipoprotein (LDL). This lipoprotein only contains apoB100, is low in triglyceride, but rich in cholesterol (80% of lipoprotein) being the major carrier of cholesterol in the circulation. It is essential for the delivery of cholesterol to different cell types for various biological processes, including regulating cell membrane fluidity and synthesis of various hormones (Mahley, Innerarity et al. 1984).

1.1.7.3.3 Reverse Cholesterol Transport

Reverse cholesterol transport describes the process whereby HDL particles pick up cholesterol from the arterial wall and transport it back to the liver, where it is excreted in bile (Bruce, Chouinard et al. 1998; Sviridov and Nestel 2002; von Eckardstein, Hersberger et al. 2005). HDL particles are the smallest and most dense lipoproteins, and can be subdivided into 2 subfractions, HDL_2 (larger, less dense) and HDL_3 (smaller, more dense) (McPherson, Young et al. 2007). Apo A-I, A-II and A-IV are the major apolipoproteins of HDL and are responsible for modulating HDL metabolism.

Both the liver and intestine synthesise and secrete nascent, discoid HDL, which consists mainly of apoC's, apoE, phospholipids and cholesterol. ApoA proteins are subsequently incorporated and these allow the particle to utilise lecithin:cholesterol acyltransferase (LCAT) and adenosine triphosphate-binding cassette protein A1 (ABCA1). The ABCA1 transporter protein facilitates the efflux of intracellular cholesterol (Fielding and Fielding 2001). LCAT then esterifies cholesterol, and as CE is more hydrophobic, it migrates from the surface of the HDL particle to the core, making room for the particle to absorb more free cholesterol. Thus the HDL particle gradually enlarges and becomes more spherical.

As mentioned above, the both the intestinal-derived TRL, chylomicrons, and hepatic-derived TRL, VLDL have the ability to accept cholesterol from HDL *via* the action of CETP. But HDL is also taken up by the liver via scavenger-receptor B1 (SR-B1) receptors where its cholesterol can be converted into bile acid for excretion, or repackaged into endogenous lipoprotein particles (Van Eck, Pennings et al. 2005).

HDLs ability to remove cholesterol from the periphery back to the liver is the general quoted reason for its anti-atherogenic properties. However, HDL also exerts anti-oxidative (Watson, Berliner et al. 1995), anti-thrombotic (Naqvi, Shah et al. 1999), and anti-inflammatory (Nofer, Kehrel et al. 2002) effects. It has also been shown to affect endothelial function (Ashby, Rye et al. 1998; Bisoendial, Hovingh et al. 2003).

1.1.7.4 Abnormalities of Lipoprotein Metabolism: Dyslipidaemia

When the above described pathways and regulatory enzymes are altered either by genetic diseases or acquired conditions, dysfunctional lipoprotein metabolism occurs and is termed dyslipidaemia. There are five main inherited types of dyslipidaemia that have been described to date, with several subsets within each disorder being recognised. The main subypes, their inherited abnormal lipoprotein phenotype, defect and subsequent pathology are summarised in Table 1-4.

Secondary or acquired causes of dyslipidaemia include diabetes mellitus, hypothyroidism, nephrotic syndrome, alcohol excess, lipodystrophy and medications such as thiazide diuretics and oestrogens. However, the two commonest types of dyslipidaemia in clinical practice are raised LDL cholesterol and atherogenic dyslipidaemia, also known as atherogenic lipid profile (ALP). The latter is characterised by hypertriglyceridaemia, low levels of HDL and the presence of small dense LDL (sdLDL) particles. ALP is prevalent in insulin resistant states, such as PCOS (Berneis, Rizzo et al. 2007) and MetS (Grundy, Brewer et al. 2004).

Table 1-4: Characteristics of Inherited Dyslipidaemias

Type	Subtype	Name of Dyslipidaemia	Increased Lipoprotein	Aetiology	Associated Clinical Signs	Associated Condition	Prevalence
I	Ia	Familial hyperchylomicronaemia	CM & VLDL	LPL deficiency	Lipaemia retinalis		
	Ib	Familial apoprotein CII deficiency		Altered ApoC-II	Eruptive xanthomas	Pancreatitis	1:1,000,000
	Ic			LPL inhibitor present in plasma	hepatosplenomegaly		
II	IIa	Familial hypercholesterolaemia	LDL	LDL receptor deficiency	Xanthelasma	Atherosclerosis	1:500
	IIb	Familial combined hyperlipidaemia		↓ LDL receptor & ↑ ApoB	Tendon xanthomas		1:10
III		Familial dysbetalipoproteinaemia	IDL	Altered ApoE	Tuboeruptive xanthomas & Palmar xanthomas	Atherosclerosis	1:10,000
IV		Familial hypertriglyceridaemia	VLDL	VLDL production & ↓ clearance		Atherosclerosis	1:100
V		Hypertriglyceridaemia	VLDL & CM	VLDL production & ↓ LPL activity		Pancreatitis	

Abbreviations: CM – chylomicrons, VLDL – very low density lipoprotein, IDL – intermediate density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein, LPL – lipoprotein lipase.

1.1.7.5 Dyslipidaemia in Insulin Resistant States – the Atherogenic Lipid Profile

Insulin resistant states are commonly associated with an ALP that contributes to significantly higher risk of atherosclerosis and cardiovascular disease. Although the molecular mechanism(s) underlying ALP are far from being completely understood, it is generally recognised that the elevation in plasma TG and TRLs is central to the development of this phenotype. Indeed insulin resistance has been correlated with intracellular TG stores, and lipotoxicity – the detrimental effects of elevated lipids – has been implicated in pancreatic beta-cell failure and the development of T2DM (Ziegler, Quilliot et al. 2001).

Insulin controls hepatic sterol regulatory element binding protein (SREBP) expression, which is a key transcription factor able to activate enzymes such as fatty acid synthase (FAS) (Bennett, Lopez et al. 1995) and 3-hydroxy-3-methly-glutaryl-coenzyme-A (HMG-CoA) reductase (Brown and Goldstein 1997). Insulin enhances SREBP expression in all three major insulin target tissues, liver, adipose tissue and skeletal muscle (Kim, Sarraf et al. 1998; Foretz, Guichard et al. 1999; Guillet-Deniau, Mieulet et al. 2002).

Both fasting and post-prandial levels of FFA are often elevated in obese and IR individuals (Boden 1998) due to increased FFA release from AT and decreased FFA uptake by muscles (Arner 2002). Increased free fatty acid release from adipose tissue and visceral adipose depots enhances hepatic TG synthesis, and ultimately leads to excess VLDL production (Fried and Rao 2003). Excess VLDL secretion in turn delivers TG to muscle and other tissues, further exacerbating IR (Dresner, Laurent et al. 1999; Zammit, Waterman et al. 2001) setting up a vicious cycle.

1.1.7.5.1 Hypertriglyceridaemia and the importance of the postprandial phase

As described above, excess VLDL may result from excess circulatory FFA. There is little dispute over whether VLDL is a predominant contributor to the ALP (Adiels, Olofsson et al. 2008). However there is also a substantial body of evidence that increased chylomicrons and chylomicron remnants are also found in IR subjects (Curtin, Deegan et al. 1996; Schaefer, McNamara et al. 2002; Duez, Lamarche et al. 2006; Hogue, Lamarche et al. 2007) and that fasting hypertriglyceridaemia may actually predict this abnormal postprandial response (Lewis, O'Meara et al. 1990; Lewis, O'Meara et al. 1991; O'Meara, Lewis et al. 1992; Chen, Swami et al. 1993). There is also growing evidence that chylomicrons and chylomicron remnants are risk factors of atherosclerosis (Boquist, Ruotolo et al. 1999; Doi, Kugiyama et al. 2000; Karpe, Boquist et al. 2001; McNamara, Shah et al. 2001; Yu and Cooper 2001). Indeed, chylomicron remnants have been shown to be able to enter the subendothelial space of the vascular wall and induce or participate in the development of atherosclerotic lesions (Mamo, Proctor et al. 1998; Vine, Takechi et al. 2007).

There may be several reasons for the increased production of chylomicrons. Microsomal triglyceride transfer protein (MTP)-mediated lipidation of $apoB_{48}$ is a crucial step in the synthesis of chylomicrons (Black 2007), and its mass and activity has been shown to be increased in the enterocytes of the Syrian Golden hamster made IR by a high-fructose diet (Haidari, Leung et al. 2002; Lewis, Uffelman et al. 2005). Enterocytes which are IR, as suggested by reduced insulin receptor substrate (IRS)-1 and Akt phosphorylation, with corresponding increased ERK signalling, has been shown to secrete more chylomicrons (Federico, Naples et al. 2006). Inflammation may also play a role. Infusion of TNF-α has been shown to impair insulin signalling in the intestine and increase MTP mass and stimulate lipoprotein secretion by enterocytes (Qin, Qiu et al. 2007). Finally, *de novo* lipogenesis *in vivo* in IR hamsters have also been demonstrated (Haidari, Leung et al. 2002). This may be due to the action of the aforementioned SREBP1 (Federico, Naples et al. 2006).

Although an increase in circulating apoB48 containing lipoproteins has been demonstrated, the overall quantitative contribution to postprandial lipoprotein pool is small. VLDL particles are still the major component of TRLs after a fat load (Schneeman, Kotite et al. 1993; Karpe, Bell et al. 1995). These studies have shown that approximately 80% of the postprandial TRL increase is accounted for by VLDL particles, mainly the VLDL1 species. The different production and catabolic rates of apoB48 and apoB100 particles is thought to explain the differences in pool size (Marsh, Welty et al. 2002). However, because triglycerides in large lipoproteins are hydrolysed faster than in smaller particles, the flux of chylomicrons after a fat-rich meal will impede the lipolysis of VLDL particles due to the competition for LPL (Karpe, Olivecrona et al. 1997; Goldberg, Kako et al. 2000; Ginsberg and Illingworth 2001; Tomkin and Owens 2001). Thus the delipidation process of VLDL particles is retarded and even more so in IR states where VLDL-1 production is increased and LPL activity is attenuated (Ginsberg 2002; Panarotto, Remillard et al. 2002). There is also reduced clearance of remnant particles by the liver, *via* the LDL receptor and LDL receptor-related protein (LRP) (Goldberg, Kako et al. 2000; Tomkin and Owens 2001). Hence, postprandial chylomicrons play an important role to the overall increase in not just postprandial but also fasting hypertriglyceridaemia that is found in the ALP.

1.1.7.5.2 Low HDL cholesterol and the significance of the loss of its protection

As described above, HDL's main function appears to be to reverse cholesterol transport (Bruce, Chouinard et al. 1998; Sviridov and Nestel 2002), thus preventing excess accumulation of cholesterol in the arterial wall (Assmann, Schulte et al. 1996). However HDL has other intrinsic properties. Firstly, it prevents LDL oxidation through the action of enzymes such as paraoxinase 1 (PON1) and acetyl-hydrolase platelet activation factor (PAF-AH) (Navab, Hama et al. 2000; Navab, Hama et al. 2000). Secondly, HDL is able to suppress the expression of pro-inflammatory adhesion molecules (Sviridov, Mukhamedova et al. 2008) and pro-inflammatory prostaglandins (Jambou, Dejour et al. 1993). It also attenuates the inflammatory

activities of CRP (Wadham, Albanese et al. 2004) and LDL-derived oxidised phospholipids (Navab, Berliner et al. 2001). Finally, in atherosclerotic lesions, macrophage adhesion to the vessel wall is also reduced by HDL (Theilmeier, De Geest et al. 2000).

17β-oestradiol has also been shown to be associated with HDL in the form of fatty acyl esters that are generated by LCAT (Kanji, Kuohung et al. 1999). This association may explain why HDL isolated from women, but not men, have been shown to be capable of inducing endothelial nitric oxide synthetase (eNOS) in an SR-B1-dependent manner, promoting vaso-relaxation, thereby being more cardioprotective. Furthermore, oestradiol containing HDL may also be able to be taken up via LDLR in addition to SR-B1, making delivery of cholesterol to the liver more efficient (Badeau, Metso et al. 2007).

Several large epidemiological studies have demonstrated that the plasma concentration of HDL correlates inversely with the incidence of coronary artery disease and atherosclerosis (Gordon, Castelli et al. 1977; Miller, Thelle et al. 1977; Forte, Nichols et al. 1984; Wilson, Abbott et al. 1988; Gordon, Probstfield et al. 1989; Shah and Amin 1992). Higher HDL-chol correlated with a slower progression of atherosclerotic lesions and possible stabilisation of unstable atherosclerotic plaques (Gordon, Probstfield et al. 1989; Gordon and Rifkind 1989). The Helsinki study was one of the first studies to show the benefit of raising HDL (Manninen, Tenkanen et al. 1992). This study compared the effects gemfibrozil versus placebo. At the end of five years, the gemfibrozil group the HDL increased by 11%, although the total cholesterol, LDL-c and TG levels decreased by 10%, 11% and 35% respectively also. The improvement in lipid profile was accompanied by a reduction in cardiovascular mortality and non-fatal myocardial infarction – and subjects with the lowest HDL and highest TG benefited most (Manninen, Tenkanen et al. 1992).

However, more recently, results of other studies have challenged the view of whether a low HDL has a causal effect for cardiovascular disease. At least 2 studies have shown that low HDL levels due to heterozygosity for loss-of-function mutations in ABCA1 (Frikke-Schmidt, Nordestgaard et al. 2008) or an apolipoprotein A-I_{Milano} mutation (Roma, Gregg et al. 1993) were not associated with an increased risk of IHD. In the latter instance, subjects actually had enhanced protection against atherosclerosis despite having less than half of normal HDL levels (Roma, Gregg et al. 1993). Finally, most recently, in a large clinical trial, the CETP inhibitor torcetrapib was found to be associated with increased cardiovascular adverse events despite causing a substantial rise in HDL (Barter, Caulfield et al. 2007). The clinical trial had to be terminated early on safety grounds.

These findings have led to the thinking that qualitative changes in HDL and the differences in HDL subgroups are also important in its ability to protect against atherosclerosis (Tsompanidi, Brinkmeier et al.). Small lipid-poor HDL particles are believed to be more capable of accepting cholesterol than large lipid-rich HDL particles (Sviridov, Mukhamedova et al. 2008) although population based studies have suggested that large HDL particles are associated with protection against future CVD (Asztalos, Collins et al. 2005). Subjects who are have received transplants or who are undergoing haemodialysis often have reductions of very small lipid-poor HDL **particles including preβ-HDL and preβ1**-HDL and cholesterol efflux is reduced in these individuals (Miida, Miyazaki et al. 2003; Sviridov, Chin-Dusting et al. 2006). Conversely, fibrates, which reduce TG levels and increase HDL, have been shown to cause a shift from large to small HDL particles and promote cholesterol efflux (Guerin, Le Goff et al. 2003; Franceschini, Calabresi et al. 2007). On the other hand, CETP inhibitors increase the proportions of enlarged HDL and also increased cholesterol efflux (Yvan-Charvet, Matsuura et al. 2007). The variations in these findings are probably due to the existence of different pathways for cholesterol removal including the SR-B1 pathway and the ABCA1-dependent pathway, the latter being the predominant pathway in macrophages (Yancey, Kawashiri et al. 2004). Hence it is still unclear whether small or large HDL subtypes are more anti-atherogenic.

1.1.7.5.3 Small dense LDL particles and the importance of qualitative changes

Levels of LDL-chol as measured in routine clinical practice are not necessarily different between subjects with and without IR. Numerous studies have shown that sdLDL is an integral feature of the ALP even in normal or subnormal LDL-chol concentrations (Tilly-Kiesi, Syvanne et al. 1992; Stewart, Laker et al. 1993; Lahdenpera, Syvanne et al. 1996; Gray, Robbins et al. 1997; Guerin, Le Goff et al. 2001; Sniderman, Scantlebury et al. 2001). It is well recognised that sdLDL is linked to increased cardiovascular risk (Austin, Breslow et al. 1988; Coresh, Kwiterovich et al. 1993; Gardner, Fortmann et al. 1996; Lamarche, Tchernof et al. 1997). Therefore it is important to understand how sdLDL is formed and its role in atherosclerosis.

In normal physiology, LPL is responsible for the hydrolysis of normal-sized VLDL particles into large to medium sized LDL, particles that are regarded as less-atherogenic. However in IR states, large buoyant VLDL1 particles are produced in excess as a result of increased FFA influx. LPL enzyme activity is found to be decreased, with concomitant increase in HL activity (Kasim, Tseng et al. 1987; Taskinen 1987; Baynes, Henderson et al. 1991; Tan, Shiu et al. 1999) which results in abnormal VLDL TG lipolysis into sdLDL (Packard, Demant et al. 2000). LPL activity correlates inversely with sdLDL, whereas HL activity correlates positively with sdLDL (Jansen, Verhoeven et al. 2002). The process by which VLDL is abnormally metabolised to sdLDL is described in Figure 1-2.

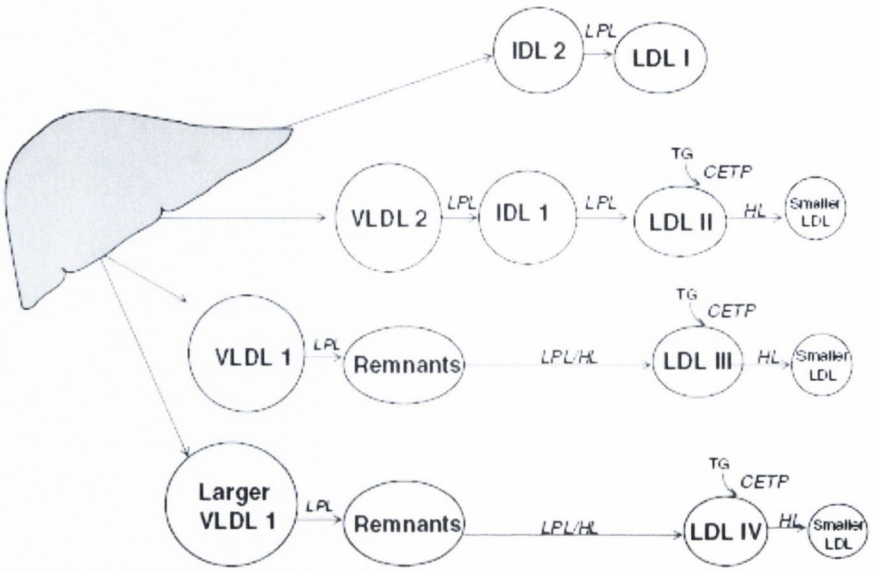

Figure 1-2: Schematic view of the production of small, dense LDL particles from large, triglyceride-rich VLDL. Decreased LPL and increased HL activity leads to the production of small, dense LDL particles, which are regarded as more atherogenic than LDL particles that are larger in size. Taken from Rizzo and Berneis (2007).

Several reasons have been suggested for the increased atherogenic risk associated with sdLDL particles. Firstly, sdLDL particles have decreased carbohydrate content, in particular reduced sialic acid content, which renders greater cellular cholesterol accumulation (Barbosa, Maranhao et al. 1995). This is due to the change in the electronegative charge of the LDL particle, causing structural alterations and impaired recognition and uptake by the native LDL receptor (Galeano, Milne et al. 1994). Secondly, sdLDL also causes endothelial dysfunction in both healthy and T2DM subjects (Makimattila, Liu et al. 1999; Tan, Ai et al. 1999; Vakkilainen, Makimattila et al. 2000). There is also an increased propensity for sdLDL to adhere directly to the endothelium via proteoglycans (Anber, Griffin et al. 1996; Galeano, Al-Haideri et al. 1998) and indirectly via LPL and decorin (Chait and Wight 2000). Thirdly, sdLDL particles can also more readily penetrate the arterial intima (Chapman, Guerin et al. 1998). Finally, sdLDL are more susceptible to oxidation than

their larger more buoyant counterparts (de Graaf, Hak-Lemmers et al. 1991; Tribble, Holl et al. 1992; Lyons and Jenkins 1997). Increased cholesterol content and glycation partly explains this, but fatty acid composition of LDL particles is probably one of the most important factors (Dimitriadis, Griffin et al. 1996; Scheffer, Bakker et al. 2001).

Once oxidised, sdLDL particles are removed from the endothelial space in an unregulated fashion by scavenger receptors on several cell types, including macrophages (Ramprasad, Fischer et al. 1995; Ramprasad, Terpstra et al. 1996) and vascular smooth muscle cells (VSMCs) (Guyton, Lenz et al. 1995; Jimi, Saku et al. 1995), leading to the development of cholesterol enriched-foam cells a key process in the formation of early atherosclerosis (Lusis 2000). Oxidised LDL has been shown to act as a chemoattractant for circulating monocytes, and can do so both directly, and indirectly, by inducing the production of monocyte chemoattractant protein-1 (MCP-1) (Wang, Deng et al. 1997). The stimulation of monocytes towards these oxidised LDL particles causes monocyte diapedesis across the endothelial wall, and their residence within the subendothelial space as shown in Figure 1-3. Oxidised LDL can cause monocytes to synthesize and release a variety of pro-inflammatory cytokines that contribute to the chemotactic recruitment of additional monocytes and also VSMCs (Jovinge, Ares et al. 1996; Svensson, Asea et al. 2006; Zhang and Wahl 2006). They also influence cell proliferation and differentiation into macrophages (Kamanna, Pai et al. 1996; Inoue, Itoh et al. 2001), probably via agents such as macrophage colony-stimulating factor (M-CSF) from EC (Rajavashisth, Andalibi et al. 1990; Fuhrman, Partoush et al. 2008). As mentioned previously, the atherosclerotic plaque continues to progress until a dense fibrous cap of connective tissue develops, covering the core of lipid and necrotic debris. However, oxidised LDL promotes the synthesis and release of matrix-digesting enzymes such as metalloproteinase-9, which contribute to plaque instability by directly digesting the fibrous plaque matrix, ultimately leading to plaque rupture and thrombus formation (Xu, Meisel et al. 1999).

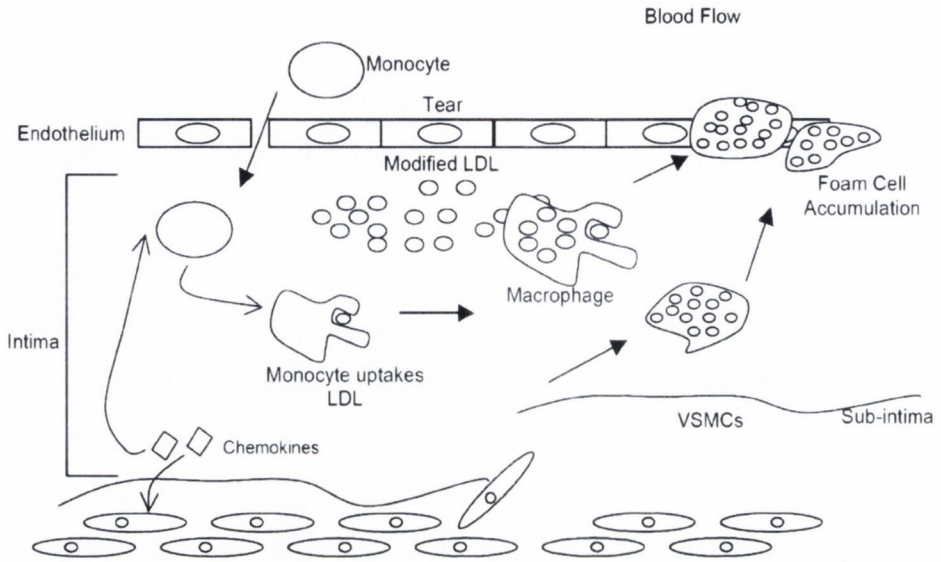

Figure 1-3: The contribution of modified LDL in atherosclerotic plaque development. An inflammatory assault in the endothelium attracts monocytes, which subsequently enter the arterial wall. Monocytes can take up modified LDL particles and differentiate into macrophages. Macrophages release cytokines that direct migration of monocytes from the blood to the sites of inflammation. Macrophages can also take up oxidised LDL and become foam cells. Taken from Lester et al. (2006).

The proportion of large and small LDL particles, that comprise total LDL, can be described as either belonging to pattern A or pattern B (Rizzo and Berneis 2006). Pattern A is characterised predominantly by the larger, more buoyant LDL particles, including a higher proportion of LDL-I and LDL-II subfractions. This pattern is accompanied by low plasma TG concentrations, typically within 0.6-1.1 mmol/L, and high HDL-chol levels, normally between 1.04-1.56 mmol/L. This pattern is most commonly found in healthy subjects. Pattern B is associated predominantly with sdLDL particles, in particular LDL-III and LDL-IV. This is accompanied by the presence of high triglyceride levels, usually >2.0 mmol/L, and low HDL-chol <1.04 mmol/L. The majority of individuals (85-90%) can be classified as having either LDL subclass phenotype A or B, with the remainder (15-10%) having an intermediate

phenotype, both equal proportions of pattern A and pattern B (Carmena, Duriez et al. 2004). Pattern A and pattern B, with their associated lipid phenotypes are depicted in Figure 1-4.

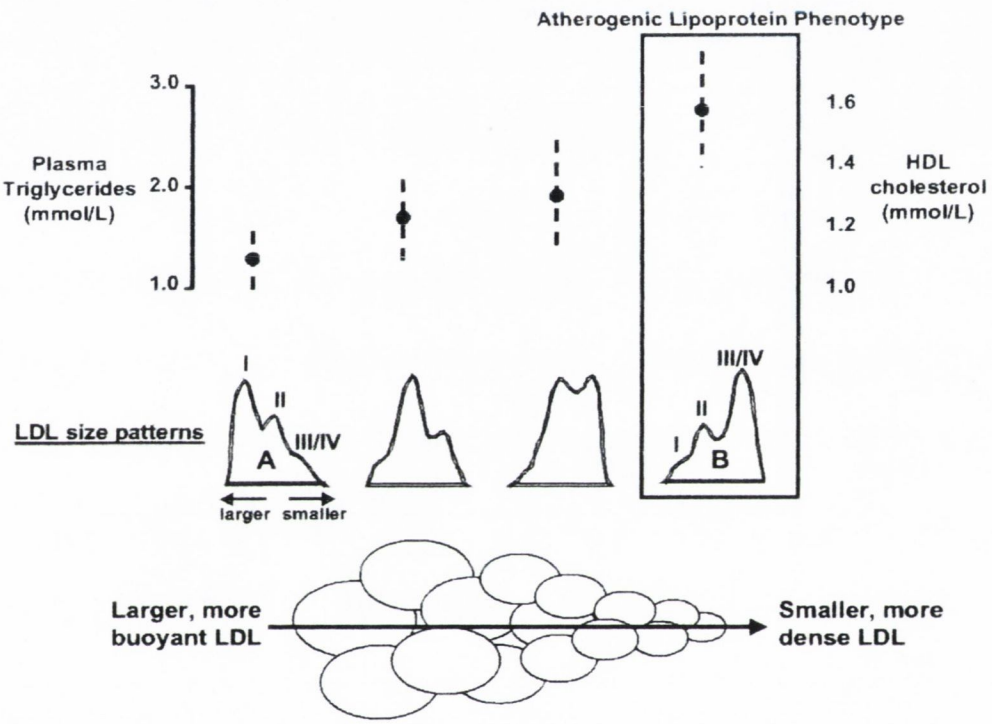

Figure 1-4: Lipoprotein phenotype of pattern A includes low levels of plasma TG, high levels of HDL cholesterol and a low preponderance of small, dense LDL particles. Pattern B demonstrates higher levels of plasma TG, low levels of HDL cholesterol and an increased preponderance of small, dense LDL particles. Taken from Rizzo et al. (2006).

The two phenotypes, with their associated varying triglyceride levels, led to the suggestion that a triglyceride level of 1.5 mmol/L (Griffin, Freeman et al. 1994) represents a threshold value, above which, LDL-II is increasingly converted to LDL-III, or, LDL-III is the preferred product of direct VLDL delipidation (Tulenko and Sumner 2002). Studies have illustrated that as triglyceride concentrations increase within the

normal range, the proportion of LDL-I decrease gradually. Similarly, McNamara et al. (1992) also demonstrated a positive correlation between LDL density and plasma triglyceride levels. Figure 1-5 demonstrates the positive association between plasma triglyceride levels and small, dense (LDL III) mass.

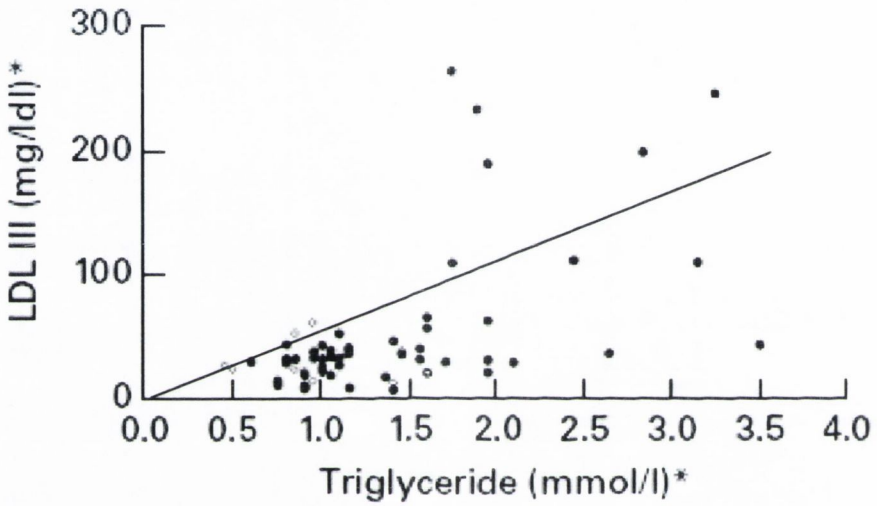

Figure 1-5: The association between plasma triglyceride concentration and small, dense (LDL III) mass. A triglyceride concentration greater than 1.5 mmol/L significantly increases the preponderance of small, dense LDL in vivo. Adapted from Pirwany et al. (2001).

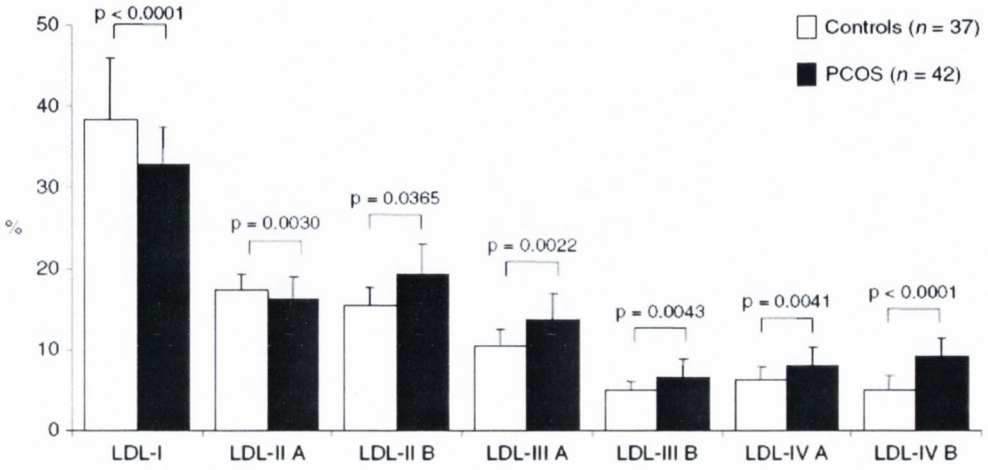

Figure 1-6: LDL subfractions 1-7. Taken from Berneis et al. (2009).

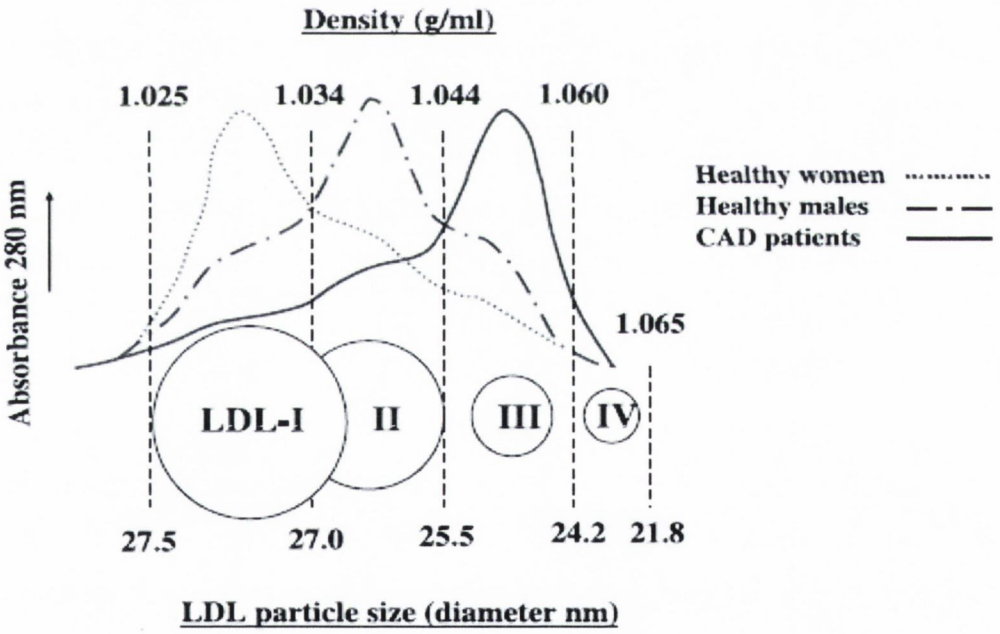

Figure 1-7: LDL subclass distribution according to size (diameter) and density. Healthy women and men have more of the larger LDL particles (LDL I and II), whereas patients suffering from CAD have a prevalence of the smaller, denser LDL particles (LDL III & IV).

1.1.7.6 Dietary fatty acids

1.1.7.6.1 Background

Fatty acids (FA) are molecules that include hydrocarbon chains and a carboxyl (COOH) and methyl (CH_3) group at opposite ends of the chain. The carboxyl group is reactive, readily forming ester bonds. The nomenclature is constructed in 3 parts: the number of carbons, number of double-bonds, and the position of the first double bond from the methyl end. For example, C20:5*n*-3, eicosapentaenoic acid contains 20 carbon atoms, 5 double bonds of which the first one is at the 3rd carbon from the methyl end.

The majority of dietary fats are long chain FA, defined as having at least 14 carbon atoms. They can be classified into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). The shape and physical properties of the unsaturated fatty acids change with the presence of double bonds, and vary according to number, position and configuration (*cis* or *trans*).

Apart from the obvious role of FA in providing energy, FA and the ratios of different types of FA are thought to exert different biological effects via a few different mechanisms. Firstly, FA composition of cell membranes influences its fluidity and permeability, thereby affecting ion pumps, cell membrane receptor function and transport of nutrients (Lund, Harvey et al. 1999). Specifically relevant to IR are the findings of numerous studies that different FA compositions can affect insulin sensitivity (Vessby 2000) and even secretion (Rojo-Martinez, Esteva et al. 2006). Secondly, FA can act as ligands for transcription factors (Duplus and Forest 2002) regulating gene expression, related to nutrient management within cells and between tissues, especially the adipose tissue (Al-Hasani and Joost 2005). Thirdly, FA have effects on inflammation and the immune response (Thies, Miles et al. 2001; Thies,

Nebe-von-Caron et al. 2001) not only because they are substrates for prostaglandin production but they can also directly affect phagocytosis, immune cell signalling and membrane trafficking (Calder 2008). Different FA will be described below.

1.1.7.6.2 Saturated fatty acids (SFA)

SFA contain no double bonds. They generally have between 12 and 18 carbons. In a typical Western diet, a third to one half of all FA are saturated. The commonest types are palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) and lauric acid (C12:0). It is common knowledge that SFA increase cholesterol levels, however, palmitic and myristic acids have the greatest effects and stearic acid has no hypercholesterolaemic effect (Hunter, Zhang et al. ; Grundy 1994). SFA also have a deleterious effect on IR (Vessby 2000).

1.1.7.6.3 Monounsaturated fatty acids (MUFA)

The major dietary MUFA is oleic acid (C18:1*n*-9) and contains one double bond at the 9th carbon from the methyl end. The food types that contain most MUFA are oleic-rich vegetable oils e.g. high-oleic-acid sunflower or safflower oil, olive oil and canola oil, nuts such as macadamia, hazelnut, pecan and almonds, and fruits such as avocado and olives. However animal fats also have a significant proportion of MUFA.

MUFA alters lipid and lipoprotein profiles. A meta-analysis of 10 studies showed that the net changes in fasting plasma concentrations of TG, VLDL-chol, LDL-chol and HDL-chol was a 19% reduction, 22% reduction, 0% change and a 4% increase, respectively (Garg 1998). The lowering of TG, without significant HDL-chol reduction has been shown in multiple studies (Kris-Etherton, Pearson et al. 1999; Curb, Wergowske et al. 2000; Rajaram, Burke et al. 2001). This contrasts the rise in TG and lowering of HDL-chol by high carbohydrate diets (Reaven 1997). Although MUFA increases early postprandial hyperlipidaemia, it is thought that CRs are cleared faster

and therefore likely to reduce atherosclerotic burden (Higashi, Ishikawa et al. 1997; Roche, Zampelas et al. 1998). A high-MUFA diet has also been shown to reduce the proportion of sdLDL (Zambon, Sartore et al. 1999) and probably reduces the susceptibility of LDL to oxidation (Reaven and Witztum 1996), although the results are conflicting (Ros 2003).

Although antioxidants are important in endothelial function, dietary fats probably have a significant role (West 2001). At least two studies have shown that MUFA improve endothelial function measured by vasoreactivity (Ryan, McInerney et al. 2000; Fuentes, Lopez-Miranda et al. 2001). A third study showed that vegetables with olive oil, rather than olive oil alone attenuated postprandial endothelial dysfunction (Vogel, Corretti et al. 2000). Results of circulating markers of endothelial function post MUFA diets are conflicting (Perez-Jimenez, Castro et al. 1999; Thomsen, Rasmussen et al. 1999). In vitro studies suggest MUFA reduces factors linked to inflammation of the vascular wall, such as monocyte chemotaxis and adhesion to endothelial cells (Mata, Alonso et al. 1996; Tsimikas, Philis-Tsimikas et al. 1999), and expression of adhesion molecules (Yaqoob, Knapper et al. 1998; Carluccio, Massaro et al. 1999). MUFA may also have beneficial effects on blood pressure (Ferrara, Raimondi et al. 2000) and coagulation profile (Roche, Zampelas et al. 1998; Larsen, Jespersen et al. 1999; Tholstrup, Marckmann et al. 1999).

1.1.7.6.4 *n*-6 Polyunsaturated Fatty Acids (*n*-6 PUFA)

n-6 PUFA are characterised by the presence of 2 or more *cis* double bonds, the first being located between the 6th and 7th carbon atoms from the methyl end of the fatty acid i.e. *n*-6 position. The main dietary *n*-6 PUFA is linoleic acid (LA; C18:2*n*-6)), which can be found in vegetable oils such as soya bean, safflower, maize and rapeseed oils. LA cannot be synthesised by humans and hence is an essential FA (Holman 1961; Williard, Nwankwo et al. 2001).

Diets rich in *n*-6 PUFA exert a hypocholesterolaemic effect (Nichaman, Sweeley et al. 1967; Shepherd, Packard et al. 1980). A meta-analysis of sixty controlled trials reported that the replacement of carbohydrates with largely *n*-6 PUFA predicted the largest change in LDL-chol and total:HDL-chol ratio (Mensink, Zock et al. 2003). Replacement of 1% of energy of carbohydrates by n-6 PUFA decreases LDL-chol by 0.02mmol/L, whereas substitution with SFA increases this by 0.03mmol/L. Another meta-analysis showed that T-chol is also reduced by PUFA (Clarke, Frost et al. 1997). Several studies have shown that diets supplemented by *n*-6 PUFA increases LDL oxidation in vitro, compared to MUFA (Berry, Eisenberg et al. 1991; Bonanome, Pagnan et al. 1992; Abbey, Belling et al. 1993). However, other studies did not find a correlation between n-6 PUFA and levels of oxLDL (Kleemola, Freese et al. 2002; Damsgaard, Frokiaer et al. 2008).

Studies regarding haemostatic parameters are not conclusive (Knapp 1997). In a recent randomised cross-over controlled trial in 45 healthy subjects consuming 3 different diets for 5 weeks each, LA increased *ex vivo* platelet aggregation time compared with stearic acid. However the 3 diets had no effect on in vitro whole-blood platelet aggregation variables, factor VII or fibrinolytic activity (Thijssen, Hornstra et al. 2005). Similarly, studies on inflammation are equivocal (Czernichow, Thomas et al.), whilst blood pressure appears to be lowered by *n*-6 PUFA (Hall 2009). Several observational (Laaksonen, Nyyssonen et al. 2005; Oh, Hu et al. 2005; Harris, Poston et al. 2007) studies have suggested that a higher *n*-6 PUFA is associated with lower risk of CVD. However, others have not found this (Ascherio, Rimm et al. 1996; Esrey, Joseph et al. 1996; Pietinen, Ascherio et al. 1997). Likewise, some interventional studies have also suggested benefits of *n*-6 PUFA (Miettinen, Turpeinen et al. 1983; Frantz, Dawson et al. 1989) but others have not, although two of these were very old studies (Rose, Thomson et al. 1965; 1968).

1.1.7.6.5 *n*-3 Polyunsaturated Fatty Acids (*n*-3 PUFA)

The main dietary *n*-3 PUFA are α-linoleic acid (ALA; C18:3*n*-3)), eicosapentaenoic acid (EPA; C20:5*n*-3) and docosahexaenoic acid (DPA; C22:5*n*-3) (Jump 2002; Arterburn, Hall et al. 2006). ALA cannot be synthesised by humans and hence is an essential FA. Dietary sources include flaxseed (linseed), rapeseed, soya-beans and walnuts. Whilst EPA and DHA can be made from ALA, in humans, the conversion rate is very low, with less DPA converted than EPA (Gerster 1998; Anderson and Ma 2009). EPA and DHA can be gotten directly from the diet in oily fish such as salmon, herring, mackerel, halibut, tuna, and sardines (Kris-Etherton, Harris et al. 2002), which consume microalgae, which make EPA and DHA in seawater. Beneficial effects of EPA and DHA, also known as fish-oils, were noted initially from studies in Greenland Eskimos, in whom fish is a daily staple, were noted to have a lower incidence of CVD (Bang, Dyerberg et al. 1971; Bang and Dyerberg 1972).

EPA and DHA reduce plasma both fasting (Harris 1997; Sacks and Katan 2002; Lewis, Lookinland et al. 2004) and postprandial (Weintraub, Zechner et al. 1988; Williams, Moore et al. 1992) TG. VLDL synthesis is reduced by the liver (Narayanan, Narayanan et al. 2003), probably because of reduced FFA release from AT and induction of genes involved in FFA oxidation (Novak, Babcock et al. 2003). Chylomicron clearance is also accelerated probably due to increased LPL activity (Park and Harris 2003). When co-administered with statins, *n*-3 PUFA improves the lipid profile of hypertriglyceridaemic patients to a greater extent than with statins alone (Nordoy, Bonaa et al. 1998; Durrington, Bhatnagar et al. 2001; Chan, Watts et al. 2002; Davidson, Stein et al. 2007).

n-3 PUFA has several other effects on cardiovascular biology. Numerous studies have shown a reduction in blood pressure (Appel, Miller et al. 1993; Morris, Sacks et al. 1993; Geleijnse, Giltay et al. 2002). There is reduced production of proatherogenic and proinflammatory proteins in human endothelial cells (De Caterina, Cybulsky et al.

1994) and inhibition of the antigen-presenting function of monocytes (Hughes and Pinder 2000). Both NO-dependent and NO-independent endothelial-dependent function is improved (Tagawa, Shimokawa et al. 1999), probably due to less production of the prostaglandin thromboxane A2 (Johansen, Seljeflot et al. 1999). Platelet aggregation and prothrombotic factors such as thrombomodulin and von Willebrand factor are also reduced, reducing thrombosis tendency (Knapp 1997; Hornstra 2001). Finally IR, and therefore T2DM, maybe preventable or reversible even by n-3 PUFA (Fedor and Kelley 2009), probably via their action on PPAR-α (Neschen, Morino et al. 2007).

1.1.8 Oxidative Stress

CVD and T2DM are associated with increased production of reactive oxygen species (ROS) and compromised endogenous anti-oxidant defence (Lankin, Lisina et al. 2005). This imbalance between the production and removal of ROS is known as oxidative stress, and is thought to be a significant cause of the development of aging and diseases such as inflammation, infection, cancer and CVD (Berlett and Stadtman 1997; Finkel and Holbrook 2000). Specifically, in vascular endothelial cells, production of ROS leads to oxidation of LDL and the expression of ROS-sensitive inflammatory genes, resulting in increased VCAM-1 and MCP-1, ultimately resulting in endothelial monocyte adhesion, infiltration and the atherosclerotic process (Ganji, Qin et al. 2009).

ROS are natural by-products of metabolism (Sies 1997) and are produced by enzymes such as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. However excessive levels lead to cell toxicity, inflammation and death (Kang, Lee et al. 2005; Lee, Li et al. 2005). Examples of ROS include hydroxyl radical (**$^{\cdot}$OH**), superoxide anion radical ($O_2^{\cdot-}$) and nitric oxide radical (**NO$^{\cdot}$**). All are highly reactive due to the presence of unpaired electrons, allowing them to interact with lipids, proteins and carbohydrates. The measurement of the collective free radical reactivity

indicates the amount of oxidants present, is known as total oxidant status (TOS) (Erel 2005).

In contrast to ROS, antioxidants include any substrate that, when present at low concentrations compared with that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate (Halliwell and Gutteridge 1995). Common antioxidants include vitamin C and E, glutathione (GSH), bilirubin and β-carotene, and enzymes with antioxidant activity such as superoxide dismutase (SOD), GSH-synthesising enzymes, GSH peroxidase and GSH S-transferase (GST). The measurement of the combined effects of these antioxidants is known as the total antioxidant capacity (TAC) (Erel 2004).

Several studies have found that obesity is associated with increased oxidative stress (Olusi 2002; Keaney, Larson et al. 2003). This is not surprising given that obesity is regarded by some as a proinflammatory state as evidenced by an increased presence of inflammatory markers such as TNF-α, IL-6 and CRP (Dandona, Aljada et al. 2005). The origin of the proinflammatory nature of obesity may in turn be explained by studies demonstrating that macronutrient intake may induce oxidative stress and inflammatory responses. In one study, a 75-g glucose challenge was shown to induce an increase in leukocyte superoxide generation by 140% (Mohanty, Hamouda et al. 2000). Similarly, fat intake also induced ROS production (Mohanty, Ghanim et al. 2002). A mixed meal has been shown to induce superoxide radical generation from mononuclear cells (Aljada, Mohanty et al. 2004). Conversely, calorie restriction and weight loss results in reduced ROS production (Dandona, Mohanty et al. 2001; Dandona, Mohanty et al. 2001).

That PCOS is associated with oxidative stress is not in itself a novel concept. Although whether this is due to obesity or PCOS *per se* is not clearly known. In one study, 27 women with PCOS had increased levels of oxidative stress as determined by

erythrocyte malondialdehyde (MDA) concentration, and lower antioxidant status as determined by erythrocyte reduced GSH concentration, GSH peroxidase and SOD compared to 18 age- and BMI-matched controls (Sabuncu, Vural et al. 2001). MDA levels correlated positively age, BMI, WHR and both systolic and diastolic BP. No other inflammatory markers were measured (Sabuncu, Vural et al. 2001). In another study, total antioxidant status (TAOS) was found to be reduced in women with PCOS compared to normal controls and correlated negatively with fasting insulin, homeostatic model assessment for IR ($HOMA_{IR}$) and CRP (Fenkci, Fenkci et al. 2003). Finally a third study showed that ROS generation from mononuclear cells (MNC) were higher in lean and obese PCOS compared with lean but not obese controls, although this could be because the number of subjects were very small (n=8 in each group) (Gonzalez, Rote et al. 2006). This was the only study to find a positive correlation between ROS generation and testosterone and androstenedione levels. In summary, whist it is generally accepted that increased oxidative stress occurs in PCOS, the mechanism is not well understood.

1.2 INSULIN RESISTANCE

1.2.1 Introduction

IR plays a crucial part in the pathogenesis of conditions such as T2DM and non-diabetic conditions such as MetS, PCOS and non-alcoholic fatty liver disease (NAFLD), as well as hypertension and dyslipidaemia – all of which are associated with increased cardiovascular risk.

1.2.2 Insulin Resistance and Atherosclerosis

IR can be defined as a state in which normal levels of insulin produce a subnormal biological response (Yalow and Berson 1970; Savage, Petersen et al. 2005). Greater insulin secretion is therefore required to achieve a normal response resulting in hyperinsulinaemia which can be accompanied by either normoglycaemia or – if pancreatic β-cell function declines – hyperglycaemia and type 2 diabetes mellitus (T2DM) (Prentki and Nolan 2006). Thus, IR is a central feature in the natural history and pathogenesis of T2DM. Studies confirm that IR precedes the development of hyperglycaemia in subjects that eventually develop T2DM (Morrison, Glueck et al. 2008). IR and impairments in insulin signalling is now recognised as one of the major underlying factor in the complex pathophysiological processes leading to the diseases such as the MetS, dyslipidaemia, hypertension and atherosclerosis (Eriksson 2007).

1.2.3 Insulin Resistance and Cardiovascular Risk

Multiple prospective studies have demonstrated that IR predicts future cardiovascular disease. In the San Antonio Heart Study (Hanley, Williams et al. 2002), 2564 non-diabetic participants were followed for 8 years. Individuals in the highest quintile of insulin resistance, as assessed by the $HOMA_{IR}$, had an approximately 2.5-fold increased incidence of CVD. Even after adjustment for multiple cardiovascular

risk factors, individuals in the highest quintile of IR still had a twofold increased incidence of CVD. In 3,606 non-diabetic participants followed for almost 7 years in the Botnia Study (Isomaa, Almgren et al. 2001), the MetS was associated with a threefold increased risk of CVD. Each component of the MetS, as well as IR (as assessed by $HOMA_{IR}$) itself, was associated with a 1.5- to 2-fold increased incidence of CVD. Similar observations have been made in the Bruneck (Bonora, Kiechl et al. 2007), Verona Diabetes (Bonora, Formentini et al. 2002) and Insulin Resistance Atherosclerosis Study (IRAS) studies (Howard, O'Leary et al. 1996). A strong relationship between $HOMA_{IR}$ and carotid intima-media thickness (IMT) has also been demonstrated (Hedblad, Nilsson et al. 2000), as has an association between IR and greater cardiovascular risk factor load (Ferrannini, Balkau et al. 2007). Furthermore, an independent role for IR in CVD has been suggested by various other studies that showed that other conventional cardiovascular risks factors could not fully account for the risk of CVD (Wilson, D'Agostino et al. 1998; D'Agostino, Grundy et al. 2001; Golden, Folsom et al. 2002).

Insulin, especially at high doses may promote atherogenesis (DeFronzo and Ferrannini 1991; Defronzo 2006; Kashyap and Defronzo 2007). Insulin promotes both *de novo* lipogenesis and hepatic VLDL synthesis (Tobey, Greenfield et al. 1981; Koopmans, Kushwaha et al. 1999) via its stimulation of sterol regulatory-element-binding protein-1c and its inhibition of actyl-CoA carboxylase (Azzout-Marniche, Becard et al. 2000). LDL-cholesterol transport (Stout 1971) into arterial smooth muscle cell, as well as smooth muscle cell proliferation (Pfeifle and Ditschuneit 1981; Nakao, Ito et al. 1985) and both processes are augmented by insulin. Insulin also serves as a growth factor, stimulating collagen synthesis, and is capable of turning on genes involved in inflammation (Coletta, Balas et al. 2008).

Further evidence of the atherogenic properties of insulin comes from animal studies (Duff, Brechin et al. 1954; Stamler, Pick et al. 1960; Cruz, Amatuzio et al. 1961). Dogs infused with insulin locally into a hind limb developed severe femoral atherosclerosis in the infused artery whilst other arteries remained free of atherosclerotic plaque

(Cruz, Amatuzio et al. 1961). Alloxan-induced diabetic rabbits fed with high cholesterol diet developed marked hypercholesterolaemia, but did not develop atherosclerotic plaques unless they were treated with insulin (Duff, Brechin et al. 1954). Non-diabetic chickens developed atherosclerosis on a high cholesterol diet, which regressed when switched to a low cholesterol diet. However this regression was inhibited by insulin administration (Stamler, Pick et al. 1960).

Hyperinsulinaemia itself appears to exacerbate insulin resistance. Rats infused with insulin became resistant to insulin mediated stimulation of glucose uptake and suppression of NEFA (Koopmans, Kushwaha et al. 1999). A similar finding has been observed in humans, whereby insulin infusions have been shown to reduce insulin sensitivity (Del Prato, Leonetti et al. 1994) and also reduce insulin's own ability to synthesis glycogen for storage (Iozzo, Pratipanawatr et al. 2001). Finally, insulin therapy is invariably associated with weight gain (Henry, Gumbiner et al. 1993; Holman, Thorne et al. 2007). Obesity is certainly a well recognised risk of IR, T2DM and ultimately leads to increased deaths from CVD (Allison, Fontaine et al. 1999; Calle, Thun et al. 1999).

1.2.4 Insulin Secretion and Action

Insulin is an anabolic hormone that affects a range of biological functions such as cell growth, glycogen and fat storage and protein synthesis depending on its target organ or tissue such as liver, muscle, adipose tissue (AT) and inflammatory cells (Pessin and Saltiel 2000; Gonzalez-Sanchez and Serrano-Rios 2007). It enables entry of glucose into body tissues and its utilisation as a fuel and for the conversion of glucose to glycogen for storage in liver and muscle cells. β-cells of the pancreas produce insulin in response to rising levels of plasma glucose levels which occur in the postprandial state. As a result, in healthy individuals, plasma glucose concentrations remain in a narrow range, typically between 4-7mmol/L, despite periods of feeding and fasting. This tight control is constantly governed by the balance of glucose absorption from

the intestines, endogenous production by the liver and uptake and metabolism by peripheral tissues (Saltiel and Pessin 2002). As glucose levels fall, insulin is suppressed resulting in the reverse conversion of glycogen to glucose, though only in the liver not in muscle tissue.

Glucose enters the β-cells through the glucose transporter (GLUT) 2 channel and is then phosphorylated into glucose-6-phosphate (G6P) by the enzyme glucokinase. This is the rate-limiting step in the metabolism of glucose. Glucose is then metabolised through pyruvate to acetly-coenzyme A (acetyl-CoA) with subsequent mitochondrial oxidation and increased adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio. This results in the closure of ATP-sensitive K^+ channels, depolarisation of the β-cell's plasma membrane, opening of voltage-dependent Ca^{2+} channels and finally Ca^{2+} mediated exocytosis of insulin granules (Prentki 1996). This early insulin secretion then declines to baseline levels but is soon followed by a second phase of insulin secretion that is sustained during hyperglycaemia. In the second phase insulin release, the mechanism above is augmented by ATP sensitive K^+ channel-independent, non-ionic signals (Komatsu, Sato et al. 2002). The magnitude of the second phase is determined by the elevation of Ca^{2+} (Bratanova-Tochkova, Cheng et al. 2002; Henquin, Ishiyama et al. 2002).

Weyer et al. demonstrated a secretory failure of insulin when comparing people with normal glucose tolerance to those with IGT and T2DM (Weyer, Bogardus et al. 1999). The experimental design as shown in Figure 1-6 initially measures the first phase insulin response (FPIR), otherwise also known as the acute insulin response to glucose (AIR_G), which is a small but rapid spike of insulin secretion that occurs within minutes of a glucose challenge.

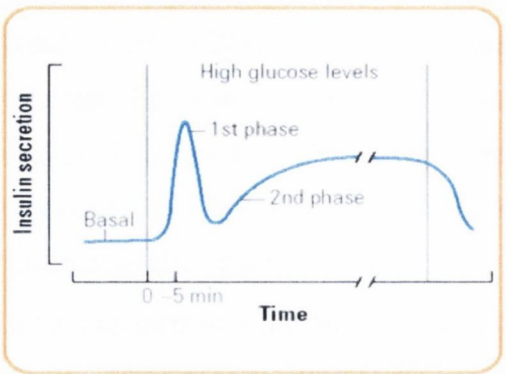

Figure 1-8: The biphasic pattern of glucose stimulated insulin secretion from the pancreatic ß-cell (Persaud & Howell, 2003).

A study in lean Japanese subjects showed that those with IGT who exhibited normal insulin sensitivity displayed a reduced insulin secretion when compared to subjects with NGT (Taniguchi, Nakai et al. 1994). A similar study in Caucasian individuals with IGT approximately 60 years of age showed impaired FPIR after intravenous glucose (Davies, Rayman et al. 1994). These results show that FPIR may be linked with IR and the development of T2DM. Indeed, individuals with T2DM have a markedly attenuated AIR_G and hence it is not surprising that postprandial glucose levels can increase to well over 11.1mmol/L in such patients (Spellman 2007). A specific alteration of FPIR after a glucose challenge is an early and progressive defect of β-cell failure and plays a crucial role in postprandial hyperglycaemia. The effects of FPIR release is primarily achieved in the liver, allowing a prompt inhibition of postprandial hepatic glucose production (Del Prato, Marchetti et al. 2002). The likely mechanisms of early β-cell demise includes oxidative stress, endoplasmic reticulum stress, increased TAG and NEFA and glucolipotoxicity (Prentki and Nolan 2006).

The dynamic regulation of insulin secretion is complex. There is a time dependent inhibition of insulin release after short glucose stimulation, which makes the β-cells refractious (Nesher and Cerasi 2002). Furthermore, this refractory period is itself subject to time-dependent potentiation to longer glucose stimuli. Long-term

hyperglycaemia under experimental conditions induces a long lasting depletion of crucial components of the insulin exocytic process distal to closure of ATP-sensitive K^+ channels (Eizirik, Jansson et al. 1997). Insulin release is pulsatile *in vivo*, in periods of 4-13 minutes, which provides a better signal to target tissues as pulsatile insulin is more effective at reducing glucose concentrations than the same amount of insulin given at a constant rate (Lang, Matthews et al. 1979). This effect may be explained by the dose-response curve of the insulin receptor and of prevention of receptor desensitisation (Kennedy, Kauri et al. 2002). A loss of regular pulsatile insulin release is an early feature of T2DM (Polonsky, Given et al. 1988).

Insulin action is mediated by signal transduction over the insulin receptor – a receptor belonging to the superfamily of tyrosine kinase receptors and consists of 2 extracellular α- subunits and 2 intracellular β-subunits. Insulin binds to the α-subunits resulting in a conformational change and leads to the activation of tyrosine kinase in the intracellular domain, ATP binding and finally receptor autophosphorylation (White 2003).

Insulin signalling involves a complex signalling cascade downstream of the insulin receptor (Jackson 2006). The signalling cascade branches into two main pathways. The first is the phosphatidylinositol-3-kinase (PI3K)-AKT, which is largely responsible for insulin action on glucose uptake. The second pathway is the Ras-mitogen activated protein kinase (MAPK) pathway which mediates gene expression, but also interacts with the P13K-AKT pathway to control cell growth and differentiation (Taniguchi, Emanuelli et al. 2006). The common intermediate of these is insulin receptor substrate-1 (IRS-1). Activation of insulin receptor leads to tyrosine phosphorylation of IRS-1, thereby initiating signal transduction. When IRS-1 is alternatively phosphorylated on serine 307, its downstream signalling ability is diminished (Aguirre, Werner et al. 2002). Serine kinases that phosphorylate serine 307 include I kappa B kinase beta (IκKβ) in the nuclear factor kappa B (NF-κB) pathway and C-jun N-terminal kinase 1 (Jnk1) in the JNK/AP-1 pathway. Additionally

inflammation-related negative regulators of IRS-1 include the suppressor of cytokine signalling (Socs). Socs1 and Soc3, which are induced in inflammation, promote the ubiquitylation and subsequent degradation of the IRS proteins (Rui, Yuan et al. 2002).

Defects in several insulin signalling pathways, such as the insulin receptor kinase activity, reduced IRS-1 tyrosine phosphorylation and decreased PI3K-AKT activity were observed in different animal models of diabetes (Shimomura, Matsuda et al. 2000; Sun, Liu et al. 2002). Glucose enters the cell by facilitated diffusion mediated by a group of structurally related GLUT proteins. To date, at least **12 GLUT's have been** described (Joost, Bell et al. 2002). In AT and skeletal muscle, GLUT1 mediates basal glucose transport, GLUT2 mediates the entry of glucose into β-cells, whereas GLUT4 is responsible for insulin-mediated glucose uptake (Tordjman, Leingang et al. 1989). In T2DM, defects in the GLUT4 and glycogen synthesis in skeletal muscle have been identified (Vestergaard, Lund et al. 1993). It has been previously shown that these defects that cause T2DM are not a result of a decrease in skeletal muscle GLUT4 gene expression, but may be related to a defect in GLUT4 intracellular trafficking (Brozinick, Yaspelkis et al. 1996). Interestingly, increased expression of GLUT4 in skeletal muscle produces changes (lowered blood glucose, increased insulin, and contractile stimulated glucose transport) that could be of benefit in the treatment of T2DM (Gibbs, Stock et al. 1995).

1.2.5 Aetiology of Insulin Resistance

The aetiology of IR is complex and multifactorial, with both genetic and environmental factors being implicated. Some of the well recognised factors contributing to IR are discussed as follows.

1.2.5.1 Obesity

Obesity if often associated with IR, and develops when chronic over-nutrition conspires with genetic susceptibility to negatively impact insulin signalling (Muoio and Newgard 2008). How obesity exactly leads to IR is still a subject of debate but studies have clearly shown the association between the two. Studies such as the Uppsala Longitudinal Study of Adult Men (ULSAM) identified and compared long-term predictors of insulin sensitivity following adjustment for lifestyle factors and socio-economic status in a large population-based sample of non-diabetic men over the course of 20 years. This study reported that multiple lifestyle factors independently contribute to predict insulin sensitivity; however BMI was identified as the strongest predictor of insulin sensitivity, even after adjustment for other variables including lifestyle factors (Riserus, Arnlov et al. 2007). There are several ways in which obesity and adiposity can contribute to insulin resistance.

1.2.5.1.1 Adipose tissue location

Visceral adipose tissue has a greater ability to influence insulin sensitivity due to its anatomical proximity to the portal circulation. 80% of the blood directed towards the liver originates from the portal system (Schenk, Mc et al. 1962), and so the increased lipolytic activity of the visceral depot, with the resultant increase in non-esterified fatty acid (NEFA) as well as other factors such as inflammatory cytokines and glycerol, draining from the visceral adipose tissue into the portal vein can have substantial effects on hepatic metabolism (Montague and O'Rahilly 2000). Fontana and associates conducted an interesting study in which blood was obtained from both the peripheral and portal supply from obese individuals undergoing surgery for gastric bypass. The group found that IL-6 concentrations were significantly greater in the portal vein than in the peripheral circulation, and postulated that this inflammatory process may detrimentally affect hepatic glucose and insulin metabolism (Fontana, Eagon et al. 2007). Furthermore, an increase in visceral adiposity is associated with disordered glucose tolerance in humans (Boyko, Leonetti

et al. 1996; Hayashi, Boyko et al. 2003) as well as an increased T2DM risk (Boyko, Fujimoto et al. 2000).

1.2.5.1.2 Adipocyte hypertrophy and hypoxia

Blood flow through the adipose tissue is required for the local metabolic and physiological demands. Inadequate adipose tissue blood flow can affect lipid handling and the tissue responsiveness to nutrients. In obesity, hypertrophic adipocytes can reach over 140-**180μm in diameter. Optimal oxygen diffusion through the cell** decreases once its diameter exceeds **100μm** and hence, larger adipocytes are more difficult to diffuse, resulting in poor adipose tissue blood flow and hypoxia (Goossens 2008). The cellular response to low oxygen within a cell is diverse and is driven towards meeting the metabolic and survival needs of these suboptimal conditions. Hypoxia is associated with angiogenesis through release of angiogenic growth factors and cytokines such as VEGF (Kaluz, Kaluzova et al. 2008).

Adipocyte size and hypertrophy are also important determinants of adipokine secretion. It has been demonstrated that a divergent adipokine secretion profiles exists between large and small adipocytes – the larger cells showing dominance for the pro-inflammatory rather than the anti-inflammatory cytokines (Goossens 2008). Furthermore, enlarged adipocytes have a reduced capacity to respond effectively to the dynamic postprandial environment, prompting a delayed switch between fatty acid secretion and postprandial fatty acid uptake. This impaired capacity to respond effectively to the changing environment conceivably results in lipid overflow, ectopic lipid accumulation and insulin resistance (Goossens 2008). Adipocyte hypertrophy predicts the development of T2DM in this group more so than obesity alone (Weyer, Foley et al. 2000).

1.2.5.2 Inflammation

Chronic, low-grade inflammation is generally accepted as an integral element of both CVD and T2DM. This inflammatory response can be triggered by many factors including nutrients, as well as metabolic surplus, both of which engage a similar set of molecules and signalling pathways to those involved in classical inflammation (Hotamisligil 2006). Known markers of chronic low-grade inflammation such as fibrinogen and C-reactive protein (CRP) are associated with cardiovascular associated inflammation. Inflammation and inflammatory markers have already been discussed in detail earlier in Section 1.1.6.

1.2.5.3 Adipose tissue macrophage infiltration

Obesity-induced inflammation mainly originates from expanding adipose tissue mass and adipocyte hypertrophy, which result in development of a pro-inflammatory phenotype (Weisberg, McCann et al. 2003), a situation associated with a reduction in insulin sensitivity (Xu, Barnes et al. 2003). Infiltration of the adipose tissue during obesity may be related to hypoxic or necrotic lipid loaded adipocytes, and it has been suggested that the convergence of macrophages and other immune cells around these adipocytes may be primarily for clearage purposes (Cinti, Mitchell et al. 2005).

In addition, the profile of the macrophages is negatively impacted by obesity. A switch from alternatively activated, anti-inflammatory M2 macrophages, to a preponderance of classically activated, pro-inflammatory M1 macrophages is observed in obese states (Lumeng, Bodzin et al. 2007). This macrophage polarisation is important for the health of the adipose tissue as the M2 macrophages are involved in tissue remodelling, as well as the secretion of anti-inflammatory cytokines such as IL-10. M1 macrophages in contrast secrete pro-inflammatory cytokines such as IL-1β, which interfere with insulin signalling (Goossens 2008). Although this effect is generally local, due to the communication network between the AT and other organs discussed above, local inflammation within the AT may have a systemic impact.

This inflammation of the adipose tissue is associated with impairments in insulin sensitivity and may have a causal effect. An increase in inflammatory gene-expression profiles precedes the increase in both plasma insulin levels and insulin resistance in rats on a high fat diet (Xu, Barnes et al. 2003). Production of pro-inflammatory cytokines such as TNF-α and IL-β, which, through the activation of downstream kinases such as JNK, IKK and conventional protein kinase C (PKC), conspire to attenuate insulin signalling in obesity (Hotamisligil 2006). These kinases are able to phosphorylate serine, and thus inactivate, several core components of the proximal insulin signalling cascade including the insulin receptor and IRS-proteins. This inhibition in turn hinders down-stream signalling governing processes such as plasma glucose clearance, intracellular glucose transport and important insulin regulated pathways such as lipolysis, glycogen synthesis, protein synthesis and cellular growth (Cohen 2006).

1.2.5.4 Fatty acid induced insulin resistance

Over the past couple of decades, there has been a shift from a glucocentric view of insulin resistance to a more lipocentric perspective. This adjusted viewpoint considers insulin resistance to be a consequence of disordered lipid metabolism, leading the accumulation of lipid within the liver, muscle and pancreatic β-cells. This so called 'ectopic fat deposition' and the resultant lipotoxicity can induce insulin resistance in insulin target tissues (Savage, Petersen et al. 2005). The basis of this hypothesis stems from the notion that obesity and adipocyte hypertrophy represent states of inadequate storage capacity for excess/surplus energy. This represents a state of metabolic inflexibility wherein the adipose tissue is unable to optimally respond to the changing metabolic environment represented by increasing energy intake, and this reduced capacity for adipose tissue to act is as a "sink" for excess energy, can lead to "spill-over" into non-adipose tissues (Goossens 2008). Excessive or inappropriate storage of lipid in the muscle (Krssak, Falk Petersen et al. 1999) for example is closely correlated with increasing severity of insulin resistance. Additionally, ectopic lipid accumulation in the pancreatic β-cell can affect the capacity

for insulin secretion which will negatively impact circulating glucose levels (Eriksson 2007). To date, two conflicting hypotheses have been proposed regarding the *in vivo* metabolic response to increased circulating NEFAs, and particularly, how this impacts insulin sensitivity.

Randle and co-workers were among the first groups to address the aetiology of diabetes and IR in obese individuals, working from the premise that increasing fatty acid concentrations could impair carbohydrate metabolism (Randle, Garland et al. 1963). **Randle's theory suggests that fatty acids and glucose compete** for oxidation within the cell, and accordingly any increase in lipid oxidation would be to the detriment of glucose oxidation. Increases in lipid substrate and resultant oxidation would increase the mitochondrial acetyl coenzyme A (CoA):CoA as well as the NADH:NAD+ ratios, causing the subsequent inactivation of pyruvate dehydrogenase. This inactivation would increase intracellular citrate levels, therefore inhibiting phosphofructokinase, with the overall effect being the accumulation of glucose-6-phophate and the reduction of glucose uptake.

This hypothesis was challenged by a series of later experiments conducted by Schulman and colleagues, who demonstrated that the pathway, as delineated by Randle et al., does not occur in an *in vivo* environment. Infusing individuals with fatty acids over a prolonged period decreased insulin sensitivity as expected, however **contrary to that predicted by Randle's hypothesis, levels of intracellular glucose**-6-phosphate decreased by about 90% following lipid infusion. This decrease was associated with a reduction in IRS-1 associated PI3K activity, and subsequent studies within the group highlighted that this was likely secondary to the inhibitory serine phosphorylation of IRS-1 (Savage, Petersen et al. 2005).

Triacylglycerol (TAG) or TG is considered to be a relatively metabolically inert fat, however fatty acid derivatives such as long-chain acyl coenzymes A (LCCoA),

diacylglycerol (DAG) and ceramides are considered more likely candidates (Savage, Petersen et al. 2007). These fatty acid derivates could be more present in liver or muscles through either increased delivery, or reduced metabolism, and could plausibly induce insulin resistance. For example, DAG can activate a serine/threonine kinase cascade involving PKC, which leads to phosphorylation of serine/threonine sites on IRS-1 and IRS-2, thus to a decrease in insulin signalling. Current evidence would support the Schulman hypothesis over that of Randle's hypothesis; however the exact molecular mechanisms involved in insulin resistance remain to be fully elucidated.

The role of ectopic lipid accumulation particularly in the liver muscle and pancreas is being increasingly recognised, and research is focusing on aspects of lipid accumulation in the liver in the aetiology of conditions such as non alcoholic fatty liver disease (NAFLD), a condition associated with profound insulin resistance (Edens, Kuipers et al. 2009). From a clinical standpoint, there is increasing interest in the determination of plasma biomarkers of liver lipid accumulation, as liver biopsy or assessment by proton magnetic resonance spectroscopy may not be convenient, particularly in large scale research studies. Alanine amino transaminase (ALT) is a specific marker of liver pathology, and an established marker of liver fat accumulation (Westerbacka, Corner et al. 2004). A more recently discovered molecule fetuin-A can also be employed in the biochemical based determination of the liver fat content, and has been shown to correlate significantly with proton magnetic resonance spectroscopy assessments of fatty liver (Stefan, Hennige et al. 2006).

1.2.6 Measurements of Insulin Resistance

A global epidemic of obesity is driving the increased incidence and prevalence of T2DM and its CVD complications. Therefore, it is important to have reliable diagnostic methods to assess insulin sensitivity that can be readily applied in a clinical setting.

The hyperinsulinaemic-euglycaemic glucose clamp is considered the reference method for evaluating insulin sensitivity and the frequently sampled intravenous glucose tolerance test (fsIVGTT) with minimal model (MINMOD) modelling assessment is an alternative reference method (DeFronzo, Tobin et al. 1979; Monzillo and Hamdy 2003). Both tests are time-consuming and labour intensive and they have been criticised as they assess insulin sensitivity under non-physiological conditions (Breda, Cavaghan et al. 2001). However they are still widely accepted as the preferred methods of measuring insulin resistance and sensitivity in clinical studies that are done in smaller groups of patients. For larger studies, simpler methods are used. Fasting insulin concentration is probably the simplest surrogate marker for IR, but insulin alone can only be considered a reasonable measure of insulin sensitivity in steady-state postabsorptive (basal) conditions and when blood glucose levels are within the normal range. To compensate for variations in basal glucose levels, several alternatives have been proposed that use both fasting glucose and insulin concentrations to better account for conditions in which insulin response is deficient and hyperglycaemia is present. The $HOMA_{IR}$ and the quantitative insulin sensitivity check index (QUICKI) are two such measures.

1.2.6.1 Fasting insulin

It still remains elusive as to whether hyperinsulinaemia *per se* contributes to the MetS or a marker of IR (Lerman, Villa et al. 2003). In either case, fasting insulin is frequently used as a surrogate marker of IR in clinical studies (Mannucci, Bardini et al. 2003). Because direct insulin sensitivity measurement by euglycaemic clamp or MINMOD requires hours (hrs) of subject and investigator time, fasting plasma insulin is often used as an easy-to-obtain, highly correlated surrogate, with higher fasting insulin indicating lower sensitivity (Laakso 1993). Several prospective studies have reported a higher T2DM risk in relation to greater fasting insulin level, thereby demonstrating concordance with other research that included direct measurement of insulin sensitivity (Lundgren, Bengtsson et al. 1990; Skarfors, Selinus et al. 1991). The use of fasting insulin as a measure of insulin sensitivity may lead to biased

conclusions if unmeasured factors that affect this variable are related to the outcome of interest. Boyko et al. (Boyko, Leonetti et al. 1997) concluded that research on T2DM risk in relation to fasting insulin may yield biased effect measures unless adjusted for insulin secretion. Fasting insulin level is determined not only by degree of insulin sensitivity, but also β-cell secretory function and insulin clearance rate (Matthews, Hosker et al. 1985). It was reported that fasting connecting peptide (c-peptide) may better reflect insulin level than the fasting insulin measurement, because the latter undergoes hepatic extraction dependent on body fat distribution that may confound the association between diabetes risk and hyperinsulinaemia (Tillil, Shapiro et al. 1988). C-peptide is formed when proinsulin, the precursor for insulin is split into insulin and c-peptide. The physiological effects of c-peptide are still unknown, although it seems to constitute a crucial role in glucose control of T1DM (Walenciak, Fendler et al. 2007).

1.2.6.2 Homeostatic Model Assessment of Insulin Resistance ($HOMA_{IR}$)

$HOMA_{IR}$ is a model of interactions between glucose and insulin dynamics used to predict fasting steady-state glucose, insulin to c-peptide concentrations and a wide range of possible combinations of IR and β-cell function (Matthews, Hosker et al. 1985; Wallace, Levy et al. 2004). $HOMA_{IR}$ is calculated as:

$$\frac{\text{fasting glucose (mmol/L) x fasting insulin (μU/mL)}}{22.5}$$

The denominator of 22.5 is a normalizing factor. It is the product of normal fasting plasma insulin which is 5μU/mL multiplied by the typical plasma glucose of a healthy individual which is 4.5mmol/L. Therefore, for an individual with normal insulin sensitivity, $HOMA_{IR}$ equals 1. $HOMA_{IR}$ values of greater than 1 indicate IR whilst $HOMA_{IR}$ values less than 1 indicate increased sensitivity to insulin.

The $HOMA_{IR}$ model has become a widely used clinical and epidemiological tool and when used appropriately can yield valuable data. $HOMA_{IR}$ seems to be a reliable tool in the assessment of IR which can be used as a valid alternative to the glucose clamp or other sophisticated techniques in epidemiological settings, having the advantage of requiring only a single plasma sample of glucose and insulin (Bonora, Formentini et al. 2002). Yet, as with all models, the primary input data needs to be robust and interpreted carefully (Wallace, Levy et al. 2004). The coefficient of variance (CV) for $HOMA_{IR}$ varies considerably depending on the number of fasting samples obtained and the type of insulin assay used (Matthews, Hosker et al. 1985; Bonora, Formentini et al. 2002). A study in 80 subjects with T2DM treated with sulphonylurea or diet treatment alone showed that $HOMA_{IR}$ strongly correlated with the hyperinsulinaemic euglycaemic clamp in both groups (Emoto, Nishizawa et al. 1999).

1.2.6.3 Quantitative Insulin Sensitivity Check Index (QUICKI)

QUICKI is an index of insulin sensitivity based on the determination of glucose and insulin concentrations in a post-absorptive state (Katz, Nambi et al. 2000). QUICKI is calculated using the following formula:

$$1/[\log(\text{fasting insulin}) + \log(\text{fasting glucose})]$$

Previously, it has been reported to be a reliable, reproducible and accurate index of insulin sensitivity with excellent positive predictive power (Hanley, Williams et al. 2003; Chen, Sullivan et al. 2005). However, QUICKI is less correlated to the glucose clamp in non-obese, non-diabetic control subjects compared to obese T2DM patients (Katz, Nambi et al. 2000). The original QUICKI underwent revision to incorporate plasma NEFA (Brady, Gower et al. 2004). The revised QUICKI formula is:

$$1/[\log(\text{insulin}_0) + \log(\text{glucose}_0) + \log(\text{NEFA}_0)]$$

Perseghin et al. (Perseghin, Caumo et al. 2001) aimed to ascertain whether the revised QUICKI improves its association with insulin sensitivity in 57 young, healthy, non-obese subjects. The study concluded that the incorporation of fasting NEFA into QUICKI was useful to improve its correlation with the glucose clamp index of insulin sensitivity. Such findings are in line with a recent study that compared the traditional QUICKI to the revised QUICKI method and found that both methods are good indices that offer correlations similar to or higher than the $HOMA_{IR}$ method (Rabasa-Lhoret, Bastard et al. 2003). Contrary to this, significant increases in insulin sensitivity induced by weight-loss was detected by QUICKI, but not by revised QUICKI (Bastard, Jan et al. 2002). Whilst QUICKI and $HOMA_{IR}$ were derived in a completely different conceptual fashion, nevertheless, these two surrogate markers are mathematically related, wherein, QUICKI is inversely proportional to the $\log(HOMA_{IR})$. Whilst QUICKI is a well validated surrogate index of insulin sensitivity, it still requires blood sampling at more than 1 single time point, hence many researchers prefer to use a more sophisticated test if they have the resources to conduct one.

1.2.6.4 Hyperinsulinaemic-euglycaemic clamp

The hyperinsulinaemic euglycaemic clamp or **"clamp"** method is a widely accepted reference method for quantifying insulin sensitivity in humans because it directly measures the effects of insulin in promoting glucose utilisation under steady-state conditions *in vivo* (DeFronzo, Tobin et al. 1979). The validity of clamp measurements of insulin sensitivity depends on achieving steady-state conditions and the assumption that data from an arbitrary time point at the end of the clamp (2 hrs) represents steady-state values. However, defining the steady-state as a period greater than 30 minutes (at least 2 hrs after initiation of insulin infusion) during which the CV for blood glucose, plasma insulin and glucose infusion rate are less that 5% is a more rigorous approach than may be determined by more values for glucose disposal rate and insulin sensitivity (Katz, Nambi et al. 2000). It has been reported that these steady-state insulin levels may be supra-physiological, which may result in a reversal of the normal portal to peripheral insulin gradient. Therefore, the glucose clamp may

not as accurately reflect insulin action and glucose dynamics under physiological conditions compared to an oral meal or oral glucose load.

Although the hyperinsulinaemic euglycaemic clamp is considered the "gold-standard" for technique for measuring insulin sensitivity it has many limitations. Relative to other techniques, it is time-consuming, labour intensive, expensive and requires an experienced operator to manage the technical difficulties. Thus, for epidemiological studies, large clinical investigations or routine clinical applications, such as following changes in IR after therapeutic intervention in individual patients, the glucose clamp may not be appropriate. In addition, if measuring insulin sensitivity/resistance is not a primary study outcome, the cost/benefit ratio for the glucose clamp may not be favourable.

1.2.6.5 Frequently Sampled Intravenous Glucose Tolerance Test (fsIVGTT)

The minimal model fsIVGTT provides an indirect measure of metabolic insulin sensitivity/resistance based on glucose and insulin data (Bergman, Ider et al. 1979). This technique involves an **infusion of glucose tailored to the individual's body weight** at baseline, followed by the measurement of insulin and glucose at selected time points over 3 hrs (Pacini and Bergman 1986). Beard et al. (Beard, Bergman et al. 1986) proposed a modified protocol which requires an infusion of exogenous insulin or tolbutamide, 20 minutes after the infusion of glucose at baseline (Yang, Youn et al. 1987; Saad, Steil et al. 1997). This is to ensure an adequate endogenous plasma insulin response which is critical for determining insulin sensitivity (S_I) using the minimal model technique. The MINMOD Millennium® is a software package that computes estimates of S_I, glucose effectiveness (S_G) and β-cell function for each individual profile by curve fitting techniques (Bergman, Ider et al. 1979; Pacini and Bergman 1986; Boston, Stefanovski et al. 2003). The minimal model is defined by two coupled differential equations with four model parameters. The first equation describes plasma glucose dynamics in a single compartment. The second describes

insulin dynamics in a remote compartment. The structure of the minimal model allows MINMOD to uniquely identify model parameters that determine a best fit to glucose disappearance during the modified fsIVGTT. S_I is calculated from two of these model parameters and is defined as fractional glucose disappearance per insulin concentrations unit.

Unlike the hyperinsulinaemic euglycaemic clamp method which depends on steady-state conditions, the fsIVGTT technique uses dynamic data. Valid minimal model analysis of the modified fsIVGTT requires several important assumptions. First, instantaneous distribution of the glucose bolus in a monocompartmental space is assumed to occur. Second, glucose disappearance in response to glucose and/or insulin is assumed to occur at a monoexponential rate. Third, the glucose concentrations at the end of the fsIVGTT are assumed to be identical to the beginning. Finally, to achieve a valid estimate of S_I the minimal model assumes that total insulin secretion (endogenous and exogenous) during the fsIVGTT is above a certain threshold (Yang, Youn et al. 1987).

However, the fsIVGTT method is more advantageous compared to the glucose clamp as it is significantly less labour intensive. The steady-state conditions of the hyperinsulinaemic euglycaemic clamp are not obligatory and hence there are no intravenous infusions that require constant adjustments. It is suitable for use when studies involve larger numbers of volunteers. Furthermore, information about S_I, S_G and β-cell function can all be obtained from a single dynamic test. The fsIVGTT model has been reported to generate excellent predictions of glucose disappearance and the CV for S_I is comparable to glucose clamp estimates of insulin sensitivity (Ferrari, Alleman et al. 1991; Steil, Murray et al. 1994).

A summary of these four methods of assessing IR is presented in Table 1-5.

Table 1-5: Summary of Different Methods of Assessing IR

Assessment method	Main advantages	Main disadvantages
$HOMA_{IR}$	Simple single time point measurement Easy to perform Inexpensive Suitable for large population and epidemiological studies	Accuracy dependent on number of measurements Static measurement means that dynamic β-cell function cannot be determined Results skewed over a wide range of IR
QUICKI	Simple single time point measurement Easy to perform, Inexpensive Good linear correlation with the clamp and fsIVGTT	Simlar to $HOMA_{IR}$
Hyperglycaemic-Euglycaemic Clamp	Measures whole body glucose disposal. Ability to distinguish between hepatic and peripheral IR with use of radioactive labelled isoptopes Excellent test characteristics	Labour intensive Time consuming Expensive Experienced operator required Utilises supraphysiological insulin level
fsIVGTT	Easier than Clamp method No requirement for steady state or continuous intravenous infusions Ability to assess glucose effectiveness and β-cell function in the one test	More intensive than $HOMA_{IR}$ & QUICKI Monocompartmental representation of glucose dynamics resulting in possible overestimation of S_G and underestinmation of S_I.

1.3 POLYCYSTIC OVARY SYNDROME (PCOS)

1.3.1 Introduction

The typical morphological structure of the polycystic ovary (PCO) was first described by Chereau (Chereau, 1844) and Rokitansky (Rokitansky, 1844) in 1844. However it was in the 1930's that two physicians, Irving Stein and Michael Leventhal, linked these polycystic appearing ovaries to several clinical symptoms found in a small number of female patients (Stein and Leventhal 1935). Symptoms included a combination of oligomenorrhoea, hirsutism and obesity. The associated manifestations in this subgroup of female patients led to the identification of the condition which we now recognise as PCOS.

1.3.2 Prevalence

PCOS is probably the commonest endocrine disorder, at least in women of reproductive age group (Carmina and Lobo 1999). It has been suggested that between 6-8% of premenopausal women suffer from PCOS, even when the more stringent National Institute of Health (NIH) criteria of 1990 is used (Azziz, Woods et al. 2004). However, the true prevalence of PCOS has not been fully determined, mainly due to the on-going controversy surrounding the correct diagnostic criteria (Azziz 2006). The prevalence also varies with different populations. For example, the prevalence in Mexican-American population has been estimated to be about 13%, twice that of other populations (Goodarzi, Quinones et al. 2005). Also immigrant populations from the Indian subcontinent to the United Kingdom, and Australians of Aboriginal heritage, have a higher prevalence of PCOS. Rodin et al. (Rodin, Bano et al. 1998) found that the prevalence rates of PCOS among the South Asian female immigrants in Britain, in 1998 was 52 %, with the majority of them (49.1 %) suffering from menstrual disturbances. The time-scale at which symptoms are experienced can also vary between ethnic groups and geographical location. Female UK immigrants

originating from South Asia generally present their symptoms at an earlier age than their white counterparts, and symptoms may also be more severe in this subgroup of women (Wijeyaratne, Balen et al. 2002).

1.3.3 Definition & Diagnostic Criteria

Various diagnostic criteria have been proposed in order to correctly confirm the presence of PCOS. The four most widely used criteria are:

1. National Institute for Health (NIH) criteria (1990)
2. Rotterdam Consensus, compiled by the European Society of Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) (2003) (2004; 2004)
3. Androgen Excess Society criteria (2006) (Azziz, Carmina et al. 2006)
4. Androgen Excess-Polycystic Ovary Syndrome (AE-PCOS) criteria (2009)

All the above criteria require the exclusion of other hyperandrogenic syndromes e.g. **Cushing's syndrome, androgen**-secreting tumours and non-classical congenital adrenal hyperplasia.

1.3.3.1 National Institute of Health (NIH) criteria (1990)

The proceedings of an expert conference sponsored by the National Institutes of Health (NIH) in April 1990 suggested that the diagnosis of PCOS rested on two main features:

1. Hyperandrogenism (either biochemical or clinical) and
2. Chronic oligo- or anovulation

The presence of PCO is consistent with, but not essential for the diagnosis of PCOS.

1.3.3.2 Rotterdam criteria (2003)

The 2003 Rotterdam criteria (2004; 2004) requires the presence of two of the following three features:

1. Chronic anovulation
2. Clinical and/or biochemical signs of hyperandrogenism
3. Presence of PCO

However, using the Rotterdam consensus has resulted in significant controversy regarding whether or not PCOS can be diagnosed in the absence of hyperandrogenism, as many of the clinical manifestations associated with PCOS arise from hyperandrogenism (Azziz 2006). Also, the prevalence of polycystic-appearing ovaries has been noted with as many as 20%–25% of the general young healthy female population who have no other clinical symptomatology or subfertility. Hence from an endocrine and metabolic perspective, this criterion is less satisfactory.

1.3.3.3 Androgen Excess Society (AES) criteria (2006)

The AES criteria (Azziz, Carmina et al. 2006) requires hyperandrogenaemia or clinical hyperandrogenism as a mandatory criteria, in addition to either one or both of:

1. Chronic anovulation
2. Presence of PCO.

The emphasis on androgen excess is based on the fact that the presence of PCO is common, even in women with no apparent metabolic disturbances (Taylor 1998).

More recently, the Androgen Excess Society, was renamed the Androgen Excess and PCOS (AE-PCOS) Society, and in 2009, recommended the acceptance of the NIH criteria, with a few modifications (Azziz, Carmina et al. 2009).

1.3.3.4 Androgen Excess-Polycystic Ovary Syndrome (AE-PCOS) criteria (2009)

This criteria is based on the original NIH criteria and similar to the previous AES criteria with a few modifications:

1. Hyperandrogenism: hirsutism and/or hyperandrogenaemia.
2. Ovarian dysfunction: oligo-anovulation and/or polycystic ovaries.

As with previous criterion, other causes of androgen excess or related disorders have to be excluded.

1.3.4 Features of Polycystic Ovary Syndrome

1.3.4.1 Oligomenorrhoea and Anovulatory Cycles

Most women with PCOS have some degree of anovulation which presents clinically as either oligomenorrhoea or amenorrhoea. Oligomenorrhoea is classified as having < 8 spontaneous episodes of menstrual bleeding per year (Carmina and Lobo 1999), whilst amenorrhoea is the complete absence of menstruation for at least 6 months (Norman, Dewailly et al. 2007). A few proportion of PCOS women have regular menstrual cycles or even have increased frequency, but the cycles are anovulatory i.e. without the release of an ovum. Anovulation, regardless of the frequency of menstrual cycle, results in subfertility and is a common reason for seeking medical advice.

1.3.4.2 Hyperandrogenism

Hyperandrogenism is the most constant and prominent diagnostic component of PCOS. It can be assessed either by biochemical measurements or clinical manifestations can in the majority of patients with PCOS, and indeed, according to both the NIH criteria and AES criteria, have to be present for the diagnosis of PCOS to be made (see above). Although both the ovaries and adrenal glands of women with

PCOS produce excess androgens, the ovaries are thought to be the more significant contributor (Carmina 2006).

1.3.4.2.1 Ovarian androgen excess

It is now well recognised that there is a deregulation of the hypothalamic-pituitary-ovarian axis, especially with regards to abnormal patterns of gonadotrophin and gonadotrophin releasing hormone (GnRH) pulsatility (Waldstreicher, Santoro et al. 1988; Eagleson, Gingrich et al. 2000). Increased GnRH pulsatility may be attributable to low progestin levels in PCOS women (Eagleson, Gingrich et al. 2000) and results in increased secretion of luteinising hormone (LH), one of two gonadotrophins from the anterior pituitary (Haisenleder, Dalkin et al. 1991). This is sometimes noted in blood measurements of LH and follicle stimulating hormone (FSH) – FSH is usually higher than LH but in PCOS women, LH levels are frequently higher than FSH – increasing the LH/FSH ratio. However, because of the pulsatile nature of gonadotrophin release, a single blood sample may not detect any significant changes in the LH/FSH ratio, and frequent (e.g. every 10 minutes) and prolonged (12-24 hr) serial blood sampling studies are necessary to detect the increased LH/FSH ratio.

The mechanism by which the theca cells produce excess androgens is complex. Luteinising hormone (LH) has been shown to stimulate ovarian theca cells in PCOS to convert androgenic precursors to testosterone more readily than normal theca cells in both *in vivo* and *in vitro* studies (Nelson, Legro et al. 1999; Nelson, Qin et al. 2001). Following LH stimulation, activation of cAMP and increased expression of several key enzymes involved in steroidogenesis including cytochrome P450 **11A (CYP11A)**, **3β**-hydroxysteroid-dehydrogenase **(3β-HSD)**, **17α**-hydroxylase/C_{17-20}lyase cytochrome P450 (CYP17), and steroid acute regulatory (StAR) protein result in cholesterol being metabolised into androstenedione (Magoffin 2005). Androstenedione is converted to testosterone by **17β**-hydroxysteroid-**dehydrogenase (17β**-HSD).

Insulin and hyperinsulinaemia is also thought to contribute to hyperandrogenism and gonadotrophin abnormalities. High values of insulin reduce SHBG (Plymate, Matej et al. 1988; Nestler, Powers et al. 1991), thereby increasing the bioavailability of testosterone (Nestler, Powers et al. 1991; Pugeat, Crave et al. 1991). Insulin may also be a co-factor in the synthesis of adrenal and ovarian androgens, thereby contributing to abnormal gonadotrophin concentrations (Nestler and Jakubowicz 1996; Azziz, Ehrmann et al. 2003). There may also be a direct gonadotrophin releasing regulatory properties of insulin on the hypothalamus, pituitary or both (Adashi, Hsueh et al. 1981), but the contribution of IR to gonadotrophin abnormalities in PCOS remains uncertain (Dunaif 1997).

1.3.4.2.2 Adrenal androgen excess

Adrenal androgen also contributes to hyperandrogenism and is prevalent in about 25-60% of women with PCOS (Dumesic, Abbott et al. 2007). The exact reason for increased adrenal androgen production is unclear but it has been proposed that an altered cortisol metabolism with a reduced negative feedback to the hypothalamus may result in activation of the hypothalamic-pituitary-adrenal axis that maintains cortisol levels but also increases adrenal androgens (Carmina 2006).

1.3.4.2.3 Biochemical Manifestations

Regardless of the cause of excess androgens, levels can be measured and are usually but not universally raised in women with PCOS. Hyperandrogenaemia refers to the finding of supranormal levels or estimates of circulating endogenous androgens. Testosterone (T) is possibly the most important androgen in women. However, it is bound to SHBG and only the unbound or free T (FT) is able to enter the cells of target tissues. FT can be measured by direct radioimmunoassay or by equilibrium dialysis. However, the former is highly inaccurate (Vermeulen, Verdonck et al. 1999; Miller, Rosner et al. 2004) and the latter is not widely available due to its technical complexity and cost (Van Uytfanghe, Stockl et al. 2004). Hence a different measure of

unbound testosterone is usually used: the free androgen index (FAI), which is the ratio of measured serum total testosterone to SHBG multiplied by 100 (T/SHBG x 100) (Mathur, Moody et al. 1981). Given the well accepted fact that SHBG is usually suppressed by hyperinsulinaemia in IR states which is commonly found in PCOS, FAI is often found to be raised, even if total testosterone is not elevated.

Androstenedione (4A) and dehydroepiandrosterone (DHEA) are two other androgens that are raised in some women with PCOS (Azziz, Black et al. 1998). Androstenedione can be produced from either the adrenal cortex or ovaries. However there are only a few studies assessing the prospective value of this androgen. DHEA has been more extensively studied. Between 95 and 97% of the circulating DHEA is produced from the zona glomerulosa of the adrenal cortex. However, DHEA concentration in plasma is low, varies with time of day, and may be elevated by any degree of stress, including the anticipation of venesection. Hence, its much more stable metabolite dehydroepiandrosterone sulphate (DHEAS) is the preferred measured substrate for assessment of adrenal androgen production. DHEAS is 97% to 99% adrenal in origin (Abraham and Chakmakjian 1973; Abraham 1974), its half life is longer (Wang, Bulbrook et al. 1967; Gant, Hutchinson et al. 1971) and its levels are more constant during the day and throughout the menstrual cycle (Abraham 1974). Measuring DHEAS levels therefore is also helpful in the diagnosis of hyperandrogenaemia.

1.3.4.2.4 Clinical Manifestations

1.3.4.2.4.1 Hirsutism

Hirsutism is defined as the presence of terminal hairs on the face and/or body in a male-like pattern in a female. The most commonly used method for assessment is the modified Ferriman-Gallwey score (Ferriman and Gallwey 1961; Hatch, Rosenfield et al. 1981). This score is derived based on the assessment of hair growth in 9 regions of

the body: upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, upper arm and inner thigh. Each region is given a score between 0 and 4, giving a total range of scores between 0 and 36. Whilst various have been proposed for the diagnosis of hirsutism, a value of 8 or more is generally accepted upper limit (Hatch, Rosenfield et al. 1981). However, there are clearly observed differences between various racial and ethnic groups (Archer and Chang 2004). The overall prevalence of hirsutism is probably approximately 65% to 75% of women with PCOS.

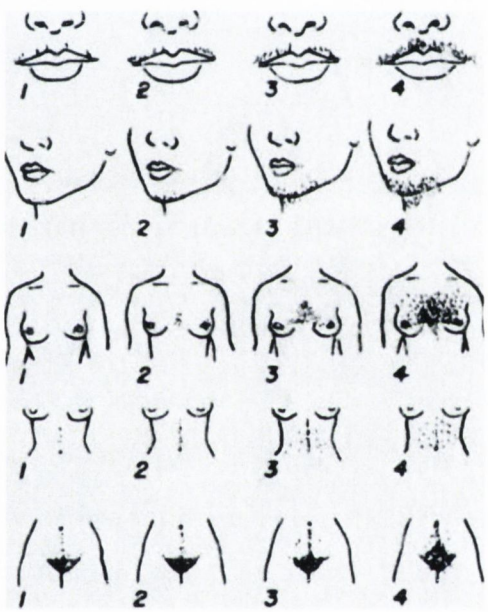

Figure 1-9: Ferriman-Gallwey Scoring System illustrating the extent of hair growth in androgen-sensitive regions of the female body (King, 2006).

The rate of hair growth is genetically determined **by the hair follicle enzyme 5α-**reductase, an enzyme that converts testosterone to its more potent metabolite dihydrotestosterone (DHT), which transforms vellus hair into terminal hair in the areas that are androgen sensitive. Women with PCOS presenting with hirsutism are **found to have an increased activity of this 5α-**reductase enzyme (Figure 1-10) and subsequently are found to have excessive growth of terminal hair (Archer and Chang 2004).

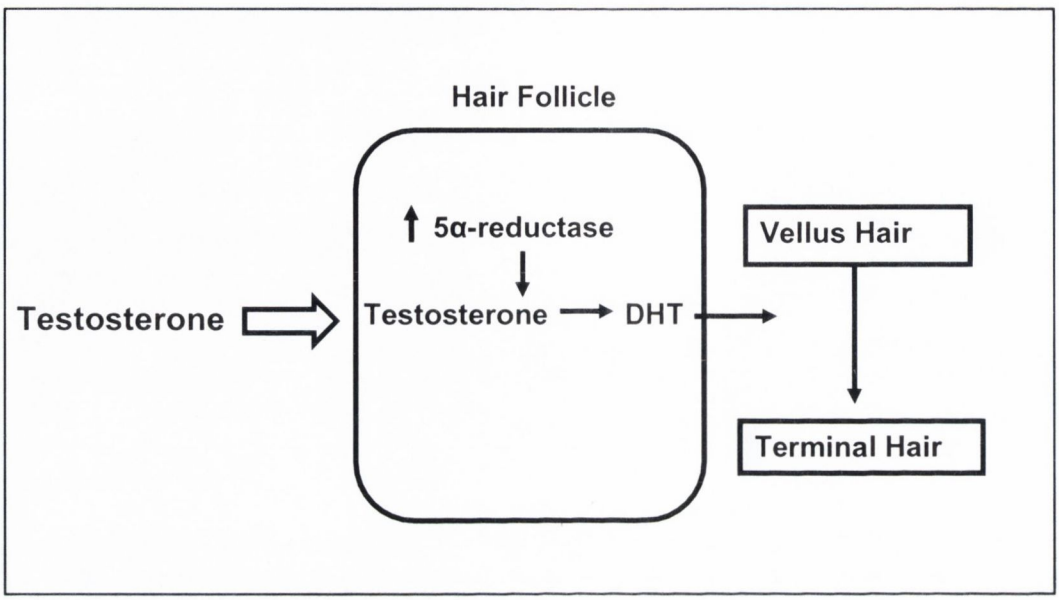

Figure 1-10: Increased activity of 5α-reductase leads to the conversion of testosterone to DHT which transforms soft vellus hair to dark, course terminal hair. Adapted from Archer and Chang (2004).

1.3.4.2.4.2 Acne

Acne affects approximately 12% to 14% of white women with PCOS (Azziz, Sanchez et al. 2004; Azziz, Woods et al. 2004), although like hirsutism, the prevalence also varies with ethnicity (Williamson, Gunn et al. 2001; Wijeyaratne, Balen et al. 2002). The prevalence has been noted to be higher in Italy – almost 24% in one study (Falsetti, Gambera et al. 2002). However, acne is a relatively prevalent in the general population – ranging from approximately 20% of individuals in teenage years, and gradually decreasing to 5% in their 6th or 7th decade (Johnson and Roberts 1978; Cunliffe and Gould 1979; Dalgard, Svensson et al. 2004). It is therefore not entirely clear whether the prevalence of acne is significantly higher in PCOS than the general population, however it is still regarded as one of the clinical manifestations of hyperandrogenism.

1.3.4.2.4.3 Androgenic Alopecia

Androgenic alopecia is defined as hair loss in women in a male-like pattern, and is a well recognised clinical feature of PCOS (Ludwig 1977; Conway, Honour et al. 1989; Cela, Robertson et al. 2003; Azziz, Sanchez et al. 2004). It is usually, but not always associated with hirsutism and acne, and correlates poorly with biochemical hyperandrogenism (Deplewski and Rosenfield 2000; Rosenfield 2001). Similar to hirsutism, alopecia is thought to be a maladaptive response of the pilosebaceous unit to endogenous androgens, with an increased activity of 5α-reductase and subsequent response to DHT (Schweikert and Wilson 1974).

The pattern of hair loss generally involves thinning of the crown with preservation of the anterior hairline (Futterweit, Dunaif et al. 1988; O'Driscoll, Mamtora et al. 1994). Understandably, this clinical feature contributes significantly to psychological morbidity in women.



Figure 1-11: Progressive hair loss over the crown area of the scalp of women with PCOS. (King, 2006).

1.3.4.3 Polycystic Ovaries (PCO)

The presence of PCO is not required for the diagnosis for PCOS (Ehrmann, Rosenfield et al. 1992) and conversely their presence, in the absence of other features of the syndrome does not establish the diagnosis (Polson, Adams et al. 1988; Michelmore, Balen et al. 1999). The presence of PCO is common in the general population. Approximately 20% of non-hirsute, normoandrogenic women with regular menses have PCO (Clayton, Ogden et al. 1992). PCO may develop at any stage of the fertile female life span and may be induced potentially by weight gain or the onset of IR (Carmina and Lobo 1999). However, without the additional presence of hyperandrogenism or menstrual cycle irregularities, these women are not considered to have PCOS (Dunaif 1997).

The Rotterdam criterion defines PCO as the presence of 12 or more follicles throughout the ovary measuring 2 to 9 mm in diameter and/or an increased ovarian volume of > 10 mL, in at least one ovary (2004; 2004). This definition cannot be applied to women taking oral contraceptives because ovarian morphology can be altered by such medications (Christensen, Boldsen et al. 1997).

The pathology behind the abnormal morphology of these PCO is thought to be attributable to the over expression of circulatory androgens, as androgens encourage the development of primary follicles to the stage of pre-antral and small antral follicles. These localised androgens exert a direct inhibitory effect on follicular maturation by reducing FSH stimulation, causing the follicles to continue to develop without maturing fully. This gives rise to immature follicular cysts which are arranged on the ovaries in a distinct pearl necklace configuration and are visible during ultrasound (Homburg 2008), as shown in Figure 1-12 and has been described **as having a "pearl necklace" appearance.**

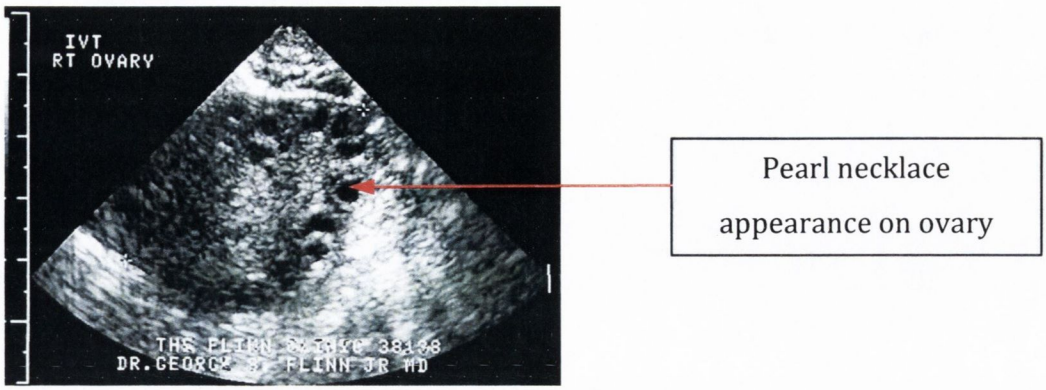

Figure 1-12: Ultrasonography view of a polycystic ovary (Homburg, 2008).

Overall, the prevalence of PCO is high among patients with androgen excess or PCOS (Rajkhowa, Talbot et al. 1995; Falsetti and Eleftheriou 1996; van Santbrink, Hop et al. 1997; Alborzi, Khodaee et al. 2001; Laven, Imani et al. 2001; Williamson, Gunn et al. 2001; Amer, Li et al. 2002; Jonard, Robert et al. 2003; Orio, Matarese et al. 2003; Hahn, Tan et al. 2005; Legro, Myers et al. 2006) but we should also recognise that the false positive rate is also relatively high.

1.3.5 Cardiovascular Risk in Polycystic Ovary Syndrome

The association between PCOS and CVD has been the subject of much controversy (Srikanthan, Korenman et al. 2006). Women with PCOS, particularly obese women with PCOS are thought to have a higher cardiovascular risk burden (Legro, Kunselman et al. 1999; Cibula, Cifkova et al. 2000; Legro, Kunselman et al. 2001; Talbott, Zborowski et al. 2004; Macut, Damjanovic et al. 2006; Shaw, Bairey Merz et al. 2008). Dahlgren et al., using a model based upon a prospective study suggested that women with PCOS had a seven-fold increased risk of myocardial infarction than women without PCOS (Dahlgren, Janson et al. 1992). IR, obesity and possibly hyperandrogenaemia are thought to be implicated, but there are other features which are thought to contribute.

1.3.5.1 Insulin Resistance in PCOS

Between 50% and 70% of women have demonstrable IR, but this increases up to 95% in obese women with PCOS (Carmina and Lobo 2004; DeUgarte, Bartolucci et al. 2005), when compared to weight-matched non-PCOS women (Dunaif, Segal et al. 1989). Both PCOS and obesity appear to synergistically worsen insulin resistance (Carmina and Lobo 2004). Impaired β-cell function (O'Meara, Blackman et al. 1993; Dunaif and Finegood 1996) and insulin signalling defects (Dunaif 1997) have also been demonstrated, and in the setting of IR, results in an increased likelihood of both IGT and MetS (Ehrmann, Barnes et al. 1999; Legro, Kunselman et al. 1999). Up to 40% of women with PCOS develop IGT and T2DM by the forth decade of life (Norman, Masters et al. 2001; Legro, Gnatuk et al. 2005) and even adolescents with PCOS have alarmingly high prevalence of IGT and T2DM (Palmert, Gordon et al. 2002). The cause of IR and abnormalities of insulin secretion and action are complex and multifactorial with both genetic and environmental contributions (Diamanti-Kandarakis and Papavassiliou 2006). A more detailed discussion of IR and insulin action has already been covered elsewhere in this thesis.

IR in PCOS women and the resultant hyperinsulinaemia is the factor that drives many of the phenotypic features of the disorder including ovarian hyperandrogenaemia and acanthosis nigricans. It is well accepted that hyperinsulinaemia has been implicated in the pathogenesis of increased ovarian theca androgen production (Barbieri, Makris et al. 1986; Nestler, Jakubowicz et al. 1998) and decreased hepatic SHBG production (Plymate, Matej et al. 1988; Nestler, Powers et al. 1991). The latter contributes to the finding of elevated free testosterone even though the measured total testosterone may be normal.

Assessment of IR in PCOS includes the various techniques described in the previous section 1.2.6. It is generally accepted that for larger epidemiologic studies, detection of IR by $HOMA_{IR}$ or QUICKI would be reasonable, whereas dynamic testing by the

OGTT, fsIVGTT or hyperinsulinaemia euglycaemic clamp should be used, where possible, if studies involving smaller numbers of volunteers should be used (Azziz, Carmina et al. 2009).

1.3.5.2 Obesity in PCOS

Approximately 50% of PCOS women are affected by obesity (Azziz, Sanchez et al. 2004). Obesity in PCOS is more prevalent in the United States of America (USA) (Azziz, Ehrmann et al. 2001) compared to other countries (Balen, Conway et al. 1995; Taponen, Martikainen et al. 2004; Carmina, Chu et al. 2005; Hahn, Tan et al. 2005). An increased background prevalence of obesity, to the extent of epidemic proportions is likely to be one of the main reasons for this difference. Lifestyle and dietary factors have been implicated as well (Carmina, Legro et al. 2003). Weight gain post adolescence are associated with an increased prevalence of PCOS symptoms (Laitinen, Taponen et al. 2003). Central adiposity has long been recognised to increase risk of diabetes and cardiovascular disease (Vague 1956) and can be detected even in lean women with PCOS (Kirchengast and Huber 2001; Yildirim, Sabir et al. 2003).

The risk of PCOS appears to increase with obesity (Alvarez-Blasco, Botella-Carretero et al. 2006) although the effects of obesity may be modest (Yildiz, Knochenhauer et al. 2008). Similarly, obesity exacerbates the metabolic and reproductive derangements, and symptoms of PCOS may become apparent especially if the individual is at increased susceptibility due to a positive family history (Legro, Bentley-Lewis et al. 2002). This appears to be particularly true for glucose intolerance (Plymate, Matej et al. 1988; Talbott, Clerici et al. 1998; Wallace, Levy et al. 2004). IR is almost always present in obese women with PCOS (Carmina and Lobo 2004; DeUgarte, Bartolucci et al. 2005), and frequently results in IGT (Dunaif, Segal et al. 1989; Corbould 2008).

Obesity and concomitant hyperinsulinaemia in women with PCOS results in lower SHBG levels (Nestler, Powers et al. 1991) thereby increasing FT and FAI levels. Excess adiposity has also been implicated in abnormal adipokine secretion profiles (O'Connor, Phelan et al. ; Carmina, Orio et al. 2005; Barber, Hazell et al. 2008).

1.3.5.3 Hypertension in PCOS

Although hypertension appears to be an integral part of the MetS, women with PCOS have only occasionally been noted to have increased blood pressure, with many clinical studies reporting normal levels (Legro 2003). However, it has been postulated that women who suffer from lengthy durations of menstrual disturbances or irregularities (> 40 days) have a 2-fold increase risk of developing hypertension. Also a small percentage of young women with PCOS demonstrate an increased prevalence of labile day-time blood pressure which may be associated with sustained hypertension in later life (Cattrall and Healy 2004). By the time of the menopause, women with PCOS are 2.5 times more likely to develop hypertension, than their age and weight-matched counterparts (Elting, Korsen et al. 2001). However, in most cases this increased risk has been attributed to the presence of obesity, associated with PCOS, with weight loss reducing blood pressure to within a normal range.

1.3.5.4 Dyslipidaemia in PCOS

Up to 70% of women have dyslipidaemia, when assessed according to the National Cholesterol Education Program (NCEP) guidelines (Wild, Painter et al. 1985; Legro, Kunselman et al. 2001). Different patterns have been observed, including low HDL-chol, increased TG, increased total and LDL-chol, and increased sdLDL particle. (Wild, Painter et al. 1985; Graf, Richards et al. 1990; Talbott, Clerici et al. 1998; Legro, Azziz et al. 2003; Berneis, Rizzo et al. 2007; Essah, Nestler et al. 2008; Valkenburg, Steegers-Theunissen et al. 2008). The different patterns may be related to the associated effects of IR and hyperandrogenaemia, combined with genetic and environmental factors (Essah, Nestler et al. 2008; Valkenburg, Steegers-Theunissen et al. 2008).

Hypertriglyceridaemia, decreased HDL-chol and increased small dense LDL-chol levels is probably the commonest dyslipidaemia pattern noted in PCOS (Rizzo and Berneis 2005; Berneis, Rizzo et al. 2009). This pattern, often referred to as the atherogenic lipoprotein phenotype (Austin, King et al. 1990), is similar to that found in T2DM (Brunzell and Ayyobi 2003), and is mainly the consequence of IR that impairs the ability of insulin to suppress lipolysis and inhibition of fatty acid release from adipose stores. Obesity itself exacerbates IR so this is more common in obese women with PCOS (Dunaif 1997), and it is less common in countries where the mean body weight is lower (Essah, Nestler et al. 2008).

LDL-chol has also been shown to be increased in women with PCOS (Conway, Agrawal et al. 1992; Legro, Kunselman et al. 2001; Essah, Nestler et al. 2008; Valkenburg, Steegers-Theunissen et al. 2008), although its prevalence is lower – ranging between 24% to 40% (Essah, Nestler et al. 2008; Valkenburg, Steegers-Theunissen et al. 2008). Lp(a) has also been found to be elevated in women with PCOS (Rizzo, Berneis et al. 2009).

Postprandial dyslipidaemia, in particular hypertriglyceridaemia is also thought to increase cardiovascular risk (Bansal, Buring et al. 2007), and this has been studied in two separate studies in PCOS women (Velazquez, Bellabarba et al. 2000; Bahceci, Aydemir et al. 2007). However both of these studies included non-obese women for normal controls and the role of androgens was not explored.

1.3.5.5 The Role of Hyperandrogenism in CVD in PCOS

Normal menstruating premenopausal women are significantly protected against cardiovascular morbidity and mortality, compared to their age-matched male counterparts (Baker, Meldrum et al. 2003). The reason for this disparity between the two sexes is not fully elucidated although oestrogen is known to have

cardioprotective properties and is certainly a key factor (Mendelsohn and Karas 1999).

The presence of oestrogen in women of reproductive age is thought to down-regulate PAI-1 activity and reduce coagulation tendency in comparison with postmenopausal women (Gebara, Mittleman et al. 1995). Oestrogen has also been shown to regulate endothelial function by enhancing the nitric oxide (NO) synthase production. NO helps to regulate vasodilatation and reduce hypertension (Baker, Meldrum et al. 2003). Other effects include a decrease in adhesion molecules (Caulin-Glaser, Watson et al. 1996), LDL-chol and increased HDL-chol (Nabulsi, Folsom et al. 1993).

In contrast, men express higher levels of androgens, a hormone thought to exacerbate the risk of cardiovascular complications during the male life span (Wu and von Eckardstein 2003). Studies examining hyperandrogenaemic women have demonstrated that these women have a greater risk of developing cardiovascular complications, despite being young and of reproductive age (Wu and von Eckardstein, 2003). However, the question as to whether or not this increase in cardiovascular risk is due to excessive androgen levels, *per se*, is still unanswered. The few studies that have examined this association have been weak and have not demonstrated that androgens are significantly associated with increased cardiovascular risk (Cussons, Stuckey et al. 2006). Subsequently, it is believed that it is the presence of the associated metabolic risk factors, frequently present in PCOS, such as IR, dyslipidaemia and obesity that may be more important (Wild 1995).

1.3.5.6 Surrogate Markers of Atherosclerosis in Polycystic Ovary Syndrome

Numerous studies have investigated surrogate markers of atherosclerosis in PCOS. Carotid IMT has been positively associated with the risk of strokes and myocardial infarction (Bots, Hoes et al. 1997) and studies have shown that women with PCOS

have an increased carotid IMT in both older (Guzick, Talbott et al. 1996; Talbott, Guzick et al. 2000) and younger (Lakhani, Hardiman et al. 2004; Orio, Palomba et al. 2004; Vryonidou, Papatheodorou et al. 2005; Vural, Caliskan et al. 2005; Luque-Ramirez, Mendieta-Azcona et al. 2007) age groups. Another surrogate marker of atherosclerosis is coronary artery calcification (CAC) (Christian, Dumesic et al. 2003; Talbott, Zborowski et al. 2004) which reflects the plaque burden in these arteries (Detrano, Wong et al. 1997; Valabhji and Elkeles 2002). Both carotid IMT and CAC measurements utilises techniques that are non-invasive, although the latter would involve radiation exposure. Endothelial dysfunction, as assessed by flow-mediated dilatation (FMD) and arterial stiffness, as assessed by pulse wave velocity (PWV) has also been implicated in the pathogenesis of atherosclerosis and have also been shown to be impaired in women with PCOS (Orio, Palomba et al. 2004; Meyer, McGrath et al. 2005; Meyer, McGrath et al. 2005; Carmina, Orio et al. 2006).

Some of these studies suggested that these abnormalities are explained mainly by IR and/or obesity (Christian, Dumesic et al. 2003; Carmina, Orio et al. 2006) but others have implied that the derangements are caused by hyperandrogenism and/or PCOS status (Talbott, Guzick et al. 2000; Luque-Ramirez, Mendieta-Azcona et al. 2007). Clearly further research is required in this area to investigate the determinants of increased cardiovascular burden.

1.3.6 Cardiovascular Events in Polycystic Ovary Syndrome

Given the challenges with interpreting epidemiological studies which have used different criteria for the diagnosis of PCOS, evidence for increased CVD morbidity and mortality is inconclusive, yet suggestive. Initial studies suggested an increased risk in cerebrovascular (Wild, Pierpoint et al. 2000) but not coronary artery disease (Pierpoint, McKeigue et al. 1998; Wild, Pierpoint et al. 2000). A more recent study – the Women's Ischemia Syndrome Evaluation (WISE) study – showed a 1.7-fold increased risk of finding multivessel CVD in postmenopausal women who had

irregular menses and elevated androgen measurements, suggestive of the diagnosis of premenopausal PCOS (Shaw, Bairey Merz et al. 2008). The increased risk correlated with several factors including free testosterone. Higher cerebrovascular events were noted also in this study. Conversely, in the Nurses' Health Study, the incidence of cardiovascular disease was significantly increased in women with irregular menses, whilst that of cerebrovascular study was non-significantly increased (Solomon, Hu et al. 2002).

In summary, the present epidemiological data suggest more frequent CVD in PCOS, especially those with irregular menses and obesity, probably mediated through increased adiposity and perhaps interacting with hyperandrogenism.

1.4 THE METABOLIC SYNDROME (MetS)

1.4.1 Introduction

In 1988, Gerald M Reaven highlighted the existence of a syndrome due to the escalating incidence of several metabolic disorders in unison, including glucose intolerance, hyperinsulinaemia and hypertriglyceridaemia (Reaven 1988). The then unknown syndrome, soon referred to as Syndrome X was subsequently called the Metabolic Syndrome (MetS) because it was associated with various metabolic abnormalities and found to be associated with an increased risk of CVD. Various other names were suggested: Insulin Resistance Syndrome, Cardiometabolic Syndrome and Dysmetabolic syndrome (Reaven 2004; Sarafidis and Nilsson 2006). Currently, the MetS is still the most frequently used name and it is thought to arise due to the presence of IR and obesity mainly, and accompanied by glucose intolerance, hypertension and dyslipidaemia although there are slight international variations as to its exact definition (Assmann, Guerra et al. 2007).

It has also been hypothesised that the MetS is also characterised by the presence of several other metabolic abnormalities including pro-inflammatory and pro-thrombotic states as illustrated in Figure 1-13. Increased inflammatory markers such as CRP (Ridker, Hennekens et al. 2000) and TNF-α (Yudkin, Juhan-Vague et al. 2004), oxidative stress (Keaney, Larson et al. 2003), endothelial dysfunction (Suzuki, Hirata et al. 2008) and hypercoagulability (Nieuwdorp, Stroes et al. 2005) have all been reported in MetS.

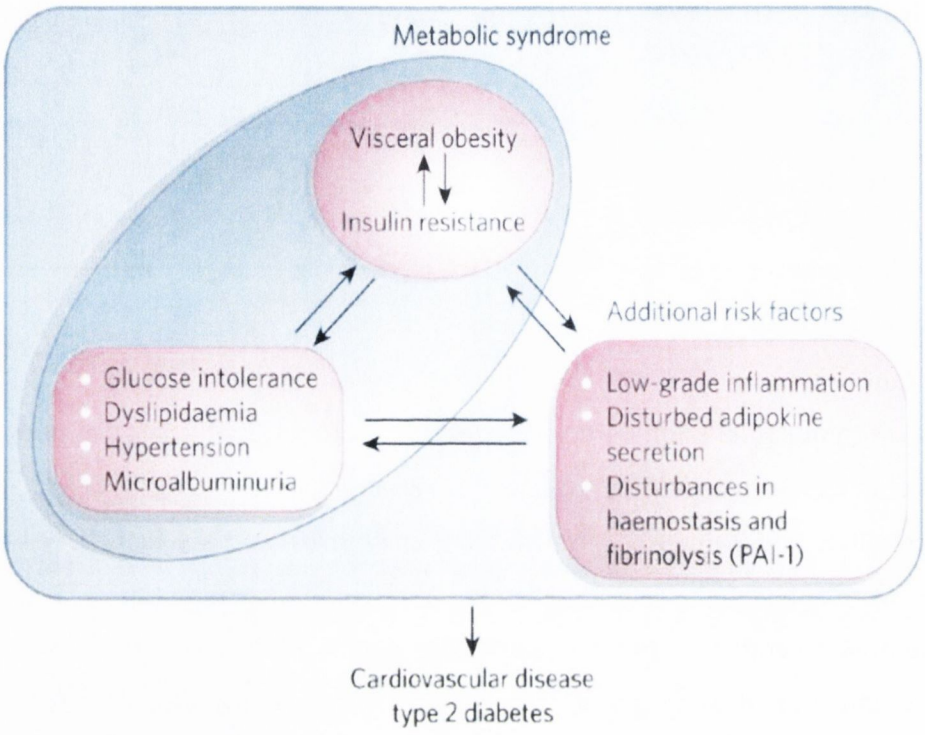

Figure 1-13: The inter-relationship between various components of the metabolic syndrome.

1.4.2 Prevalence

Similar to PCOS, the prevalence of the MetS will depend on the definition applied. Definitions vary considerably and IR is not necessarily the core criterion. Making IR a mandatory requirement for the diagnosis of the MetS is based on results from animal studies (Shafrir 1996; Zimmet, Boyko et al. 1999)), and from human metabolic studies (Karter, Mayer-Davis et al. 1996; Howard, Mayer-Davis et al. 1998; Hanley, Wagenknecht et al. 2003) which suggest that components of the MetS are almost always found in IR subjects.

Whilst the various definitions yield different estimates of the prevalence of the MetS, the WHO and the NCEP-ATPIII criteria have been shown to correlate strongly with being able to identify people at risk of CVD (Eckel, Grundy et al. 2005). Also, despite

the differences in criteria, it is generally well accepted that the prevalence of the MetS is increasing significantly (Ford 2003). Also, similar to that of T2DM, the risk of developing the MetS increases with age and are higher in certain ethnic groups such as Pima Indians (Royer, Castelo-Branco et al. 2007).

1.4.3 Definition & Diagnostic Criteria

Various diagnostic criteria have been proposed in order to correctly confirm the presence of MetS:

1. World Health Organisation (WHO) criteria (1999)
2. European Group for the study of Insulin Resistance (EGIR) criteria (1999)
3. Third Report of the National Cholesterol Education Programme Adult Treatment Panel III (NCEP-ATPIII) criteria (2001)
4. International Diabetes Foundation (IDF) criteria (2005)

1.4.3.1 World Health Organisation (WHO) criteria (1999)

The WHO criteria for MetS is based on using an OGTT to detect IR individuals (Alberti and Zimmet 1998). An abnormal OGTT result is central to the diagnosis and 2 of the following criteria are required:

1. Increased arterial blood pressure **≥140/90** mmHg
2. Increased plasma TG **≥1.7** mmol/L (150 mg/dL) and/or low HDL cholesterol <0.9 mmol/L (35 mg/dL) in men; <1.0 mmol/L (39 mg/dL) in women
3. Central obesity waist to hip ratio (WHR) >0.90 in males, >0.85 in females and/or BMI >30 kg/m^2
4. Microalbuminuria i.e. **urinary albumin excretion rate ≥20** µg/min or albumin:**creatinine ratio ≥30** mg/g

1.4.3.2 European Group for the study of Insulin Resistance (EGIR) criteria (1999)

Following the 1999 report by the WHO, the European Group for the Study of Insulin Resistance (EGIR) published their definition of the MetS (Balkau and Charles 1999). As with the WHO criteria, IR, defined as hyperinsulinaemia (upper 25 percentile of fasting insulin values among the non-diabetic population) was a central feature of the criteria. Two of the following criteria were also required:

1. Central obesity: waist circumference (WC) ≥94 cm in males, ≥80 cm in females
2. Increased TG **≥2.0** mmol/L and/or low HDL-cholesterol <1.0 mmol/L or treatment
3. Fasting **plasma glucose ≥6.1** and <7.0 mmol/L and/or 2 hour post OGTT plasma glucose of ≥7.8 and <11.1 mmol/L
4. Raised ar**terial pressure (≥140/90** mmHg) or treatment

Hence the WHR was replaced by WC and microalbuminuria was omitted, whilst DM and glucose intolerance could be included as a criterion separate to IR.

1.4.3.3 Third Report of the National Cholesterol Education Programme Adult Treatment Panel III (NCEP-ATPIII) criteria (2001)

In 2001, the American Heart Association (AHA) and National Heart, Lung and Blood Institute (NHLBI) in the USA published the NCEP-ATP III report (2001). It included only measurements available to routine practice, and measurement of IR *per se* was not included. 3 of the following 5 criteria are needed for diagnosis of the MetS:

1. Central obesity – waist circumference >102 cm in males, >88 cm in females
2. Increased plasma TG **≥1.7** mmol/L (150 mg/dL)
3. Decreased HDL cholesterol <1.03 mmol/L (40 mg/dL) in males; <1.29 mmol/L (50 mg/dL) in females
4. Blood pressure **≥130/≥85** mmHg
5. **Fasting glucose** ≥6.1 mmol/L (110 mg/dL)

1.4.3.4 International Diabetes Foundation (IDF) criteria (2005)

Finally, the International Diabetes Foundation (IDF) also published their criteria for diagnosing the MetS (http://www.idf.org/webdata/docs/Metac_syndrome_def.pdf). The essential criterion was central obesity as determined by the waist circumference and according to gender and ethnic specific reference ranges:

Table 1-6: Gender and Ethnic specific normal reference ranges for waist circumference:

Ethnicity	Upper limit of normal Waist Circumference for males (cm)	Upper limit of normal Waist Circumference for females (cm)
Europeans	94	80
USA	102	88
South Asians	90	80
Chinese	90	80
Japanese	85	90

A BMI of >30 kg/m^2 was an alternative to waist circumference for the fulfilment of the central obesity criteria. Two other criteria were required:

1. Raised triglycerides: >150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality
2. Reduced HDL cholesterol: <40 mg/dL (1.03 mmol/L) in males, <50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality
3. Raised blood pressure: systolic BP >130 or diastolic BP >85mmHg, or treatment of previously diagnosed hypertension
4. Raised fasting plasma glucose (FPG) >5.6 mmol/L (100 mg/dL), or previously diagnosed type 2 diabetes. If FPG >5.6 mmol/L (100 mg/dL), an OGTT is strongly recommended but not necessary to define presence of the Syndrome

The IDF definition was found to have a tendency towards higher prevalence rates of the MetS (Athyros, Ganotakis et al. 2005; Lawlor, Smith et al. 2006). It has also been suggested that the IDF criteria was inferior compared to the NCEP criteria in identifying the metabolically abnormal but non-obese groups known to be predisposed to T2DM and CVD (Yoon, Lee et al. 2007). However, recently, there has been a serious effort made by the different organisations / institutions / foundations to try to reach a consensus on the definition of the MetS (Alberti, Eckel et al. 2009). This last consensus mainly sought to reconcile the differences between the IDF and AHA/NHLBI – the IDF states that increased waist circumference is mandatory, and that gender and ethnic specific references be used, whilst the AHA/NHLBI states that an increased waist circumference should not be a mandatory criterion. The consensus is that waist circumference is no longer mandatory, but that gender and ethnic specific references should be used (Alberti, Eckel et al. 2009). The other 4 criteria are exactly the same for both definitions and remain so. This new agreement will pave the way for agreement between investigators who are studying the MetS.

1.4.4 Features of Metabolic Syndrome

1.4.4.1 Insulin Resistance

Although a measurement of IR is not included in any of the different criteria for MetS, it is clearly a central feature and is intricately related to all the other criteria. The aetiology and effects of IR has been discussed fully Section 1.2.

1.4.4.2 Central adiposity

Obesity is usually defined by the body mass index (BMI) which is determined by the ratio between weight in kilograms and the square of one's height in meters i.e. kg/m^2. The WHO published a report in 2000, advocating the international use of BMI to define obesity, overweight and normal weight: Normal weight is defined as having a

BMI of 18.5 – 24.9 kg/m^2, overweight is defined as a BMI of 25.0 – 29.9 kg/m^2, and obesity is a BMI of 30 kg/m^2 (2000). Whilst the BMI has still retained its usefulness as a clinical and research tool, it is now increasingly recognised that the waist to hip ratio (WHR) and waist circumference (WC) may be more predictive of cardiovascular disease (Yusuf, Hawken et al. 2005). WC is an anthropometric index of abdominal visceral AT and a strong predictor of the lipid and non-lipid abnormalities associated with MetS (Case, Jones et al. 2002). Hence, it has become the tool for diagnosing central adiposity in the later definitions of the MetS (Balkau and Charles 1999; 2001).

The pathophysiology behind the risk factor clustering is complex and unlikely to be caused by one single underlying factor, and has been discussed in full detail above in the section 0. Regardless, obesity is a major public health concern and is stated as the second leading cause of preventable death in the USA accounting for approximately 300,000 deaths per year (Aronne 2002). Obesity should be treated as a complex, multifactorial disease that involves both lifestyle (including behavioural, dietary and exercise) and pharmacological or surgical therapy, although the latter may be the most effective (Bray 2008).

1.4.4.3 Hypertension

Several studies over the last 2-3 decades have shown that patients with hypertension are resistant to insulin-stimulated glucose uptake and are hyperinsulinaemic compared to normotensive controls, independently of obesity (Pollare, Lithell et al. 1990; Reaven 1991; Reaven 2003). In addition, it has been shown that the prevalence of hypertriglyceridaemia and low levels of HDL-c is increased in hypertensive subjects (Pollare, Lithell et al. 1990). Hypertension in IR states is generally attributed to selective IR, chiefly by skeletal muscle with preservation of the renal and sympathetic nervous system sensitivity to insulin. Accordingly, hyperinsulinaemia resulting from this selective IR causes increased sympathetic neural output and renal sodium retention and may thereby increase peripheral vascular resistance (Reaven

1988; Rocchini 1991). Insulin is known to promote sodium retention in the kidneys, to stimulate sympathetic nervous system activity and may be a stimulant of the growth of smooth muscles cells (SMC) in the vessel walls (Reaven, Lithell et al. 1996). However, hyperinsulinaemia during euglycaemic clamp studies acutely stimulates both noradrenalin release and increases indirectly measured sympathetic neural activity with concomitant vasodilatation resulting in no change in blood pressure (Anderson, Balon et al. 1992). Regardless of the mechanism, hypertension being proven cardiovascular risk factor, rather than a marker of risk, needs to be adequately treated.

1.4.4.4 Dyslipidaemia

As mentioned previously, IR states are commonly associated with dyslipidaemia and MetS is no exception. Raised triglycerides, lowered HDL-chol and increased sdLDL, are all typically found in MetS. Since sdLDL is not usually measured in routine clinical practice, raised triglycerides and low HDL-c are included in the definitions for the Mets. However, as previously discussed, small, dense LDL-c particles are associated with increased risks for coronary heart disease (CHD) and atherosclerosis. Although the molecular mechanism(s) underlying ALP are far from being completely understood, it is generally recognised that the elevation in plasma TG and TRLs is central to the development of this phenotype. Indeed insulin resistance has been correlated with intracellular TG stores, and lipotoxicity – the detrimental effects of elevated lipids – has been implicated in β-cell failure and the development of T2DM (Ziegler, Quilliot et al. 2001). Insulin controls hepatic sterol regulatory element binding protein (SREBP) expression, which is a key transcription factor able to activate enzymes such as fatty acid synthase (FAS) (Bennett, Lopez et al. 1995) and 3-hydroxy-3-methly-glutaryl-coenzyme-A (HMG-CoA) reductase (Brown and Goldstein 1997). Insulin enhances SREBP expression in all three major insulin target tissues, liver, adipose tissue and skeletal muscle (Kim, Sarraf et al. 1998; Foretz, Guichard et al. 1999; Guillet-Deniau, Mieulet et al. 2002).

1.4.4.5 Glucose Intolerance

In IR states, the biological response to insulin is impaired (Yalow and Berson 1970; Savage, Petersen et al. 2005). Greater insulin secretion is therefore required to achieve a normal response resulting in hyperinsulinaemia which is initially able to maintain normoglycaemia in the early stages of IR. However, the subsequent and almost inevitable decline in β-cell function results in hyperglycaemia, prediabetes and T2DM (Prentki and Nolan 2006). Studies confirm that IR precedes the development of hyperglycaemia in subjects that eventually develop T2DM (Morrison, Glueck et al. 2008). As mentioned previously, IR and impairments in insulin signalling are now recognised key factors in the complex pathophysiological processes leading to diseases such as MetS, dyslipidaemia, hypertension and atherosclerosis (Eriksson 2007). Therefore MetS patients often have hyperglycaemia or even overt T2DM and hence their inclusion in all 4 definitions of the MetS.

1.4.5 Cardiovascular Risk in Metabolic Syndrome

Clearly, IR is a central component of the MetS, and is a cardiovascular risk as discussed above. The basic cellular/molecular cause of IR not only promotes inflammation and atherogenesis, but also leads to and/or aggravates other components of the MetS (Bajaj and Defronzo 2003), which are themselves cardiovascular risks (Reaven 1988; Miranda, DeFronzo et al. 2005). This is similar to patients with even lean T2DM or obesity without diabetes, who are also found to have increased IR and manifest features of MetS such as hypertension and dyslipidaemia (Reaven 1988; Stamler, Vaccaro et al. 1993; Miranda, DeFronzo et al. 2005; Rana, Nieuwdorp et al. 2007). As already described, hypertension is common in patients with IR, and patients with **"essential hypertension" are frequently found to have** IR (Ferrannini, Buzzigoli et al. 1987; DeFronzo and Ferrannini 1991; Reaven 2003; Reaven 2003). The glycogen synthesis pathway has been implicated in the development of hypertension in IR (Ferrannini, Buzzigoli et al. 1987; DeFronzo and Ferrannini 1991; Reaven 2003; Reaven 2003). Dyslipidaemia is also associated with IR (DeFronzo and Ferrannini 1991; Galvan, Santoro et al. 1993; Sheu, Shieh et al.

1993; Jeppesen, Hollenbeck et al. 1995) with hypertriglyceridaemia being more prevalent than hypercholesterolaemia (Stamler, Vaccaro et al. 1993). However, LDL-cholesterol, if elevated, synergistically accelerates atherosclerosis (Howard, Robbins et al. 2000).

In summary, each component of the MetS is related to IR and IR, as well as the individual components of the MetS are cardiovascular risks which are present even though the individual may not have diabetes.

Chapter 2

Materials and Methods

"Give me a lever long enough and a fulcrum on which to place it, and I shall move the world."

Archimedes

2.1 SUBJECTS

2.1.1 PCOS subjects

PCOS was defined according to the NIH criteria as women having:

1. Chronic oligomenorrhoea (fewer than nine menstrual periods per year) and
2. Clinical and/or biochemical evidence of hyperandrogenism, in the absence of other disorders causing the same phenotype (Zawadzki and Dunaif 1992). Clinical criteria included hirsutism with a Ferriman Galwey score greater than 9, acne or male pattern alopecia; biochemical criteria included total testosterone, FAI, androstenedione, or DHEAS greater than the laboratory reference range.

Other inclusion criteria included:

1. Age 18-45 years
2. BMI 20-40 kg/m^2
3. Caucasians
4. Medications such as anxiolytics and antidepressants were allowed provided subjects were on stable doses prior to and throughout the study.
5. Regular consumers of alcohol, which is not excessive as defined by elevated liver enzymes (AST and ALT)
6. Smokers and non-smokers

Exclusion criteria include acute and chronic medical conditions and medications that might affect inflammatory markers, lipid profiles and glucose homeostasis. A full list of exclusion criteria is included in Appendix 1.

Clinical investigations were within 10 days of a spontaneous menstrual bleed done or after exclusion of ovulation as determined by a normal LH level. PCOS subjects were recruited from the general endocrine and reproductive endocrine clinics in AMNCH, Tallaght.

2.1.2 Control subjects for PCOS studies

The control group was comprised of normal volunteers on no medications recruited from the general population. All normal subjects were eumenorrhoeic with testosterone levels within the normal female range and were studied in the follicular phase of the menstrual cycle. Exclusion criteria were age <18y or >45y; peri- or post-menopausal status (with normal FSH and LH values); pregnancy or planned pregnancy; concurrent illness that may influence results. Clinical investigations were performed within 10 days of a normal regular menstrual bleed. Subjects were recruited by local advertisement. Prevalence of smoking, weekly alcohol intake were matched in both groups.

2.1.3 Metabolic Syndrome subjects – cohort from LIPGENE – Human Dietary Intervention Study

The analysis on subjects with MetS was part of the European Union (EU) Framework 6 Integrated Project LIPGENE – **"Diet, genomics and the metabolic s**yndrome: an integrated nutrition, agro-**food, social and economic analysis"** (Shaw, Tierney et al. 2009). 15,000 individuals were screened over the telephone using a volunteer suitability questionnaire which assessed dairy food consumption, fish consumption and willingness to consume sandwiches during the study, to ensure alteration of composition of fatty acids in the diets. 483 subjects with the MetS participated in the 8 research centres throughout the EU. (See appendix 2.2 for a list of the 8 EU partners.)

Confirmation of diagnosis of MetS was based on the NCEP-ATP III criteria. 3 of the following 5 criteria were needed for diagnosis of MetS:

1. Central obesity – waist circumference >102 cm in males, >88 cm in females
2. Increased plasma TG **≥1.7** mmol/L (150 mg/dL)
3. Decreased HDL cholesterol <1.03 mmol/L (40 mg/dL) in males; <1.29 mmol/L (50 mg/dL) in females
4. Blood pressure **≥130/≥85** mmHg
5. **Fasting glucose** ≥6.1 mmol/L (110 mg/dL)

Other inclusion criteria included:

1. Male or Female
2. Age 30-70 years
3. BMI 20-40 kg/m^2
4. Caucasians
5. Medications / nutritional supplements allowed, on condition that the subjects adhere to the same regimen during the intervention: anti-hypertensive medication (including beta-blockers), oral contraceptives, hormone replacement therapy, multi-vitamin supplements and other non-fatty acid based nutritional supplements (e.g. garlic, anti-oxidants, etc)
6. Regular consumers of alcohol, which is not excessive as defined by elevated liver enzymes (AST and ALT)
7. Smokers and non-smokers

Exclusion criteria include acute and chronic medical conditions and medications that might affect inflammatory markers, lipid profiles and glucose homeostasis.

2.2 ETHICS APPROVAL & INFORMED CONSENT

2.2.1 PCOS studies

The PCOS studies were approved by the Joint Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland). All subjects had given their written informed consent.

2.2.2 LIPGENE study

Joint Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland) granted approval for the study to be conducted for the TCD cohort. The study was also approved by the local ethics committees at each of the other 7 clinical intervention centres, all of which conformed to the Helsinki Declaration of 1975 (revised in 1983). All subjects gave written informed consent.

2.3 ASSESSMENT OF ANTHROPOMETRICAL PARAMETERS

2.3.1 Measurement of height and weight, calculation of BMI

All measurements were made in the fasted state prior to any further investigations. Weight was measured in duplicate to the nearest 0.1kg, using calibrated scales located in the diabetes day centre of the Adelaide and Meath Hospital, incorporating the National Children's Hospital (AMNCH), Dublin 24, Ireland. Subjects were weighed in light clothing after voiding, and were requested to remove shoes and any heavy items from their person. Height was measured in the upright position to the nearest 0.1cm using a Harpenden stadiometer. BMI was calculated using the equation:

$$\text{BMI (kg/m}^2\text{)} = \text{weight (kg)/height squared (m}^2\text{)}.$$

BMI is a very simple and convenient method of estimating excess adiposity. It has been advocated by the WHO as a measure of overweight and obesity, there are significant limitations to this. The most important and incorrect assumption is that %BF remains the same for individuals across a range of heights. However, lean and physically active individuals may often be overweight based on their BMI but in fact have a higher proportion of lean muscle mass which contributes to their weigth and hence BMI. Similarly, BMI does not take in to account body frame size. Furthermore, mathematically, the weight of a person should increase cubically in proportion to the height. However, since on average, a taller person have a slimmer build relative to their height, the average quotient has been estimated to be between 2 and 3, probably around 2.6 for an average Caucasian. Therefore in general, BMI tends to overestimate adiposity in lean individuals, and underestimate adiposity in overweight or obese individuals. Some studies have concluded that BMI is less useful in predicting CVD than WC (Czernichow, Kengne et al. 2011) but not all have agreed on these findings, especially in different ethnic groups (Qiao and Nyamdorj 2010). Despite these limitations, the BMI is still a widely used clinical measurement both in routine clinical practice and also in clinical research trials and studies.

2.3.2 Measurement of waist and hip circumferences, calculation of the waist:hip ratio (WHR)

Waist circumference (WC) was measured directly on the skin, and was taken as the mid-point between the supra iliac crest and lower ribs margin following a normal expiration. Hip circumference (HC) was taken as the largest diameter over the buttocks. Both measurements were taken in duplicate to the nearest 0.1cm and were made using a non-distensible flexible tape. WHR as a measure of central adiposity was calculated as:

waist circumference (cm)/ hip circumference (cm)

The reason for including the WC in measures of obesity is because it more accurately reflects central adiposity. Studies that showed a positive correlation between WC or WHR and health outcomes date back to the 1980s (Lapidus, Bengtsson et al. 1984; Larsson, Svardsudd et al. 1984). In fact, it has been suggested that WC is superior to BMI in predicting cardiovascular risk (Yusuf, Hawken et al. 2005), but not all studies agree with this (Gelber, Gaziano et al. 2008). Currently, only the WHO (1999) has continued to allow the use of BMI to be one of the criteria for the MetS, whereas the EGIR (1999) and NCEP-ATEP III (2001) guidelines use only WC as a criterion for central obesity without referring to BMI. The IDF has stated that the WC, rather than BMI, is the core feature of the MetS. WC is a simple and convenient measurement. It is particularly useful in persons whose mobility is impeded and in whom it may be difficult to transfer on to a set of weighing scales (sitting or standing). WC is also easier to perform than WHR. The main drawback is accuracy and reproducibility but this can be overcome if repeated measures are taken and an average is obtained.

2.3.3 Bioelectrical impedance analysis (BIA) protocol

BIA was undertaken on all subjects using a body fat analyser bioimpedance analysis using the Bodystat 1500 system (Bodystat Ltd, UK). The subject was placed in a

supine position with arms comfortably abducted from the body at a 15° angle, and legs comfortably separated. Two current-injection electrodes were placed at the right hand and foot on the dorsal surfaces proximal to the metacarpal-phalangeal and metatarsal-phalangeal joints, respectively. The centres of two voltage-detector electrodes were placed on the midline between the prominent ends of the right radius and ulna of the wrist, and midline between the medial and lateral maleoli of the right ankle. The black current-injection and red voltage detector electrodes were at least 5cm apart, respectively. The black current-injection lead alligator clips and the red voltage-detector lead alligator clips were connected to the electrodes placed on the right hand and foot and right wrist and ankle, respectively. The results are expressed as percentage of total body mass.

2.3.4 Assessment of systolic (SysBP) and diastolic blood pressure (DiasBP)

Blood pressure measurements were recorded in duplicate in the seated position after a five-minute rest, using an appropriately sized cuff and an automated blood pressure analyser.

2.4 SURROGATE MEASURES OF INSULIN SENSITIVITY AND INSULIN RESISTANCE

2.4.1 Homeostasis model assessment – Insulin Resistance ($HOMA_{IR}$)

$HOMA_{IR}$ was calculated as a surrogate marker of insulin resistance from fasting glucose and insulin concentrations as previously described (Matthews, Hosker et al. 1985). $HOMA_{IR}$ was calculated using the following formula:

$$\frac{\text{fasting glucose (mmol/L) x fasting insulin (}\mu\text{U/mL)}}{22.5}$$

2.4.2 Frequently sampled intravenous glucose tolerance test (fsIVGTT)

Subjects attended the Clinical Investigation Unit after a 12-hour overnight fast and were requested to refrain from vigorous exercise and alcohol on the day prior to their fsIVGTT. On the morning of the fsIVGTT one cannula was inserted into the antecubital veins of both forearms. A fasting blood sample was taken from cannula 1. Then a bolus of 50% glucose solution (0.3g/kg body weight (BW)) was infused into cannula 2 over a 1-minute period followed by 20ml of saline. Twenty minutes later, a dose of insulin (0.03U/kg BW) was infused into the same cannula (cannula 2), which was then removed at 30 minutes after initiation of glucose administration. Meanwhile, blood was sampled through cannula 1 at frequent intervals over a 3-hour period (-5, 0, 2, 4, 8, 19, 22, 30, 40, 50, 70, 90 and 180 minutes after the start of the glucose injection). A total volume of 6mls of blood was collected at each time point (2mls into a fluoride oxalate tube for subsequent glucose analysis and 4mls into lithium heparin tube for subsequent analysis of insulin). Blood samples were spun at 3000rpm for 10 minutes after which the plasma was aliquoted and stored at -80°C until required for future analysis. Insulin sensitivity (S_I), glucose effectiveness (S_G) – the effect of glucose to enhance its own disposal), acute insulin response (AIR_G) and disposition index (DI) – the ability of beta-cells to compensate for changes in IR, were estimated using the MINMOD computer program (version 3.0, copyright R.N. Bergman).

2.5 DESIGN OF PHYSIOLOGICAL TEST MEAL

Following a 12 hour overnight fast, subjects came to the Clinical Investigation Unit for blood sampling and a high calorific meal. The fat meal was designed and analysed by a qualified dietician. It consisted of 948kcal. 48% of the total calories was derived from fat (20% saturated, 16% monounsaturated, 8% polyunsaturated). 36% & 15% of the calories were derived from carbohydrate and protein respectively. The meal was ingested within 20 minutes after baseline bloods. Bloods were taken at 2, 4, 6 and 8 hours after ingestion of the meal. Subjects were advised to restrict their physical activity to the minimum and were permitted to drink up to 250mls of water. The physical composition of the meal is included in Appendix 9.1.4

2.6 BLOOD SAMPLING

2.6.1 Collection of blood samples

Subjects were cannulated following minimal compression with a tourniquet by the author or another clinical researcher. Vaccutaniners® obtained from Becton & Dickinson (Oxford, UK) were used during the course of the study for preparation of blood samples for analysis of different biomarkers, as detailed in Table 2-1.

Table 2-1: Vaccutainers® used for the preparation of blood samples

Tube	Colour	Analyte
Serum Separators	**Red**	Adipokines, inflammatory markers, **hormones, apo's**
K3-EDTA	**Purple**	Plasma FAs, NEFA, full blood count
Fluoride Oxalate	**Grey**	Plasma glucose
Lithium Heparin	**Green**	Plasma lipid profile

2.6.2 Processing of blood samples

K3-EDTA, fluoride oxalate and lithium heparin Vaccutainers® were gently inverted 8-10 times to ensure the coagulant and blood sample was thoroughly mixed prior to centrifugation. Serum separator tubes were allowed to clot for 25 minutes prior to centrifugation. Centrifugation was performed at 3000 rotations per minute (rpm) for 10min at 4°C. Plasma and serum were removed and aliquoted appropriately. Samples for later analyses were stored at -80°C until further analysis. Samples for analysis by the team in the clinical chemistry laboratory in AMNCH hospital were sent immediately to the lab with the appropriate patient details.

2.7 PLASMA BIOCHEMICAL ANALYSIS

Unless stated otherwise, all the following analyses were performed in the Clinical Chemistry laboratory in AMNCH, Dublin 24. All chemicals were of at least analytical grade and obtained from the Sigma Chemical, Ireland, unless stated otherwise. All coefficients of variation (CV) were < 5% unless stated otherwise.

2.7.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a technique which allows the detection of the presence of antibodies or antigens in a sample. Sandwich ELISAs were used extensively in this thesis to assess the concentrations of various biomarkers in plasma. ELISA employs monoclonal antibodies to detect the presence of a particular antigen in a sample. A fixed amount of specific monoclonal antibody is adsorbed onto the surface of a 96-well microtitre plate. Serum is added and if antigen is present, it will bind to the antibody attached to the base of the plate. Excess serum is removed and the plate is washed gently with PBS/0.05% Tween to ensure the removal of all unbound substances, leaving only serum antigen bound to the specific capture antibody bound to the plate. A biotinylated monoclonal detection antibody is then added and the plate is incubated for a defined period of time. This detection antibody will bind to any immobilised antigen captured during the first incubation period. Excess unbound antibody is removed by washing. Streptavidin conjugated to horseradish-peroxidase (HRP) is then added. Streptavidin binds to the biotin component of the detection antibody. This HRP-conjugated antibody is considered a reporter antibody as it will result in a colour change when it interacts with an appropriate substrate. Following the removal of unbound HRP-conjugated antibody and thorough washing of the plate, a substrate solution is added. TMB (tetra-methyl benzidine) is the most commonly used substrate for this purpose and will effect a colour change (blue) when it interacts with HRP. The colour change indicates the presence of enzyme labelled antibody and bound antigen, indicating the presence of the antigen of interest in the sample, with the intensity of colour formed proportional to the amount of antigen in the sample. The reaction is stopped, generally by the

addition of a strong acid such as HCL or H_2SO_4 to the plate. This stabilises the reaction allowing the plate to be read accurately using spectrophotometry at a specified optical density in a micro-plate reader. The concentration of a given antigen within a sample can be determined by comparing the absorbance value of the sample to that of a linear standard curve, generated from a stock solution of known concentration, generally provided as part of the ELISA kit. Internal controls of known concentrations can be used to test the linearity of the standard curve.

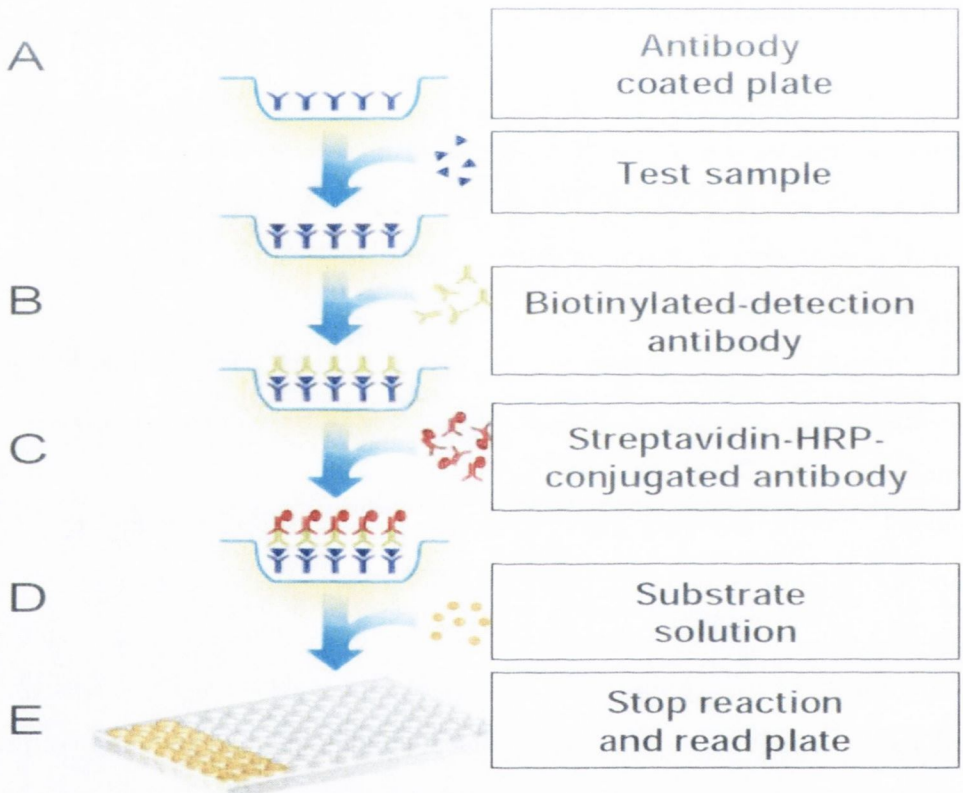

Figure 2-1: Workflow of an ELISA assay. Test sample is added to a microtitre plate coated with specific antibody (A), biotinylated detection antibody is added and will bind to captured antigen in the test sample (B). Streptavidin conjugated to HRP is added and the streptavidin component will bind with the biotinylated detection antibody (C). Substrate solution is added which will interact with the bound HRP and effect colour change (D). Stopping the reaction will allow the intensity of the colour change to be read accurately by spectrophotometry (E). Image adapted from www.panoramics.com

2.7.2 Glucose

Glucose (Roche Catalogue No. 11876899 216) analysis was performed on the Roche Hitachi MODULAR P® autoanalyser. All reagents and calibration material was provided by Roche Diagnostics Ltd. Quality control material was supplied by Technopath™, (Multichem 1, 2 and 3). The principle of the assay is the enzyme hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate by ATP (adenosine triphosphate). Glucose-6-phosphate dehydrogenase oxidises glucose-6-phosphate in the presence of NADP (nicotinamide adenine dinucleotide phosphate) to gluconate-6-phosphate. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and can be measured photometrically at 340nm (http://cobas-rocheco.uk/site/productcatalogue.aspx).

2.7.3 Glycosylated Haemoglobin (HbA1c)

HbA1c measurement was performed on the A1c 2.2 Plus Glycohemoglobin® (Tosoh Medics) Analyzer. Using principles of high performance liquid chromatography (HPLC), the analyser employs a cation exchange column and elution in a gradient with three fluid steps created by buffers with different salt concentrations. The analyser separates the haemoglobin components and measures them in six fractions, as percentages of total along with the chromatogram and the result is also quoted in percentages. (http://www.diagnostics.us.tosohbioscience.com).

2.7.4 Lipid profile

Cholesterol, TG, and HDL-chol were measured by a standard enzymatic colourimetric method on the Roche P Module. Reagents and calibration material for cholesterol and TG analyses were provided by Roche Diagnostics Ltd UK. Quality control material was supplied by Technopath™ (Multichem 1, 2 and 3). LDL-chol was calculated by the Friedewald formula:

$$\text{LDL-Chol} = \text{T-Chol} - \text{HDL-Chol} - \text{TG}/5.$$

2.7.5 NEFA

NEFA (Roche Product code FA 113) analysis was performed on the Roche Hitachi MODULAR P® autoanalyser. All control, calibration and reagent material were supplied by Randox laboratories Ltd UK. The sample type used was EDTA (1.8mg/ml blood). The quantification of NEFA is a colorimetric analysis involving three enzymatic steps. The enzymes used are acyl CoA synthetase, acyl CoA oxidase and peroxidase. Final product absorbance is read at 550nm (http://www.randox.com).

2.7.6 ApoA-I, ApoA-II, ApoB

All apolipoprotein measurements were performed on the Nephlometer Dade Behring BN II®. All reagents and quality control material was supplied by Dade Behring Inc. The sample type used for analysis was serum. The principle of the assay is an antibody/antigen assay based on the use of antibodies to each apolipoprotein particle which results in the formation of immune complexes. These complexes scatter a beam of light passed through the sample. The concentration of the measured protein is proportional to the intensity of the scattered light. The result is evaluated by comparison with a standard of known concentration. Apolipoproteins measured were apo A-I (product code QUED), apo A-II (product code OQBA) and apo B (product code OSAN) (http://www.siemens.com/diagnostics). This was performed by Ms Jane Fogarty in AMNCH, Tallaght.

2.7.7 ApoC-II and apoC-III

Plasma apoC-II and apoC-III concentrations were measured by immuno-turbidimetry on a Hitatchi 911 system with commercial kits (Diasys, Bouffmont, France). This was only done for the LIPGENE study and the analysis was carried out by Ms. Olfa Helal (Inserm, France). The intra- and inter-assay CVs were < 3%.

2.7.8 ApoB$_{48}$

Analysis of apoB$_{48}$ concentrations in plasma was carried out using a standard sandwich ELISA (Vendor). This analysis was conducted with the assistance of Dr. Anne McGowan. Inter-assay CV was 14% and intra-assay CV was 9%.

2.7.8.1 Reagents and equipment

The Human ApoB$_{48}$ ELISA Kit (AKHB48, Gentaur BVBA corporation) is an ELISA kit for the measurement of human apoB$_{48}$ using an indirect sandwich assay technique (http://www.clonagen.com/clonagen). The kit includes an anti-apoB48 coated plate, standard human apoB$_{48}$, buffer solution, Biotin conjugated anti-apoB$_{48}$ antibody, peroxidise-conjugated avidin®, chromogenic substrate (TMB), reaction stopper (1M H_2SO_4) and concentrated washing solution. Other equipment required included an EL$_x$50 Auto Strip (BioTek® Instruments, Inc.) Plate washer, EL$_x$800 Universal Microplate reader (BioTek® Instruments, Inc.) and Gen5™ Microplate Data Collection and Analysis Software (BioTek® Instruments, Inc.) for reading the colour changes.

2.7.8.2 Sample type

Serum was used as recommended by the supplier. Samples were stored at -80C.

2.7.8.3 Assay procedure

The anti-apoB$_{48}$ coated plate is initially washed to remove buffer. The appropriately diluted samples are added to the plate and left to react for one hour. Samples were diluted 1:250 to ensure the concentration was not above the assay range. The plate is subsequently gently washed four times and biotin-conjugated anti-apoB$_{48}$ antibody is added. This binds to human apoB$_{48}$ bound to the anti-apoB$_{48}$ antibody immobilised on

the coated plate as shown in Figure 2-2. This reaction is stopped after 1 hour and the plate is washed again four times. Finally a peroxidase-conjugated avidin is then added to the plate. The avidin and biotin readily bind to ensure that the horseradish peroxidase enzyme is immobilised on the plate. After washing, the chromogenic substrate tetramethyl benzidine (TMB) is added. This forms a blue colour on reaction with horseradish peroxidase enzyme. The reaction stopper (1M H_2SO_4) is added, resulting in a yellow colour formation which is proportional to substrate concentration.

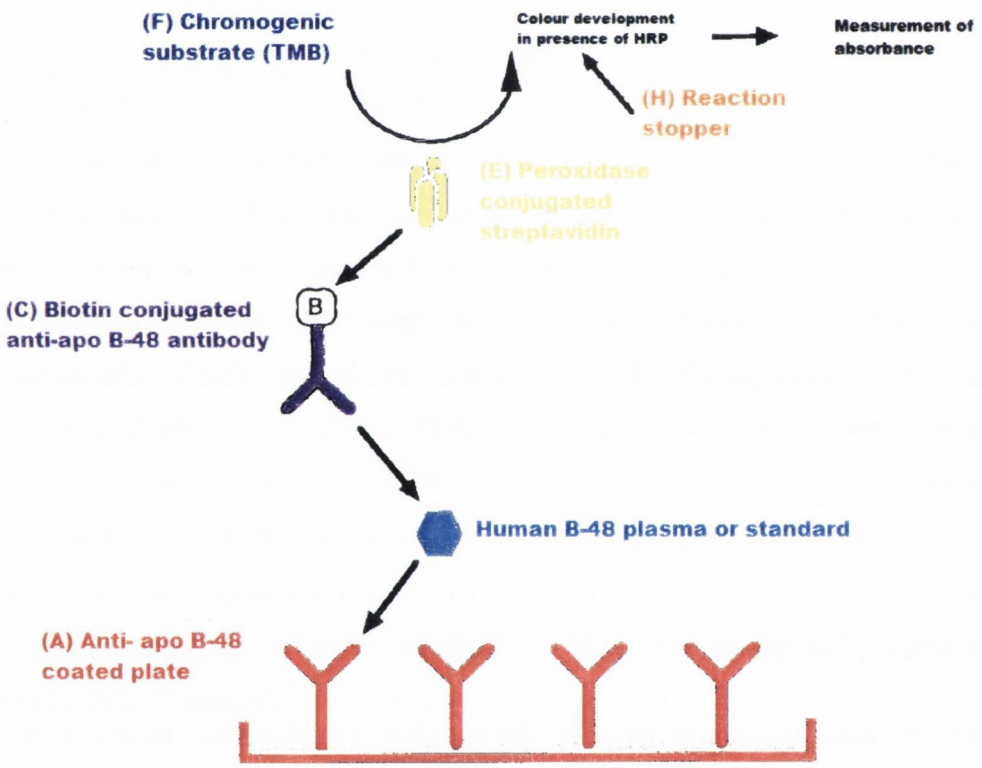

Figure 2-2: Outline of indirect sandwich assay technique used in the Human ApoB$_{48}$ ELISA Kit.

2.7.8.4 Sample concentration

A standard curve of concentration versus absorbance from a standard material was constructed from a stock standard supplied with the kit in accordance with manufacturer's instructions. The plate was read at an absorbance of 450 nm with a

minus absorbance of 620 nm. The standard curve is used to calculate the concentration of $apoB_{48}$ for a sample from the sample absorbance. The result is then multiplied by the dilution factor. Samples were diluted 1:250 to ensure the concentration was not above the upper limit of the range of the assay. (http://www.clonagen.com/clonagen/human_apo_b_48/).

2.7.8.5 Assay performance

As a confirmatory check on assay performance as outlined by the manufacturer, samples were analysed in duplicate initially to determine intra assay variation. In addition pooled frozen samples were analysed with each assay batch in order to determine inter batch variation. To determine assay specificity varying concentrations of $apoB_{100}$ were added to pooled serum and analysed.

2.7.9 Isolation of Triglyceride-Rich Lipoprotein fraction (TRL)

Isolation of TRL is based on the density difference between TRL and TG-poor lipoprotein fractions. The technique is based on ultracentrifugation and the various densities of each fraction which have been well characterised. TRLs have a density of < 1.006 kg/L and therefore to separate them a density solution is required. This was done only in the LIPGENE study and was performed by Dr. Jolene McMonagle.

2.7.9.1 Preparation of sodium chloride 1.006 kg/L density solution containing 10 µmol/L EDTA

11g of NaCl is to be dissolved in 995ml of distilled water, bringing up the total volume to 1000ml. 100µL of 0.1M EDTA is added. A Paar densitometer is used to verify the density. The solution is stored at 4°C.

2.7.9.2 Ultracentrifugation

1. Empty polycarbonate tubes which are to be used in the rotor are labelled, weighed and recorded.
2. Pipette 3ml of plasma into a polycarbonate tube, and then carefully pipette 3ml of 1.006 NaCl density solution over the plasma.
3. A type 70.1 Ti rotor is used in this method. Tubes opposite each other in the rotor should be of equal weights as much as possible, and certainly no more than 0.1g of each other to ensure the rotor is balanced. The position of each tube in the rotor is recorded.
4. The rotor is carefully placed in the centrifuge and is spun at 50,000 rotations per minute for 530min at 4°C using maximum acceleration and deceleration.
5. After the spin, the rotor is carefully taken out of the ultracentrifuge and each tube is carefully taken out of the rotor.
6. The upper 1.5ml of the solution is the TRL fraction. A glass Pasteur pipette is used to carefully extract the upper layer which is then placed into a pre-weighed graduated tube. The graduated tube with the TRL is weighed again and the weight of TRL noted.
7. The TRL is aliquoted into different tubes.
8. The TRL concentrations are to be corrected back to their respective concentration in plasma by multiplying it with the weight of the TRL / 3 (ml).

2.7.10 Hormones

2.7.10.1 Insulin

Insulin and C-peptide were measured in a referral laboratory (Central Pathology Laboratory, St James's Hospital Dublin). Measurements were carried out on the AutoDELFIA® Automatic Immunoassay System.

The principle of both assays is similar. It utilises a solid phase fluoroimmunometric assay based on a direct sandwich technique. Two monoclonal antibodies are involved. One is an immobilized antibody and the other a europium labelled antibody. Both are directed against different sites on the insulin or C-peptide molecule. Enhancement solution dissociates the europium ion from the antibody where they form fluorescent chelates with components of solution added. The fluorescence is proportional to concentration of insulin or C-peptide (http://www.perkinelmer.com).

2.7.10.2 Testosterone

Total testosterone was measured by electrochemiluminesence immunoassay on the Roche E Module. This was performed by the biochemistry laboratory in AMNCH.

2.7.10.3 SHBG and DHEAS

SHBG and DHEAS, were measured by standard chemiluminesence immunoassays on the DPC Immulite 2000. These analyses were conducted by the team in the Endocrine laboratory in St. James hospital, Dublin 8.

2.7.10.4 Androstenedione and 17-OHP

Androstenedione and 17-OHP were measured by radioimmuno-assay on the DPC Coat-A-Count. These analyses were conducted by the team in the Endocrine laboratory in St. James hospital, Dublin 8.

2.7.10.5 Free androgen index (FAI)

Concentrations of free and bioavailable testosterone more accurately reflect the level of bioactive testosterone than do total concentrations of this hormone. Testosterone

circulates in plasma either as free (2-3%) unbound hormone, tightly bound to SHBG, or loosely bound to non-specific protein such as albumin. To obtain a measure of bioavailable testosterone, we used the FAI, which was calculated by the formula:

FAI = testosterone (nmol/L) / SHBG (nmol/L) x 100.

2.7.10.6 Other hormones

Oestradiol (E2), progesterone, luteinising hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), free thyroxine (fT4), prolactin (PRL) and cortisol were all measured by chemiluminesence immunoassay on DPC Immulite 2500. These were performed by the biochemistry laboratory in AMNCH.

2.7.11 Adipokines

2.7.11.1 Total and oligomeric adiponectin

Concentrations of total- and oligomeric-adiponectin were determined by sandwich ELISA (ALPCO Diagnostics, NH, USA). This assay classifies adiponectin into four categories; total-adiponectin, HMW-adiponectin, MMW-adiponectin and LMW-adiponectin. Total- and HMW-adiponectin are assayed directly on the plate. MMW-adiponectin is an inferred value obtained by subtracting the concentration of HMW-adiponectin from the concentration of combined MMW + HMW-adiponectin which is assayed directly on the plate. LMW-adiponectin concentration is also an inferred value calculated by subtracting the combined concentration of MMW + HMW-adiponectin from total-adiponectin, both of which are assayed directly on the plate. Samples were treated with specific proteases selective for the LMW-adiponectin (to obtain the combined MMW + HMW-adiponectin), as well as for both the LMW- and the MMW-adiponectin oligomers (to obtain the HMW-adiponectin), prior to addition to the plate. Specific conditions as outlined by the manufacturer's instructions were

followed strictly and the absorbance of the plate read at 402nm. All samples were assayed in duplicate. Intra- and inter-assay CVs were 6.0% and 6.6%.

2.7.11.2 Leptin and resistin

Plasma levels of resistin and leptin were determined by ELISA using a commercially available kit (R&D Systems Inc, MN, USA) according to the manufacturer's instructions. Absorbance of the plate was read at 450nm. All samples were assayed in duplicate. Intra- and inter-assay CVs for resistin were 5.2% and 4.8% and that of leptin were 8.8% and 9.8%. Leptin analysis was carried out by Dr. Niamh Phelan. Resistin analysis was carried out by Dr. Anna-Louise O'Connor.

2.7.12 Markers of Inflammation

hsCRP was measured in plasma on an automated ILab system in AMNCH, Tallaght. MPO, PAI-1, SAA and neopterin, were analysed using a Triturus® EIA Analyser (Grifols S.A. Barcelona, Spain) system in the Royal Victoria Hospital (RVH), Belfast. Both automated systems were pre-programmed with assay procedures, which subsequently performed all standard/sample dilutions, and all wash and incubation steps, according to the manufacturer's instructions for each kit. Wash and diluent buffers were prepared manually, according to manufacturer's instructions. The system software provided an automatic calculation of standard curves and sample readings, whilst factoring in dilution factors. TNF-α, IL-6, sICAM-1 and sVCAM-1 were measured as part of the LIPGENE study and measured in Trinity College Dublin by Dr. Jolene McMonagle. All CVs were < 5% unless stated otherwise.

2.7.12.1 High-sensitive C-reactive protein (hsCRP)

hsCRP was measured using a sandwich ELISA kit (BioCheck Inc, Burlingame, CA, U.S.A.). Specific conditions as outlined by the manufacturer's instructions were

followed strictly and the absorbance of the plate read at 450nm. All samples were assayed in duplicate. Intra- and inter-assay CVs were <10%.

2.7.12.2 Plasminogen Activator Inhibitor-1 (PAI-1)

PAI-1 was measured in plasma using PAI-1 sensitive ELISA kits, manufactured by Trinity Biotech (Wicklow, Ireland). The measurement of PAI-1 in plasma was relatively similar to that of other inflammatory markers, using a similar ELISA methodology. However, there were some modifications. In summary, there are two sets of wells, the A-wells and the N-wells. The A-well contained a monoclonal antibody against PAI-1, immobilised onto the well surface, as well as a soluble anti-PAI-1 antibody. The N-well contained the same monoclonal antibody against PAI-1 immobilised onto the well surface, as well as a soluble non-immune antibody. During sample incubation, PAI-1 quantitatively binds to the antibodies coated in the N-wells, but not in the A-wells. An anti-PAI-1 antibody, conjugated to horseradish peroxidise was then added to both wells. Similarly, a sandwich between the two antibodies was subsequently formed. After several incubation and washing steps, an Ortho-phenylenedianine (OPD)/hydrogen peroxide (H_2O_2) substrate was then added, subsequently converting the sample to a yellow colour. The difference in response between the A-well and the N-well was directly proportional to the levels of PAI-1 in the plasma sample. A standard curve was prepared from a PAI-1 standard (40 ng/mL), which was diluted to obtain 20, 10 and 0 ng/mL PAI-1 concentrations. This analysis was performed with the assistance of Dr. Sarah Blair in the RVH, Belfast.

2.7.12.3 Myeloperoxidase (MPO)

MPO was measured in plasma using MPO sensitive ELISA kits, manufactured by Biocheck Inc. (Foster City, California, USA). Analysis was performed in duplicate, in a 96-well plate (NUNC, Maxisorp microwell, NY). A standard curve was prepared from a stock solution of MPO provided by the manufacturer (40 ng/mL), which was diluted to obtain 20, 10, 5, 2.5 and 0 ng/mL MPO concentrations. The concentration of MPO in

each serum sample was expressed as ng/mL. This analysis was performed with the assistance of Dr. Sarah Blair in the RVH, Belfast.

2.7.12.4 Serum Amyloid A (SAA)

SAA was measured in serum using SAA sensitive ELISA kits, manufactured by Invitrogen (Camarillo, California, USA). The measurement of SAA in serum was similar to that of MPO, again using an ELISA methodology. A standard curve was prepared from a SAA standard (600 μg/mL), which was diluted to obtain 300, 150, 75, 37.5, 18.8 and 9.4 μg/mL SAA concentrations. The concentration of human SAA in each serum sample was expressed as μg/mL. This analysis was performed with the assistance of Dr. Sarah Blair in the RVH, Belfast.

2.7.12.5 Neopterin

Neopterin was measured in serum using neopterin sensitive ELISA kits, manufactured by IBL (Hamburg, Germany). The measurement of neopterin in serum was similar to that of MPO, using an ELISA methodology. A standard curve was prepared from standards that were supplied and contained 111, 37, 12, 4, 1.35 and 0 nmol/L neopterin concentrations. The concentration of neopterin in each serum sample was expressed as nmol/L. This analysis was performed with the assistance of Dr. Sarah Blair in the RVH, Belfast.

2.7.12.6 Tumour Necrosis Factor-alpha (TNF-α)

TNF-α kits were used (R&D Systems, MN, USA) that bind the TNF-α by the immobilized antibody. After incubation of the diluted specimens the plates were washed and any unbound substances were removed, following which an enzyme-linked polyclonal antibody was added. Following another wash a substrate solution was added and the colour developed. This kit used an amplification system in which

alkaline phosphate reaction provides a cofactor that activates a redox cycle leading to the formation of a coloured product. In this amplification system, alkaline phosphatase dephosphorylates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) which is the substrate, to reduce nicotinamide adenine dinucleotide (NADH). Subsequently, the NADH serves as a specific colour that activates a redox cycle driven by a secondary enzyme system consisting of alcohol dehydrogenase and diaphorase. Catalyzed by diaphorase, NADH reduces a tetrazolium salt (INT-violet or iodonitrotetrazolium violet) to produce an intensely coloured forazan dye and NAD+. Then NAD+ is reduced by ethanol in the alcohol dehydrogenase-catalyzed reaction, to regenerate NADH which can then re-enter the redox cycle. The rate of reduction of the tetrazolium salt and thus the amount of coloured product formed are directly proportional to the amount of TNF-α bound in the initial step. A high and low internal control was run with each plate. The optical density reading was set to 450nm and wavelength correction was set to 650nm (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK). This analysis was performed by Dr. Jolene McMonagle. The CVs were less than 5%.

2.7.12.7 Interleukin-6 (IL-6)

Human IL-6 ultrasensitive ELISA kits (Biosource International Inc, CA, USA) were used for the determination of IL-6 from plasma. A monoclonal antibody specific for human IL-6 was coated onto the wells of a microtitre plate before purchase. Standards and controls, provided by Biosource were diluted and pipetted into the appropriate wells along as well as the human plasma. During the first incubation, the human IL-6 antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for human IL-6 was added. During the second incubation the antibody binds to the immobilized human IL-6 captured during the first incubation. After removal of the excess second antibody, Streptavidin-Peroxidase enzyme was added. This binds to the biotinylated antibody to complete the four-member sandwich. After the third incubation and wash to remove the entire unbound enzyme, substrate solution was added, which was acted

upon by the bound enzyme to produce colour. The intensity of the colour produced was directly proportional to the concentration of human IL-6 present in the human plasma. The plate was read at 450nm (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK). This analysis was performed by Dr. Jolene McMonagle. The CVs were <10%.

2.7.12.8 Soluble Intracellular Adhesion Molecule-1 (sICAM-1) and Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)

Human sICAM-1 and sVCAM-1 (R&D Systems, Abington, UK) were measured using an assay that employs the quantikine sandwich ELISA technique. A monoclonal antibody specific for sICAM-1 and sVCAM-1 conjugated to horseradish-peroxide with preservatives was added to each well. Both internal control contained human serum. The sICAM-1 standard contained recombinant human sICAM-1 along with preservatives and blue dye whereas, sVCAM-1 standard contains recombinant human sVCAM-1 in a buffer with preservatives lyophilized. Any human sICAM-1 or sVCAM-1 present was sandwiched by the immobilised antibody and the enzyme linked monoclonal antibody specific for sICAM-1 and sVCAM-1. Following a wash any unbound substances and/or antibody-enzyme reagent were removed. For sICAM-1 substrate solution was directly added, this contains TBM. Whereas, for sVCAM-1, the substrate solution was prepared by adding colour A (stabilized hydrogen peroxide) and colour B (stabilised chromogen or tetramethybenzidine) at a ratio of 1:1, after which the colour was allowed to develop. For sICAM-1, stop solution was added containing acid solution and for sVCAM-1, 2N sulphuric acid was included. OD was **read immediately at 450nm and λ correction is 540nm.** This analysis was performed by Dr. Jolene McMonagle. The CVs were <9% for sICAM-1 and <5% for sVCAM-1.

2.7.13 Markers of Oxidative Stress

2.7.13.1 Total Oxidant Status (TOS)

The measurement of TOS was performed on serum samples using a modified technique based on the original method developed by Erel (Erel 2005). This method was based on a colorimetric assay, in which the oxidants present in a serum sample cause the oxidation of ferrous ions to ferric ions. The oxidation of ferrous ions subsequently causes a change in colour, which is measured spectrophotometrically at 560 nm. The change in colour is directly proportional to the levels of total oxidants present in a particular sample. A standard curve was prepared from a H_2O_2 standard (200 μM) and diluted appropriately to obtain 200, 100, 50, 25, 12.5, 6.25 and 0 μM H_2O_2 concentrations. Two reagents were prepared prior to the measurement of TOS. Both reagents were stable for at least 6 months at 4°C (Erel 2004).

2.7.13.1.1 TOS Reagent 1

This was prepared by dissolving 0.114 g of xylenol orange and 8.18 g of NaCl in 900 mL of 25 mM sulphuric acid (H_2SO_4). 100 mL of glycerol was added and subsequently stirred until a homogenous solution was achieved. Reagent 1 therefore comprised 150 μM xylenol orange, 140 μM NaCl and 1.35 m glycerol, with a pH of 1.75.

2.7.13.1.2 TOS Reagent 2

This was prepared by dissolving 1.96 g of ferrous ammonium sulphate and 3.17 g of o-dianisidine dihydrochloride in 1 L of 25 mM H_2SO_4. Reagent 2 therefore comprised 5 mM ferrous ammonium sulphate and 10 mM o-dianisidine dihydrochloride.

TOS levels were analysed in duplicate as follows:

1) 267 μL of reagent 1 was added to each well of a 96 well, non-UV plate (NUNC).

2) 41 μL of serum sample was then added to each well.

3) The plate was then shaken gently for 20 seconds on a plate shaker (Labsystems, Wellmix).

4) Absorbance was read at 560 nm in a 96 well automated plate reader (Molecular Devices Spectra Max 190), at 1 minute intervals for 5 minutes. This represented the sample blank.

5) 20 µL of reagent 2 was then added to each well.

6) Absorbance was read at 560 nm for a further 15 minutes, until the reaction reached a plateau phase.

7) An endpoint absorbance was taken upon completion of the reaction and data analysed using the SoftMax Pro Version 4.8, Molecular Devices Corp.

8) The results obtained were read against a standard curve and values were expressed as µmol H_2O_2 Equiv/L.

The inter-assay CV was 7.6%, whilst the intra-assay CV was 3.3%. This analysis was performed in the RVH, Belfast, by Dr. Sarah Blair.

2.7.13.2 Total Antioxidation Capacity (TAC)

The measurement of TAC was performed on serum samples using a modified technique based on the original method developed by Erel (Erel 2004). This method was based on a colorimetric assay, in which a 2,2'–azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\cdot+}$ radical) is reduced and subsequently bleached in colour when added to a sample containing antioxidants. This change in colour was measured spectrophotometrically at 660 nm. The bleaching rate is proportional to the concentration of antioxidants present in a sample, and is inversely related to the level of TAC in the sample. A standard curve was prepared from a Trolox standard (10 µM) and diluted to obtain 2.5, 2.0, 1.5, 1.0, 0.5, and 0 µM Trolox concentrations.

Two reagents were prepared prior to the measurement of TAC. Both reagents were stable for at least 6 months at 4°C (Erel 2004).

2.7.13.2.1 TAC Reagent 1

This was prepared by dissolving 32.8 g of sodium acetate (CH_3COONa) in 1 L of ddH_2O, (final concentration of CH_3COOH was 0.4 mol/L). The two solutions were used in a ratio of approximately 940 CH_3COONa: 60 CH_3COOH, to obtain a pH of 5.8.

2.7.13.2.2 TAC Reagent 2

This was prepared by dissolving 2.46 g of CH_3COONa in 1 L of ddH_2O (final concentration of CH_3COONa was 30 mmol/L). A second solution was prepared by adding 1.750 mL of reagent-grade glacial acetic acid in 1 L of ddH_2O (final concentration of CH_3COOH was 30 mmol/L). 75 mL of the CH_3COONa solution was then mixed with 925 mL of the CH_3COOH solution to obtain a pH of 3.6. 318 µL of 30 % H_2O_2 was then added to the acetic acid-sodium acetate buffer solution (final concentration of H_2O_2 was 2.8 mmol/L). 100 mL of this solution was then removed **and 0.549 g of 2,2'**–azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was added, allowed to dissolve and left to incubate at room temperature for 1 hour, until the characteristic deep green colour of the $ABTS^{\cdot+}$ was apparent.

TAC levels were analysed in duplicate as follows:

1) 200 µL of reagent 1 was added into each well of a 96 well, non UV plate.

2) 5 µL of serum sample was then added to each of the wells.

3) The plate was then shaken gently for 20 seconds on a plate shaker (Labsystems, Wellmix).

4) Absorbance was read at 660 nm in a 96 well automated plate reader (Molecular Devices Spectra Max 190), at 1 minute intervals for 5 minutes. This represented the sample blank.

5) 20 µL of reagent 2 was then added to each well.

6) Absorbance was read at 660 nm for a further 5 minutes, until the reaction reached a plateau phase.

7) An endpoint absorbance was taken upon completion of the reaction and data analysed using the SoftMax Pro Version 4.8, Molecular Devices Corp.

8) The results obtained were read against a standard curve and values were expressed as µmol Trolox Equiv/L.

The inter-assay CV was 2.1%, whilst the intra-assay CV was 1.3%. This analysis was performed in the RVH, Belfast by Dr. Sarah Blair.

2.7.14 Plasma fatty acid composition analysis

2.7.14.1 Extraction of fatty acids from plasma

Extraction of lipids from plasma was carried out using an adaptation of the method outlined by Folch and colleagues (Folch, Lees et al. 1957). **400µls of plasma was** transferred into 16ml borosilicate glass, screw-**capped tubes and 400µls of milliQ** water was added. Lipid extraction was achieved by the addition of solvents to **solubilise the lipid, namely 2mls of methanol and 1ml of chloroform. 40µls of 5nM** BHT (butylated hydroxytoluene) in ethanol was added. BHT is a lipophilic organic compound which behaves in a manner analogous to Vitamin E and hence has anti-oxidant abilities, primarily acting as a terminating agent to suppress auto-oxidation, and also preserve the integrity of the lipid fractions during extraction. **100µls of stock**

solution heptadecanoic acid (C17:0) was added to this initial mixture and acted as an internal standard.

Samples were vortexed for 1 minute and 1ml of milliQ water and 1ml of chloroform were immediately added. The tubes were closed and vortexed for a further 30 seconds and centrifuged in a Beckman Avanti G25 equipped with a JLA rotor. They were spun at 3000rpm for 15 minutes at 20°C. Infranatants (organic phase) which contained the lipid components of the sample, were carefully removed with a Pasteur glass pipette and transferred to new 16ml borosilicate glass tubes. The supernatants (aqueous phase), which contained the non-lipid components of the sample, were then supplemented with 2mls of chloroform to increase the efficiency of the extraction, and were vortexed for 30 seconds and centrifuged at 3000rpm for 15 minutes at 20°C. Infranatants were removed as previously indicated, pooled with the previous infranatants and dried under nitrogen. The dried samples were flushed with nitrogen prior to sealing to prevent lipid oxidation and stored at -20°C.

2.7.14.2 Transmethylation method

Free fatty acids often show adsorption and tailing on columns and quantitative results are not reliable, especially for the longer chain unsaturated fatty acids. To circumvent this problem, derivatisation of fatty acids, that is the preparation of non-reactive volatile fatty acid esters are required to increase the detection. The formation of methyl esters is one of the most commonly used derivates for fatty acid analysis by GC. Methyl esters have a lower molecular weight than other possible derivates and so will elute from the column at lower temperatures.

BF3 (Boron trifluoride)-Methanol is a convenient methanol catalyst system and when used in excess with heat, can quickly convert carboxylic acids to their methyl esters. To each tube containing the dried lipid extract, 1ml of methanol and 1ml of BF3-

methanol (14%) were added and vortexed for 1 minute. The tubes were placed in a boiling bath (100°C) for 40 minutes and then cooled at room temperature. The resulting methyl esters were then extracted by adding 2mls of hexane and 2mls of milliQ water, vortexed for 10 seconds and centrifuged at 2000rpm for 5 minutes at 10°C. Supernatants (hexane phase) containing the lipid fraction were collected. For optimisation of the extraction, 2mls of hexane was added to the lower phase, vortexed for 30 seconds and centrifuged at 2000rpm for 5 minutes. The supernatants were removed, pooled with the first supernatants, dried under nitrogen and stored at -20°C.

2.7.14.3 Gas chromatographic analysis of total plasma lipids

Samples were reconstituted with 50µls of hexane and fatty acid methyl esters (FAME) were analysed by gas chromatography (GC) on a Shimadzu GC 2010 (Shimadzu, Japan) using a fused silica capillary column Omegawax 250, (30 m x 0.25 mm inner **diameter, 0.25µm** film) (Supelco, Saint Quentin Fallavier, France).

The GC was equipped with a flame ionisation detector and GC solutions software (Shimadzu, Japan). Heptadecanoic acid (C17:0) was used as an internal standard. Helium was used as the carrier gas with pressure of 0.8 psig. Injector and flame ionisation detector temperatures were 250°C. The following temperature program was used: 215°C (43 minutes) and 10°C/minute to 260°C (2.5 minutes). Fatty acids were identified by comparing the relative retention times of plasma FAME with FAME standards (SUPELCO, Saint Quentin Fallavier, France). PUFA-2 (from Menhaden oil) and PUFA-3 (from an animal source), (both from Supelco), were included with each run to determine the relative retention times of the fatty acids. The retention time of C17:0 was obtained from the blank sample from each batch. Fatty acid mass was determined as a relative percentage of the total quantified fatty acids. Individual fatty acid types were determined as follows: SFA (C14:0, C16:0, C18:0), MUFA (C16:1, C18:1, C20:1), PUFA (*n*-3 + *n*-6 PUFAs), *n*-3 PUFAs (C18:3:3, C18:4, C20:4:3, C20:5,

C22:5, C22:6) and *n*-6 PUFAs (C18:2, C18:3:6, C20:3, C20:4:6, C22:4). The fatty acid masses were calculated based on the blank C17:0 values for each batch. The efficiency of the experiment was also calculated from the C17:0 values. Samples with an extraction efficiency of less than 30% were repeated. To ensure consistency between batches, a standard serum sample, was extracted as part of each batch. Batches were considered to be acceptable if the standard serum had a variance below 10%. The analysis was done by Dr. Annalouise O'Connor.

2.7.15 Quantimetrix Lipoprint™ system

LDL and HDL sub-fractions were analysed using the Quantimetrix Lipoprint™ system. The system consists of the following components: Kit of 100 Lipoprint™ Gels (catalogue number 48-7002, catalogue number 48-9002): gel preparation rack, photopolymerization light, electrophoresis chamber(s), power supply, digital scanner or densitometer unit, computer and colour printer. Results are analysed and reported using the Lipoprint™ patient risk reporting software.



Figure 2-3: Quantimetrix LipoprintTM instrumentation

The technique is based on polyacrylamide gel electrophoresis and can resolve up 12 bands when analysing for LDL sub-fractions: VLDL, IDL-A, IDL-B, IDL-C, and 7 LDL subfractions including 2 large LDL subfractions (1-2) and 5 small LDL subfractions

(3-7) (Figure 2-4 and Figure 2-5). LDL phenotype is determined based on the absence or presence of sdLDL (Hoefner, Hodel et al. 2001).

The numbers on the top row under the horizontal axis represents the absolute values of each subfraction in mg/dL. The bottom row represents the refernce ranges for each subfraction. The peaks are the graphic representation of the density bands as scanned by the machine.

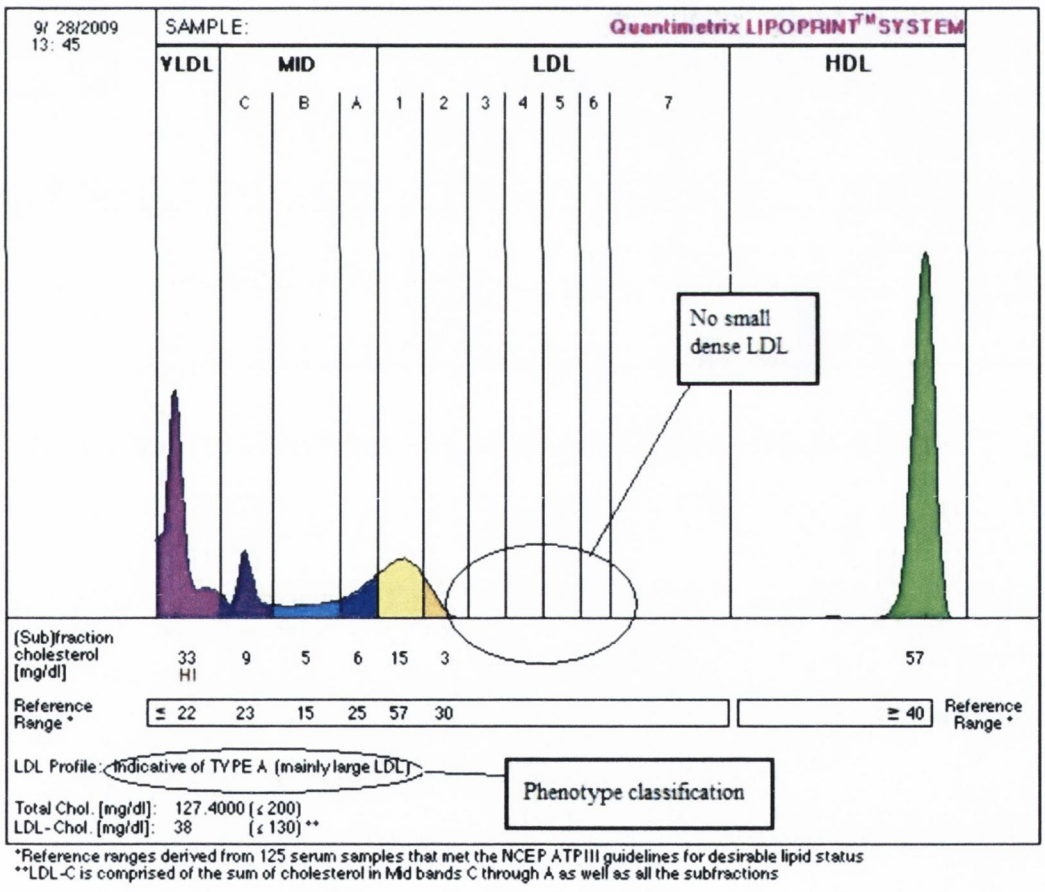

Figure 2-4: Example of a LDL Quantimetrix Lipoprint™ report (Type A)

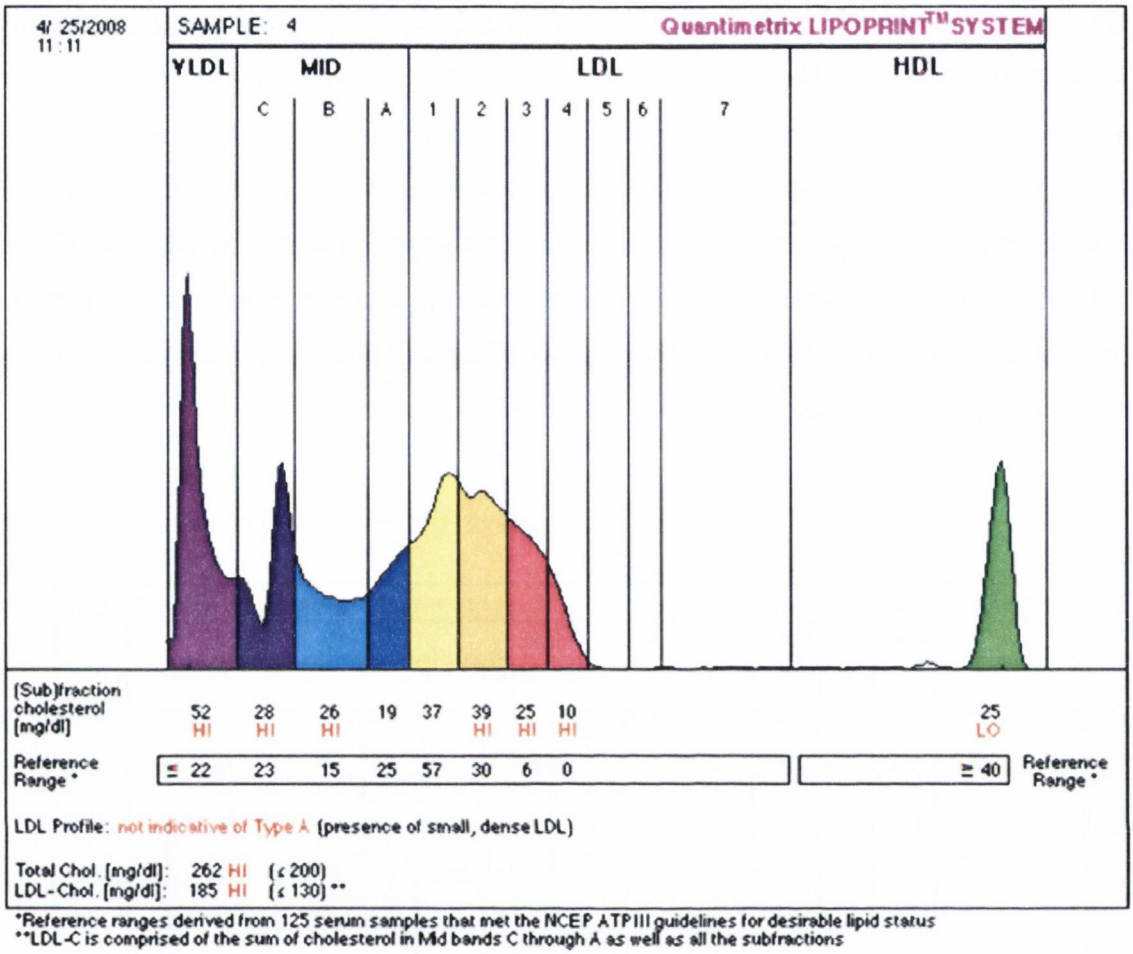

Figure 2-5: Example of a LDL Quantimetrix Lipoprint™ report (Type non-A)

The report highlights the 12 bands achieved in the LDL separations. sdLDL is represented in red and consists of LDL bands 3-7.

Lipoprint™ HDL sub-fraction analysis can resolve ten HDL fractions: HDL fractions 1-3 represent large HDL, HDL fractions 4-7 represent intermediate HDL and HDL fractions 8-10 represent small HDL (Morais & Muniz 2005). Examples of Lipoprint™ HDL analysis are shown in Figure 2-6 and Figure 2-7.

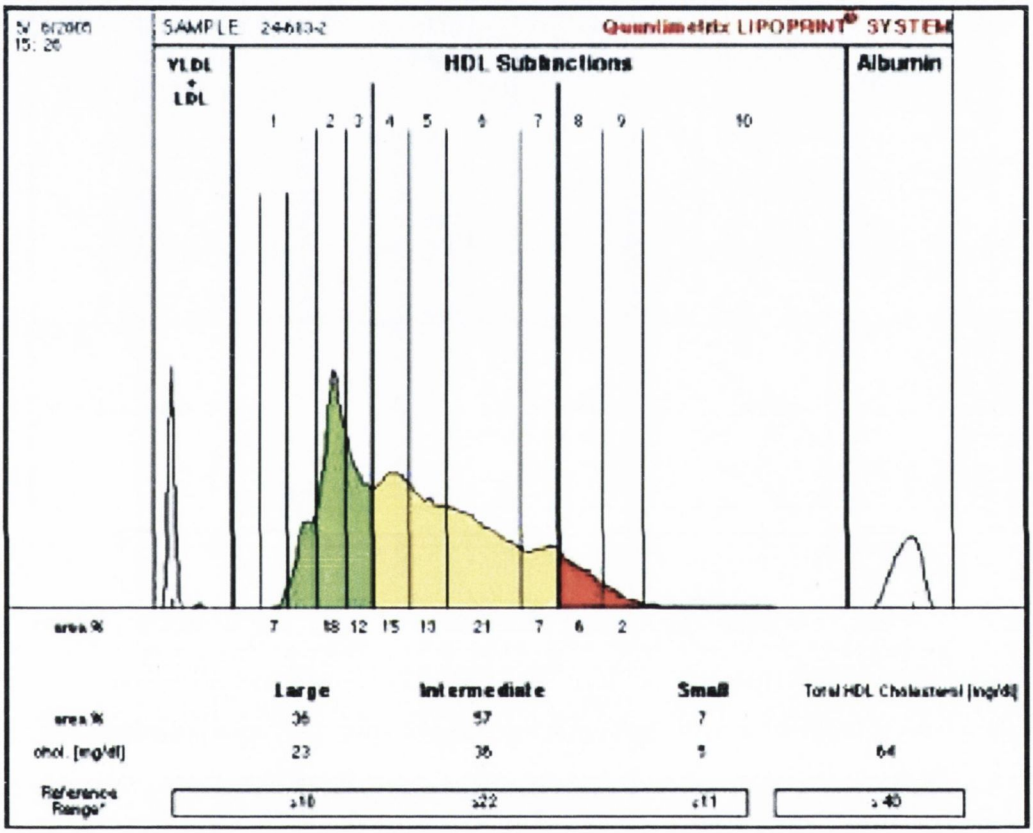

Figure 2-6: Quantimetrix Lipoprint™ system report for normal HDL sub-fractions (Morais & Muniz 2005). The HDL fractions are divided into three groups: Large fractions 1-3 intermediate fractions 4-7 and small fractions 8-10. Although small dense HDL fractions are present (denoted by the red component on the report) the large HDL fraction is still within the normal reference range.

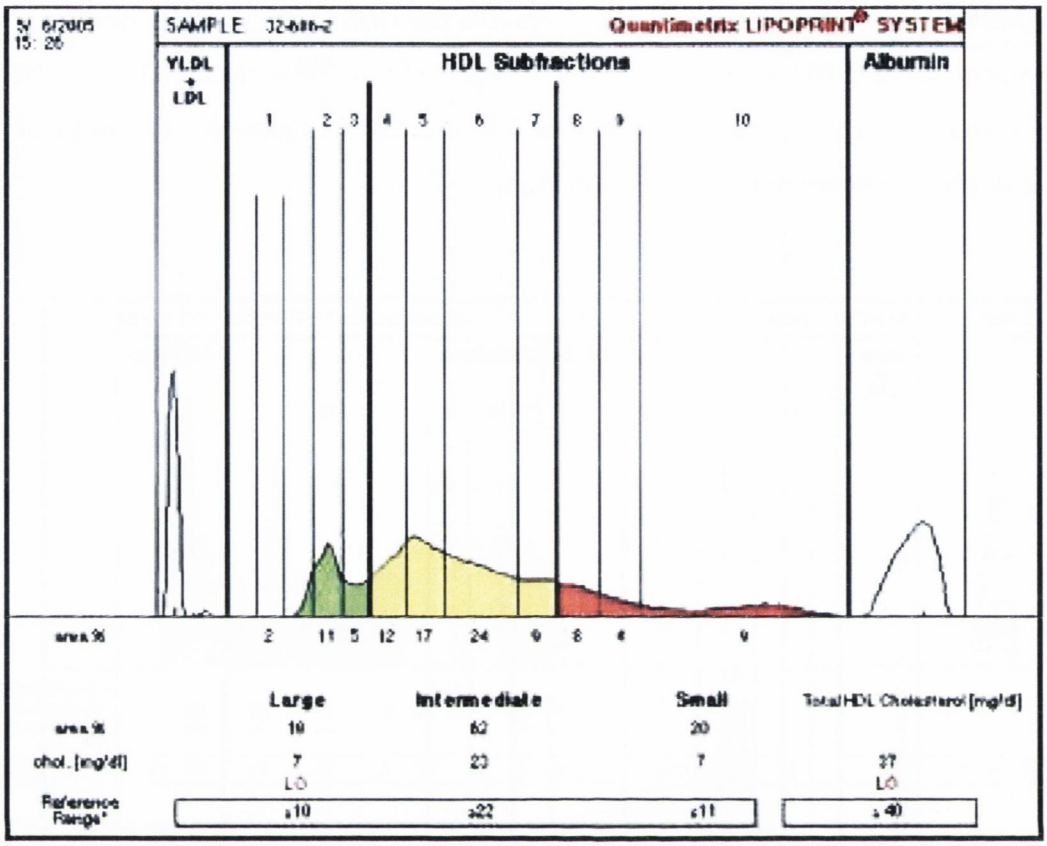

Figure 2-7: Quantimetrix Lipoprint™ system report for abnormal HDL sub-fractions (Morais & Muniz 2005). The HDL fractions are divided into three groups: Large fractions 1-3, intermediate fractions 4-7, and small fractions 8-10. The small dense HDL fractions present is denoted by the red component on the report.

2.8 STASTICAL ANALYSIS

2.8.1 Power Calculations

2.8.1.1 PCOS studies

Previous studies in PCOS have included cohorts ranging from as little as 9 subjects in any given cohort. Velazquez *et al.* studied postprandial lipid metabolism in 18 PCOS and compared them to just 9 control subjects (Velazquez, Bellabarba et al. 2000). Bahceci compared 20 woment with PCOS with 20 control subjects (Bahceci, Aydemir et al. 2007). Studies investigating adipokines, inflammatory markers and oxidation have used sample sizes that are usually between 20 and 40 although some do have larger cohorts of up to 45 subjects in each group (Ardawi and Rouzi 2005). It should be noted that different molecular weight adiponectin, the novel cardiovascular risk markers such as PAI-1 and neopterin, and TOS and TAC have not been studied previously and hence there is no pilot data which can be used to calculate the sample size required for study to be adequately powered. However as mentioned above, studies have generally shown results which are a clear separation from normal subjects using similar numbers of subjects than those planned for the curent studies and thereby it is expected that differences, if any, will be demonstrable using the current sample sizes.

2.8.1.2 LIPGENE study

The LIPGENE MetS study is a large European-wide study. 483 subjects with the MetS participated in the 8 research centres throughout the EU. The current analyses included samples from 160 volunteers across 7 of the 8 sites who had completed the 12 week dietary intervention. Dietary studies assessing the impact of dietary supplements vary greatly in cohort size. The current study of 4 dietary groups of approximately 40 subjects each is very similar to the previously published KANWU

study. The KANWU study included 162 healthy subjects chosen at random to receive a controlled, isoenergetic diet for 3 months containing either a high proportion of saturated (SAFA diet) or monounsaturated (MUFA diet) fatty acids. Within each group there was a second assignment at random to supplements with fish oil (3.6 g n-3 fatty acids/d) or placebo (Vessby, Uusitupa et al. 2001) i.e. 4 groups in total. The main outcomes were insulin sensitivity and the presence of Lp(a) which is very similar to the current LIPGENE study. Therefore it is expected that any significant differences should be demonstrable with a similar sample size of 160 subjects.

2.8.2 Use of SPSS

Statistical analysis for this thesis was conducted with SPSS (Statistical Package for the Social Sciences) version 16.0, a computer programme designed for statistical analysis. SPSS has recently been re-launched and repackaged under the name PASW (Predictive Analytics SoftWare).

2.8.3 Data preparation and statistical analysis

Normally distributed data is a common assumption in many of the statistical tests. To ensure this assumption was not violated, all data was checked for Gaussian distribution using the standard measures of skew and kurtosis. Data within the range of +1 and -1 for the measurements of skewness and kurtosis were considered to be normally distributed, and data outside of this range were transformed to encourage normal distribution. Logarithmic transformation was used to achieve this.

2.8.4 Statistics to compare mean values

2.8.4.1 T-tests

The student's t-test judges the difference between two means relative to the spread of the variability in their scores. To compare the mean scores of two groups an independent t-test was used. P-values generated by SPSS and were used to assess statistical significance between the groups.

2.8.4.2 Analysis of variance (ANOVA)

One-way between groups analysis of variable (ANOVA) was used to compare means for a continuous independent variables for which more than two groups were present or if the dependant variable had multiple levels. ANOVA calculates the variance between the different groups believed to be caused by the independent variable, and compares it with the variability within each of the groups which is believed to be as a result of chance. This is done by means of an F-ratio calculation. An F-ratio will represent the variance between groups divided by the variance within the group, and so a large F-ratio will indicate that there is more variance between the groups than there is within a group. A *P*-value corresponding to this F-test is calculated by SPSS, with a significant *P*-value meaning that the null hypothesis can be rejected and that the means are unequal.

As this analysis will indicate overall differences only, post hoc analysis was conducted to determine the location of the between-**group significant differences. Tukey's HSD** (honestly significant difference) post hoc test was used for these comparisons. This is a single step, multiple comparisons method which was designed for use in conjunction with ANOVA. This test is applied simultaneously to the set of all pair-wise comparisons and compares the means of every treatment to the means of every other treatment to identify differences between groups which are greater than the standard error would allow. **The benefit of using a post hoc test such as Tukey's HSD rather** than multiple t-tests is that it corrects for experiment-wise error rate when multiple comparisons are made, ensuring the possibility of Type 1 error does not increase.

This makes Tukey's HSD an attractive post-hoc test for situations in which multiple comparisons are likely to be made.

2.8.4.3 Analysis of Covariance (ANCOVA)

Analysis of Covariance (ANCOVA) was used in situations where the influence of a variable was thought to have a confounding influence on the between-group differences observed. ANCOVA is a more sophisticated method than ANOVA as it permits the inclusion of supplementary variables (covariates) into the model, allowing the assessment of inter-group variation associated with the dependant variables but not with the covariates. A covariate is a source of variation that affects the response variable, but which was not controlled for in the study design.As part of this analysis, SPSS will calculate estimated marginal means, which are means adjusted for specified variables such as covariates in the model.

2.8.5 Statistics to explore the relationship between variables

2.8.5.1 Correlations

Correlation analysis was used to explore, and describe the strength and direction of a linear relationship between two variables of interest. Pearson-product moment correlation coefficient was the statistic of choice in this regard as it was designed for use with normally distributed continuous variables. Zero-order correlation was used which gave a simple bivariate (between two variables) correlation coefficient. These coefficients (r) can take on values from -1 to +1 with the sign indicating whether a negative or positive correlation exists, respectively, and the absolute size giving an indication of the strength of the relationship. Interpretation of the strength of this relationship was described by many authors such as Cohen (1988), who categorised the relationship strength as small as r=0.1 to 0.29, medium as r=0.3 to 0.49, and large as r=0.5 to 1.0.

SPSS also provides a two-tailed *P*-value corresponding to each correlation coefficient generated which indicates the statistical significance of the correlation, as well the degree of confidence which can be had in the results obtained. The significance value is strongly influenced by the sample size, with significance more difficult to reach in smaller sample sizes (Pallant, 2007).

2.8.5.2 Multiple linear regression

Multiple linear regression analysis was used to explore the relationship between one continuous dependant variable, and a number of continuous independent or predictor variables. Multiple linear regression analysis is based on correlation analysis. However it allows a more sophisticated exploration of the underlying relationships between the variables. Standard multiple regression analysis was used in which all independent variables which correlated with the dependent variable were entered into the model simultaneously, and each variable evaluated in terms of its predictive power over and above that of the other independent variables.

Regression analysis makes a number of assumptions about the data, of which violations to the assumption of normality and collinearity are the most serious. All variables included in the regression models were normally distributed. In addition, care was taken to ensure that multicollinearity, a situation in which the independent variables are very highly correlated (r=0.9 or over) did not occur within the final model. Multicollinearity was checked using the collinearity diagnostics function in SPSS and by inspection of the correlation matrix generated for correlation coefficients over 0.9.

SPSS allows for various methods of performing multiple regression: Enter, Stepwise, Forward, Backward and Remove. The Stepwise method was chosen as this is regarded as the most comprehensive yet robust form of multiple regression without

increasing the risk of Type 1 error i.e. rejecting the null hypothesis when actually it is true. To determine which variables to enter, the predictive ability of all variables measured was assessed using bivariate correlations analysis. Those variables which were correlated were next grouped into sub-models of related variables, for example anthropometrical measurements, lipid profiles, inflammatory markers and so on. These variables were entered into the final regression model. SPSS enters these variables and excludes them if they are not deemed to be independent predictors of the dependent variable. The remaining variable or variables are then deemed the independent predictors and the strength of prediction is evaluated by the value given for R^2. This value indicates how much the independent variable(s) are able to explain the variance of the dependent variable.

2.9 SUMMARY OF AUTHOR'S CONTRIBUTION TO THE MATERIAL AND METHODS

2.9.1 PCOS studies:

1. Project conceptualisation, proposal and protocol preparation.
2. Ethics submission and approval.
3. Recruitment and screening of subjects with and without PCOS
4. Clinical aspects of the studies including:
    a. Anthropometric measurements
    b. fsIVGTT
    c. Fat meal preparation and administration
5. Sampling of bloods at all time points, preparation of aliquots and storage
6. Analyses of the following:
    a. Apolipoproteins, including apolipoprotein B_{48} (with the assistance of Ms. Jane Fogarty and Dr. Anne McGowan)
    b. Adipokines, including different oligomers of adiponectin (with the assistance of Dr. Niamh Phelan and Dr. **Annalouise O'Connor)**
    c. Inflammatory markers (with the assistance of Dr. Sarah Blair, RVH, Belfast)
7. Statistical analyses of all results

2.9.2 LIPGENE study (Irish cohort):

1. Assisted in recruitment of subjects with MetS
2. Screening of all subjects with MetS
3. Clinical aspects including:
    a. Anthropometric measurements
    b. fsIVGTT
4. Sampling of bloods at all time points
5. Analyses of the LDL and HDL subfractions using Quantimetrix Lipoprint™ system (with the assistance of Ms. Laura Meyler)
6. Statistical analyses of all results

Chapter 3

Insulin Resistance and Postprandial Lipoprotein Metabolism in PCOS

"When written in Chinese, the word "crisis" is composed of two characters. One represents danger and the other represents opportunity."

John F. Kennedy

3.1 CHAPTER SYNOPSIS

3.1.1 Context

Postprandial dyslipidaemia may be a plausible mechanism by which PCOS increases cardiovascular risk. Whether PCOS *per se* affects postprandial dyslipidaemia or whether IR, obesity or hyperandrogenaemia are the main determinants is unknown.

3.1.2 Aims

To investigate whether the postprandial glucose, insulin, lipid and lipoprotein responses, including that of B_{48} containing chylomicrons, to a mixed meal is different in obese women with PCOS when compared to obese control subjects, and whether differences, if any, were mainly related to obesity, IR, hyperandrogenaemia or PCOS status.

3.1.3 Methods

26 women with PCOS (age 30.4 ± 1.2 years, BMI 36.8 ± 1.5 kg/m^2) and 26 non-PCOS subjects (age 34.1 ± 0.9 years, BMI 31.5 ± 1.0 kg/m^2) were studied before and up to 8 hours following a standard mixed meal. Postprandial responses were calculated as area-under-the-curve (AUC) and incremental area-under-the cover (iAUC) values. Both the $HOMA_{IR}$ and the fsIVGTT were used. T-tests were used to compare the two groups. Further comparisons were made using ANCOVA with BMI, $HOMA_{IR}$ and S_I as covariates to determine the affects of each of these.

3.1.4 Results

AUC-TG was higher and AUC-HDL was lower in PCOS women compared to obese control subjects although these differences were no longer apparent when comparisons were made using BMI as a covariate. S_I, AUC-B_{48} and AUC-apoB were found to be independent predictors of AUC-TG, accounting for 55% of the variance. AUC-glucose and AUC-insulin were also much higher in women with PCOS compared with controls, but only AUC-insulin remained significantly higher following adjustment for BMI. AUC-insulin correlated negatively with S_I and DI.

3.1.5 Conclusions

Obesity is the main determinant of postprandial abnormalities seen in women with PCOS. Obesity related IR explains postprandial hypertriglyceridaemia, postprandial suppression of HDL and exaggerated postprandial hyperinsulinaemic responses. The **latter is associated with a reduction in β-cell** function as assessed by DI, a strong predictor of future risk of diabetes. Management of obesity in premenopausal women with PCOS is likely to reduce cardiovascular risk burden in such women.

3.2 INTRODUCTION

There is considerable evidence from epidemiological research, meta-analysis (Hokanson and Austin 1996; Austin 1999) and prospective clinical trials (Fontbonne, Eschwege et al. 1989; Eberly, Stamler et al. 2003) to support an independent role for fasting and postprandial plasma TG as a risk factor for CVD. Increased plasma remnants of TRLs, caused by delayed elimination of the same, has been shown to be prospectively associated with angiographic evidence of atherosclerosis and cardiac events (Phillips, Waters et al. 1993). Postprandial hypertriglyceridaemia is associated with two other well established cardiovascular risks – IR and obesity, both of which are prevalent in PCOS. An increase in postprandial lipoproteins is often found in insulin resistant states (Jeppesen, Hollenbeck et al. 1995), and hyperinsulinaemia itself appears to delay and exacerbate postprandial accumulation of intestinally derived chylomicrons (Harbis, Defoort et al. 2001). Obesity, especially visceral adiposity also contributes to a magnified postprandial TG response (Couillard, Bergeron et al. 1999; Couillard, Bergeron et al. 2002).

Postprandial hypertriglyceridaemia may be a plausible mechanism by which PCOS increases cardiovascular risk. While numerous studies have investigated plasma lipid profile under fasting conditions in women with PCOS, few have investigated changes in the postprandial setting. In a previous study by Valazquez et al a strong positive correlation between postprandial triglycerides and increasing waist-to-hip ratio was demonstrated (Velazquez, Bellabarba et al. 2000). That study evaluated overweight (BMI 27.41 ± 0.50 kg/m^2) PCOS women and compared them to lean PCOS women and lean controls. There were no obese controls and there was no analysis of the influence of androgens on postprandial hypertriglyceridaemia (Velazquez, Bellabarba et al. 2000). A separate study by Bahceci et al evaluated postprandial responses to an oral fat tolerance test, comparing lean PCOS women with lean controls (BMI 23.5 ± 2.6 kg/m^2 vs. 23.1 ± 4.0 kg/m^2; $p > 0.05$) (Bahceci, Aydemir et al. 2007). There were no obese or overweight controls in this study. They showed that PCOS women had higher baseline insulin, IR as assessed by $HOMA_{IR}$, AUC-TG, AUC-total-chol, AUC-

VLDL-chol, and AUC-apoB. Surprisingly though, AUC-insulin did not differ between the two groups. Again, as with the study by Valazquez et al, no analyses were reported between androgens and postprandial lipids (Bahceci, Aydemir et al. 2007).

It is also unclear whether postprandial hypertriglyceridaemia is mainly due to the contribution intestinally-derived $apoB_{48}$ containing chylomicrons or hepatically-derived $apoB_{100}$ containing VLDL particles. Both VLDL and chylomicrons share a common lipolytic pathway and are hydrolysed by lipoprotein lipase (LPL), an enzyme predominantly found on the endothelial surfaces of the capillaries of adipose tissue, heart and skeletal muscle. Hydrolysis results in the formation of a spectrum of smaller, denser particles. There are a number of described receptor mediated pathways through which these particles may eventually be cleared from the circulation (Mahley and Ji 1999). Traditionally it has been difficult to assess how what proportion of TRLs are chylomicrons and VLDLs. However, using ELISA, it is now possible to directly measurement the quantity of $apoB_{48}$, and hence chylomicron particles, in whole plasma (Lorec, Juhel et al. 2000; Sakai, Uchida et al. 2003).

3.3 AIMS

We investigated the postprandial response, including that of $apoB_{48}$ containing chylomicrons, to a mixed meal in obese women with PCOS and obese control subjects, and whether differences, if any, were mainly related to IR, obesity, hyperandrogenaemia or PCOS status.

3.4 METHODS

3.4.1 Subjects

52 Caucasian women were studied, including 26 women with PCOS and 26 women without PCOS, who were recruited from the Endocrinology and Reproductive Endocrinology outpatients and by local advertisement. PCOS subjects fulfilled the NIH criteria for diagnosis and met all the inclusion criteria as described in Section 2.1.1. The control group was comprised of normal volunteers on no medications recruited from the general population as described in Section 2.1.2. All control subjects were studied in the follicular phase of their menstrual cycle. PCOS subjects had both study days completed within 2 weeks of a spontaneous bleed. All study subjects gave their written signed consent to the study, which was approved by the Joint Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland).

3.4.2 Clinical protocol

This was a cross-sectional study. All subjects had their height measured with a Harpenden stadiometer and weight measured in light clothing. BMI was calculated as weight (kg) / height squared (m^2). WC and HC were measured with a non-distensible flexible tape, and WHR calculated accordingly. All subjects also underwent estimation of body composition including percentage body fat using bioimpedance analysis using the Bodystat 1500 system (Bodystat Ltd, UK) described in Section 2.3.3.

3.4.2.1 Frequently sampled intravenous glucose tolerance test (fsIVGTT)

Each subject underwent an fsIVGTT. This procedure has been described in Section 2.4.2.

3.4.2.2 Mixed meal and sampling

Following a 12-hour overnight fast, subjects came to the Clinical Investigation Unit for blood sampling and a high calorific meal. The mixed meal was designed and analysed by a qualified dietician. It consisted of 948kcal. 48% of the total calories was derived from fat (20% saturated, 16% monounsaturated, 8% polyunsaturated). 36% & 15% of the calories were derived from carbohydrate and protein respectively. The meal was ingested within 20 minutes after baseline bloods. Bloods were taken at 2, 4, 6 and 8 hours after ingestion of the meal. Subjects were advised to restrict their physical activity to the minimum and were permitted to drink up to 250mls of water until the end of the sampling period.

3.4.3 Laboratory methods

3.4.3.1 Hormones, glucose, HbA1c, lipids

Insulin and testosterone were measured by electrochemiluminesence immunoassay on the Roche E Module. E2, progesterone, LH, FSH, TSH, fT4, PRL, cortisol were all measured by chemiluminesence immunoassay on DPC Immulite 2500. SHBG and DHEAS were measured by chemiluminesence immunoassay on DPC Immulite 2000 in **St James's Hospital**. FAI was calculated by the equation: FAI = total testosterone (nmol/L) / SHBG (nmol/L) x 100. Androstenedione and 17-OHP were measured by radioimmunoassay on the DPC Coat-A-Count. Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module. HbA1c was measured by ion exchange chromatography on the Tosoh 2.2. Cholesterol, TG, HDL-chol and NEFA were measured by a standard enzymatic colourimetric method on the Roche P Module. LDL-chol was calculated by the Friedewald formula. Laboratory methods of all these are described in full in section 2.7.

3.4.3.2 Apolipoproteins A-I, A-II, B

All apolipoprotein measurements were performed on the Nephlometer Dade Behring BN II®, as described in section 2.7.6.

3.4.3.3 ApoB$_{48}$ ELISA

The human apolipoproteinB$_{48}$ ELISA Kit (AKHB48) is an ELISA kit for the measurement of human apoB$_{48}$ using an indirect sandwich assay technique (http://www.clonagen.com/clonagen). This is a modified version of the method described by Lorec et al. (Lorec, Juhel et al. 2000). The anti-apo B48 coated plate is initially washed to remove buffer. The appropriately diluted samples are added to the plate and left to react for one hour. Samples were diluted 1:250 to ensure the concentration was not above the assay range. The plate is subsequently washed 4 times, and biotin-conjugated anti apoB48 antibody is added. This binds to human apoB48 bound to the anti-apo B48 antibody immobilised on the coated plate, as shown in Figure 2-2. This reaction is stopped after 1 hour and the plate is washed 4 times. Finally a peroxidase-conjugated avidin is then added to the plate. The avidin and biotin readily bind to ensure that the horseradish peroxidise enzyme is immobilised on the plate. After washing, the chromogenic substrate tetramethyl benzidine (TMB) is added. This forms a blue colour on reaction with horseradish peroxidase enzyme. The reaction stopper (1M H_2SO_4) is added, resulting in a yellow colour formation which is proportional to substrate concentration. The plate was read at an absorbance of 450 nm.

3.4.4 Statistical analysis

Data are presented as mean ± SEM. Skewed variables were logarithmically transformed to normalise data prior to analysis. Initial comparisons between groups were performed using independent t-test. ANCOVA was used to compare differences between the two groups adjusting for 3 separate covariates: BMI, HOMA$_{IR}$ and S_I.

Correlations were made using Pearson's correlation co-efficient. Multiple linear regression analysis was used to identify independent contributors to postprandial TG, $apoB_{48}$ and HDL-chol. Independent variables that correlated significantly in univariate analysis were entered into the multiple regression models in forward stepwise fashion. Statistical significance was defined as $p<0.05$.

3.5 RESULTS

3.5.1 Baseline demographic data and hormonal profile

Women with PCOS had a greater BMI, waist and hip circumferences, and percentage of body fat (Table 3-1). All androgens were higher whilst SHBG and FSH were lower in women with PCOS. As expected, women with PCOS were more insulin resistant with a higher $HOMA_{IR}$ (1.37 ± 0.09 in PCOS vs. 0.80 ± 0.07 in controls) and lower Si (0.57 ± 0.11 in PCOS vs. 1.28 ± 0.11 in controls).

3.5.2 Postprandial lipids, NEFA, glucose, insulin and apolipoproteins

Postprandial TG at 2 hours was significantly greater in PCOS women (0.68 ± 0.47 vs. 0.40 ± 0.36, p=0.019) and non-significantly greater at the other time points compared to controls (Table 3-2 and Figure 3-1). AUC-TG, but not the iAUC-TG, was also significantly higher in women with PCOS. Conversely, HDL-chol was significantly lower in PCOS women compared to controls at T = 0, 2, 4 and 6 hours (Table 3-2 and Figure 3-2). AUC-HDL was also significantly lower in PCOS compared with controls. Both the postprandial hypertriglyceridaemia and suppressed HDL-chol levels are consistent with the dyslipidaemic status of PCOS women. NEFA levels did not differ significantly between the two groups (Table 3-2 and Figure 3-3).

Table 3-1: Baseline characteristics of all subjects

	PCOS (n=26)	Controls (n=26)	p
Age (yrs)	30.4 ± 1.2	34.1 ± 1.5	0.063
Systolic BP (mmHg)	126.3 ± 1.8	121.6 ± 2.5	0.131
Diastolic BP (mmHg)	79.0 ± 1.1	77.8 ± 2.3	0.652
Waist (cm)	112.0 ± 2.2	99.8 ± 2.4	<0.001
Hip (cm)	120.5 ± 1.7	110.9 ± 1.9	<0.001
Waist : Hip	0.930 ± 0.014	0.900 ± 0.014	0.139
Weight (kg)	95.4 ± 2.6	83.2 ± 2.8	0.003
BMI (kg/m^2)	36.8 ± 0.9	31.5 ± 1.0	<0.001
Smoking (%)	26	31	0.696
% Body fat mass (%)	40.4 ± 1.3	33.6 ± 2.0	0.006
T (nmol/L)*	1.06 ± 0.10	0.34 ± 0.10	<0.001
SHBG (nmol/L)*	3.33 ± 0.07	3.79 ± 0.07	<0.001
FAI*	2.33 ± 0.12	1.15 ± 0.12	<0.001
Androstenedione (nmol/L)	15.58 ± 0.84	11.82 ± 0.84	0.003
DHEAS (µmol/L)	7.62 ± 0.71	4.98 ± 0.49	0.003
FSH (IU/L)*	1.64 ± 0.08	2.09 ± 0.09	<0.001
LH (IU/L)*	1.63 ± 0.16	1.73 ± 0.14	0.654
$HOMA_{IR}$ ($\mu mol^2/L^2$)*	1.37 ± 0.09	0.80 ± 0.07	<0.001
S_I ($\times 10^4$ $(mU.L^{-1})^{-1}.min^{-1}$)*	0.57 ± 0.11	1.28 ± 0.11	<0.001
S_G (min^{-1})	0.02 ± 0.00	0.02 ± 0.00	0.352
AIR_G ($mU.L^{-1}.min$)*	6.51 ± 0.20	6.16 ± 0.18	0.207
DI	1756 ± 281	2308 ± 358	0.226

* log transformed

Table 3-2: Postprandial triglycerides, HDL-chol, NEFA

	PCOS (n=26)	Controls (n=26)	p
TG 0 hrs (mmol/L)*	0.34 ± 0.09	0.13 ± 0.06	0.075
TG 2 hrs (mmol/L)*	0.68 ± 0.09	0.40 ± 0.07	0.019
TG 4 hrs (mmol/L)*	0.77 ± 0.10	0.53 ± 0.08	0.057
TG 6 hrs (mmol/L)*	0.54 ± 0.10	0.26 ± 0.11	0.074
TG 8 hrs (mmol/L)*	0.39 ± 0.11	0.16 ± 0.09	0.129
AUC-TG (mmol/L.8hr)*	2.00 ± 0.09	1.75 ± 0.07	0.041
iAUC-TG (mmol/L.8hr)	1.93 ± 0.28	1.35 ± 0.24	0.127
HDL-chol 0 hrs (mmol/L)	1.34 ± 0.06	1.51 ± 0.06	0.046
HDL-chol 2 hrs (mmol/L)	1.27 ± 0.06	1.44 ± 0.05	0.037
HDL-chol 4 hrs (mmol/L)	1.22 ± 0.06	1.37 ± 0.05	0.043
HDL-chol 6 hrs (mmol/L)	1.25 ± 0.06	1.42 ± 0.05	0.034
HDL-chol 8 hrs (mmol/L)	1.30 ± 0.06	1.44 ± 0.05	0.092
AUC-HDL-chol (mmol/L.8hr)	5.06 ± 0.23	5.71 ± 0.21	0.041
iAUC-HDL-chol (mmol/L.8hr)	-0.32 ± 0.03	-0.33 ± 0.07	0.883
NEFA 0 hrs (mmol/L)	0.65 ± 0.04	0.61 ± 0.05	0.252
NEFA 2 hrs (mmol/L)	0.19 ± 0.02	0.18 ± 0.05	0.468
NEFA 4 hrs (mmol/L)	0.33 ± 0.03	0.36 ± 0.05	0.058
NEFA 6 hrs (mmol/L)	0.62 ± 0.05	0.67 ± 0.05	0.184
NEFA 8 hrs (mmol/L)	0.81 ± 0.05	0.84 ± 0.05	0.239
AUC NEFA (mmol/L.8hr)	1.87 ± 0.10	1.94 ± 0.05	0.157
iAUC NEFA (mmol/L.8hr)	-0.72 ± 0.14	-0.52 ± 0.05	0.796

* log transformed

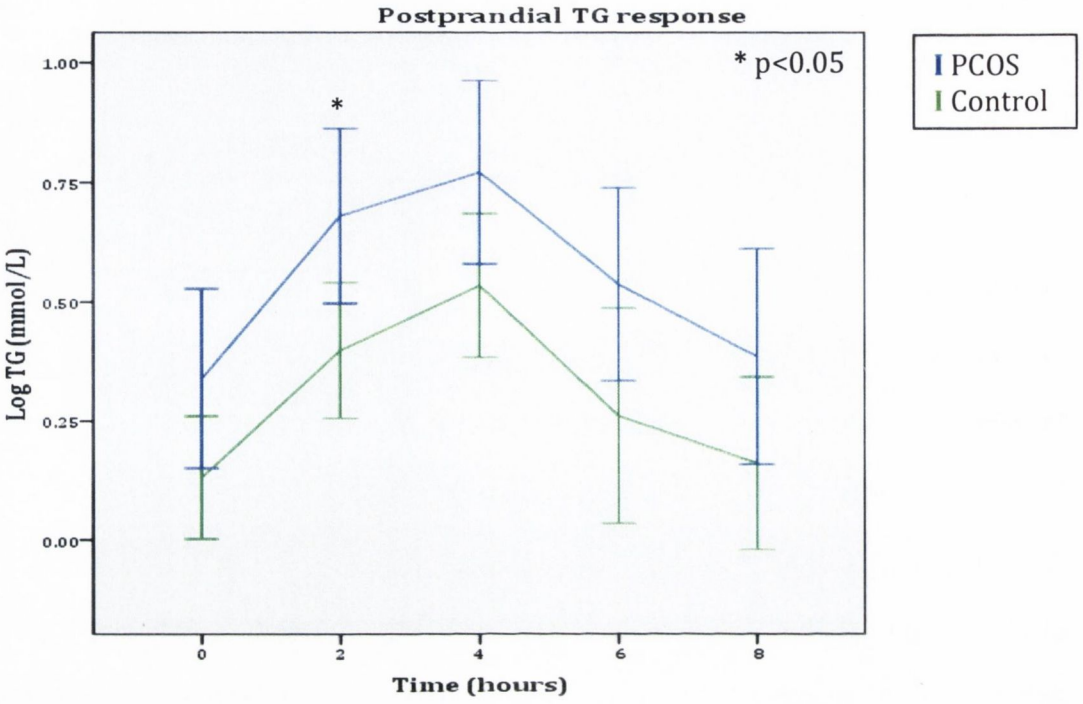

Figure 3-1: Postprandial triglyceride response in PCOS and control subjects

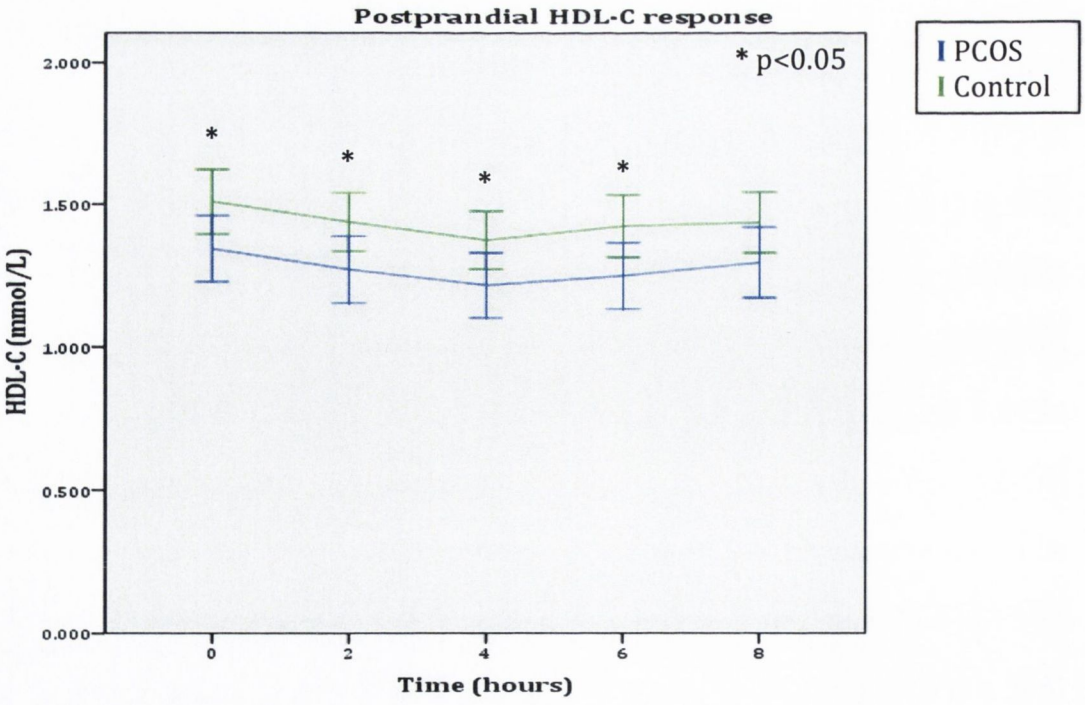

Figure 3-2: Postprandial HDL-chol response in PCOS and control subjects

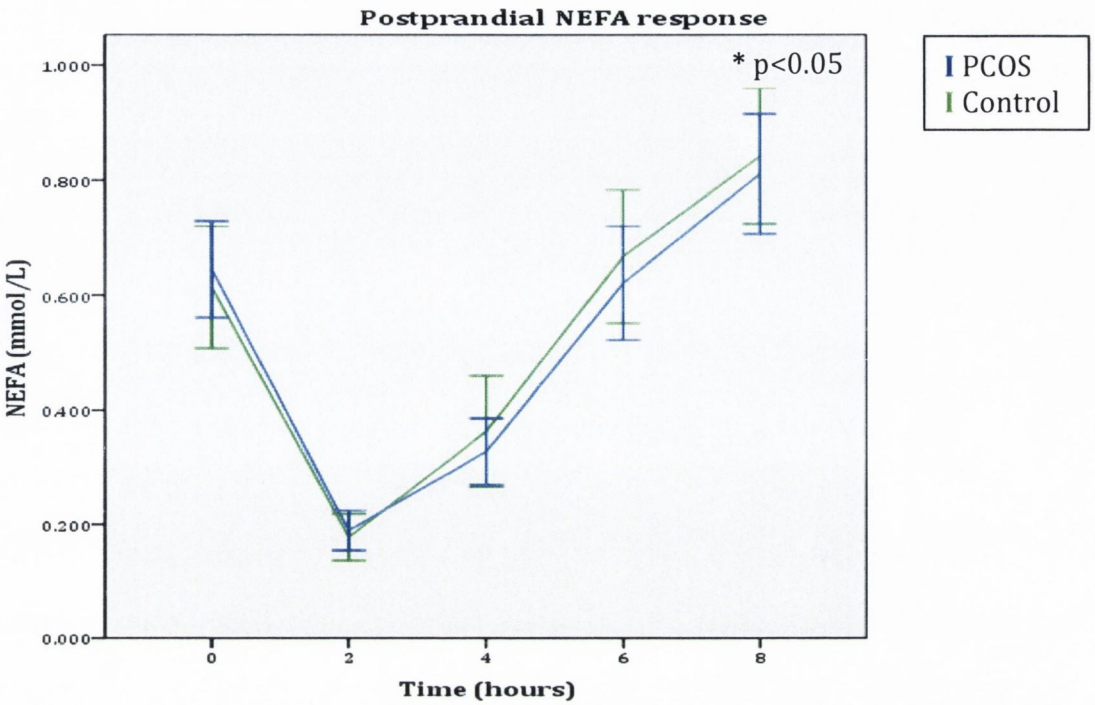

Figure 3-3: Postprandial NEFA response in PCOS and control subjects

Glucose levels were non-significantly greater at 2 hours and significantly greater at 4 hours in women with PCOS compared to controls (Table 3-3 and Figure 3-4). AUC-glucose was also greater in PCOS. With the exception of insulin at 8 hours, insulin level at all other time points, AUC-insulin and iAUC-insulin were all greater in women with PCOS (Table 3-3 and Figure 3-5). This finding would be consistent with the hyperinsulinaemia that accompanies IR.

Table 3-3: Postprandial glucose and insulin

	PCOS (n=26)	Controls (n=26)	p
Glucose 0 hrs (mmol/L)	5.18 ± 0.11	4.99 ± 0.10	0.191
Glucose 2 hrs (mmol/L)	5.67 ± 0.30	4.90 ± 0.25	0.053
Glucose 4 hrs (mmol/L)	5.18 ± 0.18	4.73 ± 0.12	0.044
Glucose 6 hrs (mmol/L)	4.66 ± 0.08	4.66 ± 0.06	0.959
Glucose 8 hrs (mmol/L)	4.66 ± 0.06	4.62 ± 0.09	0.713
AUC-glucose (mmol/L.8hr)	20.50 ± 0.48	19.10 ± 0.38	0.024
iAUC-glucose (mmol/L.8hr)	-0.33 ± 0.32	-0.86 ± 0.40	0.306
Insulin 0 hrs (mU/L)*	2.86 ± 0.09	2.34 ± 0.09	<0.001
Insulin 2 hrs (mU/L)*	4.43 ± 0.14	3.70 ± 0.11	<0.001
Insulin 4 hrs (mU/L)*	3.71 ± 0.12	2.91 ± 0.11	<0.001
Insulin 6 hrs (mU/L)*	2.95 ± 0.10	2.25 ± 0.09	<0.001
Insulin 8 hrs (mU/L)*	2.60 ± 0.10	2.33 ± 0.11	0.084
AUC insulin (mU/L.8hr)*	5.12 ± 0.11	4.42 ± 0.09	<0.001
iAUC insulin (mU/L.8hr)*	4.49 ± 0.15	3.52 ± 0.18	<0.001

* log transformed

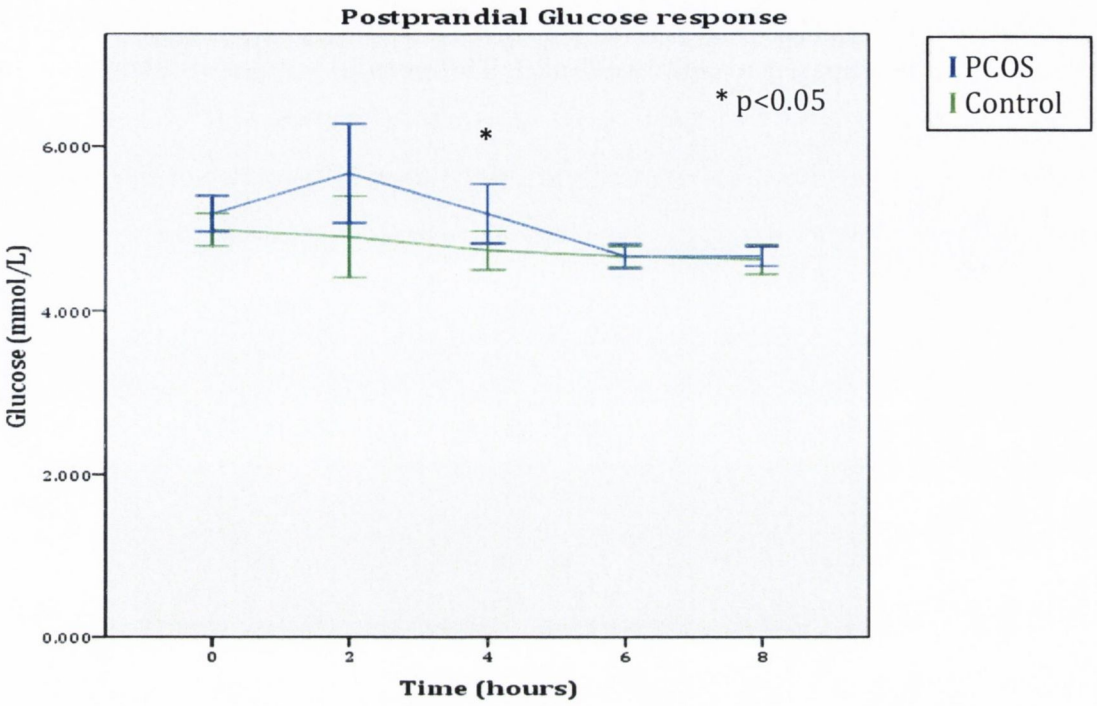

Figure 3-4: Postprandial glucose response in PCOS and control subjects

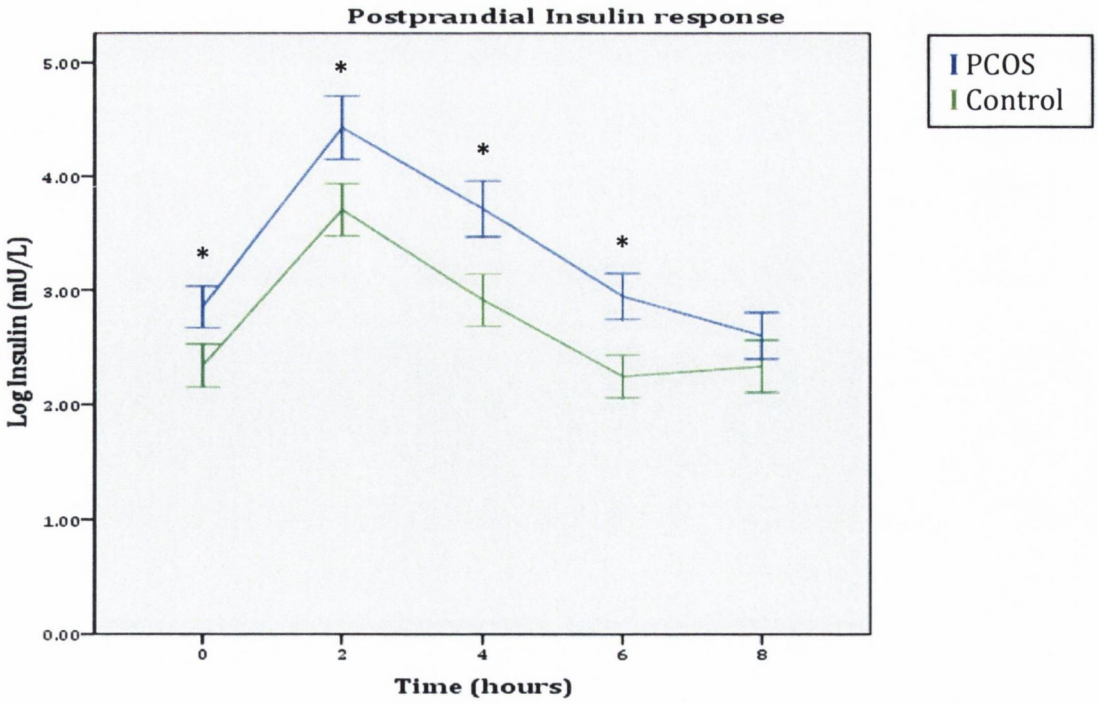

Figure 3-5: Postprandial insulin response in PCOS and control subjects

$ApoB_{48}$ was non-significantly (p=0.058) greater at 4 hours in PCOS women (2.88 ± 0.39 μg/mL) compared to controls (2.62 ± 0.56 μg/mL) but not at other time points (Table 3-4 and Figure 3-6). There were no significant differences in apoB, apoA-I or apoA-II at any of the time points or in the AUC or iAUC between the two groups (Table 3-4 and Figure 3-7, Figure 3-8 and Figure 3-9).

Table 3-4: Postprandial $apoB_{48}$, apoB, apoA-I and apoA-II

	PCOS (n=26)	Controls (n=26)	p
$ApoB_{48}$ 0 hrs (μg/mL)*	2.26 ± 0.14	2.01 ± 0.17	0.252
$ApoB_{48}$ 2 hrs (μg/mL)*	2.76 ± 0.08	2.69 ± 0.07	0.468
$ApoB_{48}$ 4 hrs (μg/mL)*	2.88 ± 0.08	2.62 ± 0.11	0.058
$ApoB_{48}$ 6 hrs (μg/mL)*	2.81 ± 0.09	2.64 ± 0.09	0.184
$ApoB_{48}$ 8 hrs (μg/mL)*	2.43 ± 0.10	2.24 ± 0.12	0.239
AUC-$apoB_{48}$ (μg/mL.8hr)*	4.14 ± 0.07	3.99 ± 0.08	0.157
iAUC-$apoB_{48}$ (μg/mL.8hr)	19.35 ± 3.38	18.27 ± 2.45	0.796
ApoB 0 hrs (g/dL)	0.73 ± 0.07	0.82 ± 0.06	0.328
ApoB 2 hrs (g/dL)	0.76 ± 0.07	0.73 ± 0.06	0.816
ApoB 4 hrs (g/dL)	0.83 ± 0.06	0.76 ± 0.06	0.364
ApoB 6 hrs (g/dL)	0.79 ± 0.05	0.77 ± 0.05	0.779
ApoB 8 hrs (g/dL)	0.83 ± 0.05	0.79 ± 0.05	0.603
AUC-apoB hrs (g/dL.8hr)	3.16 ± 0.17	3.07 ± 0.14	0.688
iAUC-apoB (g/dL.8hr)	0.22 ± 0.22	-0.22 ± 0.19	0.143
ApoA-I 0 hrs (g/dL)	1.15 ± 0.07	1.10 ± 0.10	0.679
ApoA-I 2 hrs (g/dL)	1.04 ± 0.09	1.14 ± 0.09	0.437
ApoA-I 4 hrs (g/dL)	1.19 ± 0.08	1.28 ± 0.08	0.465
ApoA-I 6 hrs (g/dL)	1.18 ± 0.08	1.22 ± 0.08	0.715
ApoA-I 8 hrs (g/dL)	1.08 ± 0.08	1.16 ± 0.09	0.481
AUC-apoA-I hrs (g/dL.8hr)	4.52 ± 0.19	4.75 ± 0.18	0.391
iAUC-apoA-I (g/dL.8hr)	-0.08 ± 0.30	0.35 ± 0.34	0.342
ApoA-II 0 hrs (g/dL)	0.32 ± 0.02	0.30 ± 0.03	0.544
ApoA-II 2 hrs (g/dL)	0.29 ± 0.03	0.30 ± 0.02	0.796
ApoA-II 4 hrs (g/dL)	0.26 ± 0.03	0.25 ± 0.03	0.764
ApoA-II 6 hrs (g/dL)	0.26 ± 0.02	0.30 ± 0.02	0.195
ApoA-II 8 hrs (g/dL)	0.25 ± 0.02	0.26 ± 0.02	0.761
AUC-apoA-II hrs (g/dL.8hr)	1.10 ± 0.04	1.13 ± 0.04	0.657
iAUC-apoA-II (g/dL.8hr)	-0.19 ± 0.09	-0.08 ± 0.09	0.410

* log transformed

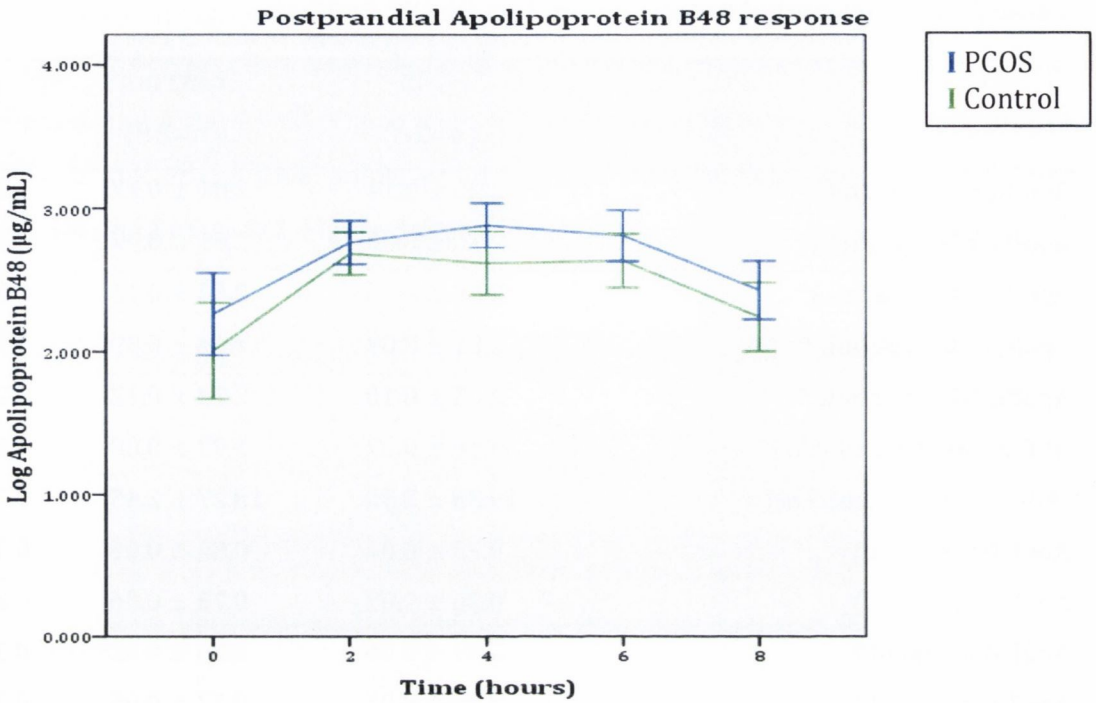

Figure 3-6: Postprandial apolipoprotein B48 response in PCOS and control subjects

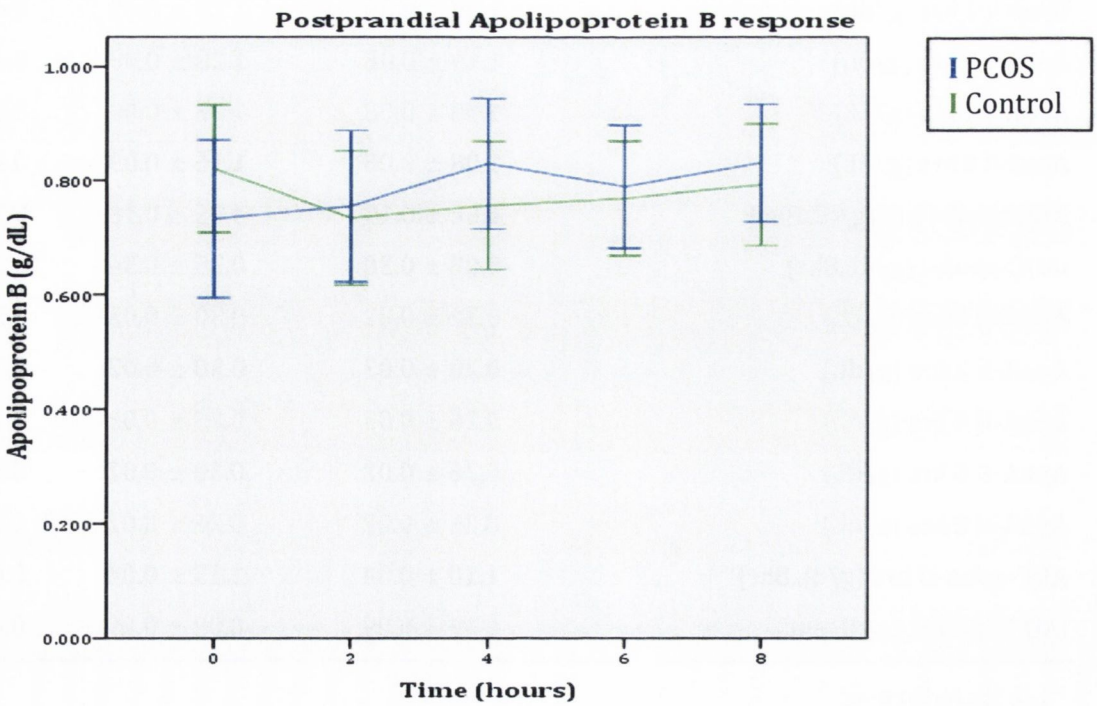

Figure 3-7: Postprandial apoB response in PCOS and control subjects

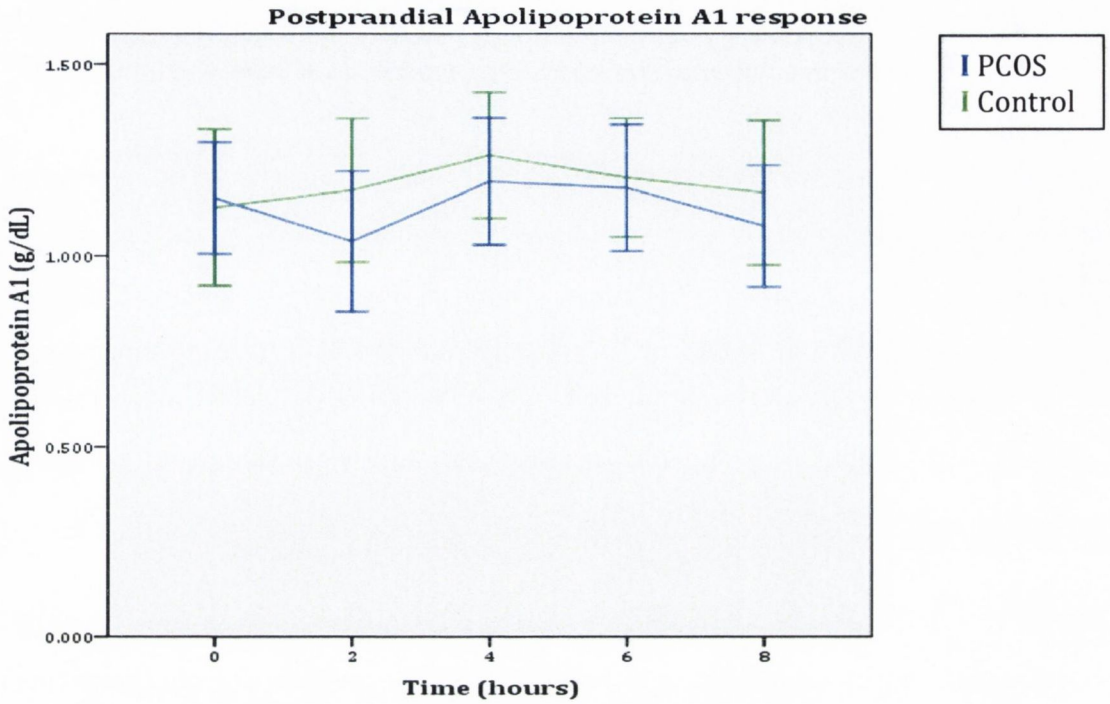

Figure 3-8: Postprandial apoA-I response in PCOS and control subjects

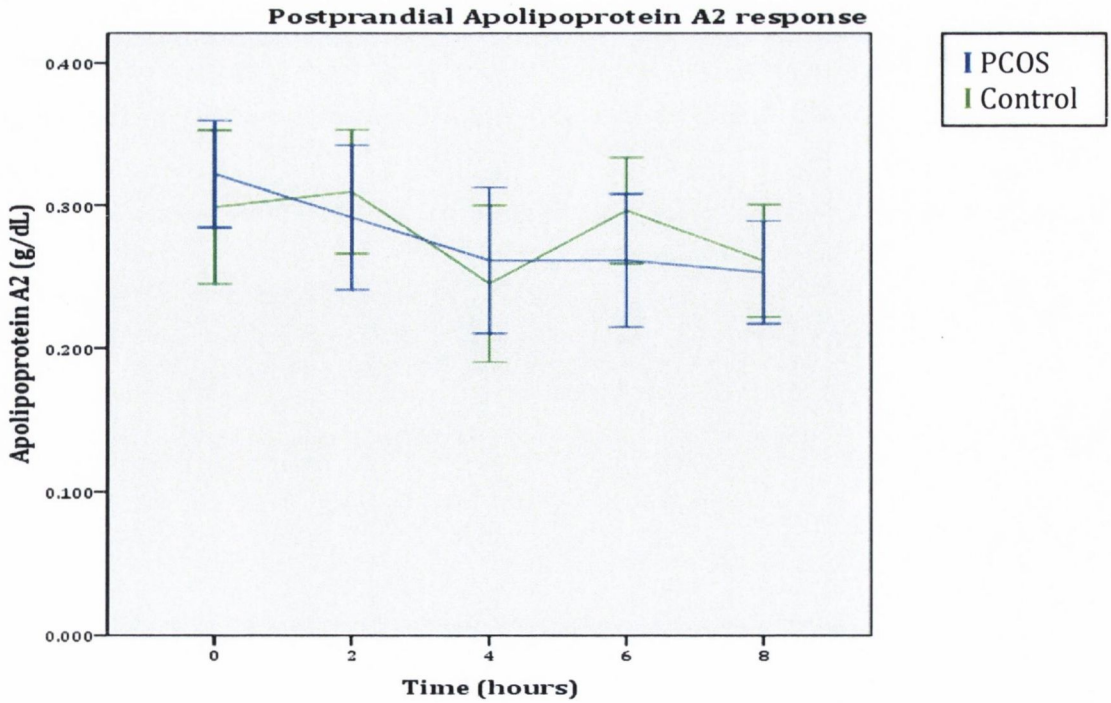

Figure 3-9: Postprandial apoA-II response in PCOS and control subjects

Because the PCOS and control groups were not matched for BMI, which is strongly related to IR, a subgroup analyses of women matched for BMI was performed. The results of these are presented in the next few pages.

Subgroup analyses of 21 pairs of subjects matched for BMI revealed results similar to those when the whole group of 52 subjects. WC and %BF was no longer different between the two groups although HC was still greater in the PCOS cohort (Table 3-5). As with the unmatched group analyses, $HOMA_{IR}$ and S_I were significantly different but S_G, AIR_G and DI were not (Table 3-5).

TG at T=2 hours and glucose at T=4 hours, which was different when comparing non-BMI-matched cohorts was not significantly different in the subgroup analyses of the BMI-matched cohorts (Table 3-6 and Table 3-7). However even with the subgroup analyses, insulin levels were significantly different between the two BMI-matched groups at all time points except for T=8 hours (Table 3-7).

Results for ApoB48, ApoB, ApoA-I and ApoA-II were the same in the subgroup analyses as the entire cohort. There were no differences in any of the apolipoproteins (Table 3-8).

Table 3-5: Baseline characteristics of 21 pairs of BMI-matched subjects

	PCOS (n=21)	Controls (n=21)	p
Age (yrs)	30.5 ± 1.4	34.8 ± 1.8	0.067
Systolic BP (mmHg)	124.6 ± 2.0	123.0 ± 2.3	0.592
Diastolic BP (mmHg)	78.0 ± 1.2	78.9 ± 2.6	0.753
Waist (cm)	109.0 ± 2.1	103.3 ± 2.4	0.082
Hip (cm)	118.2 ± 1.6	112.6 ± 2.2	0.048
Waist : Hip	0.9 ± 0.0	0.9 ± 0.0	0.849
Weight (kg)	90.9 ± 2.2	87.1 ± 2.9	0.298
BMI (kg/m^2)	35.3 ± 0.8	32.8 ± 1.1	0.083
Smoking (%)	29	31	0.696
% Body fat mass (%)	37.91 ± 1.01	35.67 ± 2.33	0.396
T (nmol/L)*	1.10 ± 0.12	0.30 ± 0.12	0.000
SHBG (nmol/L)*	3.38 ± 0.07	3.71 ± 0.07	0.002
FAI*	2.32 ± 0.14	1.19 ± 0.13	0.000
Androstenedione (nmol/L)	16.00 ± 0.95	12.07 ± 0.94	0.005
DHEAS (µmol/L)	8.12 ± 0.83	4.81 ± 0.51	0.002
FSH (IU/L)*	1.65 ± 0.09	2.08 ± 0.10	0.002
LH (IU/L)*	1.66 ± 0.20	1.71 ± 0.16	0.841
$HOMA_{IR}$ ($\mu mol^2/L^2$)*	1.28 ± 0.10	0.90 ± 0.11	0.015
S_I ($x\ 10^4\ (mU.L^{-1})^{-1}.min^{-1}$)*	0.70 ± 0.10	1.15 ± 0.09	0.002
S_G (min^{-1})	0.02 ± 0.00	0.02 ± 0.00	0.861
AIR_G ($mU.L^{-1}.min$)*	6.50 ± 0.24	6.15 ± 0.20	0.268
DI	1961 ± 330	1967 ± 321	0.990

* log transformed

Table 3-6: Postprandial triglycerides, HDL-chol, NEFA in 21 BMI-matched pairs

	PCOS (n=21)	Controls (n=21)	p
TG 0 hrs (mmol/L)*	0.24 ± 0.10	0.14 ± 0.07	0.401
TG 2 hrs (mmol/L)*	0.61 ± 0.10	0.41 ± 0.08	0.115
TG 4 hrs (mmol/L)*	0.69 ± 0.11	0.55 ± 0.09	0.286
TG 6 hrs (mmol/L)*	0.44 ± 0.10	0.26 ± 0.14	0.301
TG 8 hrs (mmol/L)*	0.28 ± 0.11	0.20 ± 0.10	0.637
AUC-TG (mmol/L.8hr)*	1.91 ± 0.10	1.77 ± 0.08	0.258
iAUC-TG (mmol/L.8hr)	1.82 ± 0.29	1.42 ± 0.29	0.339
HDL-chol 0 hrs (mmol/L)	1.36 ± 0.06	1.43 ± 0.05	0.360
HDL-chol 2 hrs (mmol/L)	1.29 ± 0.06	1.37 ± 0.05	0.270
HDL-chol 4 hrs (mmol/L)	1.23 ± 0.06	1.30 ± 0.04	0.341
HDL-chol 6 hrs (mmol/L)	1.26 ± 0.06	1.35 ± 0.05	0.285
HDL-chol 8 hrs (mmol/L)	1.31 ± 0.07	1.36 ± 0.05	0.562
AUC-HDL-chol (mmol/L.8hr)	5.11 ± 0.25	5.42 ± 0.18	0.325
iAUC-HDL-chol (mmol/L.8hr)	-0.31 ± 0.03	-0.29 ± 0.08	0.823
NEFA 0 hrs (mmol/L)	0.63 ± 0.05	0.65 ± 0.06	0.770
NEFA 2 hrs (mmol/L)	0.20 ± 0.02	0.19 ± 0.02	0.657
NEFA 4 hrs (mmol/L)	0.34 ± 0.03	0.37 ± 0.05	0.577
NEFA 6 hrs (mmol/L)	0.61 ± 0.06	0.70 ± 0.05	0.275
NEFA 8 hrs (mmol/L)	0.81 ± 0.06	0.85 ± 0.07	0.671
AUC NEFA (mmol/L.8hr)	1.86 ± 0.12	2.00 ± 0.13	0.431
iAUC NEFA (mmol/L.8hr)	-0.65 ± 0.16	-0.59 ± 0.21	0.837

* log transformed

Table 3-7: Glucose and insulin results in 21 BMI-matched pairs

	PCOS (n=21)	Controls (n=21)	p
Glucose 0 hrs (mmol/L)	5.11 ± 0.13	5.04 ± 0.12	0.701
Glucose 2 hrs (mmol/L)	5.71 ± 0.36	5.10 ± 0.26	0.176
Glucose 4 hrs (mmol/L)	5.20 ± 0.21	4.79 ± 0.09	0.084
Glucose 6 hrs (mmol/L)	4.67 ± 0.08	4.68 ± 0.07	0.946
Glucose 8 hrs (mmol/L)	4.69 ± 0.07	4.64 ± 0.11	0.754
AUC-glucose (mmol/L.8hr)	20.56 ± 0.54	19.42 ± 0.35	0.083
iAUC-glucose (mmol/L.8hr)	0.02 ± 0.30	-0.75 ± 0.45	0.160
Insulin 0 hrs (mU/L)*	2.78 ± 0.10	2.44 ± 0.10	0.020
Insulin 2 hrs (mU/L)*	4.35 ± 0.16	3.88 ± 0.11	0.018
Insulin 4 hrs (mU/L)*	3.64 ± 0.14	3.06 ± 0.10	0.002
Insulin 6 hrs (mU/L)*	2.89 ± 0.12	2.40 ± 0.08	0.001
Insulin 8 hrs (mU/L)*	2.56 ± 0.11	2.39 ± 0.13	0.316
AUC insulin (mU/L.8hr)*	5.05 ± 0.12	4.57 ± 0.07	0.002
iAUC insulin (mU/L.8hr)*	4.41 ± 0.17	3.81 ± 0.11	0.006

* log transformed

Table 3-8: Postprandial apoB$_{48}$, apoB, apoA-I and apoA-II

	PCOS (n=21)	Controls (n=21)	p
ApoB$_{48}$ 0 hrs (μg/mL)*	2.25 ± 0.15	1.98 ± 0.20	0.299
ApoB$_{48}$ 2 hrs (μg/mL)*	2.77 ± 0.09	2.68 ± 0.09	0.503
ApoB$_{48}$ 4 hrs (μg/mL)*	2.84 ± 0.09	2.63 ± 0.13	0.188
ApoB$_{48}$ 6 hrs (μg/mL)*	2.79 ± 0.10	2.67 ± 0.11	0.438
ApoB$_{48}$ 8 hrs (μg/mL)*	2.38 ± 0.12	2.29 ± 0.14	0.625
AUC-apoB$_{48}$ (μg/mL.8hr)*	4.11 ± 0.09	4.01 ± 0.09	0.424
iAUC-apoB$_{48}$ (μg/mL.8hr)	19.97 ± 3.25	19.10 ± 2.97	0.844
ApoB 0 hrs (g/dL)	0.72 ± 0.07	0.82 ± 0.06	0.300
ApoB 2 hrs (g/dL)	0.73 ± 0.06	0.81 ± 0.06	0.433
ApoB 4 hrs (g/dL)	0.78 ± 0.06	0.75 ± 0.06	0.699
ApoB 6 hrs (g/dL)	0.75 ± 0.06	0.80 ± 0.05	0.481
ApoB 8 hrs (g/dL)	0.82 ± 0.05	0.84 ± 0.06	0.756
AUC-apoB hrs (g/dL.8hr)	3.04 ± 0.16	3.18 ± 0.15	0.492
iAUC-apoB (g/dL.8hr)	0.15 ± 0.25	-0.09 ± 0.22	0.474
ApoA-I 0 hrs (g/dL)	1.21 ± 0.07	1.09 ± 0.11	0.358
ApoA-I 2 hrs (g/dL)	1.02 ± 0.11	1.14 ± 0.09	0.406
ApoA-I 4 hrs (g/dL)	1.14 ± 0.10	1.20 ± 0.09	0.653
ApoA-I 6 hrs (g/dL)	1.11 ± 0.09	1.25 ± 0.06	0.221
ApoA-I 8 hrs (g/dL)	1.08 ± 0.09	1.11 ± 0.11	0.805
AUC-apoA-I hrs (g/dL.8hr)	4.42 ± 0.22	4.67 ± 0.16	0.369
iAUC-apoA-I (g/dL.8hr)	-0.41 ± 0.26	0.32 ± 0.33	0.094
ApoA-II 0 hrs (g/dL)	0.33 ± 0.02	0.31 ± 0.03	0.688
ApoA-II 2 hrs (g/dL)	0.27 ± 0.03	0.31 ± 0.02	0.311
ApoA-II 4 hrs (g/dL)	0.26 ± 0.03	0.23 ± 0.03	0.442
ApoA-II 6 hrs (g/dL)	0.25 ± 0.03	0.30 ± 0.02	0.172
ApoA-II 8 hrs (g/dL)	0.25 ± 0.02	0.26 ± 0.02	0.565
AUC-apoA-II hrs (g/dL.8hr)	1.07 ± 0.04	1.12 ± 0.05	0.462
iAUC-apoA-II (g/dL.8hr)	-0.23 ± 0.08	-0.13 ± 0.10	0.421

* log transformed

3.5.3 Comparisons following adjustment for BMI, $HOMA_{IR}$ and S_I

Table 3-9 shows results following adjustment for BMI, $HOMA_{IR}$ and S_I separately. Androgens were higher and SHBG remained lower in PCOS women even after adjusting for BMI. There were no differences in TG, HDL, and $apoB_{48}$ at any time points nor AUC-TG, AUC-HDL, AUC-$apoB_{48}$. Insulin remained significantly higher at all time points except at 8 hours. AUC-insulin and iAUC-insulin were also significantly higher (Table 3-10). There were no differences in the other postprandial apolipoproteins.

Following adjustment for $HOMA_{IR}$, androgens and SHBG were again significantly different between the two groups (Table 3-9). $apoB_{48}$ at 4 hours was significantly ($p=0.034$) higher in the PCOS women (2.92 ± 0.11 μg/mL) compared to controls (2.58 ± 0.10 μg/mL) and insulin at 4 and 6 hours, as well as AUC-insulin and iAUC-insulin also remained significantly higher (Table 3-10).

Finally, following adjustment for S_I, androgens remained significantly greater in PCOS women, but SHBG was no longer significantly different (Table 3-9). Only insulin at 4 hours was higher in the PCOS women, but AUC-insulin and iAUC-insulin were not different between the two groups (Table 3-10).

Table 3-9: Baseline characteristics adjusted for BMI, $HOMA_{IR}$ and log S_I

	Covariate: BMI		Covariate: $HOMA_{IR}$		Covariate: S_I	
	PCOS	Controls	PCOS	Controls	PCOS	Controls
Systolic BP (mmHg)	124.6 ± 2.2	123.3 ± 2.2	124.4 ± 2.2	123.5 ± 2.2	125.0 ± 2.4	123.0 ± 2.6
Diastolic BP (mmHg)	78.0 ± 1.9	78.9 ± 1.9	78.6 ± 2.0	78.2 ± 2.0	78.6 ± 2.1	78.9 ± 2.3
Waist (cm)	107.3 ± 1.5	104.5 ± 1.5	109.0 ± 2.2	102.8 ± 2.2	108.8 ± 2.4	104.3 ± 2.6
Hip (cm)	116.6 ± 1.1	114.8 ± 1.1	118.7 ± 1.9 [a]	112.6 ± 1.9	118.4 ± 2.0	113.3 ± 2.2
Waist : Hip	0.920 ± 0.015	0.910 ± 0.015	0.917 ± 0.015	0.913 ± 0.015	0.919 ± 0.016	0.921 ± 0.017
Weight (kg)	89.2 ± 1.4	89.5 ± 1.4	91.2 ± 2.5	87.4 ± 2.5	91.0 ± 2.8	88.9 ± 3.0
BMI (kg/m^2)	n/a		35.3 ± 1.0	32.9 ± 0.9	35.4 ± 1.1	33.7 ± 1.2
% Body fat mass (%)	37.6 ± 1.2	36.2 ± 1.2	38.2 ± 1.7	35.7 ± 1.7	38.5 ± 1.8	36.2 ± 1.9
T (nmol/L)*	1.12 ± 0.11 [b]	0.33 ± 0.10	1.08 ± 0.11 [b]	0.31 ± 0.11	1.19 ± 0.11 [b]	0.16 ± 0.12
SHBG (nmol/L)*	3.40 ± 0.08 [b]	3.73 ± 0.07	3.39 ± 0.08 [b]	3.74 ± 0.08	3.44 ± 0.08	3.69 ± 0.09
FAI*	2.32 ± 0.12 [b]	1.21 ± 0.12	2.30 ± 0.13 [b]	1.18 ± 0.13	2.36 ± 0.14 [b]	1.08 ± 0.15
Androstenedione (nmol/L)	15.67 ± 0.9 [b]	11.99 ± 0.89	15.07 ± 0.92 [a]	12.30 ± 0.90	15.98 ± 0.99 [b]	11.37 ± 1.05
DHEAS (µmol/L)	8.00 ± 0.68 [b]	4.80 ± 0.66	7.88 ± 0.66	4.72 ± 0.66	8.55 ± 0.66 [b]	3.80 ± 0.72
$HOMA_{IR}$ ($\mu mol^2/L^2$)*	1.26 ± 0.09 [a]	0.93 ± 0.09	n/a		1.18 ± 0.09	1.10 ± 0.10
S_I ($x\ 10^4$ $(mU.L^{-1})^{-1}.min^{-1}$)*	0.65 ± 0.10 [b]	1.19 ± 0.11	0.73 ± 0.09 [a]	1.09 ± 0.09	±	n/a
S_G (min^{-1})	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
AIR_G ($mU.L^{-1}.min$)*	6.48 ± 0.20	6.19 ± 0.21	6.40 ± 0.20	6.29 ± 0.21	6.41 ± 0.21	6.28 ± 0.23
DI	1846 ± 321	2206 ± 345	1893 ± 326	2151 ± 351	2174 ± 312	1831 ± 338

* log transformed. [a] $p < 0.05$ vs. controls. [b] $p < 0.01$ vs. controls.

Table 3-10: Postprandial triglycerides, HDL-chol, apolipoprotein B48s and insulins adjusted for BMI, $HOMA_{IR}$ and S_I

	Covariate: BMI		Covariate: $HOMA_{IR}$		Covariate: S_I	
	PCOS	Controls	PCOS	Controls	PCOS	Controls
TG 0 hrs (mmol/L)*	0.32 ± 0.09	0.17 ± 0.08	0.23 ± 0.08	0.24 ± 0.08	0.22 ± 0.08	0.27 ± 0.09
TG 2 hrs (mmol/L)*	0.66 ± 0.09	0.44 ± 0.09	0.57 ± 0.08	0.51 ± 0.08	0.56 ± 0.09	0.51 ± 0.10
TG 4 hrs (mmol/L)*	0.74 ± 0.09	0.58 ± 0.09	0.65 ± 0.08	0.66 ± 0.08	0.63 ± 0.09	0.71 ± 0.10
TG 6 hrs (mmol/L)*	0.52 ± 0.12	0.30 ± 0.11	0.42 ± 0.11	0.38 ± 0.11	0.40 ± 0.12	0.44 ± 0.13
TG 8 hrs (mmol/L)*	0.39 ± 0.11	0.20 ± 0.11	0.23 ± 0.10	0.31 ± 0.10	0.25 ± 0.11	0.36 ± 0.12
AUC-TG (mmol/L.8hr)*	1.98 ± 0.09	1.80 ± 0.09	1.88 ± 0.08	1.88 ± 0.08	1.87 ± 0.08	1.91 ± 0.09
iAUC-TG (mmol/L.8hr)	1.87 ± 0.29	1.45 ± 0.29	1.63 ± 0.27	1.65 ± 0.27	1.64 ± 0.31	1.82 ± 0.33
HDL-chol 0 hrs (mmol/L)	1.40 ± 0.06	1.45 ± 0.06	1.41 ± 0.06	1.44 ± 0.06	1.40 ± 0.06	1.38 ± 0.07
HDL-chol 2 hrs (mmol/L)	1.33 ± 0.06	1.38 ± 0.06	1.34 ± 0.05	1.37 ± 0.05	1.34 ± 0.06	1.33 ± 0.07
HDL-chol 4 hrs (mmol/L)	1.27 ± 0.06	1.32 ± 0.05	1.28 ± 0.05	1.31 ± 0.05	1.28 ± 0.06	1.27 ± 0.06
HDL-chol 6 hrs (mmol/L)	1.30 ± 0.06	1.37 ± 0.06	1.32 ± 0.06	1.36 ± 0.06	1.31 ± 0.06	1.31 ± 0.07
HDL-chol 8 hrs (mmol/L)	1.35 ± 0.06	1.38 ± 0.06	1.36 ± 0.06	1.37 ± 0.06	1.36 ± 0.06	1.32 ± 0.07
AUC-HDL-chol (mmol/L.8hr)	5.28 ± 0.23	5.49 ± 0.22	5.33 ± 0.22	5.44 ± 0.22	5.31 ± 0.24	5.26 ± 0.26
iAUC-HDL-chol (mmol/L.8hr)	-0.33 ± 0.06	-0.31 ± 0.06	-0.33 ± 0.06	-0.32 ± 0.06	-0.30 ± 0.06	-0.27 ± 0.07

(Continued from above)						
$ApoB_{48}$ 0 hrs (μg/mL)*	2.35 ± 0.18	1.95 ± 0.17	2.32 ± 0.17	1.95 ± 0.17	2.27 ± 0.20	1.99 ± 0.21
$ApoB_{48}$ 2 hrs (μg/mL)*	2.78 ± 0.09	2.68 ± 0.08	2.76 ± 0.08	2.69 ± 0.08	2.74 ± 0.09	2.75 ± 0.10
$ApoB_{48}$ 4 hrs (μg/mL)*	2.91 ± 0.11	2.59 ± 0.10	2.92 ± 0.11†	2.58 ± 0.10	2.89 ± 0.12	2.67 ± 0.12
$ApoB_{48}$ 6 hrs (μg/mL)*	2.86 ± 0.10	2.61 ± 0.10	2.80 ± 0.10	2.65 ± 0.10	2.79 ± 0.11	2.73 ± 0.12
$ApoB_{48}$ 8 hrs (μg/mL)*	2.49 ± 0.13	2.21 ± 0.12	2.39 ± 0.12	2.28 ± 0.12	2.37 ± 0.13	2.34 ± 0.14
Log $ApoB_{48}$ (μg/mL.8hr)*	4.17 ± 0.08	3.97 ± 0.08	4.13 ± 0.08	3.99 ± 0.08	4.11 ± 0.09	4.07 ± 0.10
iAUC B_{48} (μg/mL.8hr)	19.02 ± 3.33	18.31 ± 3.18	17.57 ± 3.19	19.98 ± 3.12	17.92 ± 3.70	21.06 ± 3.91
Insulin 0 hrs (pmol/L)*	2.77 ± 0.09 [a]	2.45 ± 0.08	2.59 ± 0.02	2.60 ± 0.02	2.69 ± 0.09	2.61 ± 0.09
Insulin 2 hrs (pmol/L)*	4.29 ± 0.12 [a]	3.88 ± 0.11	4.20 ± 0.11	3.93 ± 0.11	4.20 ± 0.11	4.04 ± 0.12
Insulin 4 hrs (pmol/L)*	3.59 ± 0.12 [b]	3.06 ± 0.11	3.53 ± 0.11 [a]	3.10 ± 0.11	3.53 ± 0.11 [b]	3.16 ± 0.12
Insulin 6 hrs (pmol/L)*	2.86 ± 0.10 [b]	2.35 ± 0.10	2.77 ± 0.08 [b]	2.43 ± 0.08	2.76 ± 0.08	2.50 ± 0.09
Insulin 8 hrs (pmol/L)*	2.52 ± 0.11	2.43 ± 0.11	2.48 ± 0.11	2.46 ± 0.11	2.50 ± 0.12	2.48 ± 0.13
AUC insulin (pmol/L.8hr)*	5.00 ± 0.09 [b]	4.56 ± 0.09	4.91 ± 0.08 [a]	4.63 ± 0.08	4.91 ± 0.08	4.71 ± 0.09
iAUC insulin (pmol/L.8hr)*	4.31 ± 0.16 [a]	3.72 ± 0.16	4.33 ± 0.17 [a]	3.67 ± 0.17	4.20 ± 0.16	3.86 ± 0.18

* log transformed. [a] $p < 0.05$ vs. controls. [b] $p < 0.01$ vs. controls.

As there were no major differences in results between using the subgroup analyses of 21 BMI-matched pairs and the entire cohort of 26 non-BMI-matched pairs, subjsequent analyses was performed on the entire cohort.

3.5.4 Correlations and multiple regression analyses

Table 3-11 shows the correlations between AUC-TG, AUC-HDL, AUC-apoB_{48}, AUC-apoB, AUC-glucose, AUC-insulin and all other variables. Taking both groups as a whole, AUC-TG correlated positively with WC, weight, BMI and body fat content, and negatively with body water content and SHBG. AUC-TG did not correlate with androgens except for FAI and this is likely to be a result of the inverse correlation with SHBG, which renders FAI to be higher. As expected, AUC-TG also correlated strongly with $HOMA_{IR}$, S_I, and AUC-HDL. Insulin at 0, 2, 4 and 6 hours, AUC-insulin and iAUC-insulin also correlated significantly with AUC-TG. There were also positive correlations between AUC-TG and apoB_{48} at 0, 2, 6, 8 hours, AUC-B_{48}, apoB at 2, 4, 6, 8 hours and AUC-apoB suggesting that both chylomicrons and VLDL contributes to postprandial hypertriglyceridaemia. Glucose at 0 and 2 hours, AUC-glucose, S_G and DI also correlated with AUC-TG. Stepwise multiple regression analysis revealed that S_I, AUC-apoB_{48} and AUC-apoB were independent predictors of AUC-TG, accounting for 55% of the overall variance. This suggests that postprandial chylomicron particles probably play an important role in contributing towards postprandial hypertriglyceridaemia, together with apoB and insulin sensitivity. AUC-TG was the only independent predictor of AUC B48, explaining 25.8% of the variance.

AUC-HDL correlated significantly negatively with WC, HC, weight, BMI, body fat and water percentages, SHBG, FAI, $HOMA_{IR}$, S_I, TG at all time points, AUC-TG, iAUC-TG, iAUC-B48, apoA-I at 2, 4, 6 hours and AUC-apoA-I, glucose at 0 and 2 hours, AUC-glucose, insulin at 0, 2, 4, 6 hours, AUC and iAUC insulin. There was a non-significant negative correlation with androstenedione ($r = -0.27$, $p=0.055$). AUC-TG and WC were the only independent predictors of AUC-HDL, explaining 37.2% of the variance using multiple regression analysis.

As expected, significant correlations were found between AUC-insulin and SHBG. AUC-insulin also correlated strongly with FAI, AUC-TG, AUC-HDL, AUC-glucose, PCOS

status and anthropometrics. Finally AUC-insulin was negatively correlated with DI, suggesting a correlation with possible subsequent β-cell function failure and development of diabetes.

Table 3-11: Correlations between AUC-TG, AUC-HDL-chol, AUC-B_{48}, AUC-ApoB, AUC-glucose, AUC-insulin and other variables

	AUC-TG* (mmol/L.8hr)	AUC-HDL (mmol/L.8hr)	AUC-B_{48}* (μg/mL.8hr)	AUC-ApoB (g/dL.8hr)	AUC-glucose (mmol/L.8hr)	AUC-insulin* (pmol/L.8hr)
AUC-TG* (mmol/L.8hr)	1.000	-0.540 [b]	0.524 [b]	0.484 [b]	0.304 [a]	0.542 [b]
AUC-HDL-chol (mmol/L.8hr)	-0.540 [b]	1.000	-0.229	-0.225	-0.309 [a]	-0.488 [b]
AUC-B_{48}* (μg/mL.8hr)	0.524 [b]	-0.229	1.000	0.298 [a]	0.171	0.165
AUC-ApoB (g/dL.8hr)	0.484 [b]	-0.225	0.298 [a]	1.000	0.038	0.206
AUC-glucose (mmol/L.8hr)	0.304 [a]	-0.309 [a]	0.171	0.038	1.000	0.634 [b]
AUC-insulin* (pmol/L.8hr)	0.542 [b]	-0.488 [b]	0.165	0.206	0.634 [b]	1.000
Age, PCOS status						
Age (years)	0.125	-0.010	-0.025	0.134	0.342 [a]	-0.014
PCOS	0.284 [a]	-0.284 [a]	0.201	0.057	0.315 [a]	0.579 [b]
Androgens, SHBG						
T* (nmol/L)	0.133	-0.163	0.115	-0.023	0.092	0.331
SHBG* (nmol/L)	-0.378 [b]	0.443 [b]	-0.277 [a]	-0.277 [a]	-0.305 [a]	-0.550 [b]
FAI*	0.294 [a]	-0.350 [a]	0.227	0.125	0.226	0.530 [b]
Androstenedione (nmol/L)	0.078	-0.271	-0.014	-0.056	0.146	0.416
DHEAS (μmol/L)	-0.068	-0.048	0.108	-0.289 [a]	0.125	0.158

Anthropometrics						
Waist (cm)	0.314 [a]	-0.483 [b]	0.063	0.080	0.372 [b]	0.661 [b]
Hip (cm)	0.216	-0.285 [a]	-0.064	0.154	0.356 [a]	0.609 [b]
Waist : Hip	0.237	-0.439 [b]	0.163	-0.047	0.193	0.360 [a]
Weight (kg)	0.357 [a]	-0.378 [b]	0.059	0.276	0.271	0.639 [b]
BMI (kg/m^2)	0.316 [a]	-0.456 [b]	0.033	0.184	0.414 [b]	0.686 [b]
% Body fat mass (%)	0.378 [a]	-0.444 [b]	0.009	0.164	0.368 [a]	0.539 [b]
% Body lean mass (%)	-0.128	0.240	0.085	-0.152	-0.348 [a]	-0.381 [a]
% Body water (%)	-0.342 [a]	0.389 [a]	-0.031	-0.178	-0.314	-0.487 [b]
IR, insulin sensitivity, and variables derived from the fsIVGTT						
$HOMA_{IR}$* ($\mu mol^2/L^2$)	0.568 [b]	-0.499 [b]	0.130	0.229	0.366 [b]	0.800 [b]
S_I ($\times 10^4$ $(mU.L^{-1})^{-1}.min^{-1}$)*	-0.601 [b]	0.454 [b]	-0.223	-0.262	-0.453 [b]	-0.810 [b]
S_G (min^{-1})	-0.364 [a]	0.159	-0.266	-0.232	-0.083	-0.171
AIR_G ($mU.L^{-1}.min$)*	0.029	-0.090	-0.282	0.100	-0.410 [b]	0.153
DI	-0.364 [a]	0.226	-0.328 [a]	-0.139	-0.558 [b]	-0.391 [b]

* log transformed

[a] $p < 0.05$ vs. controls. [b] $p < 0.01$ vs. controls.

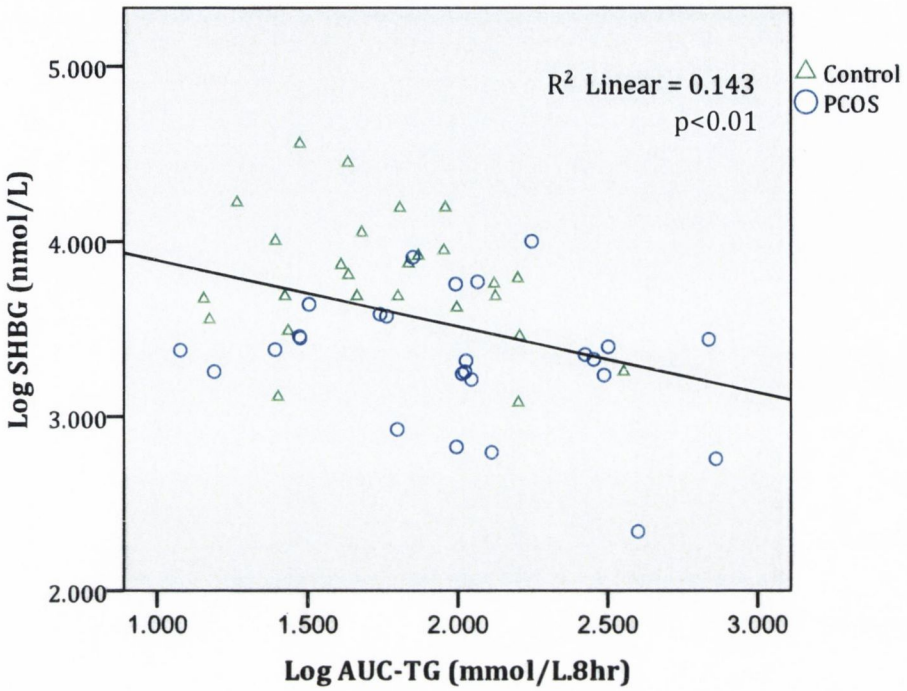

Figure 3-10: Correlation between AUC-TG and SHBG.

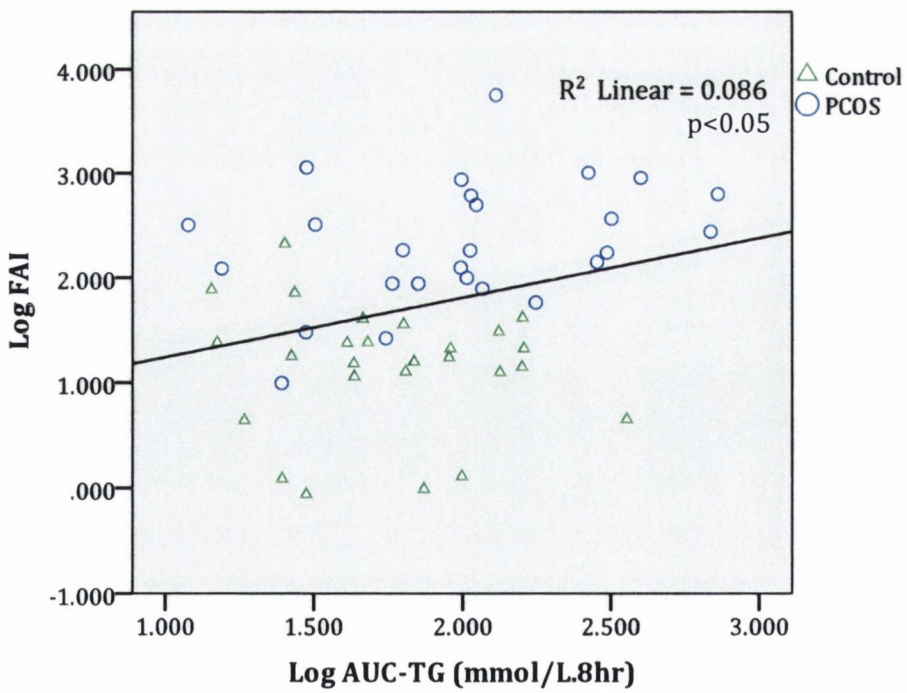

Figure 3-11: Correlation between AUC-TG and FAI.

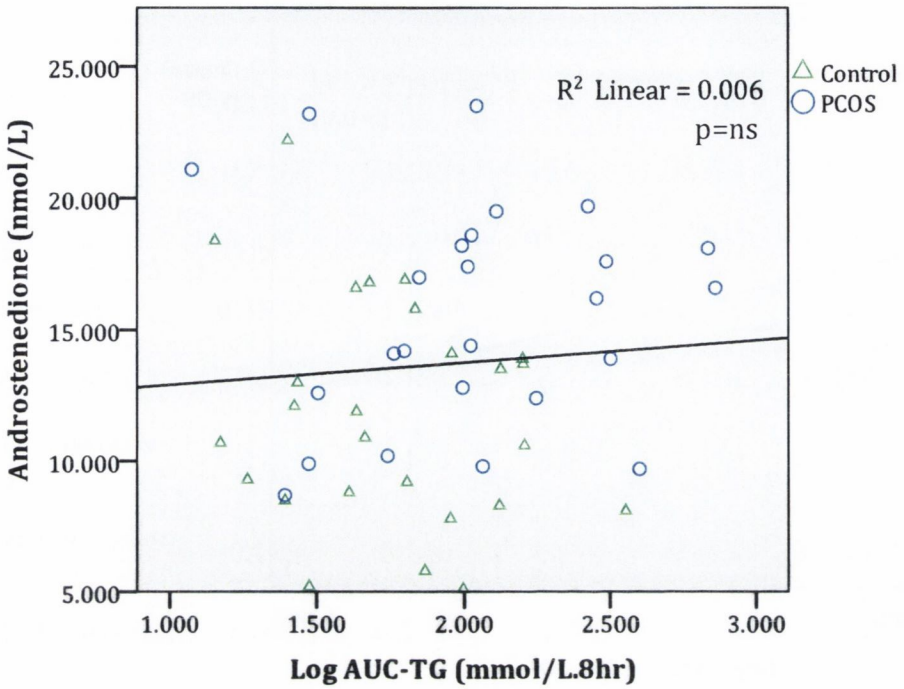

Figure 3-12: Correlation between AUC-TG and Androstenedione.

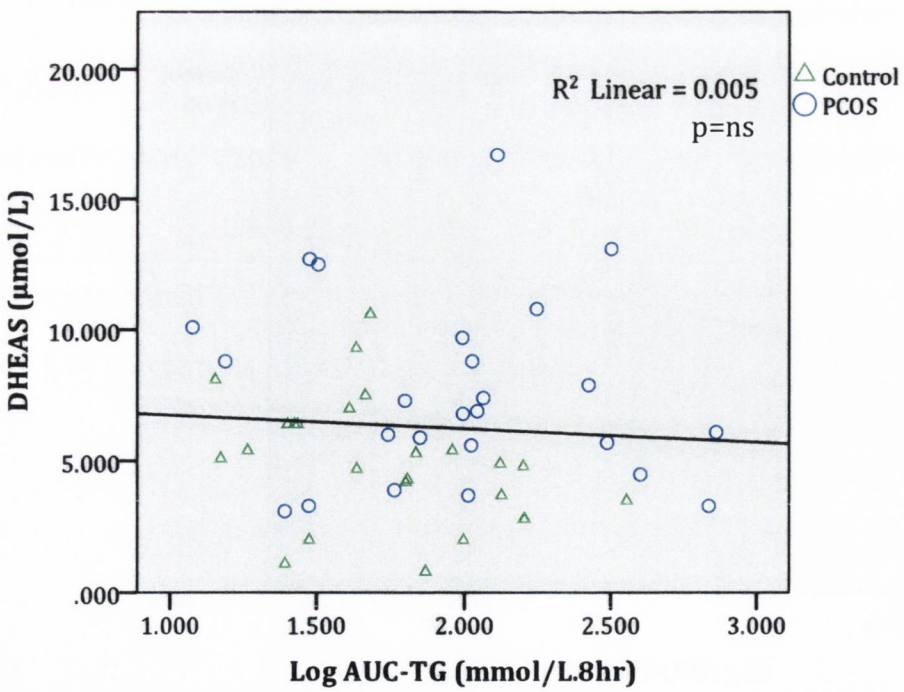

Figure 3-13: Correlation between AUC-TG and DHEAS.

3.6 DISCUSSION

Dyslipidaemia, including both increased TG and low HDL cholesterol is common in PCOS (Legro, Kunselman et al. 2001; Apridonidze, Essah et al. 2005), is a well recognized feature of the metabolic syndrome which confers increased risk of cardiovascular disease (Isomaa, Almgren et al. 2001; Ford 2004). Hypertriglyceridaemia signifies the presence of excess TRL. TRL consists of hepatically derived VLDL characterised by the presence of $apoB_{100}$, and intestinally derived chylomicrons, which contain $apoB_{48}$. Both VLDL and chylomicrons share a common lipolytic pathway and are hydrolysed by lipoprotein lipase (LPL) and there are a number of described receptor mediated pathways through which these particles may eventually be cleared from the circulation (Mahley and Ji 1999). It is postulated that an excess production and / or a delay in clearance of postprandial lipoproteins of both TRL particles maybe implicated in atherosclerosis (Zilversmit 1979; Groot, van Stiphout et al. 1991).

To the best of **the author's** knowledge, this is the first study to compare the postprandial responses in obese PCOS women with that of obese control subjects. Although some postprandial studies in PCOS women have been done previously, this is the first one to evaluate the contribution of the two main metabolic / endocrine disturbances in PCOS women – IR and hyperandrogenaemia – to postprandial dyslipidaemia in obese women with PCOS. Both are implicated in accelerated atherosclerosis but the relative contribution of either is a subject of controversy (Dunaif 1997; Morin-Papunen, Vauhkonen et al. 2000; Coviello, Legro et al. 2006). Obesity, in particular central obesity is strongly associated with insulin resistance, whilst androgens are also associated with an android distribution of fat. In a previous study by Valazquez et al a strong correlation between postprandial triglycerides and waist to hip ratio was demonstrated. That study evaluated overweight (BMI 27.41 ± 0.50 kg/m^2) PCOS women and compared them to lean PCOS women and lean controls. Only free testosterone was measured, and there was no analysis between this and postprandial hypertriglyceridaemia (Velazquez, Bellabarba et al. 2000). A

separate study by Bahceci et al evaluated postprandial responses to an oral fat tolerance test, in lean PCOS women with lean controls (BMI 23.5 ± 2.6 kg/m^2 vs. 23.1 ± 4.0 kg/m^2; $p > 0.05$). They showed that PCOS women had higher baseline insulin, $HOMA_{IR}$, AUC-TG, AUC-total-cholesterol, AUC-VLDL-cholesterol, and AUC-apoB. Surprisingly though, AUC-insulin did not differ between the two groups. Again, no analyses were reported between androgens and postprandial lipids (Bahceci, Aydemir et al. 2007).

In this study, comparing obese women with PCOS with obese control subjects, AUC-TG, AUC-HDL, AUC-glucose and AUC-insulin were all significantly different. HDL-chol and insulin were different at most of the time points, but TG was only different at 2 hours. Independent predictors of AUC-TG included S_I, AUC-apoB48 and AUC-apoB, accounting for 55% of the variance. This suggests that postprandial chylomicron particles have an important role to play in postprandial hypertriglyceridaemia, together with insulin sensitivity and apoB. On the other hand, AUC-TG was the only independent predictor of AUC-$apoB_{48}$, explaining 25.8% of the variance.

Following adjustment for BMI, lipid differences were no longer noted but insulins at all time points and AUC-insulin and iAUC-insulin were still much greater in PCOS women consistent with the fact that PCOS women are more insulin resistant than their BMI matched counterparts (Dunaif, Segal et al. 1989; Carmina and Lobo 2004). In this study, IR was determined by the more convenient $HOMA_{IR}$ and insulin sensitivity was determined by the more sophisticated fsIVGTT derived S_I. When comparisons were made adjusting for $HOMA_{IR}$, postprandial insulins were still significantly higher in the PCOS group. However, these differences were largely no longer present when adjustments were made for S_I, which may be a better marker for insulin sensitivity than $HOMA_{IR}$, since the latter is calculated from fasting values of insulin and glucose.

There was only weak or no correlation between androgens and TG, HDL-chol, B48, apoA-I, apoA-II and apoB. There were very strong correlations between SHBG and TG, HDL-chol, AUC-apoB_{48}, AUC-apoB, and AUC-apoA-II. FAI but not testosterone *per se* also strongly correlated with HDL at all time points, TG at 0 and 2 hours, and both AUC-HDL-chol and AUC-TG. This suggests that IR, and perhaps obesity, both of which are strongly negatively correlated with SHBG, rather than androgens, may be the major determinant of postprandial lipids.

This study had certain limitations. PCOS women and the controls were not entirely matched for BMI. However, both were groups were obese and results were analysed with BMI as a covariate. The two groups did not match for IR as assessed by $HOMA_{IR}$ nor S_I as assessed by the fsIVGTT. However this is to be expected based on the well accepted notion that women with PCOS are more insulin resistant than BMI-matched counterparts. In addition, after using $HOMA_{IR}$ and S_I as covariates, results still revealed that hyperinsulinaemia, and possibly a low SHBG level are the main factors influencing postprandial hypertriglyceridaemia. Postprandial chylomicrons also appear to play a significant role in postprandial Hypertriglyceridaemia in premenopausal women with and without PCOS.

In conclusion, this study provides evidence that in obese women with PCOS, IR plays a more important role than hyperandrogenaemia in postprandial dyslipidaemia and cardiovascular risk. Targeting obesity and thereby improving IR by lifestyle measures and perhaps the use of agents such as metformin should be a priority in the treatment of obese PCOS women.

Chapter 4

Insulin Resistance, β-Islet Cell Function and Adipokines in PCOS

"It is the mark of an educated mind to be able to entertain a thought without accepting it."

Aristotle

4.1 CHAPTER SYNOPSIS

4.1.1 Context

Hypoadiponectinaemia might contribute to the association between androgen levels and IR in premenopausal women.

4.1.2 Aims

To determine the relationships between androgens, adiponectin and glucose homeostasis in premenopausal women with and without PCOS.

4.1.3 Methods

28 women with PCOS and 36 women without PCOS underwent an fsIVGTT. Adipokines including total and oligomeric forms of adiponectin, leptin and resistin were evaluated and assessed in relation to variables derived from fsIVGTT and androgens.

4.1.4 Results

BMI-adjusted insulin sensitivity (S_I) was reduced in PCOS, and correlated negatively ($R=-0.39$, $P<0.01$) with free androgen index (FAI). Disposition index (DI) did not differ between groups. Total adiponectin correlated positively ($P<0.01$) with S_I ($R=0.51$) and DI ($R=0.35$), but not with androgens. High molecular weight-adiponectin correlated with S_I ($R=0.42$, $P<0.01$), while medium molecular weight-adiponectin correlated ($P<0.01$) with both S_I ($R=0.45$) and DI ($R=0.46$). Multiple regression analysis revealed PCOS status and adiponectin to be independent predictors of S_I. Adiponectin is the only independent predictor of DI.

4.1.5 Conclusions

Hypoadiponectinaemia in premenopausal women is independently associated with increased IR and reduced DI. PCOS status is also associated with IR but this association is independent of adiponectin levels. Oligomeric forms of adiponectin might exert differential effects on different components of glucose homeostasis.

4.2 INTRODUCTION

Women with PCOS are at increased risk of T2DM (Dahlgren, Johansson et al. 1992; Cibula, Cifkova et al. 2000; Salley, Wickham et al. 2007). Established diabetes is already present in up to 10% of overweight women with PCOS while impaired glucose tolerance, which confers a 50% risk of progression to diabetes within 5 years, can be detected in up to 35% (Ehrmann, Barnes et al. 1999; Legro, Kunselman et al. 1999; Gambineri, Pelusi et al. 2004). Longitudinal studies have demonstrated an approximately 7-fold increase in development of diabetes by the time of menopause in women with PCOS compared to normal women (Dahlgren, Johansson et al. 1992).

Since it is clear that not all women with PCOS develop diabetes, identification of other variables that potentially contribute to diabetes risk is important. In addition to well-established risk factors such as body composition (Ferrannini, Nannipieri et al. 2004; Bunt, Krakoff et al. 2007) and dietary fat intake (Lindstrom, Peltonen et al. 2006), low serum levels of the adipokine, adiponectin have recently emerged as a strong and independent determinant of insulin resistance and predictor of diabetes (Lindsay, Funahashi et al. 2002; Krakoff, Funahashi et al. 2003; Snehalatha, Mukesh et al. 2003; Spranger, Kroke et al. 2003; Duncan, Schmidt et al. 2004; Daimon 2005; Mather, Funahashi et al. 2008). Adiponectin circulates as 3 major oligomeric forms: a low molecular weight (LMW) trimer, a middle molecular weight (MMW) hexamer, and a high molecular weight (HMW) 12-18-mer adiponectin (Kadowaki, Yamauchi et al. 2006). The HMW form, is thought to the be the most active form (Kadowaki, Yamauchi et al. 2006) and appears more strongly associated with insulin resistance than total adiponectin (Hara, Horikoshi et al. 2006; Lara-Castro, Luo et al. 2006), but hexameric i.e. MMW and trimeric i.e. LMW adiponectin have also been shown to exert potentially important effects at cellular level (Tsao, Tomas et al. 2003; Waki, Yamauchi et al. 2003). Notably, HMW-adiponectin levels are greater in women than men (Waki, Yamauchi et al. 2003) while studies in mice have demonstrated a direct effect of testosterone to reduce HMW-adiponectin (Xu, Chan et al. 2005), raising

hyperandrogenaemia as a plausible mechanism through which PCOS might increase IR.

Individual susceptibility to diabetes, however, reflects not only insulin resistance but also the ability of β-cells to compensate for increased IR (Chiasson and Rabasa-Lhoret 2004; Weir and Bonner-Weir 2004; Abdul-Ghani, Tripathy et al. 2006). The fsIVGTT is a well-validated approach to determine both insulin sensitivity and DI, an estimate of the ability of β cells to compensate for a reduction in insulin sensitivity by increasing insulin secretion (Bergman 2007). In high-risk population groups, DI has been shown to be a major predictor of progression to diabetes (Weyer, Funahashi et al. 2001; Kharroubi, Rasschaert et al. 2003; Festa, Williams et al. 2006).

4.3 AIMS

The aim of this study was to determine the impact of adipokines on variables derived from the fsIVGTT in premenopausal women with and without PCOS. S_I and DI were evaluated in relation to circulating androgens and the adipokines: leptin, resistin and adiponectin. The relationship between one or more of the three major oligomeric forms of adiponectin and glucose homeostasis were also explored.

4.4 METHODS

4.4.1 Subjects

64 premenopausal women with (n=28) and without (n=36) PCOS were recruited. All subjects with PCOS and 22 subjects without PCOS were obese (BMI ≥30 kg/m^2) while 14 subjects without PCOS were non-obese (BMI <30 kg/m^2). PCOS subjects fulfilled the NIH critieria for diagnosis and met all the inclusion criteria as described in Section 2.1.1. The control group was comprised of normal volunteers on no medications recruited from the general population as described in Section 2.1.2. All control subjects were studied in the follicular phase of their menstrual cycle. PCOS subjects were studied within 2 weeks of a spontaneous bleed. All study subjects gave their written signed consent to the study, which was approved by the Joint Research Ethics Committee of the Adelaide and Meath Hospital **and St. James's Hospital** (Dublin, Ireland).

4.4.2 Clinical protocol

This was a cross-sectional study. All subjects had their height measured with a Harpenden stadiometer and weight measured in light clothing. BMI was calculated as weight (kg) / height squared (m^2). WC and HC were measured with a non-distensible flexible tape, and WHR calculated accordingly. All subjects also underwent estimation of body composition including percentage body fat using bioimpedance analysis using the Bodystat 1500 system (Bodystat Ltd, UK) described in Section 2.3.3.

4.4.2.1 Frequently sampled intravenous glucose tolerance test (fsIVGTT)

Each subject underwent an fsIVGTT. This procedure has been described in Section 2.4.2.

4.4.3 Laboratory methods

4.4.3.1 Hormones, glucose, HbA1c, lipids

Insulin and testosterone were measured by electrochemiluminesence immunoassay on the Roche E Module. E2, progesterone, LH, FSH, TSH, fT4, PRL, cortisol were all measured by chemiluminesence immunoassay on DPC Immulite 2500. SHBG and DHEAS were measured by chemiluminesence immunoassay on DPC Immulite 2000 in St James's Hospital. FAI was calculated by the equation: FAI = total testosterone (nmol/L) / SHBG (nmol/L) x 100. Androstenedione and 17-OHP were measured by radioimmunoassay on the DPC Coat-A-Count. Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module. HbA1c was measured by ion exchange chromatography on the Tosoh 2.2. Cholesterol, TG, HDL-chol and NEFA were measured by a standard enzymatic colourimetric method on the Roche P Module. LDL-chol was calculated by the Friedewald formula. Laboratory methods of all these are described in full in section 2.7.

4.4.3.2 Total and oligomeric adiponectin

Plasma levels of total, high, medium and low molecular weight adiponectin were determined using a commercially available ELISA kit (ALPCO Diagnostics, Salem, NH, USA) according to manufacturer's instruction. A full description of the method for total and the various oligomeric forms of adiponectin can be found in Section 2.7.11.1. Intra- and inter-assay CVs were 5.96% and 6.6%.

4.4.3.3 Leptin and Resistin

Plasma levels of resistin and leptin were determined by ELISA using a commercially available kit (R&D Systems Inc, MN, USA) according to the manufacturer's instructions. Absorbance of the plate was read at 450nm. All samples were assayed in

duplicate. Intra- and inter-assay CVs for resistin were 5.2% and 4.8% and that of leptin were 8.81% and 9.78%. Leptin analysis was carried out by Dr. Niamh Phelan. Resistin analysis was carried out by Dr. Anna-Louise O'Connor.

4.4.4 Statistical analyses

Data are presented as mean ± SEM. Skewed variables were logarithmically transformed to normalise data prior to analysis. Statistical analysis of clinical characteristics was made using ANOVA with post-hoc analysis according to the Bonferroni method. Correlations were performed using Pearson's correlation coefficient. To determine the effect of PCOS status, which is a dichotomous variable, on adipokines and variables derived from the fsIVGTT, subjects with PCOS were compared to normal subjects (obese and non-obese combined) using ANCOVA to adjust for BMI. Multiple linear regression analysis was used to identify independent contributors to variables derived from the fsIVGTT. Independent variables that correlated significantly in univariate analysis were entered into the multiple regression models using the Stepwise method. Statistical significance was defined as $p<0.05$.

4.5 RESULTS

4.5.1 Baseline demographic data and hormonal profile

Anthropometric and biochemical characteristics of the study subjects are displayed in Table 4-1. Women with PCOS were significantly younger than obese control subjects but did not differ in age from non-obese control subjects. Body weight, BMI, body fat content, WC and HC were greater and SHBG lesser in women with PCOS and obese control subjects compared to non-obese control subjects. Systolic blood pressure was greater in women with PCOS compared to non-obese control subjects. Total testosterone, FAI, androstenedione and DHEAS were all greater in women with PCOS compared to both of the control groups.

Table 4-1: Anthropometric and biochemical characteristics of obese patients with and without PCOS and non-obese patients without PCOS

	Obese PCOS (n=28)	Obese Controls (n=22)	Non-obese Controls (n=14)
Age (yrs)	30.7 ± 1.2 [a]	36.6 ± 1.4	33.4 ± 1.9
Systolic BP (mmHg)	126.8 ± 1.6 [b]	124.3 ± 3.0 [c]	113.9 ± 3.4
Diastolic BP (mmHg)	80.0 ± 1.2	82.0 ± 3.4	75.8 ± 2.9
Waist (cm)	113.3 ± 2.1 [b]	106.8 ± 1.9 [c]	82.9 ± 2.7
Hip (cm)	122.4 ± 2.0 [b]	116.8 ± 2.2 [c]	101.6 ± 1.6
Waist : Hip	0.9 ± 0.0 [b]	0.9 ± 0.0 [c]	0.8 ± 0.0
Weight (kg)	97.8 ± 2.7 [b]	91.2 ± 2.5 [c]	65.7 ± 2.6
BMI (kg/m^2)	38.2 ± 1.1 [b]	35.1 ± 0.9 [c]	25.0 ± 0.7
% Body fat mass (%)	41.5 ± 1.1 [b]	41.4 ± 1.1 [c]	23.7 ± 2.1
T (nmol/L)*	1.06 ± 0.09 [a,b]	0.19 ± 0.14	0.40 ± 0.13
SHBG (nmol/L)*	3.35 ± 0.07 [b]	3.55 ± 0.12 [c]	4.04 ± 0.12
FAI*	2.32 ± 0.10 [a,b]	1.26 ± 0.18	0.96 ± 0.18
Androstenedione (nmol/L)	15.83 ± 0.78 [a,b]	12.28 ± 1.07	9.78 ± 0.83
DHEAS (µmol/L)	7.53 ± 0.66 [a,b]	4.95 ± 0.66	4.29 ± 0.46
S_I (x 10^4 $(mU.L^{-1})^{-1}.min^{-1}$)*	0.50 ± 0.10 [a,b]	1.09 ± 0.09 [c]	1.80 ± 0.10
S_G (min^{-1})	0.02 ± 0.00 [b]	0.02 ± 0.00	0.02 ± 0.00
AIR_G ($mU.L^{-1}.min$)*	6.53 ± 0.18	6.30 ± 0.13	5.89 ± 0.21
DI	1665 ± 252 [b]	1926 ± 253	2956 ± 565

* log transformed

[a] $p < 0.05$ PCOS vs. obese controls

[b] $p < 0.05$ PCOS vs. non-obese controls

[c] $p < 0.05$ obese controls vs. non-obese controls

4.5.2 fsIVGTT, anthropometrics and androgens

Correlations between age, body composition and androgen status with variables derived from the fsIVGTT are presented in Table 4-2. Age correlated negatively with AIR_G but was not significantly associated with any other component of glucose homeostasis. Body weight, BMI, waist circumference and %BF all correlated negatively with both S_I and S_G while only waist circumference correlated negatively with DI. Of the androgens, total testosterone did not correlate with any of the fsIVGTT variables. FAI, androstenedione and DHEAS correlated negatively with S_I while FAI also correlated positively with AIR_G. None of the androgens correlated with either S_G or DI.

Table 4-2: Correlations between age, body composition and androgens with components of glucose/ insulin homeostasis

	S_I *	S_G	AIR_G *	DI
Age	0.01	-0.00	-0.22 [a]	-0.21
Body weight	-0.56 [b]	-0.49 [b]	0.20	-0.23
BMI	-0.59 [b]	-0.38 [b]	0.21	-0.25
Waist circumference	-0.61 [b]	-0.42 [b]	0.15	-0.32 [a]
% body fat	-0.50 [b]	-0.32 [a]	0.24	-0.19
Total T*	-0.18	-0.23	0.17	-0.01
FAI*	-0.39 [b]	-0.20	0.30 [a]	-0.03
Androstenedione	-0.31 [a]	-0.10	0.19	-0.07
DHEAS	-0.30 [a]	0.01	0.05	-0.19

* log transformed

[a] $p < 0.05$. [b] $p < 0.01$.

Because PCOS status is a dichotomous variable, to determine its effect on glucose homeostasis ANCOVA was performed comparing results obtained from fsIVGTT in women with and without PCOS following adjustment for age and body composition. These results are shown in Table 4-3. Only S_I was reduced in women with PCOS following adjustment for age BMI but AIR_G, S_G and DI did not differ between the groups. Similar results were seen following adjustments for age and WC or %BF.

Table 4-3: Comparison of fasting glucose, fasting insulin and components of glucose/insulin homeostasis in women with and without PCOS following adjustment for age and BMI

	PCOS (n=28)	Controls (n=34)
Fasting glucose (mmol/l)	5.14 ± 0.57	4.77 ± 0.45
Fasting insulin (mU/l)	2.93 ± 0.42	2.48 ± 0.37
S_I (x 10^4 $(mU.L^{-1})^{-1}.min^{-1}$)*	0.653 ± 0.096 [a]	1.233 ± 0.084
S_G (min^{-1})	0.018 ± 0.002	0.020 ± 0.001
AIR_G ($mU.L^{-1}.min$)*	6.384 ± 0.175	6.265 ± 0.153
DI	1813 ± 316	2218 ± 276

* log transformed

[a] $p<0.05$ PCOS vs. Controls

4.5.3 Adipokines, anthropometrics and androgens

Adipokine results are shown in Table 4-4. Total adiponectin was lower in lower in obese PCOS compared to obese controls (p=0.001) and in obese controls compared with non-obese controls subjects (p=0.019). Both HMW and MMW adiponectin were also lower in obese PCOS subjects but only when compared with non-obese controls (p=0.040 and p=0.010 respectively). There were no differences in LMW adiponectin between any of the three groups and there were no differences in any of the

adiponectins between obese PCOS and obese controls. Resistin levels were not statistically different between any of the three groups. Leptin was higher in obese PCOS compared with obese controls (p=0.002) and also compared with non-obese controls (p<0.001). These results are also shown in the graphs in Figures 4-1 to 4-6.

Table 4-4: Adipokine levels of obese patients with and without PCOS and non-obese patients without PCOS

	Obese PCOS (n=28)	Obese Controls (n=22)	Non-obese Controls (n=14)
Total adiponectin (μg/mL)	4.19 ± 0.38 [b]	4.60 ± 0.53 [c]	6.62 ± 0.61
HMW adiponectin (μg/mL)	1.65 ± 0.28 [b]	1.87 ± 0.39	2.66 ± 0.39
MMW adiponectin (μg/mL)	1.03 ± 0.11 [b]	1.13 ± 0.18	1.72 ± 0.28
LMW adiponectin (μg/mL)	1.74 ± 0.22	1.68 ± 0.20	2.29 ± 0.37
Leptin (ng/ml)	42.60 ± 2.31 [a,b]	28.92 ± 3.55	20.22 ± 2.62
Resistin (ng/ml)	13.52 ± 1.14	13.31 ± 1.23	12.72 ± 1.07

[a] $p < 0.05$ PCOS vs. obese controls

[b] $p < 0.05$ PCOS vs. non-obese controls

[c] $p < 0.05$ obese controls vs. non-obese controls

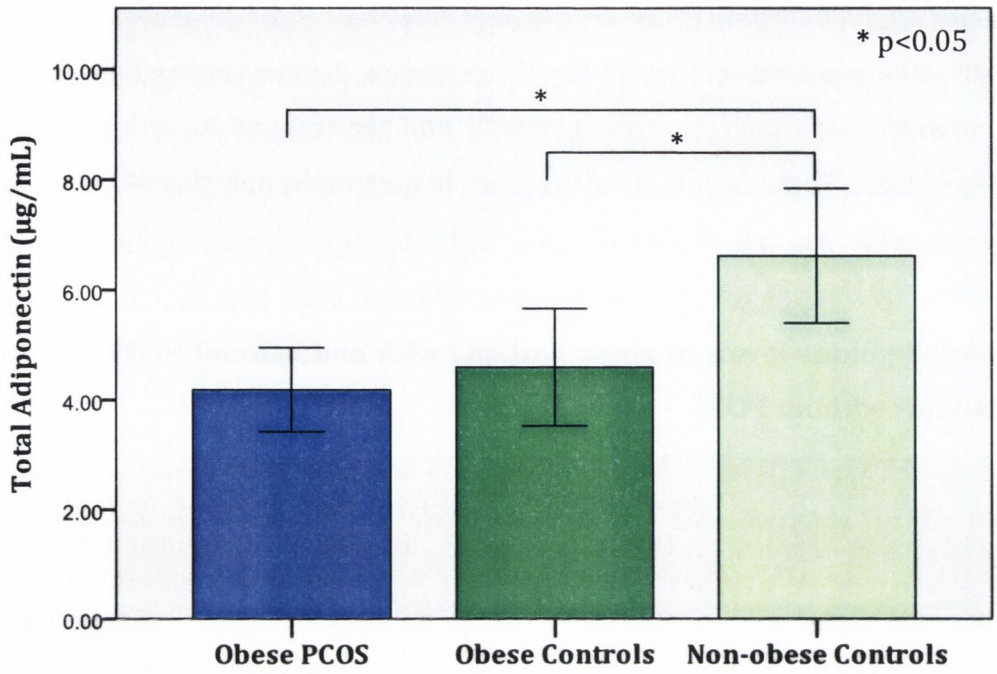

Figure 4-1: Total adiponectin levels in PCOS, obese controls and lean controls

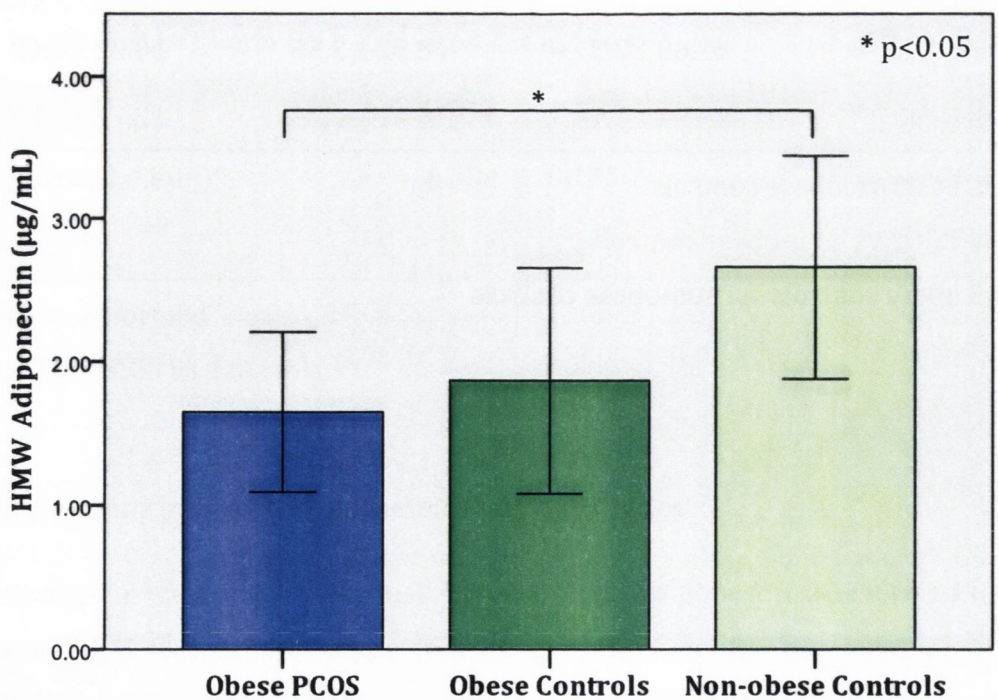

Figure 4-2: HMW adiponectin levels in PCOS, obese controls and lean controls

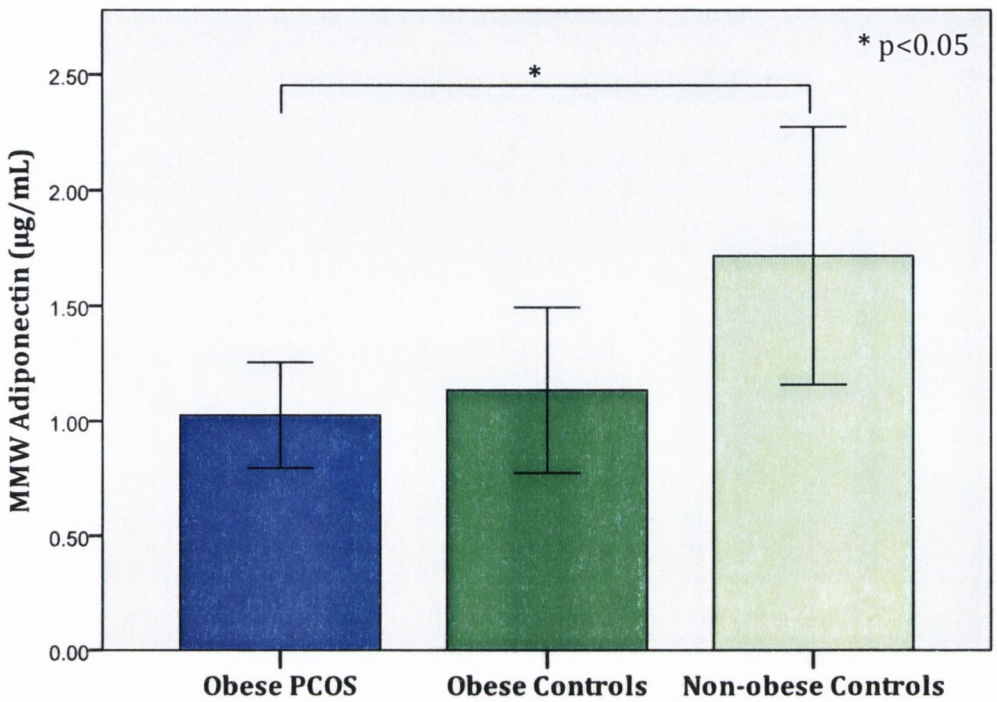

Figure 4-3: MMW adiponectin levels in PCOS, obese controls and lean controls

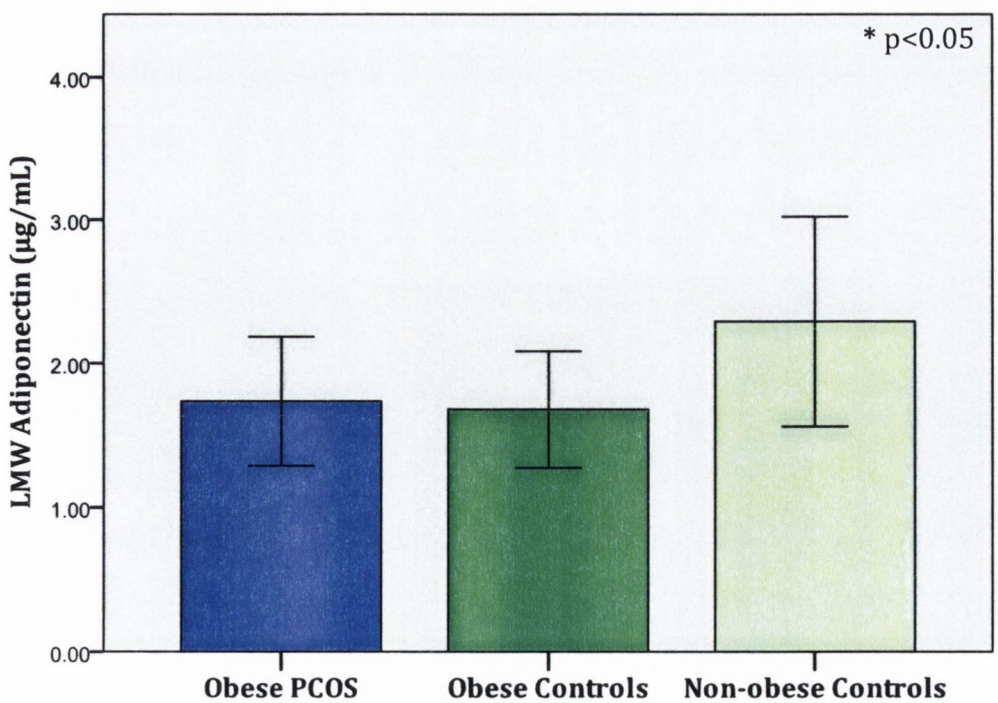

Figure 4-4: LMW adiponectin levels in PCOS, obese controls and lean controls

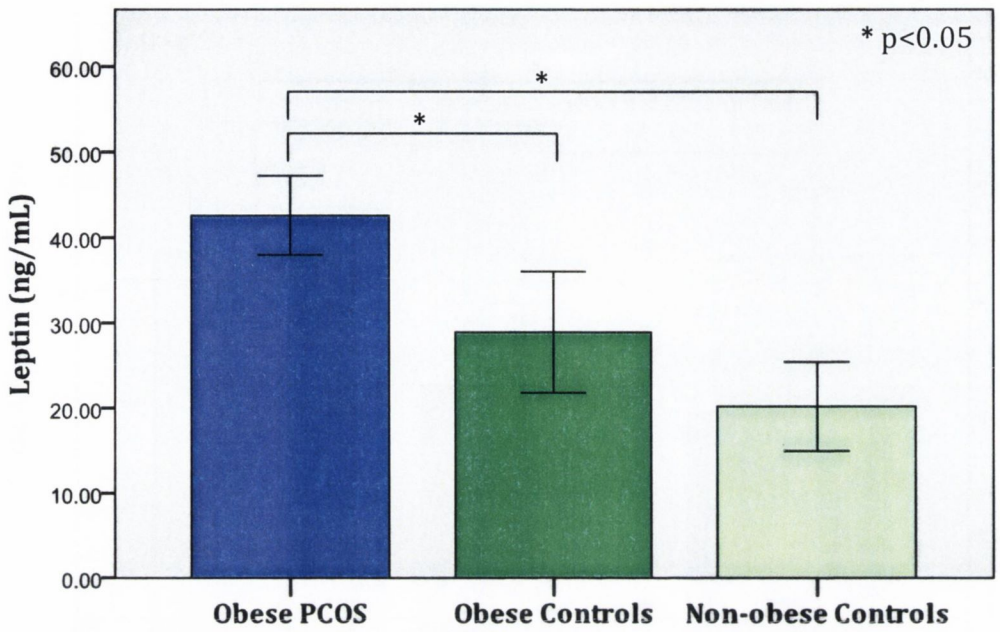

Figure 4-5: Leptin levels in PCOS, obese controls and lean controls

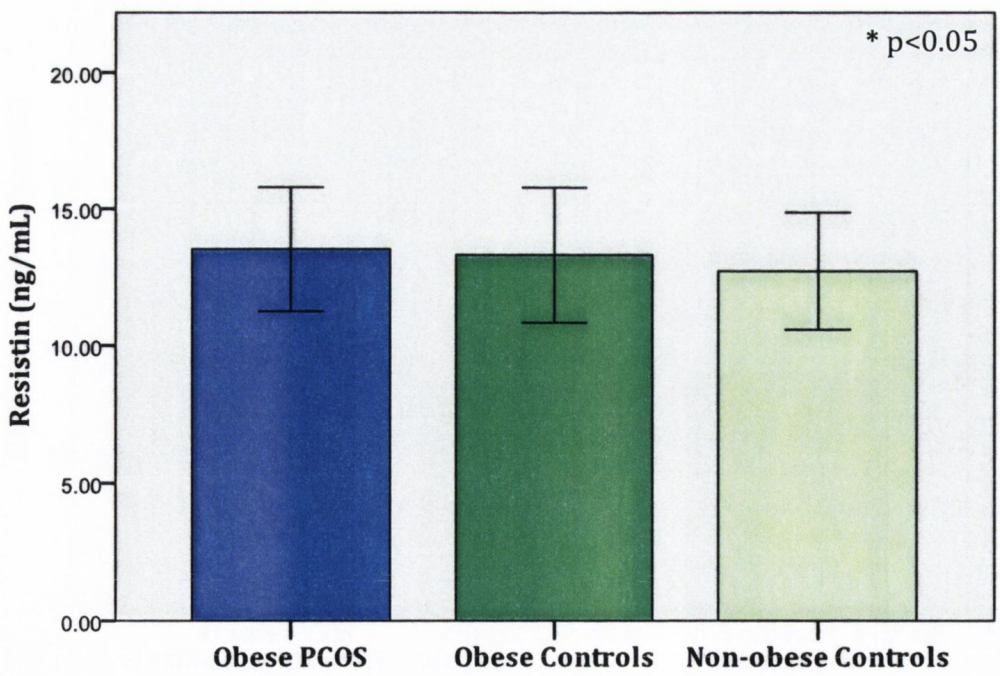

Figure 4-6: Resistin levels in PCOS, obese controls and lean controls

Following adjustment of age and BMI, only leptin levels were found to be higher in PCOS women compared to control subjects (Table 4-5). Adiponectins and resistin were not significantly different between PCOS and controls.

Table 4-5: Comparison of adipokines following adjustment for age and BMI

	PCOS (n=28)	Controls (n=34)
Total adiponectin (μg/mL)	4.69 ± 0.46	5.04 ± 0.40
HMW adiponectin (μg/mL)	1.84 ± 0.34	2.04 ± 0.29
MMW adiponectin (μg/mL)	1.08 ± 0.17	1.30 ± 0.15
LMW adiponectin (μg/mL)	1.95 ± 0.25	1.80 ± 0.22
Leptin (ng/ml)	38.85 ± 3.15 [a]	26.30 ± 2.48
Resistin (ng/ml)	13.08 ± 1.14	13.46 ± 1.14

* log transformed

[a] p=0.01, PCOS vs. Controls

Since there were no differences between PCOS and control subjects, correlation analyses were performed for the whole group. Correlations between age, weight-related anthropometry and androgens with adipokine concentrations are presented in Table 4-6. Age correlated negatively with leptin but was not significantly associated with any other adipokine. Body weight, BMI, WC and %BF correlated positively with leptin and negatively with total, HMW-and MMW-adiponectin, while BMI and %BF correlated negatively with LMW-adiponectin. There were no significant associations between body composition and resistin. There were no significant associations of androgen levels with total adiponectin or its isoforms other than weak negative associations between FAI and androstenedione with LMW-adiponectin. FAI and androstenedione also correlated positively with leptin while DHEAS correlated positively with resistin.

Table 4-6: Correlations between age, body composition and androgens with serum adipokines in all patients

	Total adiponectin	HMW-adiponectin	MMW-adiponectin	LMW-adiponectin	Leptin	Resistin
Age	-0.17	-0.20	-0.20	-0.02	-0.37 [a]	-0.11
Body weight	-0.46 [b]	-0.34 [b]	-0.28 [a]	-0.23	0.62 [b]	0.13
BMI	-0.50 [b]	-0.36 [b]	-0.32 [a]	-0.26 [a]	0.59 [b]	0.09
WC	-0.48 [b]	-0.34 [b]	-0.37 [b]	-0.23	0.65 [b]	0.15
% body fat	-0.51 [b]	-0.37 [b]	-0.31 [b]	-0.33 [b]	0.40 [b]	0.13
Total T*	-0.14	-0.02	0.01	-0.21	0.26	-0.04
FAI*	-0.18	0.01	0.02	-0.28 [a]	0.35 [b]	0.01
Androstenedione	-0.19	-0.00	-0.06	-0.32 [a]	0.43 [b]	0.17
DHEAS	-0.03	0.03	-0.06	0.02	0.10	0.31 [a]

* log transformed.

[a] $p < 0.05$, [b] $p < 0.01$

4.5.4 fsIVGTT and adipokines

Total, HMW- and MMW- adiponectin correlated positively with S_I while total and MMW- adiponectin also correlated positively with DI (Table 4-7). There were no significant associations between adiponectin or any of its isoforms with AIR_G or S_G. Leptin correlated negatively with S_I ($r=-0.593$, $p<0.01$) and S_G ($r=-0.449$, $p<0.01$) and positively with AIR_G ($r=0.352$, $p<0.05$). There were no associations of resistin with any component of glucose homeostasis.

Table 4-7: Correlations between fsIVGTT variables and serum adipokines in all patients

	Total adiponectin	HMW-adiponectin	MMW-adiponectin	LMW-adiponectin	Leptin	Resistin
$S_I(x10^4(mU.L^{-1})^{-1}.min^{-1})$*	0.501 [b]	0.418 [b]	0.448 [b]	0.108	-0.593 [b]	0.047
S_G (min^{-1})	0.245	0.248	0.236	-0.031	-0.449 [b]	-0.021
$AIR_G(mU.L^{-1}.min)$*	-0.041	-0.114	0.105	0.044	0.352 [a]	-0.075
DI	0.351 [b]	0.231	0.411 [b]	0.146	-0.208	-0.017

* log transformed

[a] $p < 0.05$, [b] $p < 0.01$

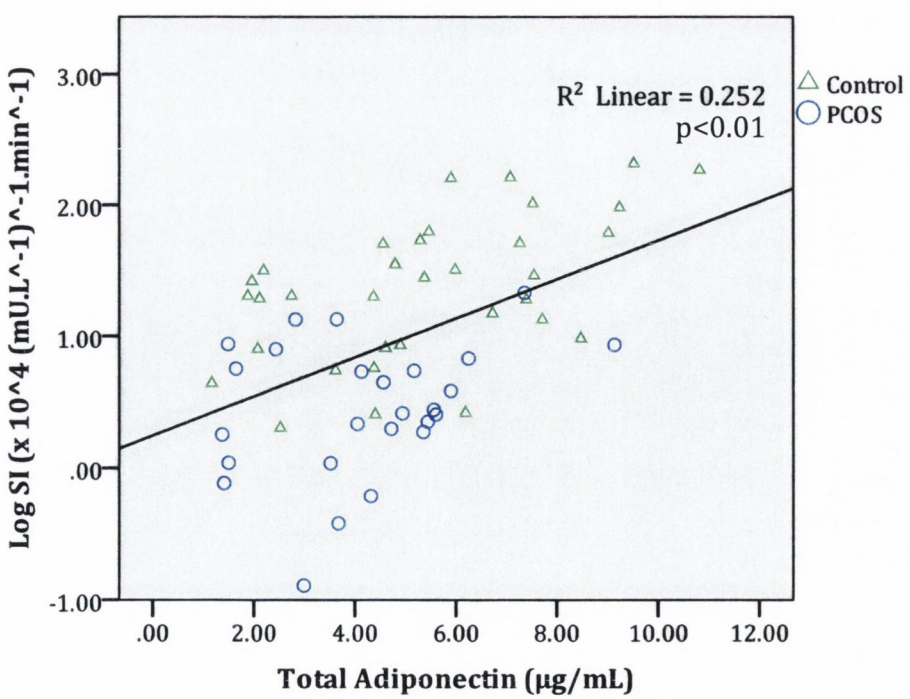

Figure 4-7: Correlation between total adiponectin and S_I

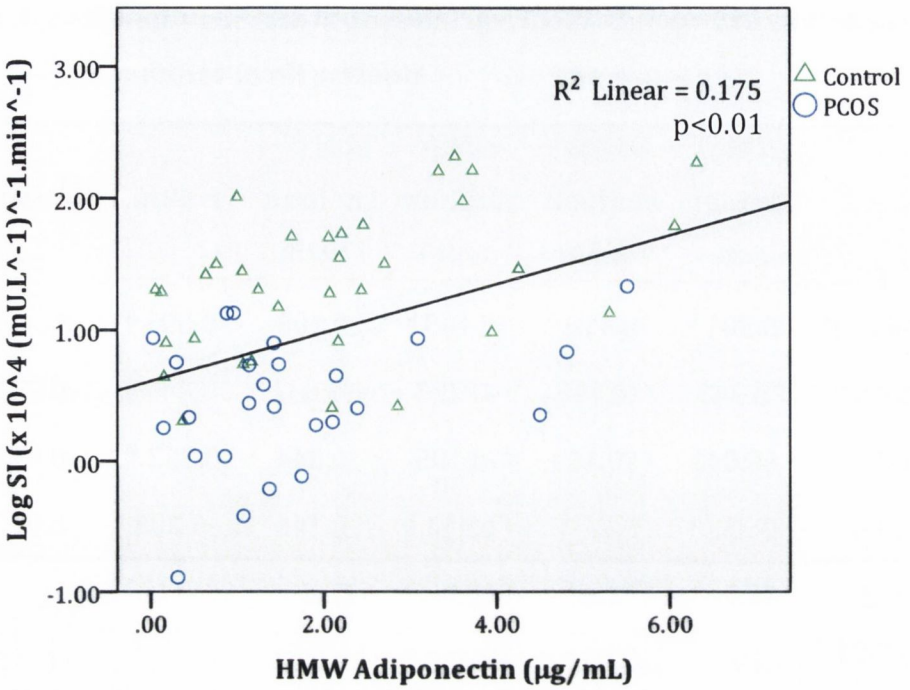

Figure 4-8: Correlation between HMW adiponectin and S_I

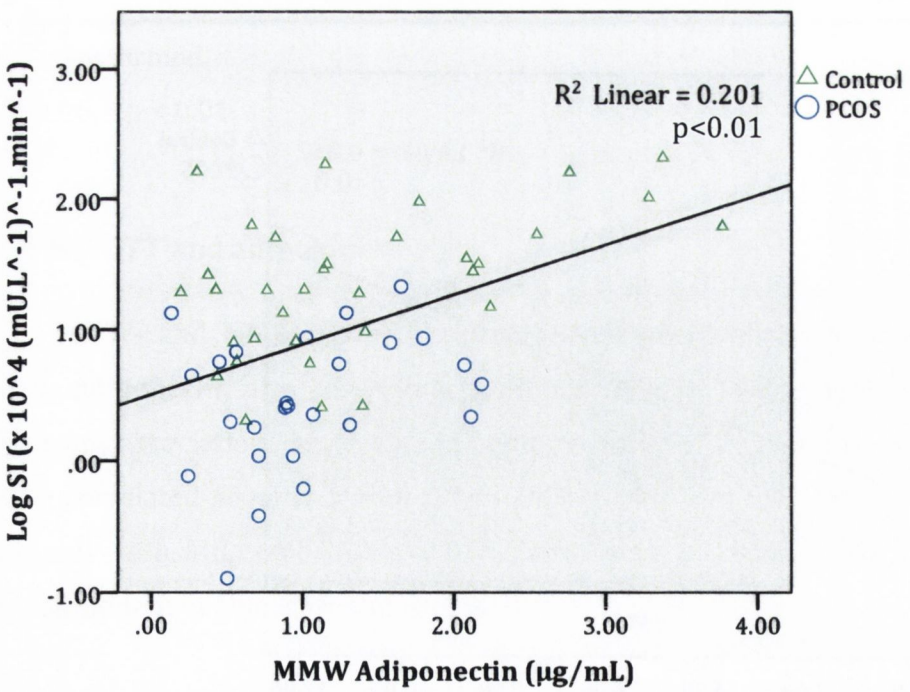

Figure 4-9: Correlation between MMW adiponectin and S_I

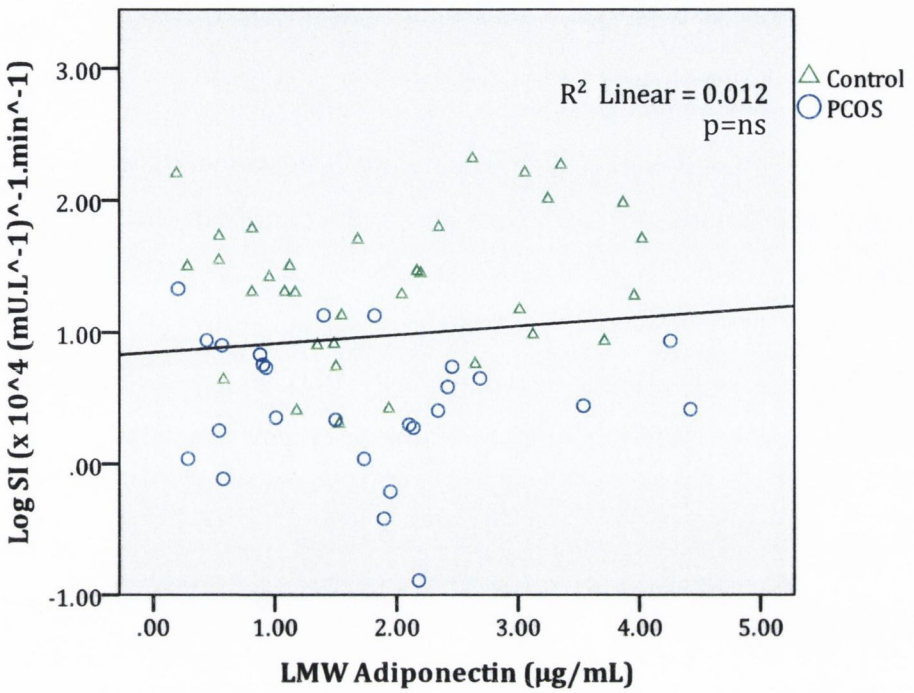

Figure 4-10: Correlation between LMW adiponectin and S_I

Multiple regression analysis revealed BMI, PCOS status and MMW adiponectin to be independent predictors of S_I, explaining 63% of the overall variance. Interestingly, MMW adiponectin was the only predictor of DI, explaining 17% of the overall variance. Leptin was the only independent predictor of S_G and AIR_G, explaining 20% and 12% of the overall variance respectively.

4.6 DISCUSSION

This cross-sectional study demonstrates that serum adiponectin levels are associated with both IR and the ability of β-cells to compensate for IR in premenopausal women with and without PCOS. Among the oligomers of adiponectin, both the HMW- and MMW- forms were associated with insulin resistance while only the MMW- form was significantly associated with beta-cell function. Circulating androgens were also associated with IR, which was greater in women with PCOS independent of other variables. Androgens, however, did not appear to influence β-cell function.

Increased IR has long been recognised in PCOS but effects on other variables derived from the fsIVGTT are less clear. In women with a family history of diabetes, no significant difference in first-phase insulin secretion relative to IR (a similar measure to DI) was observed between subjects with and without PCOS (Ehrmann, Sturis et al. 1995). However, when women with PCOS and a family history of diabetes were compared to a similar cohort with PCOS, but without a family history of diabetes, evidence of reduced first-phase insulin secretion in relation to IR was observed (Ehrmann, Sturis et al. 1995). Another study indicated reduced DI in both non-obese and obese women with PCOS compared respectively with non-obese and obese women without PCOS (Dunaif and Finegood 1996). However, within the obese cohort, subjects with PCOS were significantly heavier and were more likely to have a first-degree relative with diabetes than control subjects and statistical adjustments were not reported for these differences. Finally, in a group of normal weight women with PCOS, no difference in S_I or DI was observed compared to normal subjects (Gennarelli, Rovei et al. 2005). Taken together, the findings of these studies as well those of the current study confirm that IR is increased in women with PCOS, but provide little evidence that PCOS status or circulating androgens contribute independently to the ability of β-cells to compensate for increased IR.

Low levels of adiponectin, a hormone almost exclusively secreted by adipocytes, have recently emerged as one of the most important variables associated with IR and risk of progression to diabetes (Lindsay, Funahashi et al. 2002; Krakoff, Funahashi et al. 2003; Snehalatha, Mukesh et al. 2003; Spranger, Kroke et al. 2003; Duncan, Schmidt et al. 2004; Daimon 2005; Kadowaki, Yamauchi et al. 2006; Mather, Funahashi et al. 2008). As expected, we found serum total adiponectin to be strongly and independently associated with IR. This association was demonstrable with both HMW- and MMW- oligomers, although consistent with previous studies, the association was stronger with HMW-adiponectin (Mather, Funahashi et al. 2008). In contrast to the clear association of adiponectin levels with IR, data concerning the putative association of adiponectin with β-cell function are conflicting. Studies in cellular and animal models suggest a direct effect of adiponectin on β-cells (Kharroubi, Rasschaert et al. 2003; Winzell, Nogueiras et al. 2004; Staiger, Stefan et al. 2005; Liu, Michael et al. 2007), but in-vivo studies in human subjects have reported apparently conflicting findings. Significant correlation between adiponectin and β-cell function has been reported in normal-weight and obese adolescents (estimated using fasting proinsulin-to-insulin ratio) (Hung, Chu et al. 2006), women in late pregnancy (using a model derived from OGTT) (Retnakaran, Hanley et al. 2005) and patients with non-alcoholic steatohepatitis (using fsIVGTT) (Musso, Gambino et al. 2005). However, one study in a group of normal subjects using insulin secretagogues in conjunction with glucose clamping did not find any difference (Staiger, Stefan et al. 2005). Differences between these findings might reflect differences in methodology or alternatively differences in patient groups. The current study provides further evidence that adiponectin is associated with β-cell function, but also demonstrates for the first time an association of MMW-adiponectin with β-cell function.

Despite evidence from animal models in which administration of testosterone has been shown to reduce HMW-adiponectin (Xu, Chan et al. 2005) and the observation that adiponectin levels are greater in women than in men, we found no association of circulating androgens with total adiponectin or any of its oligomers. This is consistent with most although not all studies in human subjects, which have suggested no effect,

independent of body composition, of circulating androgens on adiponectin (Orio, Palomba et al. 2003; Panidis, Kourtis et al. 2003; Spranger, Mohlig et al. 2004; Ardawi and Rouzi 2005; Escobar-Morreale, Villuendas et al. 2006; Glintborg, Andersen et al. 2006; Aroda, Ciaraldi et al. 2007; Barber, Hazell et al. 2008). Studies, in which adiponectin oligomers have been specifically measured, however have included a relatively small number of subjects and therefore it is possible that the inverse association between testosterone and HMW-adiponectin that would be predicted based on the mouse model would be observed in a larger cohort of subjects.

Leptin plays an important role in obesity. It helps maintain satiety, and levels increase substantially postprandialy (Sinha and Caro 1998). Whilst it is well recognised that obesity is associated with increased leptin levels, it is unclear if this is the case in PCOS (Jacobs and Conway 1999). A number of studies have found similar levels of plasma leptin between women with PCOS and weight-matched controls (Chapman, Wittert et al. 1997; Laughlin, Morales et al. 1997; Mantzoros, Dunaif et al. 1997). Another recent study found that serum leptin levels were non-significantly higher in overweight/obese PCOS compared to BMI-matched controls (Lecke, Mattei et al. 2011). Other studies have also found that obesity, rather than PCOS appears to be the main determinant of leptin levels (Escobar-Morreale, Villuendas et al. 2006; Knerr, Herzog et al. 2006). Our findings of higher leptin levels in the obese PCOS compared to obese controls maybe primarily due to the non-statistically significantly greater BMI in the PCOS cohort compared to controls (38.2 ± 1.1 kg/m^2 vs. 35.1 ± 0.9 kg/m^2). Differences in the criteria used for diagnosing PCOS and including such subjects in studies might account for differences in reported findings in leptin levels (Jacobs and Conway 1999). It has also been suggested that hyperinsulinaemia, rather than PCOS status *per se*, influences leptin levels (Krassas, Kaltsas et al. 1998).

Leptin may also have implications on fertility in PCOS. Transcipts which encode isoforms of the leptin receptor have been found in human granulosa and theca cells (Karlsson, Lindell et al. 1997). It was noted in this study that leptin was able to inhibit

LH-stimulated oestradiol production by rat granulosa cells. Further studies are required to investigate whether there is any causal relationship between leptin and PCOS.

In summary, therefore, this study demonstrates that PCOS status and adiponectin are independently associated with IR in pre-menopausal women. The associations are stronger in obese women, but androgens do not appear to play a major role. Furthermore, adiponectin is also associated with the ability of β-cells to compensate for increased IR. Finally, while we have confirmed that the HMW oligomer of adiponectin is closely associated with IR, we have also demonstrated a novel association between MMW adiponectin and both IR and β-cell function.

Chapter 5

Classic and Novel Inflammatory Biomarkers and Oxidative Stress in PCOS

"Discipline is the bridge between goals and accomplishment"

Jim Rohn

5.1 CHAPTER SYNOPSIS

5.1.1 Context

Oxidative stress and inflammation are two metabolic manifestations that may predispose PCOS subjects to increased cardiovascular risk. However, it is uncertain whether PCOS influences oxidative stress and inflammation, independent of IR and obesity, both of which are prevalent in this common endocrine condition.

5.1.2 Aims

To assess oxidative stress, classical and novel inflammatory markers in women with PCOS and compare them to age, BMI and IR-matched controls.

5.1.3 Methods

30 women with PCOS and 30 control subjects matched for age, BMI and $HOMA_{IR}$ were assessed for oxidative stress and classic and novel inflammatory markers. Oxidative stress was assessed by measuring TOS and TAC by spectrophotometric assays. Inflammatory markers included hsCRP, PAI-1, MPO, SAA and neopterin measured by ELISA methodologies.

5.1.4 Results

Oxidative status was increased in the PCOS subjects, relative to their weight-matched controls (TOS: obese-PCOS vs. obese-controls, 42.42±4.49 vs. 34.46±2.68; $p<0.05$: lean-PCOS vs. lean-controls, 33.73±1.34 vs. 28.69±1.09 **μmol H_2O_2** Equiv/L; $p<0.05$). Lean PCOS subjects displayed similar oxidative status to that of the obese-control group. Antioxidant capacity was lower in the lean-PCOS group, relative to their

weight-matched controls (TAC: lean-PCOS vs. lean-controls, 1.10 ± 0.09 vs. 1.49 ± 0.03 nmol Trolox Equiv/L; $p<0.05$). The inflammatory markers were unaffected by PCOS, with the exception of SAA, which displayed a trend across the groups.

5.1.5 Conclusion

These results suggest that obese PCOS subjects had highest levels of oxidative stress although PCOS status may be more important that obesity. Obesity however, is the main determinant of inflammatory markers. Weight loss is likely to be associated with reduced cardiovascular risk as assessed by inflammatory and oxidative markers.

5.2 INTRODUCTION

As previously mentioned, an array of metabolic abnormalities occurs in PCOS, some of which may contribute to increased cardiovascular risk. Oxidative stress and inflammation are two intricately linked processes which have been identified in PCOS (Sabuncu, Vural et al. 2001; Fenkci, Fenkci et al. 2003; Diamanti-Kandarakis, Paterakis et al. 2006) and play an extremely important role in the development of all phases of the atherosclerotic process, from the initial recruitment of circulating cells towards the intima of the arterial wall, to the weakening of the fibrous cap (Quehenberger 2005).

Previous studies of oxidative stress in PCOS have mainly focused on either individual oxidants or antioxidants and not the balance between the two (Sabuncu, Vural et al. 2001; Fenkci, Fenkci et al. 2003; Gonzalez, Rote et al. 2006; Verit and Erel 2008). With regards to inflammatory markers, although classic biomarkers including PAI-1 (Sampson, Kong et al. 1996; Atiomo, Fox et al. 2000; Orio, Palomba et al. 2004) and CRP (Kelly, Lyall et al. 2001; Boulman, Levy et al. 2004; Talbott, Zborowski et al. 2004) have been studied widely in PCOS, reasons for their increased presence are not conclusively confirmed (Diamanti-Kandarakis, Paterakis et al. 2006). Furthermore novel markers of inflammation have been less well studied in PCOS populations. These include myeloperoxidase (MPO), serum amyloid A (SAA) and neopterin. MPO is a heme protein expressed at high concentrations in monocytes, neutrophils and macrophages (Klebanoff 2005) has been shown to directly contribute to the development of low-grade inflammation *in vivo*, by utilising hydrogen peroxide (H_2O_2), leading to the production of ROS (Young and McEneny 2001). SAA is an amphipathic apolipoprotein produced by liver hepatocytes and adipose tissue (Poitou, Viguerie et al. 2005) stimulates inflammatory mediators from cultured vascular endothelial cells, neutrophils and monocytes (Song, Hsu et al. 2009; Song, Shen et al. 2009). Neopterin is another marker of immune system response, being released from neutrophils in response to IGN- neutrophils during inflammation (Widner, Mayr et al. 2000), but levels of this pteridine derivative have also been

shown to increase in obesity-related low-grade inflammatory and IR conditions (Ledochowski, Murr et al. 1999).

To explore the effects of PCOS on oxidative stress and low-grade inflammation independently of BMI and IR, samples were utilised from a previously described cohort of women with or without PCOS, matched for both BMI and IR (Phelan, O'Connor et al.). Both PCOS and control subjects were subdivided into obese (BMI > 30 kg/m^2) and lean (BMI < 25 kg/m^2) cohorts. Oxidative stress was assessed by the simultaneous measurements and assessment of the ratio between the TOS and TAC (Erel 2004; Erel 2005). Inflammation was assessed by measuring both classical (hsCRP and PAI-1) and novel biomarkers (MPO, SAA and neopterin).

5.3 AIMS

To assess the overall oxidation status in women with PCOS and compare them to BMI and IR matched controls. To assess the levels of inflammatory markers, both classical and novel, in women with PCOS and compare them to BMI and IR matched controls.

5.4 MATERIAL AND METHODS

5.4.1 Subjects

60 premenopausal women with (n=30) and without (n=30) PCOS were recruited. Both groups were matched for age, BMI and IR as measured by $HOMA_{IR}$. 15 PCOS women were obese (BMI > 30 kg/m^2) and 15 were lean (BMI < 25 kg/m^2). PCOS subjects fulfilled the NIH criteria for diagnosis and met all the inclusion criteria as described in section 2.1.1. Similarly, 15 control subjects were obese (BMI > 30 kg/m^2) and 15 were lean (BMI < 25 kg/m^2). The control group was comprised of normal volunteers on no medications recruited from the general population as described in 2.1.2. All control subjects were studied in the follicular phase of their menstrual cycle. PCOS subjects were studied within 2 weeks of a spontaneous bleed. All study subjects gave their written signed consent to the study, which was approved by the Joint Research Ethics Committee of the Adelaide and Meath Hospital and St. James's Hospital (Dublin, Ireland).

5.4.2 Clinical protocol

This was a cross-sectional study. All subjects had their height measured with a Harpenden stadiometer and weight measured in light clothing. BMI was calculated as weight (kg) / height squared (m^2). WC and HC were measured with a non-distensible flexible tape, and WHR calculated accordingly.

5.4.2.1 Measure of Insulin Resistance

$HOMA_{IR}$ was used to assess insulin resistance. This was because some of the subjects were part of a larger study of well-matched control subjects (Phelan, O'Connor et al.), in which it would not have been feasible to perform a large number of fsIVGTT.

5.4.3 Laboratory methods

5.4.3.1 Blood sampling and handling

All blood samples were taken after a 12-hour fast into the appropriate tubes for various analytes. Serum separator tube samples and were allowed to clot for 30 minutes prior to centrifugation at 3000rpm for 10 minutes at 4°C. Serum was removed and aliquoted appropriately. All samples were stored at -80°C until further analysis. Analyses of TOS, TAC, MPO, PAI-1, SAA and neopterin were performed in the Pathology Building, Royal Victoria Hospital, Queen's University, Belfast. Samples were transported in dry ice.

5.4.3.2 Hormones, glucose, HbA1c, lipids

Insulin and testosterone were measured by electrochemiluminesence immunoassay on the Roche E Module. E2, progesterone, LH, FSH, TSH, fT4, PRL, cortisol were all measured by chemiluminesence immunoassay on DPC Immulite 2500. SHBG and DHEAS were measured by chemiluminesence immunoassay on DPC Immulite 2000 in St James's Hospital. FAI was calculated by the equation: FAI = total testosterone (nmol/L) / SHBG (nmol/L) x 100. Androstenedione and 17-OHP were measured by radioimmunoassay on the DPC Coat-A-Count. Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module. HbA1c was measured by ion exchange chromatography on the Tosoh 2.2. Cholesterol, TG, HDL-chol and NEFA were measured by a standard enzymatic colourimetric method on the Roche P Module. LDL-chol was calculated by the Friedewald formula. Laboratory methods of all these are described in full in section 2.7.

5.4.3.3 Oxidative Stress

TOS was measured in serum samples using a modified colourimetric method, originally developed by Erel (Erel 2005). Results were expressed as μmol H_2O_2 Equiv/L and inter-and intra-assay CVs were < 10%. TAC was measured based on the same principals of measurement of TOS (Erel 2004). Results were expressed as μmol Trolox Equiv/L and inter- and intra-assay CVs were also < 10%. The measurements of both TOS and TAC are time and cost effective, and are reliable and reproducible assays. A full description of these two assays has been described in Section 2.7.13. These assays were performed by Dr. Sarah Blair.

5.4.3.4 Inflammatory Markers

hsCRP was measured on an automated ILab system in AMNCH. PAI-1, MPO, SAA and neopterin measurements were carried out using an automated enzyme immunoassay system (Triturus EIA analyser; Grifols, Spain) in QUB. This automated system was pre-programmed with assay procedures and performed all standard/sample dilutions, and all wash and incubation steps, according to the manufacturer's instructions. Wash and diluent buffers were prepared manually, according to manufacturer's instructions. The system software provided an automatic calculation of standard curves and sample readings, whilst factoring in dilution factors. MPO was measured in plasma using MPO sensitive enzyme-linked immunosorbent assay (ELISA) kits, manufactured by Biocheck Inc. (Foster City, CA). PAI-1 was measured in plasma using PAI-1 sensitive ELISA kits, manufactured by Trinity Biotech (Wicklow, Ireland). SAA was measured in serum using SAA sensitive ELISA kits, manufactured by Invitrogen (Camarillo, CA). Neopterin was measured in serum using neopterin sensitive ELISA kits, manufactured by IBL (Hamburg, Germany). A full description of these methods has been described in section 2.7.12.

5.4.4 Statistical analyses

Data are presented as mean ± SEM. Skewed variables were logarithmically transformed to normalise data prior to analysis. Initial comparisons between groups were performed using independent t-test. Trends across groups were performed using ANOVA with post-hoc analysis according to the Bonferroni method. Correlations were made using Pearson's correlation co-efficient. Multiple regression analyses using the Stepwise method was used to determine the independent predictors of markers of oxidative stress and inflammation. Statistical significance was defined as $p<0.05$.

5.5 RESULTS

5.5.1 Baseline demographic data and hormonal profile

Subject characteristics and hormonal status are shown in Table 5-1. No statistical differences were observed in age, BMI and WHR between each of the PCOS groups and their weight-matched controls. As expected, compared to their BMI matched control groups, PCOS subjects had significantly elevated testosterone and FAI (both groups and both analyses, $p<0.001$) and DHEAS levels (obese groups, $p=0.002$ and lean groups; $p=0.007$). SHBG was significantly lower in the obese PCOS group compared to their control group. However, although the lean PCOS group had reduced SHBG compared to the lean control group, this was not significant. No differences were observed in the above four hormonal parameters within the two PCOS groups; or within the two control groups. LH levels were significantly higher in the lean PCOS group, compared to the lean controls ($p=0.007$). Also, LH levels were significantly higher within the lean PCOS group, compared to their obese counterparts ($p=0.022$). No differences in LH levels were observed between the two obese groups. FSH levels were similar in the two obese groups; however, the lean PCOS group displayed lower levels of this hormone compared to their lean counterparts ($p=0.018$). No differences in FSH levels were demonstrated between the two PCOS groups; or between the two control groups.

Fasting glucose was higher in both obese groups, compared to their lean counterparts (obese PCOS vs. lean PCOS, $p=0.009$; obese controls vs. lean controls, $p=0.033$). Whilst fasting insulin was also higher in both obese groups, compared to their lean counterparts, this was only significant within the PCOS subjects (obese PCOS vs. lean PCOS, $p=0.001$; obese controls vs. lean controls, $p=0.081$). Similarly, obese groups were more IR than their lean counterparts (obese PCOS vs. lean PCOS, $p=<0.001$; obese controls vs. lean controls, $p=0.049$). There were no differences in $HOMA_{IR}$ between obese PCOS and obese controls or between lean PCOS and lean controls.

Table 5-1: Baseline characteristics of PCOS and control subjects

	Obese PCOS (n=15)	Obese Controls (n=15)	Lean PCOS (n=15)	Lean Controls (n=15)
Age (yrs)	32.6 ± 1.5 [c]	36.6 ± 1.9 [d]	26.5 ± 1.0	28.6 ± 0.8
Systolic BP (mmHg)	126.0 ± 2.5 [c]	122.7 ± 2.8 [d]	117.3 ± 2.9	109.5 ± 3.1
Diastolic BP (mmHg)	77.9 ± 1.5 [c]	79.3 ± 3.3	79.4 ± 2.2	71.4 ± 2.6
Waist (cm)	109.2 ± 2.4 [c]	103.8 ± 2.6 [d]	83.3 ± 2.2	82.7 ± 2.3
Hip (cm)	118.7 ± 1.9 [c]	112.0 ± 2.6 [d]	98.2 ± 1.7	101.8 ± 1.2
Waist : Hip	0.920 ± 0.021[c]	0.929 ± 0.017[d]	0.846 ± 0.016	0.812 ± 0.017
Weight (kg)	90.1 ± 2.6 [c]	87.4 ± 3.4 [d]	63.3 ± 1.9	63.8 ± 1.8
BMI (kg/m^2)	34.3 ± 0.9 [c]	33.5 ± 1.4 [d]	23.1 ± 0.6	24.1 ± 0.6
T (nmol/L)*	2.81 ± 0.25 [a]	1.33 ± 0.16	2.97 ± 0.22	1.74 ± 0.15
SHBG (nmol/L)*	31.83 ± 2.76[a,c]	41.09 ± 3.21 [d]	48.44 ± 4.94	64.93 ± 6.95
FAI*	9.54 ± 1.05 [a]	3.59 ± 0.61	7.29 ± 0.93	3.56 ± 0.75
ΔA4 (nmol/L)	7.46 ± 0.75 [a]	4.21 ± 0.52	6.91 ± 0.60	4.77 ± 0.40
DHEAS (μmol/L)	15.95 ± 0.99 [a]	10.95 ± 1.09	17.50 ± 1.70	9.45 ± 0.77
FSH (IU/L)*	6.01 ± 0.42	8.87 ± 1.40	5.75 ± 0.49	7.55 ± 0.48
LH (IU/L)*	7.10 ± 0.95 [c]	7.99 ± 2.29	14.11 ± 2.85	7.21 ± 1.42
Fasting Glucose (mmol/L)	5.06 ± 0.18 [c]	4.91 ± 0.14 [d]	4.48 ± 0.08	4.58 ± 0.05
Fasting Insulin (mU/L)	14.18 ± 1.60 [c]	13.20 ± 2.30	7.42 ± 0.79	7.42 ± 2.22
$HOMA_{IR}$ (μmol^2/L^2)	3.20 ± 0.37 [c]	2.92 ± 0.54 [d]	1.48 ± 0.16	1.50 ± 0.43

* log transformed

[a] $p<0.05$, obese PCOS vs. obese controls

[b] $p<0.05$, lean PCOS vs. lean controls

[c] $p<0.05$, obese PCOS vs. lean PCOS

[d] $p<0.05$, obese controls vs. lean controls

5.5.2 Measures of Oxidative Stress

The markers of oxidative stress, TOS and TAC, are presented in Table 5-2. TOS levels were higher in the PCOS groups compared to IR- and BMI-matched controls (obese groups, p=0.043; lean groups, p=0.047) suggesting that TOS levels were elevated independent of BMI or IR. TOS levels were also higher in the obese PCOS group compared to the lean control group (p=0.002). There was a significant trend of TOS levels, highest in the PCOS groups and lowest in the control groups: obese PCOS > lean PCOS > obese control > lean control.

TAC levels were highest in the lean control subjects, and were significantly higher than obese PCOS (p=0.003), obese controls (p=0.029) and lean PCOS (p=0.001). The latter finding suggests that the presence of PCOS in lean women reduces TAC. However, in obese subjects, the presence of PCOS does not influence TAC levels.

The TAC:TOS ratio yielded similar findings to TAC levels alone. Lean control subjects had the highest levels, and were significantly higher than obese PCOS (p=0.002), obese controls (p=0.046) and lean PCOS (p=0.003). Again there were no differences between obese PCOS and obese control subjects.

5.5.3 Markers of Inflammation

Levels of hsCRP, PAI-1, MPO, SAA and neopterin are also presented in Table 5-2. There were no differences in any of these measures biomarkers between obese PCOS and obese control subjects. hsCRP was increased in both obese groups, compared to their lean counterparts, (PCOS groups, p=0.001; control groups, p=0.001). This finding was also mirrored by PAI-1 (PCOS groups, p=0.001; control groups p=0.008), MPO (PCOS groups, p=0.001; control groups, p=0.002), SAA (PCOS groups, p=0.006; control groups, p=0.006) and neopterin (control groups only, p=0.009). Whilst inflammatory markers were higher in obese PCOS compared to lean controls as

expected, they were, with the exception of neopterin, also higher in obese controls compared to lean PCOS. There was also a trend for SAA to decrease across the groups, with the obese PCOS group having the highest level and the lean control group having the lowest level, obese PCOS > obese control > lean PCOS > lean control (45.48±6.88 vs. 32.40±4.35 vs. 20.9±3.91 vs. 17.32±3.55 μg/mL SAA, respectively; trend across the groups, $p<0.001$).

Table 5-2: Markers of oxidative stress and inflammation

	Obese PCOS (n=15)	Obese Controls (n=15)	Lean PCOS (n=15)	Lean Controls (n=15)
TOS (μmol H_2O_2 Equiv/L)	42.42 ± 4.49 [a,c,e]	34.46 ± 2.68 [d]	33.73 ± 1.34 [b]	28.69 ± 1.09
TAC (nmol Trolox Equiv/L)	1.28 ± 0.06 [e]	1.36 ± 0.04 [d,f]	1.10 ± 0.09 [b]	1.49 ± 0.03
TAC : TOS	0.034 ± 0.003 [e]	0.043 ± 0.003 [d]	0.034 ± 0.003 [b]	0.053 ± 0.003
hsCRP (ng/mL)	5.35 ± 0.84 [c,e]	4.58 ± 0.55 [d,f]	1.08 ± 0.17	1.17 ± 0.14
PAI-1 (ng/mL)	49.11 ± 4.28 [c,e]	43.99 ± 4.19 [d,f]	23.07 ± 2.25	26.67 ± 4.20
MPO (ng/mL)	93.39 ± 11.00 [c,e]	92.79 ± 10.06 [d,f]	31.04 ± 4.69	47.35 ± 8.12
SAA (μg/mL)	45.48 ± 6.88 [c,e]	32.40 ± 4.35 [d]	20.92 ± 3.91	17.32 ± 3.55
Neopterin (nmol/L)	5.99 ± 0.40 [e]	6.45 ± 0.42 [d]	5.61 ± 0.51	4.99 ± 0.20

* log transformed

[a] $p<0.05$, obese PCOS vs. obese controls

[b] $p<0.05$, lean PCOS vs. lean controls

[c] $p<0.05$, obese PCOS vs. lean PCOS

[d] $p<0.05$, obese controls vs. lean controls

[e] $p<0.05$, obese PCOS vs. lean controls

[f] $p<0.05$, obese controls vs. lean PCOS

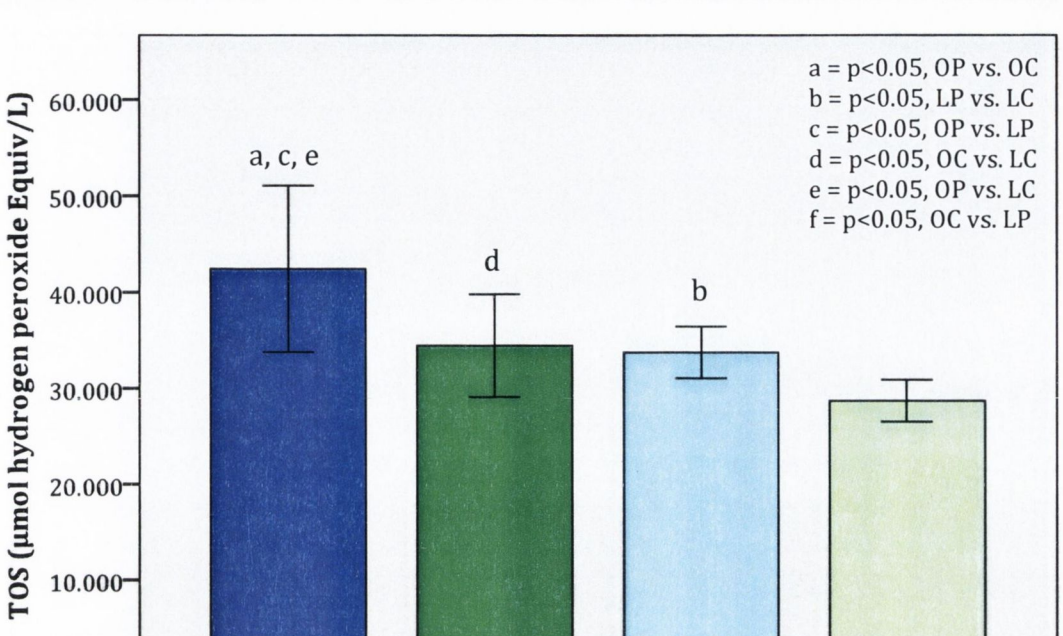

Figure 5-1: TOS levels in PCOS and control subjects

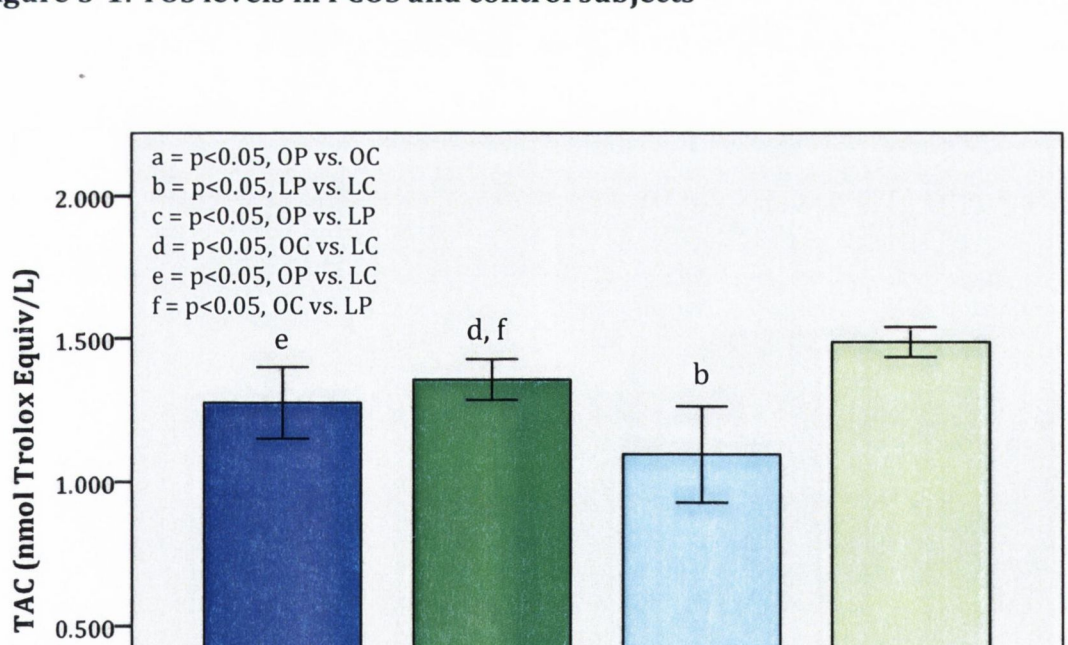

Figure 5-2: TAC levels in PCOS and control subjects

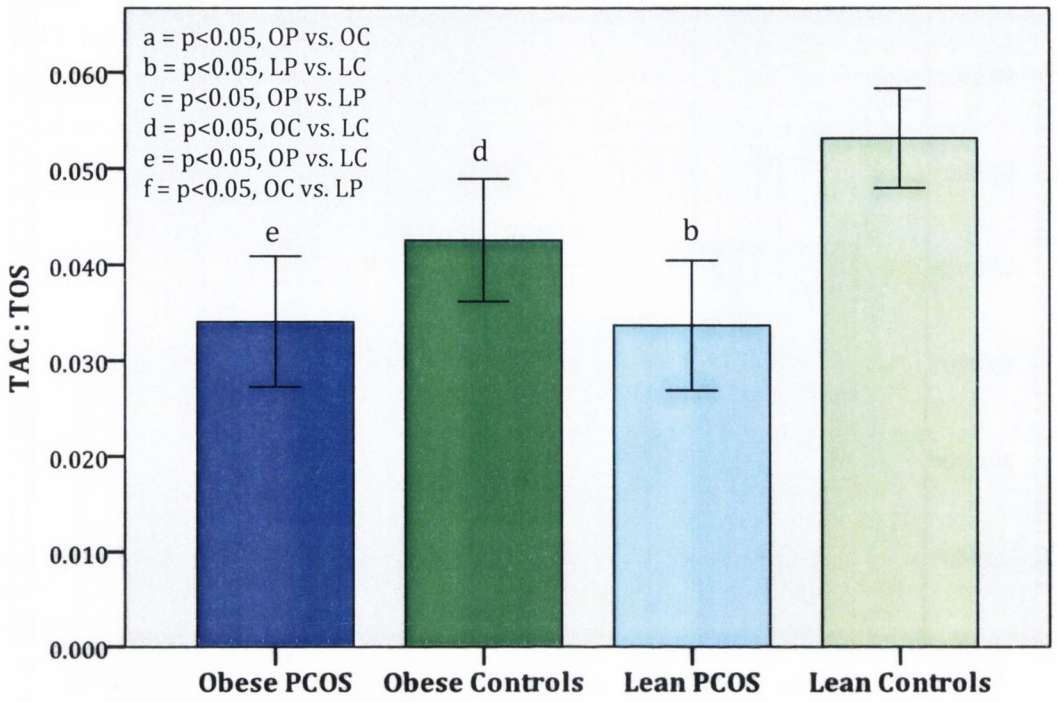

Figure 5-3: TAC:TOS ratios in PCOS and control subjects

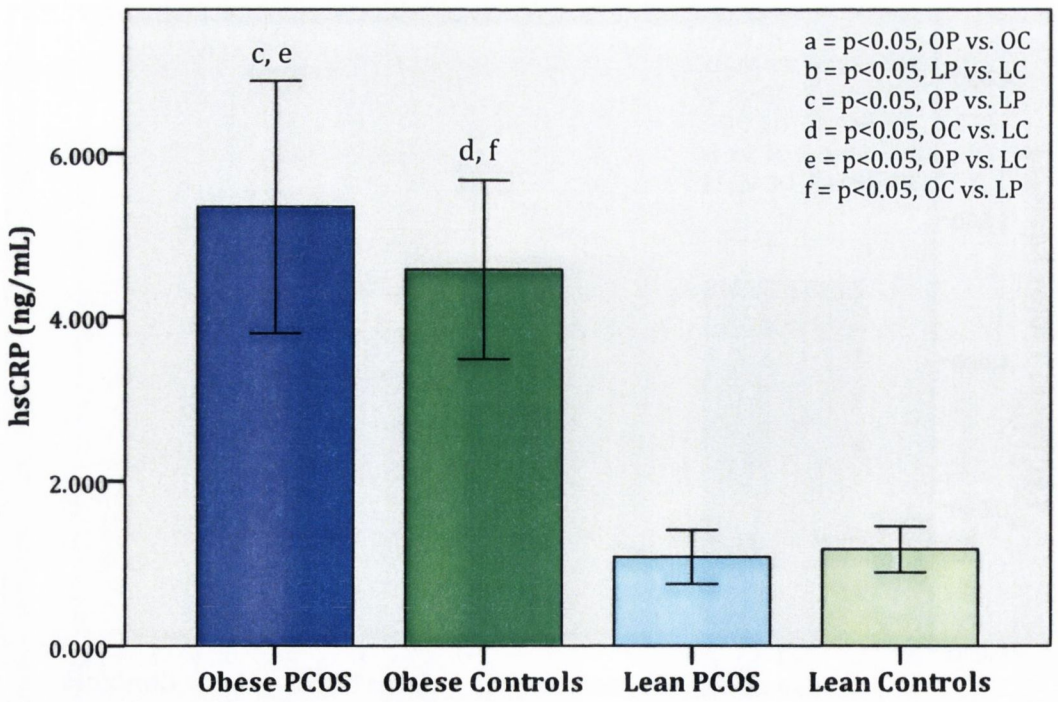

Figure 5-4: hsCRP levels in PCOS and control subjects

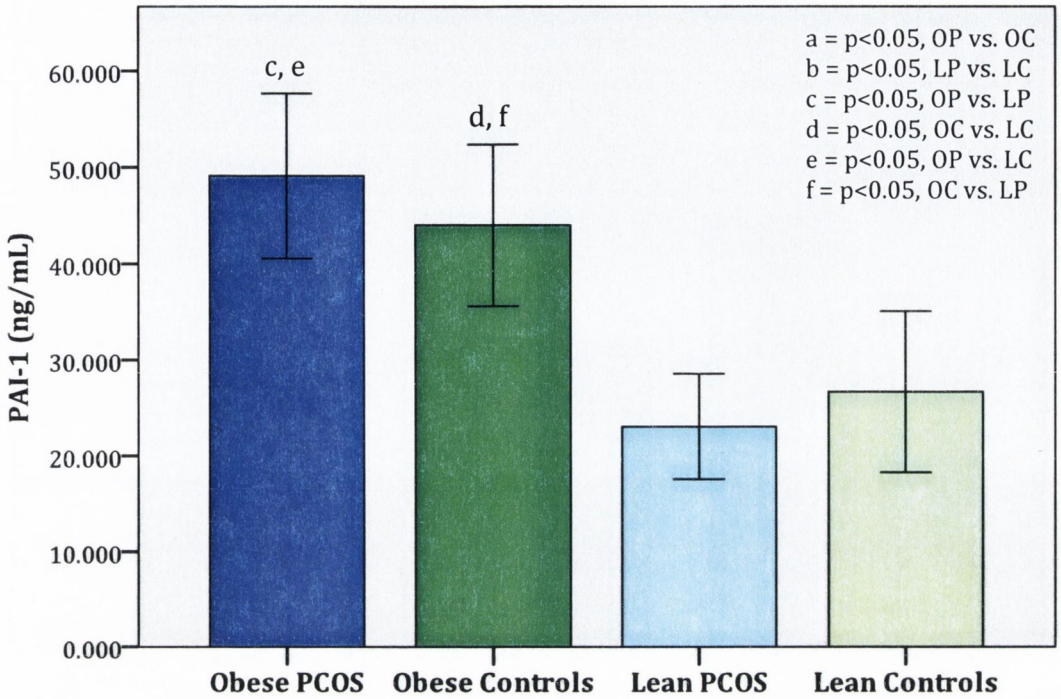

Figure 5-5: PAI-1 levels in PCOS and control subjects

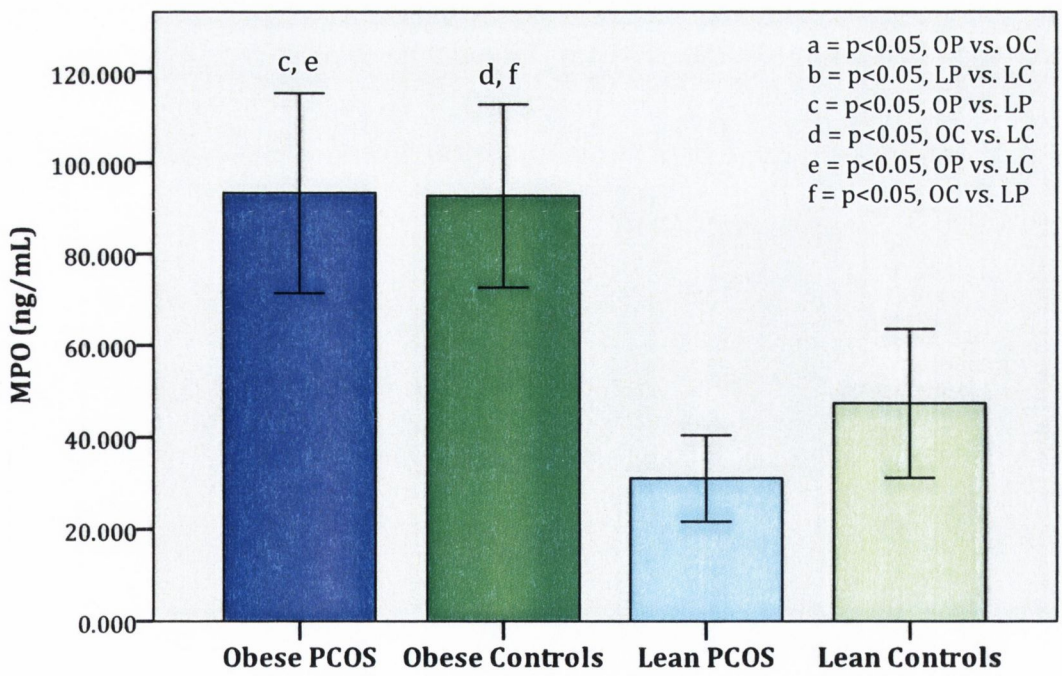

Figure 5-6: MPO levels in PCOS and control subjects

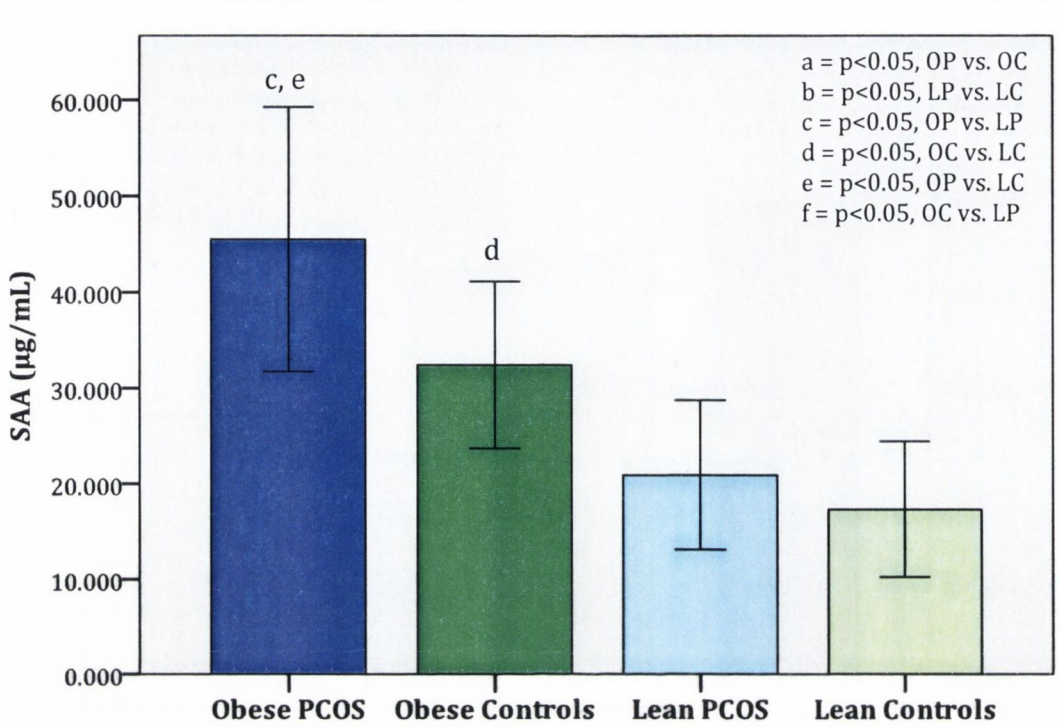

Figure 5-7: SAA levels in PCOS and control subjects

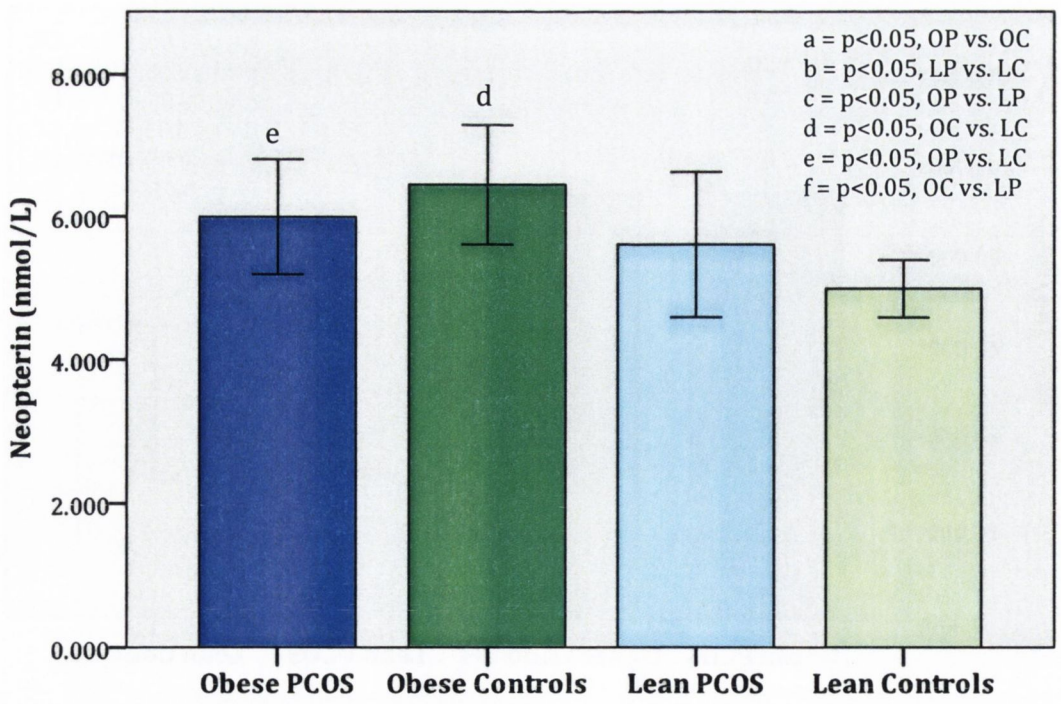

Figure 5-8: Neopterin levels in PCOS and control subjects

5.5.4 Correlations and multiple regression analyses

Correlations between markers of oxidative stress and inflammatory markers are shown in Table 5-3. TOS correlated inversely with TAC and TAC:TOS ratio. TOS correlated positively with hsCRP, PAI-1 and MPO but not with SAA and neopterin. TAC did not correlate with any inflammatory markers. TAC:TOS also did not correlate with any inflammatory markers, but correlated negatively with neopterin. hsCRP and SAA correlated positively with all other biomarkers. PAI-1 and MPO also correlated with all other biomarkers but not with neopterin.

Table 5-3: Correlations between markers of oxidative stress and inflammatory markers

	TOS	TAC	TAC : TOS	hsCRP	PAI-1	MPO	SAA	Neopterin
TOS	1.000	-0.375 [b]	-0.749 [b]	0.276 [a]	0.341 [b]	0.291 [a]	0.086	0.201
TAC	-0.375 [b]	1.000	0.817 [b]	0.025	-0.037	-0.006	0.017	-0.087
TAC : TOS	-0.749 [b]	0.817 [b]	1.000	-0.192	-0.244	-0.144	-0.078	-0.257 [a]
hsCRP	0.276 [a]	0.025	-0.192	1.000	0.534 [b]	0.395 [b]	0.534 [b]	0.277 [a]
PAI-1	0.341 [b]	-0.037	-0.244	0.534 [b]	1.000	0.392 [b]	0.297 [a]	0.158
MPO	0.291 [a]	-0.006	-0.144	0.395 [b]	0.392 [b]	1.000	0.402 [b]	0.153
SAA	0.086	0.017	-0.078	0.534 [b]	0.297 [a]	0.402 [b]	1.000	0.347 [b]
Neopterin	0.201	-0.087	-0.257 [a]	0.277 [a]	0.158	0.153	0.347 [b]	1.000

[a] $p<0.05$. [b] $p<0.01$

TOS correlated positively with body weight and BMI only. TAC and TAC:TOS did not correlate with any other variables. hsCRP correlated significantly with all variables except diastolic BP. PAI-1 and MPO correlated significantly with all variables except diastolic BP and % body fat. SAA was not correlated with age, systolic BP, diastolic BP

or % body fat but correlated positively with other variables. Neopterin only correlated positively with WHR and body weight. These results are show in Table 5-4.

Table 5-4: Correlations between oxidative stress, inflammatory markers, age and anthropometric results

	TOS	TAC	TAC : TOS	hsCRP	PAI-1	MPO	SAA	Neopterin
Age	0.076	0.208	0.010	0.516 [b]	0.278 [a]	0.266 [a]	0.226	0.142
SysBP	0.165	0.057	-0.103	0.367 [b]	0.281 [a]	0.330 [a]	0.175	-0.042
DiasBP	0.114	-0.096	-0.154	0.039	-0.077	0.015	-0.213	-0.020
Waist	0.228	0.155	-0.063	0.715 [b]	0.527 [b]	0.518 [b]	0.422 [b]	0.245
Hip	0.101	0.162	0.006	0.737 [b]	0.414 [b]	0.327 [a]	0.303 [a]	0.111
WHR	0.262	0.079	-0.104	0.381 [b]	0.421 [b]	0.486 [b]	0.355 [a]	0.295 [a]
Weight	0.288 [a]	0.033	-0.201	0.765 [b]	0.501 [b]	0.490 [b]	0.434 [b]	0.262 [a]
BMI	0.278 [a]	0.043	-0.212	0.760 [b]	0.448 [b]	0.517 [b]	0.372 [b]	0.231
% body fat	0.252	0.045	-0.215	0.620 [b]	0.104	0.187	0.281	0.180

[a] $p<0.05$. [b] $p<0.01$

Table 5-5 shows the correlations between markers of oxidative stress, inflammatory markers, hormones, glucose, insulin and IR. Testosterone correlated inversely with TAC:TOS. SHBG correlated with both TOS and TAC:TOS. SHBG also correlated inversely with all biomarkers with the exception of PAI-1. FAI correlated positively with TOS and inversely with TAC:TOS ratio whilst androstenedione also correlated negatively with TAC:TOS. However neither FAI nor androstenedione correlated with any of the biomarkers. DHEAS also did not correlate with any of the biomarkers and did not correlate with any markers of oxidative stress.

Fasting glucose correlated positively with TOS, hsCRP, PAI-1 and MPO but not with TAC, TAC:TOS, SAA or neopterin. Fasting insulin and $HOMA_{IR}$ correlated positively with TOS and negatively with TAC:TOS ratio. Both fasting glucose and $HOMA_{IR}$ also correlated positively with all biomarkers except for neopterin.

Table 5-5: Correlations between oxidative stress, inflammatory markers, hormones, glucose, insulin and IR

	TOS	TAC	TAC : TOS	hsCRP	PAI-1	MPO	SAA	Neopterin
Testosterone	0.186	-0.206	-0.262 [a]	-0.102	-0.055	-0.106	0.118	-0.035
SHBG	-0.434 [b]	0.180	0.359 [b]	-0.368 [b]	-0.207	-0.299 [a]	-0.324 [a]	-0.273 [a]
FAI	0.335 [b]	-0.241	-0.351 [b]	0.128	0.043	0.072	0.237	0.071
Δ4A	0.210	-0.225	-0.319 [a]	-0.032	-0.069	-0.003	0.228	0.223
DHEAS	0.167	-0.068	-0.142	0.000	0.060	-0.053	0.019	-0.118
Fasting gluc	0.307 [a]	-0.024	-0.250	0.409 [b]	0.355 [b]	0.408 [b]	0.126	0.019
Fasting ins	0.414 [b]	-0.091	-0.365 [b]	0.436 [b]	0.302 [a]	0.300 [a]	0.284 [a]	0.253
$HOMA_{IR}$	0.435 [b]	-0.090	-0.382 [b]	0.471 [b]	0.337 [b]	0.347 [b]	0.286 [a]	0.244

[a] $p<0.05$. [b] $p<0.01$

Table 5-6 shows the results of multiple regression analyses. $HOMA_{IR}$ and FAI were the only predictors of TOS. PCOS was the only predictor for TAC, whilst both PCOS and $HOMA_{IR}$ were found to be independent predictors of the TAC:TOS ratio. Weight was the only predictor of hsCRP and SAA whilst waist circumference was the only predictor for both PAI-1 and MPO. Finally, WHR was the only predictor of neopterin. From these results, it can be seen that PCOS status affects TAC, and IR mainly affects TOS, whilst both PCOS and IR synergistically affect oxidative stress. Of the

inflammatory markers, hsCRP was most strongly predicted by anthropometric variables whilst neopterin was least affected by measures of body fat.

Table 5-6: Results of stepwise multiple regression analyses

	Independent predictors	% variance accounted for
TOS	$HOMA_{IR}$, FAI	25.80%
TAC	PCOS	21.40%
TAC : TOS	PCOS, $HOMA_{IR}$	33.70%
hsCRP	weight	58.60%
PAI-1	waist	27.70%
MPO	waist	26.90%
SAA	weight	18.80%
Neopterin	WHR	8.70%

5.6 DISCUSSION

Markers of oxidative stress and low-grade inflammation have been found to predict atherosclerotic risk in a range of inflammatory states, including MetS and T2DM (Haffner 2006). PCOS is associated with a range of metabolic aberrations common to both MetS and T2DM, including IR and obesity (Carmina and Lobo 2004; DeUgarte, Bartolucci et al. 2005). Evidence accumulated over the last decade supports an association between PCOS, oxidative stress and low-grade chronic inflammation, in keeping with the observation that these subjects are at increased risk of cardiovascular complications. However, in most cases investigators have studied the effect of obesity in this condition, but failed to control for IR. It is unclear whether PCOS is associated with inflammation and oxidative stress independent of both obesity and IR. Therefore, this study has attempted to separate the influence of obesity and IR by examining two groups of PCOS subjects, one obese and one lean, both matched for BMI, age and IR with their control counterparts.

Oxidative stress can be investigated by an individual assessment of oxidants or antioxidants, or, as in this study, cumulatively by assessing the ratio between the two. The methods used are both time and cost effective and have the added advantage of utilising small sample volumes, a particularly attractive feature when sample volume is low. The results presented here have shown that TOS levels were significantly higher in both PCOS groups, compared to their BMI and IR matched controls. They also show that TAC and the TAC:TOS ratio was significantly lower in the lean PCOS group, compared to their lean counterparts. It is of interest to note that the degree of oxidative stress, in particular TOS, in the lean PCOS subjects was similar to that of obese subjects without PCOS. This suggests that PCOS subjects who, although lean in weight, present a similar risk of oxidative stress than normal obese women. TOS levels also correlated with SHBG and FAI, both of which are abnormal in PCOS.

Furthermore, these results confirmed the previously reported association between oxidative stress and obesity, and the parameters of the metabolic syndrome (Fenkci, Fenkci et al. 2003; Gonzalez, Rote et al. 2006; Verit and Erel 2008). Thus, they suggest that TOS and TAC, or their ratio, may be extremely useful biomarkers in predicting oxidative stress, not only in women with PCOS but also in obese individuals displaying markers of the metabolic syndrome.

The association between inflammation and PCOS was first reported by Gonzalez et al. in 1999 (Gonzalez, Thusu et al. 1999) where they found an increase in TNF-α, independent of BMI status. Contrary to this, the present study found that PCOS had limited influence on the inflammatory markers measured, which may be because the subjects were matched for both BMI and IR. We found that hsCRP levels were not influenced by PCOS, but were influenced by obesity, confirming the findings of Kelly et al., 2001 (Kelly, Lyall et al. 2001). Although several investigators have suggested that hsCRP is raised in PCOS, once adjusted for BMI this difference was removed (Talbott, Zborowski et al. 2004; Diamanti-Kandarakis, Paterakis et al. 2006). The concept that obesity is the driving force behind this increased inflammation, and not PCOS, was further confirmed by the positive correlation between hsCRP and the markers of the metabolic syndrome, parameters that are raised by the presence of obesity (Hak, Stehouwer et al. 1999). Furthermore, although a recent meta-analysis showed that CRP was raised in PCOS independent of obesity, CRP elevation attributable to PCOS is relatively small (Escobar-Morreale, Luque-Ramirez et al. 2011). Also, the role of IR in influencing hsCRP requires further investigation.

The results of this study also suggest that PCOS, per se, was not the major regulator of PAI-1; however, obesity and/or IR most probably were, thus confirming the findings of Anfosso et al. (Anfosso, Chomiki et al. 1993) and Atiomo et al. (Atiomo, Fox et al. 2000). Furthermore, the positive correlation between PAI-1 and the markers of the metabolic syndrome support this concept. However, these findings are in contrast to those of Orio et al. (2004) (Orio, Palomba et al. 2004), who reported that PAI-1

remained higher in PCOS subjects, even after adjustment for BMI, although their subjects were not match for IR, suggesting that IR, and potentially not obesity, may be the driving influence that increases PAI-1 in PCOS subjects.

To the author's knowledge, this is the first study to investigate the association between MPO and PCOS. Previous studies have shown that MPO is associated with increasing adiposity and oxidative stress (Brennan and Hazen 2003), and with markers of the metabolic syndrome (Rector, Warner et al. 2007). The results presented here suggest that it was obesity and/or IR that influenced MPO levels, rather than PCOS. Furthermore, as MPO positively correlated with the markers of the metabolic syndrome this suggests that MPO may be a useful predictor of inflammation in states of obesity and its comorbidities. However, as a marker of inflammation in PCOS, MPO may be relatively limited.

SAA, as well as being released from the liver during episodes of acute inflammation, is also released in low-levels from subcutaneous adipose tissue, making it a useful tool in identifying adipose-derived inflammation (Poitou, Viguerie et al. 2005). Furthermore, women with PCOS, regardless of BMI, display increased abdominal obesity (Horejsi, Moller et al. 2004), therefore, increasing SAA release. To the best of the author's knowledge, no other study has investigated the relationship between PCOS and SAA. This study has shown that obesity influences SAA levels and that PCOS may additionally influence its expression, as we demonstrated a trend for SAA levels to increase across the groups in the following order: lean controls > lean PCOS > obese controls > obese PCOS. In summary, elevated SAA in obesity and PCOS may be a useful marker to identify increased CV risk in women with PCOS, which is novel to PCOS.

Measuring serum levels of neopterin can be regarded as an indirect estimation of oxidative stress during cell-mediated immune responses, and is also increased during

periods of low-grade inflammation (Murr, Widner et al. 2002). Information regarding the role of neopterin in PCOS is relatively limited, with one study reporting that it may be a useful predictor of inflammation in lean PCOS subjects (Barutcuoglu, Bozdemir et al. 2006), however, in this study subjects were not matched for IR, suggesting that it may be IR that was the driving force in raising neopterin. We could not identify any differences in neopterin levels, relative to the presence of PCOS, and the only difference found was between the two control groups. Therefore, these results suggest little utility for the use of neopterin in identifying either PCOS or obesity driven oxidative stress/inflammation in PCOS subjects.

The current results showing that inflammatory markers were mainly influenced by obesity rather than PCOS is not novel. Previous studies have reported that inflammatory markers are similar in PCOS and BMI-matched controls (Talbott, Zborowskii et al. 2004; Diamanti-Kandarakis, Paterakis et al. 2006). This suggests that PCOS combined with obesty may be a more deleterious condition than PCOS without obesity, due to the increased cardiovascular burden conferred by the presence of obesity. Coversely, in the absence of obesity, there is little if any evidence of increased cardiovascular risk.

There is relatively little data on inflammation in lean PCOS subjects. In this study, inflammatory markers in lean PCOS subjects were similar to that of lean controls. Again, this confirms the findings of previous studies (Gen, Akbay et al. 2009; Samy, Hashim et al. 2009). The reasons for this are unclear, but it is generally recognised that lean PCOS subjects do not have the same cardiometabolic risk profile compared to their obese counterparts in whom a comprehensive evaluation and management of cardiovascular risk factors is paramount (Wild, Carmina et al. 2010). Therefore it is reasonable to conclude that targeting weight loss is a key component in the management of PCOS.

In summary, the present study has provided evidence to suggest that PCOS independently influenced oxidative stress. Additionally, in the absence of obesity, PCOS was as detrimental to oxidative stress as obesity alone. However, inflammatory markers were influenced only by the presence of obesity and IR and not by PCOS status. Overall, these results suggest that the regulation of the metabolic abnormalities of PCOS and the management of weight will help reduce both oxidative stress and chronic inflammation and, therefore, aid in lowering cardiovascular disease in these subjects.

Chapter 6

The Effect of Dietary Fatty Acid Supplementation on Insulin Resistance and Lipoprotein Particle Sizes in Subjects with the Metabolic Syndrome

"Education is a progressive discovery of our own ignorance."

Will Durant

6.1 CHAPTER SYNOPSIS

6.1.1 Context

The atherogenic lipid profile (ALP) consists of sdLDL, also known as LDL phenotype B, low levels of HDL and hypertriglyceridaemia. It is a common finding in subjects with MetS. It is closely associated with obesity and IR and is an important predictor of cardiovascular events. Dietary intervention may be able to improve the ALP in MetS subjects.

6.1.2 Aims

To determine the influence of four isocaloric dietary fatty acid supplementation which differ in the amount and composition of fatty acids on LDL and HDL subclasses and LDL size and phenotype in subjects with MetS.

6.1.3 Methods

The current work was conducted as part of the EU Framework 6 Integrated Project: **LIPGENE "Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis".** The Quantimetrix Lipoprint™ system was used to determine subclasses of LDL, HDL, LDL size and phenotype.

6.1.4 Results

12 weeks of dietary intervention did not result in differences in anthropometrics, basic lipids nor S_I. The proportion of sdLDL correlated positively with TG, TRL-TG, TRL-chol and TRL apoB.

6.1.5 Conclusions

In this study, a 12-week dietary intervention of different types of fatty acids did not result in any significant changes in body composition, markers of glucose homeostasis nor standard lipid measurements. Changes in the levels of n-3 PUFA were demonstrated but did not correlate with lipoprotein measurement. The proportion of sdLDL was best predicted by TG and lipids of the TRL fraction. A longer intervention maybe required to produce meaningful changes to lipids and other parameters in patients with MetS.

6.2 INTRODUCTION

LDL particle size is an important predictor of cardiovascular events and progression of coronary heart disease (Austin, Breslow et al. 1988; Rizzo and Berneis 2006). LDL size is also inversely related with carotid-IMT (Skoglund-Andersson, Tang et al. 1999) and ischaemic stroke (Slowik, Iskra et al. 2003). The sdLDL (phenotype B) is associated with both obesity and IR. Together with low levels of HDL-chol and hypertriglyceridaemia, sdLDL is recognised as being part of the ALP and is implicated in increasing the cardiovascular risk of subjects with MetS.

It is thought that both genetic and environmental factors, including diet, play an important role in the development of MetS (Phillips, Lopez-Miranda et al. 2006). Large observational studies have suggested links between dietary factors, insulin sensitivity and obesity (Pereira, Jacobs et al. 2002; Liese, Roach et al. 2003). Indeed, several studies have suggested that diet and lifestyle interventions may be effective in preventing the development of T2DM in high-risk individuals (Knowler, Barrett-Connor et al. 2002; Mensink, Blaak et al. 2003; Orchard, Temprosa et al. 2005).

Whilst high fat diets have been clearly shown to promote obesity, IR and MetS (Feskens, Virtanen et al. 1995; Marshall, Bessesen et al. 1997; Storlien, Kriketos et al. 1997; Roche 2005), the question as to whether to substitute a HFA diet with a moderate fat MUFA-enriched diet or a low-fat high complex carbohydrate (LFHCC) diet is more beneficial remains yet to be answered. Studies suggest that LFHCC diets may actually cause a more adverse lipid profile (Roche and Gibney 1996) than MUFA diets (Garg, Bonanome et al. 1988), but the addition of n-3 PUFA may negate or even yield benefits above that of MUFA.

6.3 AIMS

The aim of the study was to determine the influence of four isocaloric dietary fatty acid supplementation, which differ in the amount and composition of fatty acids on LDL and HDL subclasses and phenotype in subjects with the MetS. To correlate any changes in LDL and/or HDL subclasses with anthropometric and fsIVGTT variables, inflammatory markers and adipokines.

6.4 METHODS

6.4.1 Subjects

The current work was conducted as part of the EU Framework 6 Integrated Project LIPGENE "Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis" (Shaw, Tierney et al. 2009). In the original study, 486 Caucasian subjects, aged 35 to 70 years, BMI 20-40 kg/m^2 with MetS (defined by having 3 or more of the National Cholesterol Education Program Adult Treatment Panel (NECP-ATP) III criteria) underwent a 12-week dietary intervention period, randomised to 4 isocaloric diets: high saturated fatty acid (HSFA), high monounsaturated fatty acid (MUFA), low-fat, high complex carbohydrate (LFHCC) diet, and LFHCC with n-3 polyunsaturated fatty acid (n-3 PUFA) (LFHCC n-3) supplementation. 161 samples (from 7 of the 8 participating centres) were available for this study (n=34 in SFA, n=45 in MUFA, n=40 in LFHCC, n=42 in LFHCC n-3).

The experimental protocol was approved by the local ethics committee at each of the intervention centres. Volunteers were recruited by local and regional advertisements. Initially volunteers were screened over the telephone using a volunteer suitability questionnaire which assessed dairy food consumption, fish consumption and willingness to consume sandwiches during the study, to ensure alteration of composition of fatty acids in the diets. This was followed up by completion of a health and lifestyle questionnaire, and anthropometric and biochemical tests. The current analyses included samples from 160 volunteers across 7 of the 8 sites who had completed the 12 week dietary intervention. Each volunteer was randomly stratified (according to gender, age and fasting plasma glucose) to one of four dietary interventions for 12 weeks. A minimisation procedure was used centrally to randomise volunteers to one of four study diets as previously described.

6.4.2 Clinical protocol

All clinical measurements were done at baseline (week 0) and repeated again after 12 weeks of intervention (week 12). All subjects had their height measured with a Harpenden stadiometer and weight measured in light clothing. BMI was calculated as weight (kg) / height squared (m^2). WC and HC were measured with a non-distensible flexible tape. All subjects also underwent estimation of body composition including percentage body fat using bioimpedance analysis using the Bodystat 1500 system (Bodystat Ltd, UK) described in Section 0.

6.4.2.1 Frequently sampled intravenous glucose tolerance test (fsIVGTT)

Each subject underwent an fsIVGTT on two occasions – before and after the dietary intervention period. This procedure has been described in the Section 2.4.1.

6.4.2.2 Dietary fatty acid supplementation

Patients were asked to maintain an isocaloric but were given food and fatty acid supplements that differed in fat quantity and quality. Patients received one of four dietary regimes for 12 consecutive weeks:

(A) high-fat (38% of energy derived from fat) saturated fatty acid-rich diet (HSFA);

(B) high-fat (38% energy) monounsaturated fatty acid-rich diet (HMUFA);

(C) low-fat (28% energy) high-complex carbohydrate diet supplemented with 1.24g/d high oleic sunflower oil (LFHCC) and

(D) low-fat (28% energy) high complex carbohydrate diet supplemented with 1.24g/d linoleic acid (LC) n-3 polyunsaturated fatty acid (PUFA) diet (LFHCC n-3)

Intervention foods were provided by Unilever Best Foods (Vlaardingen, The Netherlands). The LC n-3 PUFA supplement (Marinol C-38) and placebo high-oleic acid sunflower seed oil (HOSO) supplement were provided by Loders Croklaan (Wormerveer, The Netherlands).

6.4.3 Laboratory methods

Serum and plasma samples were taken and frozen at -80°C for further biochemical analyses at baseline (week 0) and post-dietary intervention week 12.

6.4.3.1 Hormones, glucose, HbA1c, lipids

Insulin and c-peptide were measured by electrochemiluminesence immunoassay on the Roche E Module. Glucose was measured by an enzymatic (hexokinase) method on the Roche P Module. HbA1c was measured by ion exchange chromatography on the Tosoh 2.2. Cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-chol) and non-esterified fatty acid (NEFA) were measured by a standard enzymatic colourimetric method on the Roche P Module. Low density lipoprotein cholesterol (LDL-chol) was calculated by the Friedewald formula. TRLs were isolated as described in section 2.7.9 and apolipoproteins including apolipoprotein B48 were analysed as described in sections 2.7.6, 2.7.7 and 2.7.8.

6.4.3.2 LDL and HDL subfractions

The Quantimetrix Lipoprint™ System was used to analyse the proportion of sdLDL, LDL size and HDL subfractions. The Lipoprint™ System consists of the following components: Kit of 100 Lipoprint® Gels (catalogue number 48-7002, catalog number 48-9002): gel preparation rack, photopolymerization light, electrophoresis chamber(s), power supply, digital scanner or densitometer unit, computer and colour

printer. Results are analysed and reported using the Lipoprint® patient risk reporting software. Details of the method are described in section 2.7.15.

6.4.3.3 Fatty acid analyses

This has been fully described in section 2.7.14.

6.4.3.4 Inflammatory markers and adipokines

Methods for hsCRP, TNF-α, IL-6, PAI-1, sICAM-1 and sVCAM-1 have been described in full in section 2.7.12. Methods for adiponectin, leptin and resistin have been described in full in section 2.7.11. These were performed by Dr. Jolene McMonagle.

6.4.4 Statistical analyses

Data are presented as mean ± SEM. Skewed variables were logarithmically transformed to normalise data prior to analysis. To determine the effect the dietary intervention, paired t-tests were performed between the pre-intervention and post intervention data, according the dietary groups. Statistical analysis of clinical characteristics was made using ANOVA with post-hoc analysis according to the Bonferroni method. **The delta change (Δ)** (post-intervention minus pre-intervention) of each variable was also analysed using ANOVA with post-hoc analysis. Correlations **were performed using Pearson's correlation coefficient.** Multiple linear regression analysis was used to identify independent contributors to the proportion of atherogenic sdLDL and subfractions of HDL. Independent variables that correlated significantly in univariate analysis were entered into the multiple regression models using the Stepwise method. Statistical significance was defined as $p<0.05$.

6.5 RESULTS

6.5.1 Baseline demographic data, lipid profiles and apolipoproteins

There were no significant differences in the baseline measurements of anthropometric values as shown in Table 6-1. Variables derived from the fsIVGTT are presented in Table 6-2 and show that there are no baseline differences between the groups. There were no differences in routine lipid profiles, NEFA and TRL-TG, TRL-chol and TRL apoB between the 4 groups as shown in Table 6-3. There were also no differences in apolipoprotein as shown in Table 6-4. Table 6-5 shows the individual fatty acid results and Table 6-6 shows the fatty acid results grouped in to SFA, MUFA, PUFAn-6 and PUFAn-3.

Baseline lipoprotein subclasses showed slight differences only in the percentage of IDL-B subfraction and HDL measured in the LDL-Quantimetrix assay, when expressed as percentage of the total lipid fraction as shown in Table 6-7 and Table 6-8. Table 6-9 and Table 6-10 show results of lipid subfractions measured on the LDL Quantimetrix assay expressed in mg/dL. There were no pre-intervention differences between the groups except for VLDL. There were no baseline differences in any of the HDL subfractions measured by the HDL-Quantimetrix assay as shown in Table 6-11 and Table 6-12.

6.5.2 Comparisons post-dietary intervention

There were no changes in any of the anthropometric variables as shown in Table 6-1. Whilst absolute values for S_G, AIR_G and DI were different between groups, in that the LFHCCn-3 group had lower values of each of these variables compared to MUFA post-intervention, the delta changes (Δ) (**difference between post-** and pre-dietary intervention) were not significantly different between any of the groups as shown in Table 6-2.

There were no changes in standard lipid profiles, NEFA, TRL measurements and apolipoproteins either at baseline or after the 12-week dietary intervention as shown in Table 6-3 and Table 6-4.

Table 6-5 and Table 6-6 show the results of individual fatty acids and groups of fatty acids. The main differences in changes pre- and post-intervention were in eicosapentaenoic acid (C20:5 n-3) and total n-3 PUFA levels in that they were higher in the LFHCCn-3 group compared to the MUFA and LFHCC groups. This confirms compliance of volunteers in taking the n-3 PUFA supplementation.

There were no significant differences in changes in LDL and HDL subfractions between the 4 groups. Differences only existed in changes in percentage of IDL-B in that SFA groups showed a significant increase of IDL-B compared to the other groups. When expressed as actual units of mg/dL, the change in IDL-B and IDL-C were highest in SFA groups.

Table 6-1: Anthropometric results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Age (yr)		53.4 ± 1.3	53.2 ± 1.2	54.9 ± 1.3	55.2 ± 1.7	0.663
Male:Female		47.1 : 52.9	46.7 : 53.3	42.5 : 57.5	38.1 : 61.9	0.832
Systolic BP (mmHg)	pre	137.1 ± 2.3	136.7 ± 2.5	137.2 ± 2.6	136.5 ± 2.5	0.997
	post	132.2 ± 2.3	134.0 ± 2.2	133.4 ± 2.4	132.7 ± 1.9	0.943
	Δ	-4.3 ± 1.8	-2.7 ± 1.8	-3.8 ± 1.7	-4.0 ± 2.3	0.942
Diastolic BP (mmHg)	pre	87.7 ± 1.7	84.9 ± 1.5	85.6 ± 1.4	84.4 ± 1.4	0.456
	post	84.1 ± 1.4	84.2 ± 1.6	83.3 ± 1.3	82.6 ± 1.1	0.807
	Δ	-2.9 ± 1.2	-0.7 ± 0.9	-2.4 ± 1.2	-1.9 ± 1.4	0.570
Waist (cm)	pre	106.9 ± 1.8	107.2 ± 1.7	105.6 ± 1.6	106.0 ± 1.6	0.908
	post	105.2 ± 2.0	107.4 ± 1.7	105.5 ± 1.7	105.0 ± 1.7	0.737
	Δ	-0.9 ± 0.7	0.2 ± 0.6	-0.1 ± 0.6	-1.1 ± 0.7	0.420
Hip (cm)	pre	112.7 ± 1.9	113.1 ± 1.6	112.4 ± 1.5	111.9 ± 1.5	0.961
	post	110.9 ± 2.0	112.1 ± 1.9	111.2 ± 1.5	110.8 ± 1.6	0.943
	Δ	-1.4 ± 0.6	-0.9 ± 0.7	-1.3 ± 0.6	-1.2 ± 0.4	0.957
Waist : Hip	pre	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.956
	post	0.9 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	0.524
	Δ	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.347
Weight (kg)	pre	93.3 ± 2.6	94.3 ± 2.0	91.3 ± 2.3	90.7 ± 2.3	0.624
	post	91.5 ± 2.4	94.0 ± 1.9	90.2 ± 2.2	89.8 ± 2.3	0.495
	Δ	-0.6 ± 0.4	-0.3 ± 0.3	-1.1 ± 0.4	-0.6 ± 0.3	0.396
BMI (kg/m²)	pre	31.9 ± 0.8	33.3 ± 0.7	32.0 ± 0.6	31.8 ± 0.7	0.333
	post	31.5 ± 0.8	33.2 ± 0.7	31.6 ± 0.6	30.9 ± 1.0	0.172
	Δ	-0.2 ± 0.1	-0.1 ± 0.1	-0.4 ± 0.1	-0.9 ± 0.7	0.443
% Body fat mass (%)	pre	36.4 ± 1.6	36.7 ± 1.3	36.4 ± 1.6	37.6 ± 1.4	0.930
	post	37.1 ± 1.6	35.7 ± 1.3	36.4 ± 1.4	39.0 ± 1.2	0.328
	Δ	0.0 ± 0.4	-1.3 ± 0.5	-0.1 ± 0.8	0.6 ± 0.5	0.151

Table 6-2: fsIVGTT results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
	pre	0.79 ± 0.11	0.84 ± 0.09	0.91 ± 0.09	0.82 ± 0.11	0.851
S_I (x 10^4 $(mU.L^{-1})^{-1}.min^{-1}$)*	post	0.73 ± 0.11	0.87 ± 0.10	0.84 ± 0.10	0.49 ± 0.19	0.152
	Δ	-0.07 ± 0.12	0.03 ± 0.09	-0.08 ± 0.11	-0.33 ± 0.16	0.182
	pre	-3.73 ± 0.24	-3.93 ± 0.17	-3.84 ± 0.19	-3.82 ± 0.20	0.921
S_G (min^{-1})*	post	-3.46 ± 0.28	-3.94 ± 0.14	-3.55 ± 0.25	-2.90 ± 0.29	0.021
	Δ	0.27 ± 0.40	-0.01 ± 0.19	0.30 ± 0.29	0.92 ± 0.30	0.118
	pre	4.88 ± 0.33	5.20 ± 0.28	4.94 ± 0.29	5.00 ± 0.29	0.870
AIR_G ($mU.L^{-1}.min$)*	post	4.56 ± 0.39	5.32 ± 0.27	4.54 ± 0.36	3.90 ± 0.41	0.039
	Δ	-0.31 ± 0.52	0.12 ± 0.27	-0.40 ± 0.38	-1.10 ± 0.38	0.141
	pre	5.67 ± 0.39	6.01 ± 0.32	5.83 ± 0.34	5.82 ± 0.35	0.924
DI *	post	5.29 ± 0.45	6.19 ± 0.31	5.34 ± 0.41	4.39 ± 0.49	0.021
	Δ	-0.38 ± 0.59	0.18 ± 0.34	-0.48 ± 0.47	-1.42 ± 0.44	0.079

* log transformed

Table 6-3: Lipids, NEFA and TRL results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Chol (mmol/L)	pre	5.56 ± 0.14	5.18 ± 0.14	5.20 ± 0.16	5.56 ± 0.14	0.092
	post	5.56 ± 0.16	5.09 ± 0.14	5.06 ± 0.16	5.33 ± 0.16	0.094
	Δ	0.00 ± 0.02	-0.02 ± 0.01	-0.03 ± 0.02	-0.05 ± 0.02	0.327
LDL-chol (mmol/L)*	pre	1.15 ± 0.07	1.05 ± 0.07	1.04 ± 0.07	1.25 ± 0.05	0.096
	post	1.23 ± 0.04	1.09 ± 0.05	1.05 ± 0.07	1.11 ± 0.07	0.152
	Δ	0.08 ± 0.05	0.04 ± 0.05	0.00 ± 0.07	-0.13 ± 0.06	0.056
HDL-chol (mmol/L)*	pre	0.08 ± 0.04	0.07 ± 0.03	0.14 ± 0.04	0.11 ± 0.04	0.482
	post	0.12 ± 0.04	0.11 ± 0.03	0.13 ± 0.04	0.14 ± 0.04	0.950
	Δ	0.04 ± 0.02	0.04 ± 0.02	-0.01 ± 0.02	0.03 ± 0.02	0.155
TG (mmol/L)*	pre	0.58 ± 0.07	0.45 ± 0.06	0.39 ± 0.07	0.45 ± 0.06	0.273
	post	0.46 ± 0.06	0.42 ± 0.06	0.40 ± 0.08	0.35 ± 0.07	0.771
	Δ	-0.12 ± 0.07	-0.03 ± 0.05	0.01 ± 0.05	-0.10 ± 0.05	0.267
NEFA (mmol/L)*	pre	6.37 ± 0.08	6.38 ± 0.07	6.35 ± 0.06	6.48 ± 0.06	0.496
	post	6.37 ± 0.07	6.45 ± 0.06	6.34 ± 0.05	6.43 ± 0.06	0.500
	Δ	-0.01 ± 0.05	0.07 ± 0.05	0.00 ± 0.05	-0.04 ± 0.05	0.425
TRL-TG (mmol/L)*	pre	-0.21 ± 0.11	-0.29 ± 0.10	-0.52 ± 0.11	-0.35 ± 0.11	0.206
	post	-0.26 ± 0.10	-0.38 ± 0.10	-0.41 ± 0.13	-0.51 ± 0.11	0.521
	Δ	-0.06 ± 0.10	-0.09 ± 0.08	0.11 ± 0.08	-0.15 ± 0.09	0.174
TRL-Chol (mmol/L)*	pre	-1.08 ± 0.12	-1.19 ± 0.09	-1.38 ± 0.10	-1.14 ± 0.10	0.196
	post	-1.08 ± 0.11	-1.25 ± 0.10	-1.20 ± 0.15	-1.29 ± 0.10	0.653
	Δ	0.00 ± 0.12	-0.06 ± 0.07	0.18 ± 0.12	-0.15 ± 0.08	0.110
TRL-ApoB (mmol/L)*	pre	3.57 ± 0.20	3.61 ± 0.11	3.33 ± 0.17	3.55 ± 0.18	0.606
	post	3.27 ± 0.26	3.28 ± 0.18	3.45 ± 0.17	3.54 ± 0.20	0.744
	Δ	-0.30 ± 0.20	-0.33 ± 0.17	0.13 ± 0.20	-0.02 ± 0.11	0.183

* log transformed

Table 6-4: Apolipoprotein results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
ApoA-I	pre	0.34 ± 0.03	0.32 ± 0.03	0.36 ± 0.03	0.31 ± 0.02	0.605
	post	0.33 ± 0.03	0.32 ± 0.02	0.35 ± 0.03	0.31 ± 0.03	0.859
	Δ	-0.01 ± 0.01	0.01 ± 0.02	-0.01 ± 0.02	0.00 ± 0.01	0.853
ApoB	pre	0.04 ± 0.03	-0.02 ± 0.04	-0.08 ± 0.04	0.05 ± 0.03	0.057
	post	0.06 ± 0.03	-0.02 ± 0.04	-0.08 ± 0.05	0.00 ± 0.04	0.119
	Δ	0.02 ± 0.02	0.00 ± 0.03	0.00 ± 0.03	-0.05 ± 0.02	0.332
ApoC-II	pre	3.85 ± 0.06	3.65 ± 0.10	3.64 ± 0.07	3.78 ± 0.06	0.169
	post	3.82 ± 0.06	3.64 ± 0.10	3.58 ± 0.12	3.66 ± 0.11	0.453
	Δ	-0.03 ± 0.03	0.00 ± 0.04	-0.06 ± 0.09	-0.12 ± 0.10	0.670
ApoC-III	pre	5.06 ± 0.05	4.88 ± 0.12	4.96 ± 0.05	5.01 ± 0.04	0.424
	post	4.99 ± 0.05	4.85 ± 0.12	4.85 ± 0.14	4.85 ± 0.14	0.830
	Δ	-0.07 ± 0.03	-0.03 ± 0.03	-0.10 ± 0.12	-0.16 ± 0.13	0.749
ApoE	pre	3.54 ± 0.12	3.52 ± 0.09	3.67 ± 0.04	3.59 ± 0.07	0.555
	post	3.60 ± 0.04	3.54 ± 0.09	3.57 ± 0.10	3.55 ± 0.11	0.968
	Δ	0.06 ± 0.12	0.02 ± 0.04	-0.10 ± 0.09	-0.05 ± 0.11	0.587
ApoB48	pre	-0.47 ± 0.12	-0.60 ± 0.12	-0.38 ± 0.12	-0.73 ± 0.13	0.230
	post	-0.63 ± 0.13	-0.52 ± 0.12	-0.58 ± 0.13	-0.82 ± 0.12	0.363
	Δ	-0.16 ± 0.11	0.08 ± 0.09	-0.19 ± 0.10	-0.09 ± 0.09	0.176

Table 6-5: Fatty acid composition results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
C14:0	pre	-3.26 ± 0.11	-3.45 ± 0.13	-3.50 ± 0.09	-3.36 ± 0.10	0.490
	post	-2.99 ± 0.14	-3.15 ± 0.11	-3.16 ± 0.13	-3.02 ± 0.12	0.703
	Δ	0.27 ± 0.15	0.38 ± 0.14	0.25 ± 0.12	0.28 ± 0.12	0.888
C16:0	pre	-0.65 ± 0.05	-0.73 ± 0.06	-0.75 ± 0.05	-0.76 ± 0.06	0.593
	post	-0.54 ± 0.08	-0.64 ± 0.05	-0.68 ± 0.06	-0.68 ± 0.07	0.460
	Δ	0.11 ± 0.09	0.12 ± 0.06	0.04 ± 0.06	0.07 ± 0.06	0.842
C16:1	pre	-3.84 ± 0.12	-3.85 ± 0.17	-4.05 ± 0.13	-3.79 ± 0.14	0.601
	post	-3.57 ± 0.11	-3.83 ± 0.13	-3.82 ± 0.14	-3.73 ± 0.16	0.552
	Δ	0.27 ± 0.14	0.10 ± 0.14	0.13 ± 0.13	-0.01 ± 0.09	0.495
C18:0	pre	-2.56 ± 0.07	-2.61 ± 0.10	-2.62 ± 0.08	-2.85 ± 0.09	0.099
	post	-2.53 ± 0.10	-2.65 ± 0.09	-2.62 ± 0.11	-2.76 ± 0.11	0.480
	Δ	0.04 ± 0.08	0.04 ± 0.06	-0.10 ± 0.05	0.00 ± 0.06	0.384
C18:1	pre	-0.67 ± 0.07	-0.78 ± 0.08	-0.86 ± 0.07	-0.84 ± 0.08	0.324
	post	-0.51 ± 0.10	-0.58 ± 0.08	-0.71 ± 0.08	-0.72 ± 0.09	0.268
	Δ	0.15 ± 0.12	0.23 ± 0.09	0.13 ± 0.09	0.11 ± 0.09	0.814
C18:2 n6	pre	-0.62 ± 0.07	-0.73 ± 0.08	-0.75 ± 0.06	-0.76 ± 0.07	0.568
	post	-0.36 ± 0.08	-0.56 ± 0.08	-0.61 ± 0.07	-0.52 ± 0.09	0.207
	Δ	0.26 ± 0.11	0.18 ± 0.09	0.13 ± 0.08	0.22 ± 0.09	0.797
C18:3 n6	pre	-7.50 ± 0.21	-6.98 ± 0.26	-7.44 ± 0.17	-7.73 ± 0.17	0.068
	post	-7.40 ± 0.19	-7.39 ± 0.15	-7.26 ± 0.27	-7.17 ± 0.25	0.860
	Δ	0.10 ± 0.19	-0.26 ± 0.25	-0.03 ± 0.23	0.35 ± 0.17	0.224
C18:4 n3	pre	-7.81 ± 0.21	-7.61 ± 0.26	-8.03 ± 0.18	-8.02 ± 0.19	0.439
	post	-7.34 ± 0.25	-7.56 ± 0.18	-7.76 ± 0.27	-7.04 ± 0.25	0.170
	Δ	0.47 ± 0.28	0.20 ± 0.23	0.12 ± 0.18	0.78 ± 0.23	0.161

Table 6-5: Fatty acid composition results: Baseline, post-dietary supplementation and delta-changes (continued)

	pre	-6.26 ± 0.20	-6.21 ± 0.23	-5.97 ± 0.19	-5.90 ± 0.17	0.535
C20:1	post	-6.35 ± 0.21	-6.34 ± 0.21	-6.21 ± 0.24	-6.00 ± 0.28	0.693
	Δ	-0.09 ± 0.20	-0.01 ± 0.23	-0.37 ± 0.21	-0.25 ± 0.20	0.628
	pre	-4.23 ± 0.10	-4.26 ± 0.15	-4.40 ± 0.12	-4.53 ± 0.12	0.365
C20:3 n6	post	-4.05 ± 0.15	-4.21 ± 0.13	-4.14 ± 0.16	-4.37 ± 0.17	0.509
	Δ	0.18 ± 0.15	0.18 ± 0.11	0.10 ± 0.12	0.03 ± 0.10	0.755
	pre	-2.18 ± 0.11	-2.07 ± 0.13	-2.20 ± 0.11	-2.27 ± 0.12	0.652
C20:4 n6	post	-1.82 ± 0.09	-1.91 ± 0.09	-1.89 ± 0.09	-1.99 ± 0.10	0.652
	Δ	0.36 ± 0.14	0.20 ± 0.12	0.27 ± 0.12	0.23 ± 0.10	0.813
	pre	-7.55 ± 0.21	-7.32 ± 0.25	-7.60 ± 0.16	-7.69 ± 0.22	0.616
C20:4 n3	post	-7.30 ± 0.23	-7.39 ± 0.15	-7.22 ± 0.28	-6.97 ± 0.27	0.596
	Δ	0.25 ± 0.23	0.08 ± 0.23	0.20 ± 0.23	0.55 ± 0.27	0.525
	pre	-4.43 ± 0.17	-4.22 ± 0.19	-4.25 ± 0.18	-4.41 ± 0.19	0.796
C20:5 n3	post	-4.03 ± 0.14	-4.26 ± 0.14	-4.10 ± 0.17	-3.31 ± 0.15	0.000
	Δ	0.40 ± 0.17	0.07 ± 0.15	0.02 ± 0.18	1.01 ± 0.18	0.000
	pre	-6.08 ± 0.12	-5.83 ± 0.17	-6.07 ± 0.13	-6.27 ± 0.14	0.178
C22:4 n6	post	-5.81 ± 0.17	-5.72 ± 0.11	-5.86 ± 0.20	-5.73 ± 0.20	0.930
	Δ	0.27 ± 0.18	0.21 ± 0.13	0.11 ± 0.12	0.38 ± 0.16	0.604
	pre	-4.92 ± 0.10	-4.84 ± 0.18	-4.97 ± 0.09	-5.01 ± 0.17	0.841
C22:5 n3	post	-4.49 ± 0.09	-4.61 ± 0.11	-4.63 ± 0.16	-4.31 ± 0.17	0.333
	Δ	0.42 ± 0.12	0.32 ± 0.15	0.23 ± 0.11	0.59 ± 0.18	0.327
	pre	-3.40 ± 0.14	-3.33 ± 0.15	-3.44 ± 0.14	-3.52 ± 0.16	0.825
C22:6 n3	post	-3.10 ± 0.14	-3.27 ± 0.13	-3.10 ± 0.15	-3.01 ± 0.17	0.629
	Δ	0.29 ± 0.17	0.15 ± 0.14	0.27 ± 0.15	0.47 ± 0.16	0.502

Table 6-6: Fatty acid categories results: Baseline, post-dietary supplementation and delta-changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Total SFA*	pre	-0.44 ± 0.05	-0.54 ± 0.06	-0.53 ± 0.05	-0.56 ± 0.05	0.433
	post	-0.32 ± 0.08	-0.42 ± 0.06	-0.48 ± 0.06	-0.48 ± 0.06	0.292
	Δ	0.12 ± 0.09	0.13 ± 0.07	0.04 ± 0.06	0.08 ± 0.06	0.798
Total MUFA*	pre	-0.61 ± 0.07	-0.74 ± 0.08	-0.79 ± 0.07	-0.76 ± 0.08	0.423
	post	-0.45 ± 0.09	-0.52 ± 0.08	-0.67 ± 0.08	-0.66 ± 0.09	0.237
	Δ	0.16 ± 0.12	0.22 ± 0.09	0.13 ± 0.09	0.10 ± 0.09	0.822
Total n6 PUFA*	pre	-0.39 ± 0.07	-0.46 ± 0.08	-0.48 ± 0.07	-0.52 ± 0.07	0.649
	post	-0.39 ± 0.07	-0.46 ± 0.08	-0.48 ± 0.07	-0.52 ± 0.07	0.649
	Δ	0.27 ± 0.11	0.18 ± 0.09	0.15 ± 0.09	0.22 ± 0.09	0.841
Total n3 PUFA*	pre	-2.86 ± 0.12	-2.84 ± 0.13	-2.85 ± 0.13	-2.94 ± 0.14	0.948
	post	-2.52 ± 0.11	-2.65 ± 0.10	-2.64 ± 0.10	-2.26 ± 0.11	0.032
	Δ	0.34 ± 0.14	0.20 ± 0.12	0.21 ± 0.13	0.68 ± 0.15	0.036

* log transformed

Table 6-7: LDL-1 to LDL-7 subfractions as measured on the LDL Quantimetrix assay (% of total)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
LDL-1 (%)	pre	18.91 ± 0.78	19.04 ± 0.63	18.65 ± 0.60	18.68 ± 0.59	0.965
	post	19.57 ± 0.78	18.78 ± 0.70	18.85 ± 0.68	18.93 ± 0.62	0.862
	Δ	0.66 ± 0.55	-0.26 ± 0.55	0.19 ± 0.48	0.25 ± 0.40	0.640
LDL-2 (%)	pre	12.23 ± 0.65	12.42 ± 0.68	10.05 ± 0.82	11.47 ± 0.74	0.095
	post	12.18 ± 0.73	12.39 ± 0.66	9.93 ± 0.66	11.24 ± 0.68	0.047
	Δ	-0.04 ± 0.61	-0.03 ± 0.44	-0.03 ± 0.56	-0.22 ± 0.45	0.991
LDL-3 (%)	pre	2.05 ± 0.35	2.14 ± 0.38	1.13 ± 0.22	1.58 ± 0.29	0.096
	post	2.08 ± 0.39	2.26 ± 0.38	1.31 ± 0.30	1.85 ± 0.31	0.234
	Δ	0.03 ± 0.26	0.11 ± 0.29	0.21 ± 0.27	0.27 ± 0.22	0.925
LDL-4 (%)	pre	0.13 ± 0.07	0.30 ± 0.09	0.09 ± 0.05	0.10 ± 0.07	0.133
	post	0.15 ± 0.08	0.39 ± 0.12	0.23 ± 0.10	0.14 ± 0.06	0.185
	Δ	0.02 ± 0.07	0.09 ± 0.12	0.14 ± 0.10	0.04 ± 0.05	0.810
LDL-5 (%)	pre	0.00 ± 0.00	0.04 ± 0.03	0.02 ± 0.02	0.01 ± 0.01	0.265
	post	0.00 ± 0.00	0.05 ± 0.03	0.01 ± 0.01	0.00 ± 0.00	0.118
	Δ	0.00 ± 0.00	0.01 ± 0.03	0.00 ± 0.02	-0.01 ± 0.01	0.960
LDL-6 (%)	pre	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00	0.783
	post	0.00 ± 0.00	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.160
	Δ	-0.01 ± 0.01	0.02 ± 0.03	-0.02 ± 0.02	0.00 ± 0.00	0.461
LDL-7 (%)	pre	0.01 ± 0.01	0.03 ± 0.03	0.04 ± 0.04	0.09 ± 0.05	0.587
	post	0.00 ± 0.00	0.12 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.115
	Δ	-0.01 ± 0.01	0.09 ± 0.09	-0.04 ± 0.04	-0.09 ± 0.05	0.161

Table 6-8: Large and small LDL, VLDL, IDL and HDL subfractions as measured on the LDL Quantimetrix assay (% of total)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Large LDL (%)	pre	31.14 ± 0.95	31.46 ± 0.63	28.70 ± 1.08	30.14 ± 0.84	0.114
	post	31.76 ± 0.90	31.17 ± 0.77	28.78 ± 0.99	30.17 ± 0.75	0.087
	Δ	0.62 ± 0.57	-0.29 ± 0.58	0.17 ± 0.61	0.03 ± 0.42	0.719
Small LDL (%)	pre	2.21 ± 0.41	2.53 ± 0.47	1.30 ± 0.27	1.78 ± 0.35	0.124
	post	2.23 ± 0.45	2.85 ± 0.53	1.55 ± 0.39	1.99 ± 0.35	0.189
	Δ	0.02 ± 0.30	0.32 ± 0.44	0.28 ± 0.38	0.21 ± 0.25	0.942
VLDL (%)	pre	18.07 ± 1.01	16.18 ± 0.58	17.23 ± 0.78	18.01 ± 0.73	0.246
	post	17.36 ± 0.70	16.46 ± 0.61	17.15 ± 0.78	17.69 ± 0.86	0.667
	Δ	-0.71 ± 0.76	0.28 ± 0.55	0.08 ± 0.45	-0.32 ± 0.41	0.593
IDL-C (%)	pre	9.62 ± 0.34	9.60 ± 0.35	10.08 ± 0.38	10.62 ± 0.36	0.145
	post	9.95 ± 0.35	9.73 ± 0.34	9.97 ± 0.35	10.25 ± 0.37	0.753
	Δ	0.33 ± 0.27	0.12 ± 0.21	-0.11 ± 0.30	-0.36 ± 0.23	0.258
IDL-B (%)	pre	6.20 ± 0.25	7.12 ± 0.19	6.67 ± 0.25	7.38 ± 0.28	0.006
	post	6.90 ± 0.22	7.11 ± 0.28	6.46 ± 0.21	7.17 ± 0.24	0.158
	Δ	0.71 ± 0.28	-0.01 ± 0.25	-0.18 ± 0.20	-0.20 ± 0.18	0.029
IDL-A (%)	pre	7.69 ± 0.30	8.07 ± 0.27	8.67 ± 0.44	8.71 ± 0.42	0.171
	post	8.46 ± 0.34	8.27 ± 0.46	8.56 ± 0.37	8.72 ± 0.37	0.869
	Δ	0.76 ± 0.29	0.20 ± 0.46	-0.20 ± 0.34	0.01 ± 0.25	0.307
HDL (%)	pre	24.97 ± 1.15	24.86 ± 0.90	27.22 ± 1.28	23.22 ± 0.68	0.049
	post	23.17 ± 0.88	24.25 ± 0.94	27.35 ± 1.08	23.76 ± 0.83	0.012
	Δ	-1.79 ± 0.62	-0.60 ± 0.77	-0.11 ± 0.68	0.54 ± 0.54	0.115

Table 6-9: LDL-1 to LDL-7 subfractions as measured on the LDL Quantimetrix assay (mg/dL)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
LDL-1 (mg/dL)	pre	40.71 ± 2.07	37.24 ± 1.69	37.82 ± 1.83	40.12 ± 1.63	0.449
	post	42.15 ± 2.38	37.29 ± 1.75	37.60 ± 1.96	37.43 ± 1.74	0.267
	Δ	1.44 ± 1.42	0.04 ± 1.40	-0.28 ± 1.12	-2.69 ± 1.43	0.203
LDL-2 (mg/dL)	pre	26.62 ± 1.72	25.38 ± 1.85	20.56 ± 1.76	25.05 ± 1.91	0.118
	post	26.21 ± 1.86	25.11 ± 1.63	20.00 ± 1.53	23.24 ± 1.96	0.077
	Δ	-0.41 ± 1.15	-0.27 ± 0.96	-0.38 ± 1.30	-1.81 ± 1.14	0.731
LDL-3 (mg/dL)	pre	4.71 ± 0.85	4.73 ± 0.89	2.36 ± 0.48	3.55 ± 0.72	0.096
	post	4.71 ± 0.92	4.80 ± 0.85	2.78 ± 0.69	4.10 ± 0.77	0.267
	Δ	0.00 ± 0.60	0.07 ± 0.64	0.49 ± 0.62	0.55 ± 0.49	0.881
LDL-4 (mg/dL)	pre	0.29 ± 0.17	0.73 ± 0.23	0.23 ± 0.11	0.26 ± 0.18	0.147
	post	0.32 ± 0.17	0.87 ± 0.27	0.48 ± 0.21	0.33 ± 0.15	0.208
	Δ	0.03 ± 0.15	0.13 ± 0.27	0.26 ± 0.21	0.07 ± 0.13	0.884
LDL-5 (mg/dL)	pre	0.00 ± 0.00	0.13 ± 0.08	0.03 ± 0.03	0.02 ± 0.02	0.154
	post	0.00 ± 0.00	0.11 ± 0.07	0.03 ± 0.03	0.00 ± 0.00	0.119
	Δ	0.00 ± 0.00	-0.02 ± 0.09	0.00 ± 0.04	-0.02 ± 0.02	0.983
LDL-6 (mg/dL)	pre	0.03 ± 0.03	0.04 ± 0.04	0.03 ± 0.03	0.00 ± 0.00	0.760
	post	0.00 ± 0.00	0.07 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.199
	Δ	-0.03 ± 0.03	0.02 ± 0.07	-0.03 ± 0.03	0.00 ± 0.00	0.798
LDL-7 (mg/dL)	pre	0.03 ± 0.03	0.07 ± 0.07	0.10 ± 0.10	0.21 ± 0.13	0.521
	post	0.00 ± 0.00	0.27 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.109
	Δ	-0.03 ± 0.03	0.20 ± 0.19	-0.10 ± 0.10	-0.21 ± 0.13	0.141

Table 6-10: Large and small LDL, VLDL, IDL and HDL subfractions as measured on the LDL Quantimetrix assay (mg/dL)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Large LDL (mg/dL)	pre	67.32 ± 3.02	62.62 ± 2.73	58.38 ± 2.96	65.17 ± 2.77	0.173
	post	68.35 ± 3.24	62.40 ± 2.57	57.60 ± 2.89	60.67 ± 3.04	0.094
	Δ	1.03 ± 1.43	-0.22 ± 1.78	-0.67 ± 1.74	-4.50 ± 2.00	0.155
Small LDL (mg/dL)	pre	5.06 ± 0.98	5.71 ± 1.15	2.74 ± 0.60	4.05 ± 0.89	0.132
	post	5.03 ± 1.04	6.11 ± 1.19	3.28 ± 0.89	4.43 ± 0.88	0.242
	Δ	-0.03 ± 0.71	0.40 ± 0.98	0.62 ± 0.83	0.38 ± 0.57	0.960
VLDL (mg/dL)	pre	38.38 ± 2.12	31.56 ± 1.55	34.67 ± 1.89	38.69 ± 1.88	0.017
	post	36.65 ± 1.69	32.58 ± 1.50	33.45 ± 1.71	35.10 ± 2.17	0.409
	Δ	-1.74 ± 1.72	1.02 ± 1.34	-0.92 ± 1.31	-3.60 ± 1.35	0.121
IDL-C (mg/dL)	pre	20.71 ± 0.86	18.78 ± 0.88	20.41 ± 1.09	22.79 ± 0.96	0.025
	post	21.15 ± 0.96	19.13 ± 0.77	19.80 ± 1.03	20.05 ± 0.96	0.515
	Δ	0.44 ± 0.74	0.36 ± 0.57	-0.62 ± 0.94	-2.74 ± 0.85	0.013
IDL-B (mg/dL)	pre	13.38 ± 0.74	14.20 ± 0.69	13.49 ± 0.68	15.90 ± 0.78	0.055
	post	14.71 ± 0.60	14.24 ± 0.71	12.68 ± 0.59	14.50 ± 0.79	0.163
	Δ	1.32 ± 0.50	0.04 ± 0.58	-0.79 ± 0.49	-1.40 ± 0.64	0.009
IDL-A (mg/dL)	pre	16.65 ± 0.80	15.87 ± 0.75	17.69 ± 1.20	18.67 ± 0.98	0.157
	post	18.03 ± 1.01	16.38 ± 0.93	17.10 ± 1.11	17.43 ± 1.01	0.715
	Δ	1.38 ± 0.76	0.51 ± 0.92	-0.79 ± 0.83	-1.24 ± 0.79	0.125
HDL (mg/dL)	pre	53.00 ± 2.41	47.60 ± 1.77	53.21 ± 2.06	49.40 ± 1.65	0.112
	post	48.68 ± 1.89	47.18 ± 1.68	52.43 ± 1.86	46.50 ± 2.21	0.127
	Δ	-4.32 ± 1.74	-0.42 ± 1.70	-1.41 ± 1.21	-2.90 ± 1.36	0.303

Table 6-11: HDL subfractions as measured by HDL Quantimetrix assay (% of total)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
HDL-1 (%)	pre	6.32 ± 0.72	5.87 ± 0.54	7.75 ± 0.75	5.81 ± 0.65	0.134
	post	6.29 ± 0.72	5.94 ± 0.51	7.66 ± 0.79	6.20 ± 0.67	0.265
	Δ	-0.03 ± 0.54	0.07 ± 0.43	-0.08 ± 0.40	0.38 ± 0.45	0.886
HDL-2 (%)	pre	9.18 ± 0.70	9.06 ± 0.53	9.58 ± 0.64	8.00 ± 0.57	0.285
	post	8.69 ± 0.74	9.15 ± 0.55	9.56 ± 0.67	8.20 ± 0.54	0.439
	Δ	-0.49 ± 0.38	0.10 ± 0.27	-0.02 ± 0.30	0.20 ± 0.28	0.427
HDL-3 (%)	pre	6.45 ± 0.49	6.56 ± 0.38	7.09 ± 0.46	5.94 ± 0.40	0.298
	post	6.41 ± 0.52	6.74 ± 0.35	6.98 ± 0.50	6.30 ± 0.42	0.692
	Δ	-0.04 ± 0.27	0.19 ± 0.18	-0.11 ± 0.20	0.36 ± 0.21	0.363
HDL-4 (%)	pre	10.13 ± 0.46	10.20 ± 0.37	10.76 ± 0.35	9.43 ± 0.41	0.128
	post	10.01 ± 0.50	10.40 ± 0.36	10.44 ± 0.42	9.91 ± 0.40	0.733
	Δ	-0.12 ± 0.28	0.19 ± 0.22	-0.32 ± 0.22	0.48 ± 0.25	0.094
HDL-5 (%)	pre	12.51 ± 0.37	12.67 ± 0.26	12.10 ± 0.25	11.53 ± 0.40	0.058
	post	12.18 ± 0.33	13.01 ± 0.26	12.03 ± 0.31	11.60 ± 0.35	0.010
	Δ	-0.33 ± 0.23	0.35 ± 0.22	-0.07 ± 0.24	0.06 ± 0.21	0.213
HDL-6 (%)	pre	23.26 ± 0.70	23.31 ± 0.79	22.00 ± 0.62	23.36 ± 0.59	0.439
	post	22.77 ± 0.57	22.77 ± 0.91	22.21 ± 0.66	23.28 ± 0.61	0.776
	Δ	-0.49 ± 0.37	-0.54 ± 0.59	0.21 ± 0.40	-0.09 ± 0.43	0.638
HDL-7 (%)	pre	8.21 ± 0.32	8.46 ± 0.24	7.87 ± 0.30	8.58 ± 0.23	0.239
	post	8.19 ± 0.34	8.44 ± 0.27	7.84 ± 0.34	8.34 ± 0.24	0.487
	Δ	-0.02 ± 0.15	-0.02 ± 0.18	-0.03 ± 0.16	-0.24 ± 0.17	0.738

Table 6-11: HDL subfractions as measured by HDL Quantimetrix assay (% of total) (continued)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
HDL-8 (%)	pre	8.27 ± 0.36	8.46 ± 0.27	7.97 ± 0.34	8.93 ± 0.29	0.173
	post	8.45 ± 0.37	8.50 ± 0.32	8.07 ± 0.38	8.67 ± 0.29	0.635
	Δ	0.18 ± 0.20	0.03 ± 0.24	0.09 ± 0.19	-0.26 ± 0.21	0.505
HDL-9 (%)	pre	6.29 ± 0.36	6.56 ± 0.27	6.13 ± 0.33	7.28 ± 0.32	0.053
	post	6.59 ± 0.38	6.44 ± 0.33	6.23 ± 0.35	7.06 ± 0.32	0.353
	Δ	0.30 ± 0.22	-0.13 ± 0.24	0.09 ± 0.18	-0.22 ± 0.17	0.296
HDL-10 (%)	pre	9.29 ± 1.00	8.81 ± 0.56	8.71 ± 0.70	11.03 ± 1.27	0.231
	post	10.36 ± 1.02	8.53 ± 0.68	8.95 ± 0.78	10.36 ± 1.01	0.310
	Δ	1.07 ± 0.64	-0.28 ± 0.55	0.24 ± 0.63	-0.67 ± 0.68	0.260
Large HDL (%)	pre	21.94 ± 1.61	21.48 ± 1.23	24.41 ± 1.59	19.75 ± 1.46	0.158
	post	21.39 ± 1.70	21.81 ± 1.21	24.20 ± 1.62	20.69 ± 1.44	0.368
	Δ	-0.55 ± 0.95	0.33 ± 0.67	-0.21 ± 0.72	0.93 ± 0.78	0.563
Intermediate HDL (%)	pre	54.11 ± 1.13	54.65 ± 0.83	52.72 ± 0.85	52.90 ± 1.08	0.410
	post	53.14 ± 0.85	54.64 ± 0.98	52.52 ± 0.92	53.12 ± 0.96	0.405
	Δ	-0.97 ± 0.62	-0.02 ± 0.70	-0.20 ± 0.74	0.21 ± 0.69	0.688
Small HDL (%)	pre	23.86 ± 1.54	23.83 ± 0.98	22.82 ± 1.21	27.24 ± 1.71	0.110
	post	25.39 ± 1.67	23.46 ± 1.22	23.23 ± 1.34	26.09 ± 1.49	0.388
	Δ	1.54 ± 0.98	-0.37 ± 0.88	0.42 ± 0.85	-1.15 ± 0.83	0.195

Table 6-12: HDL subfractions as measured by HDL Quantimetrix assay (in mg/dL)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
HDL-1 (mg/dL)	pre	2.88 ± 0.43	2.49 ± 0.23	3.65 ± 0.43	2.69 ± 0.41	0.139
	post	2.88 ± 0.42	2.56 ± 0.23	3.50 ± 0.40	2.98 ± 0.44	0.333
	Δ	0.00 ± 0.28	0.07 ± 0.20	-0.15 ± 0.20	0.29 ± 0.23	0.575
HDL-2 (mg/dL)	pre	4.24 ± 0.51	3.98 ± 0.28	4.58 ± 0.43	3.69 ± 0.43	0.461
	post	4.21 ± 0.49	4.02 ± 0.30	4.65 ± 0.45	3.98 ± 0.44	0.648
	Δ	-0.03 ± 0.22	0.04 ± 0.16	0.07 ± 0.18	0.29 ± 0.17	0.654
HDL-3 (mg/dL)	pre	2.97 ± 0.33	2.76 ± 0.23	3.48 ± 0.33	2.83 ± 0.30	0.299
	post	3.12 ± 0.33	2.93 ± 0.20	3.38 ± 0.36	3.10 ± 0.37	0.794
	Δ	0.15 ± 0.16	0.18 ± 0.12	-0.10 ± 0.16	0.26 ± 0.17	0.364
HDL-4 (mg/dL)	pre	4.44 ± 0.34	4.33 ± 0.22	4.93 ± 0.31	4.21 ± 0.30	0.318
	post	4.59 ± 0.37	4.56 ± 0.20	4.83 ± 0.35	4.62 ± 0.35	0.932
	Δ	0.15 ± 0.20	0.22 ± 0.15	-0.10 ± 0.16	0.40 ± 0.23	0.284
HDL-5 (mg/dL)	pre	5.38 ± 0.28	5.33 ± 0.19	5.45 ± 0.20	4.98 ± 0.21	0.398
	post	5.44 ± 0.27	5.67 ± 0.17	5.35 ± 0.28	5.12 ± 0.21	0.385
	Δ	0.06 ± 0.16	0.33 ± 0.13	-0.10 ± 0.16	0.14 ± 0.15	0.212
HDL-6 (mg/dL)	pre	9.79 ± 0.39	9.76 ± 0.42	9.80 ± 0.32	10.12 ± 0.35	0.891
	post	10.03 ± 0.43	9.76 ± 0.47	9.73 ± 0.39	10.17 ± 0.35	0.843
	Δ	0.24 ± 0.27	0.00 ± 0.37	-0.08 ± 0.24	0.05 ± 0.23	0.905
HDL-7 (mg/dL)	pre	3.47 ± 0.16	3.51 ± 0.16	3.43 ± 0.12	3.69 ± 0.14	0.599
	post	3.62 ± 0.19	3.71 ± 0.18	3.43 ± 0.16	3.67 ± 0.14	0.622
	Δ	0.15 ± 0.13	0.20 ± 0.11	0.00 ± 0.11	-0.02 ± 0.09	0.380

Table 6-12: HDL subfractions as measured by HDL Quantimetrix assay (in mg/dL) (continued)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
HDL-8 (mg/dL)	pre	3.41 ± 0.15	3.53 ± 0.18	3.55 ± 0.12	3.86 ± 0.17	0.241
	post	3.68 ± 0.18	3.76 ± 0.20	3.48 ± 0.16	3.79 ± 0.15	0.590
	Δ	0.26 ± 0.11	0.22 ± 0.13	-0.08 ± 0.10	-0.07 ± 0.13	0.093
HDL-9 (mg/dL)	pre	2.56 ± 0.16	2.87 ± 0.15	2.65 ± 0.13	3.10 ± 0.18	0.081
	post	2.85 ± 0.16	2.96 ± 0.18	2.73 ± 0.15	3.10 ± 0.18	0.459
	Δ	1.12 ± 0.16	0.89 ± 0.14	0.83 ± 0.10	0.69 ± 0.15	0.221
HDL-10 (mg/dL)	pre	3.79 ± 0.34	3.76 ± 0.27	3.85 ± 0.32	4.62 ± 0.51	0.290
	post	4.29 ± 0.38	3.73 ± 0.32	3.93 ± 0.37	4.48 ± 0.45	0.489
	Δ	-0.12 ± 0.37	0.00 ± 0.22	-0.38 ± 0.28	-0.83 ± 0.45	0.293
Large HDL (mg/dL)	pre	10.15 ± 1.18	9.07 ± 0.64	11.73 ± 1.06	9.17 ± 1.06	0.186
	post	10.21 ± 1.14	9.64 ± 0.62	11.50 ± 1.07	9.98 ± 1.20	0.579
	Δ	0.06 ± 0.52	0.58 ± 0.36	-0.23 ± 0.44	0.81 ± 0.47	0.335
Intermediate HDL (mg/dL)	pre	23.12 ± 0.93	22.93 ± 0.68	23.60 ± 0.78	23.05 ± 0.81	0.936
	post	23.68 ± 1.01	23.62 ± 0.70	23.50 ± 0.97	23.62 ± 0.84	0.999
	Δ	0.56 ± 0.61	0.69 ± 0.51	-0.10 ± 0.53	0.57 ± 0.52	0.721
Small HDL (mg/dL)	pre	9.76 ± 0.53	10.16 ± 0.56	10.15 ± 0.49	11.55 ± 0.74	0.164
	post	10.79 ± 0.63	10.42 ± 0.65	10.00 ± 0.59	11.52 ± 0.69	0.384
	Δ	1.03 ± 0.41	0.27 ± 0.39	-0.15 ± 0.42	-0.02 ± 0.39	0.207

Table 6-13: Inflammatory markers and adipokines results

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
hsCRP (ng/mL)	pre	1.14 ± 0.15	1.11 ± 0.15	1.06 ± 0.15	1.33 ± 0.15	0.606
	post	1.06 ± 0.22	1.24 ± 0.13	1.33 ± 0.13	1.29 ± 0.13	0.649
	Δ	-0.08 ± 0.17	0.12 ± 0.12	0.27 ± 0.13	-0.04 ± 0.11	0.243
IL-6 (pg/mL)	pre	1.20 ± 0.16	1.09 ± 0.16	1.46 ± 0.11	1.37 ± 0.14	0.250
	post	1.24 ± 0.13	1.19 ± 0.13	1.23 ± 0.13	1.12 ± 0.15	0.924
	Δ	0.03 ± 0.14	0.10 ± 0.16	-0.23 ± 0.11	-0.25 ± 0.16	0.203
TNFα (pg/mL)	pre	1.39 ± 0.16	1.27 ± 0.14	0.89 ± 0.22	1.31 ± 0.13	0.167
	post	1.12 ± 0.18	1.17 ± 0.17	1.07 ± 0.15	1.17 ± 0.14	0.961
	Δ	-0.27 ± 0.19	-0.09 ± 0.12	0.18 ± 0.26	-0.14 ± 0.15	0.394
PAI-1 (ng/mL)	pre	3.71 ± 0.17	3.80 ± 0.16	3.92 ± 0.13	3.47 ± 0.15	0.186
	post	3.78 ± 0.18	3.75 ± 0.14	3.81 ± 0.14	3.73 ± 0.17	0.981
	Δ	0.07 ± 0.10	-0.06 ± 0.15	-0.11 ± 0.10	0.26 ± 0.19	0.281
sICAM (ng/mL)	pre	5.61 ± 0.04	5.62 ± 0.04	5.56 ± 0.04	5.62 ± 0.04	0.770
	post	5.57 ± 0.05	5.66 ± 0.04	5.57 ± 0.04	5.65 ± 0.03	0.256
	Δ	-0.04 ± 0.03	0.04 ± 0.03	0.00 ± 0.02	0.03 ± 0.03	0.332
sVCAM (ng/mL)	pre	6.31 ± 0.05	6.33 ± 0.04	6.37 ± 0.05	6.35 ± 0.03	0.833
	post	6.36 ± 0.06	6.37 ± 0.05	6.37 ± 0.05	6.42 ± 0.04	0.862
	Δ	0.05 ± 0.04	0.05 ± 0.03	0.00 ± 0.03	0.07 ± 0.02	0.379

Table 6-13: Inflammatory markers and adipokines results (continued)

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
Leptin (ng/mL)	pre	2.55 ± 0.17	2.58 ± 0.19	2.64 ± 0.14	2.65 ± 0.18	0.975
	post	2.35 ± 0.21	2.62 ± 0.15	2.48 ± 0.15	2.70 ± 0.16	0.490
	Δ	-0.20 ± 0.09	0.05 ± 0.12	-0.15 ± 0.08	0.05 ± 0.09	0.159
Resistin (pg/mL)	pre	9.16 ± 0.10	9.03 ± 0.10	9.04 ± 0.07	9.10 ± 0.09	0.741
	post	9.18 ± 0.10	9.06 ± 0.09	9.05 ± 0.13	8.91 ± 0.23	0.687
	Δ	0.02 ± 0.05	0.03 ± 0.05	0.02 ± 0.10	-0.19 ± 0.20	0.511
Adiponectin (μg/mL)	pre	1.19 ± 0.09	0.99 ± 0.11	0.87 ± 0.14	1.05 ± 0.10	0.290
	post	1.16 ± 0.12	1.13 ± 0.09	1.27 ± 0.08	1.30 ± 0.08	0.463
	Δ	-0.03 ± 0.11	0.14 ± 0.10	0.40 ± 0.11	0.25 ± 0.09	0.035

Because HSFA diet is recognised to have the most deleterious effects on cardiovascular risk burden, and the other three diets considered to have beneficial effects by comparisons, individual t-tests were performed between HSFA and the other three groups i.e. MUFA, LFHCC and LFHCC-n3. There were no significant differences between the HSFA and MUFA groups in any of the cardiovascular markers including adipokines. However delta change in adiponectin was lower in the HSFA group (-0.03 ± 0.11) compared to the LFHCC group (0.40 ± 0.11, p=0.007) and also LFHCC-n3 group (0.14 ± 0.10, p=0.039). There were no other differences in any other cardiovascular risk markers measured.

In summary, the main positive findings following dietary supplementation include a demonstrable rise in n3 PUFA due to the supplementation of C20:5 n3 fatty acid. The HSFA group had a significant rise in the presence of IDL-B. The MUFA, LFHCC and LFHCC-n3 groups demonstrated a significant rise in adiponectin compared to the HSFA group. These results are summarised in Table.

Table 6-14: Summary of positive results of pre- and post-intervention changes in all subjects

		HSFA	MUFA	LFHCC	LFHCC-n3	Sig.
C20:5 n3	pre	-4.43 ± 0.17	-4.22 ± 0.19	-4.25 ± 0.18	-4.41 ± 0.19	0.796
	post	-4.03 ± 0.14	-4.26 ± 0.14	-4.10 ± 0.17	-3.31 ± 0.15	0.000
	Δ	0.40 ± 0.17	0.07 ± 0.15	0.02 ± 0.18	1.01 ± 0.18	0.000
Total n3 PUFA*	pre	-2.86 ± 0.12	-2.84 ± 0.13	-2.85 ± 0.13	-2.94 ± 0.14	0.948
	post	-2.52 ± 0.11	-2.65 ± 0.10	-2.64 ± 0.10	-2.26 ± 0.11	0.032
	Δ	0.34 ± 0.14	0.20 ± 0.12	0.21 ± 0.13	0.68 ± 0.15	0.036
IDL-B (mg/dL)	pre	13.38 ± 0.74	14.20 ± 0.69	13.49 ± 0.68	15.90 ± 0.78	0.055
	post	14.71 ± 0.60	14.24 ± 0.71	12.68 ± 0.59	14.50 ± 0.79	0.163
	Δ	1.32 ± 0.50	0.04 ± 0.58	-0.79 ± 0.49	-1.40 ± 0.64	0.009
Adiponectin (µg/mL)	pre	1.19 ± 0.09	0.99 ± 0.11	0.87 ± 0.14	1.05 ± 0.10	0.290
	post	1.16 ± 0.12	1.13 ± 0.09	1.27 ± 0.08	1.30 ± 0.08	0.463
	Δ	-0.03 ± 0.11	0.14 ± 0.10	0.40 ± 0.11	0.25 ± 0.09	0.035

* log transformed

6.5.3 Correlations and multiple regression analyses

Due to the lack of change and differences in the groups of FAs, with the exception of n-3 PUFA, and that there were no differences in between the 4 dietary groups with regards to proportions of LDL and HDL subfractions, univariate correlations were used to further explore the predictors of LDL and HDL size and proportions of atherogenic lipoproteins with various lipids and lipoproteins. Baseline correlations were examined to explore the predictors of proportions of LDL and HDL fractions. The proportion of sdLDL particles correlated strongly with all measured lipids and lipoproteins except for apoA-I as shown in Table 6-15. The correlations between the proportion of large LDL with all lipids and lipoproteins, with the exception of apoA-I, was inversely correlated i.e. the opposite direction to sdLDL proportion.

Whilst there were some positive correlations between the baseline proportions of sdLDL with individual dietary fatty acids and even between groups of fatty acids, there were no significant correlations with proportions of groups of FA. These results are shown in Table 6-16.

Table 6-15: Correlations between the proportion of sdLDL particles with other lipids and lipoproteins

Lipids & Lipoproteins	r=	p=
Chol (mmol/L)	0.309	0.000
LDL-chol (mmol/L)	0.294	0.000
HDL-chol (mmol/L)	-0.237	0.003
TG (mmol/L)	0.476	0.000
TRL-TG (mmol/L)	0.420	0.000
TRL-Chol (mmol/)	0.464	0.000
TRL-ApoB (mmol/L)	0.333	0.000
ApoA-I (g/L)	-0.080	0.313
ApoB (g/L)	0.421	0.000
ApoC-II (mg/L)	0.376	0.000
ApoC-III (mg/L)	0.385	0.000
ApoE (mg/L)	0.181	0.023
ApoB48 (mg/L)	0.279	0.001

Table 6-16: Correlations between the proportion of sdLDL particles with FA

Fatty acids	r=	p=
C14:0	0.414	0.000
C16:0	0.324	0.000
C16:1	0.111	0.164
C18:0	0.183	0.021
C18:1	0.302	0.000
C18:2 n-6	0.240	0.002
C18:3 n-6	0.113	0.155
C18:4 n-3	0.235	0.003
C20:1	-0.134	0.092
C20:3 n-6	0.185	0.019
C20:4 n-6	0.263	0.001
C20:4 n-3	0.089	0.266
C20:5 n-3	0.219	0.006
C22:4 n-6	0.179	0.024
C22:5 n-3	0.198	0.012
C22:6 n-3	0.305	0.000
Total SFA	0.353	0.000
Total MUFA	0.297	0.000
Total n6-PUFA	0.268	0.001
Total n3-PUFA	0.274	0.000
SFA as % of total FA	-0.080	0.319
MUFA as % of total FA	0.096	0.230
n6-PUFA as % of total FA	-0.041	0.606
n3-PUFA as % of total FA	0.135	0.091

There were no significant correlations between the proportion of sdLDL and anthropometrics measurements except diastolic BP. There were also no correlations between sdLDL proportion and S_I, S_G, AIR_G or DI.

Table 6-17: Correlations between the proportion of sdLDL particles with anthropometric and fsIVGTT variables

Anthropometrics & fsIVGTT	r=	p=
Systolic BP (mmHg)	-0.020	0.798
Diastolic BP (mmHg)	0.180	0.023
Waist (cm)	-0.042	0.598
Hip (cm)	-0.116	0.143
Waist : Hip	0.087	0.276
Weight (kg)	-0.007	0.929
BMI (kg/m^2)	-0.048	0.544
Body Fat (%)	-0.107	0.187
Lean body mass (%)	0.106	0.189
Body Water (%)	0.159	0.076
S_I ($x\ 10^4\ (mU.L^{-1})^{-1}.min^{-1}$)	-0.122	0.141
S_G (min^{-1})	0.013	0.880
AIR_G ($mU.L^{-1}.min$)	-0.034	0.686
DI	-0.099	0.236

There were also no correlations between the proportions of sdLDL and any of the inflammatory markers and adipokines measured at baseline.

Table 6-18: Correlations between the proportion of sdLDL particles with inflammatory markers and adipokines

Inflammation & Adipokines	r=	p=
hsCRP (ng/mL)	-0.115	0.148
IL-6 (pg/mL)	0.027	0.731
TNF-α (pg/mL)	0.147	0.064
PAI-1 (ng/mL)	-0.014	0.863
sICAM (ng/mL)	0.132	0.096
sVCAM (ng/mL)	-0.078	0.324
Leptin (ng/mL)	-0.031	0.697
Resistin (pg/mL)	-0.037	0.646
Adiponectin (μg/mL)	-0.065	0.411

Table 6-19 shows the baseline correlations between the proportions of large, intermediate and small HDL particles with various lipids and lipoproteins. The proportion of large HDL correlated negatively with LDL-chol, TG, TRL-TG, TRL-chol, TRL apoB, apoB, apoC-II and TRL-apoB. It correlated positively with HDL-chol and apoA-I. Conversely, the proportion of intermediate HDL correlated inversely with HDL-chol and apoA-I. The proportion of small HDL also correlated inversely with HDL-chol, but correlated positively with TG, TRL-TG, TRL-chol and apoB i.e. in the opposite direction to large HDL.

Table 6-19: Correlations between proportions of large, intermediate and small HDL particles with other lipids and lipoproteins

Lipids & Lipoproteins	% of large HDL	% of intermediate HDL	% of small HDL
Chol (mmol/L)	-0.025	-0.074	0.079
LDL-chol (mmol/L)	-0.171 [a]	0.067	0.132
HDL-chol (mmol/L)	0.467 [b]	-0.304 [b]	-0.276 [b]
TG (mmol/L)	-0.340 [b]	0.130	0.266 [b]
TRL-TG (mmol/L)	-0.391 [b]	0.154	0.302 [b]
TRL-Chol (mmol/)	-0.418 [b]	0.125	0.351 [b]
TRL-ApoB (mmol/L)	-0.248 [b]	0.148	0.155
ApoA-I (g/L)	0.279 [b]	-0.273 [b]	-0.100
ApoB (g/L)	-0.248 [b]	0.080	0.204 [a]
ApoC-II (mg/L)	-0.183 [a]	0.070	0.143
ApoC-III (mg/L)	-0.128	-0.012	0.144
ApoE (mg/L)	-0.070	-0.008	0.079
ApoB48 (mg/L)	-0.017	0.050	-0.018

[a] $p<0.05$. [b] $p<0.01$

Table 6-20 shows the correlations between the percentage of HDL particles of different sizes and fatty acids. As with sdLDL, small HDL correlated with some but not all individual fatty acids. MUFA as a proportion of all FAs correlated negatively with large HDL. Both the proportions of n6-PUFA and n3-PUFA correlated positively with large HDL whilst n3-PUFA also correlated negatively with small HDL.

Table 6-20: Correlations between proportions of large, intermediate and small HDL particles FAs

Anthropometrics & fsIVGTT	% of large HDL	% of intermediate HDL	% of small HDL
C14:0	-0.240 [b]	0.090	0.189 [a]
C16:0	-0.071	0.084	0.015
C16:1	-0.223 [b]	0.215 [b]	0.082
C18:0	0.224 [b]	-0.059	-0.194 [a]
C18:1	-0.080	0.092	0.019
C18:2 n-6	0.067	-0.038	-0.043
C18:3 n-6	-0.071	0.044	0.044
C18:4 n-3	0.010	0.056	-0.050
C20:1	-0.212 [b]	0.066	0.176 [a]
C20:3 n-6	0.169 [a]	-0.007	-0.172 [a]
C20:4 n-6	0.032	0.045	-0.065
C20:4 n-3	-0.065	0.053	0.031
C20:5 n-3	0.201 [a]	-0.039	-0.184 [a]
C22:4 n-6	-0.082	0.042	0.056
C22:5 n-3	0.007	0.040	-0.035
C22:6 n-3	0.136	0.003	-0.145
Total SFA	-0.020	0.064	-0.025
Total MUFA	-0.083	0.104	0.013
Total n6-PUFA	0.081	-0.015	-0.074
Total n3-PUFA	0.205 [b]	-0.029	-0.195 [a]
SFA as % of total FA	-0.074	0.033	0.055
MUFA as % of total FA	-0.237 [b]	0.178 [a]	0.122
n6-PUFA as % of total FA	0.164 [a]	-0.137	-0.074
n3-PUFA as % of total FA	0.264 [b]	-0.095	-0.211 [b]

[a] $p<0.05$. [b] $p<0.01$

Table 6-21 shows that small HDL correlated negatively with hip circumference, and positively with WHR. Somewhat surprisingly, small HDL correlatively negatively with body fat but positively with lean body mass and body water content. Small HDL also correlated positively with AIR_G and DI.

Table 6-21: Correlations between proportions of large, intermediate and small HDL particles with anthropometric and fsIVGTT variables

Anthropometrics & fsIVGTT	% of large HDL	% of intermediate HDL	% of small HDL
Systolic BP (mmHg)	0.022	0.065	-0.069
Diastolic BP (mmHg)	0.019	0.000	-0.020
Waist (cm)	-0.233 [b]	0.229 [b]	0.083
Hip (cm)	0.094	0.080	-0.155 [a]
Waist : Hip	-0.335 [b]	0.164 [a]	0.237 [b]
Weight (kg)	-0.221 [b]	0.269 [b]	0.043
BMI (kg/m^2)	-0.051	0.182 [a]	-0.075
Body Fat (%)	0.189 [a]	0.054	-0.233 [b]
Lean body mass (%)	-0.204 [a]	-0.046	0.244 [b]
Body Water (%)	-0.168	-0.020	0.191 [a]
S_I ($x\ 10^4\ (mU.L^{-1})^{-1}.min^{-1}$)	0.106	-0.169 [a]	0.008
S_G (min^{-1})	0.076	0.044	-0.111
AIR_G ($mU.L^{-1}.min$)	-0.124	-0.069	0.179 [a]
DI	-0.074	-0.116	0.161 [a]

[a] $p<0.05$. [b] $p<0.01$

There was only a positive correlation between IL-6 and large HDL. There was also a positive correlation between adiponectin and large HDL, and an inverse correlation with small HDL Table 6-22.

Table 6-22: Correlations between proportions of large, intermediate and small HDL particles with inflammatory markers

Inflammatory markers	% of large HDL	% of intermediate HDL	% of small HDL
hsCRP (ng/mL)	0.088	0.065	-0.139
IL-6 (pg/mL)	0.174 [a]	-0.141	-0.083
TNF-α (pg/mL)	0.017	-0.117	0.065
PAI-1 (ng/mL)	-0.095	0.083	0.042
sICAM (ng/mL)	-0.121	0.016	0.116
sVCAM (ng/mL)	0.125	-0.105	-0.058
Leptin (ng/mL)	0.185 [a]	-0.028	-0.175 [a]
Resistin (pg/mL)	0.034	-0.075	0.017
Adiponectin (µg/mL)	0.053	-0.048	-0.022

[a] $p<0.05$. [b] $p<0.01$

To assess the effects of dietary intervention across all the variables, and in particular to determine the associations between these variables and lipoprotein sizes, correlation analyses were performed between the Δ of the variables with Δ of proportions of lipoproteins.

Table 6-23 shows the significant correlations between the change in proportion of sdLDL and changes in lipids and lipoproteins pre and post intervention period. There were strong positive correlations between the change in proportion of sdLDL with changes in TG, TRL-TG, TRL-chol and apoB48 and whilst the change in proportion of sdLDL correlated negatively with HDL-chol.

Table 6-23: Correlations between the change of proportion of sdLDL particles with changes in lipids and lipoproteins.

Δ Lipids & Lipoproteins	r=	p=
Chol (mmol/L)	0.138	0.087
LDL-chol (mmol/L)	0.047	0.561
HDL-chol (mmol/L)	-0.242	0.002
TG (mmol/L)	0.479	0.000
TRL-TG (mmol/L)	0.277	0.000
TRL-Chol (mmol/)	0.286	0.000
TRL-ApoB (mmol/L)	0.157	0.051
ApoA-I (g/L)	-0.013	0.868
ApoB (g/L)	0.147	0.066
ApoC-II (mg/L)	0.167	0.037
ApoC-III (mg/L)	0.136	0.090
ApoE (mg/L)	0.116	0.151
ApoB48 (mg/L)	0.251	0.002

There were no correlations between the changes in proportion of sdLDL size with changes in proportions of fatty acids (Table 6-24).

Table 6-24: Correlations between the change of proportion of sdLDL particles with changes in groups of fatty acids

Δ Fatty acids	r=	p=
SFA as % of total FA	0.155	0.052
MUFA as % of total FA	0.078	0.333
n6-PUFA as % of total FA	-0.062	0.438
n3-PUFA as % of total FA	-0.117	0.146

Table 6-25: Correlations between the change of proportion of sdLDL particles with changes in anthropometric and fsIVGTT variables

Δ Anthropometrics & IVGTT	r=	p=
Systolic BP (mmHg)	0.010	0.902
Diastolic BP (mmHg)	-0.015	0.847
Waist (cm)	0.061	0.444
Hip (cm)	0.057	0.479
Waist : Hip	-0.011	0.889
Weight (kg)	0.146	0.067
BMI (kg/m^2)	0.070	0.378
Body Fat (%)	0.058	0.496
Lean body mass (%)	-0.039	0.645
Body Water (%)	0.002	0.984
S_I ($x\ 10^4\ (mU.L^{-1})^{-1}.min^{-1}$)	-0.003	0.966
S_G (min^{-1})	-0.057	0.477
AIR_G ($mU.L^{-1}.min$)	0.048	0.543
DI	0.042	0.602

There were also no correlations between the proportion of sdLDL particles and any of the anthropometric and fsIVGTT variables (Table 6-25).

Correlation analysis with inflammatory markers showed that the change in the proportion of sdLDL also correlated positively with soluble intercellular-cell adhesion molecule (sICAM) ($r=0.268$, $p<0.001$) (Table 6-26). Multiple regression analysis indicated that plasma TG and sICAM levels were the only independent predictors of changes in levels of sdLDL, accounting for 26.9% of the overall variance.

Table 6-26: Correlations between the change of proportion of sdLDL particles with changes in inflammatory markers

Δ Inflammatory markers	**r=**	**p=**
hsCRP (ng/mL)	0.057	0.473
IL-6 (pg/mL)	0.114	0.151
TNF-α (pg/mL)	-0.069	0.384
PAI-1 (ng/mL)	-0.088	0.267
sICAM (ng/mL)	0.268	0.001
sVCAM (ng/mL)	0.050	0.532
Leptin (ng/mL)	0.096	0.225
Resistin (pg/mL)	0.037	0.640
Adiponectin (μg/mL)	-0.134	0.091

Table 6-27 shows the significant correlations between the change in large, intermediate and small HDL and changes in various lipid and lipoproteins. The changes in proportion of small HDL particles correlated positively with TG, chol, TRL-TG, TRL-chol, LDL-chol, apoA-I, apoB, apoC-II and apoC-III.

Table 6-27: Correlations between the change of proportion of large, intermediate and small HDL particles with changes in lipids and lipoproteins.

Δ Lipids & Lipoproteins	% of large HDL	% of intermediate HDL	% of small HDL
Chol (mmol/L)	-0.337 [b]	-0.018	0.306 [b]
LDL-chol (mmol/L)	-0.226 [b]	0.042	0.163 [a]
HDL-chol (mmol/L)	0.211 [b]	-0.041	-0.151
TG (mmol/L)	-0.380 [b]	-0.036	0.358 [b]
TRL-TG (mmol/L)	-0.293 [b]	-0.007	0.260 [b]
TRL-Chol (mmol/)	-0.291 [b]	0.028	0.230 [b]
TRL-ApoB (mmol/L)	-0.050	-0.072	0.099
ApoA-I (g/L)	-0.125	-0.089	0.178 [a]
ApoB (g/L)	-0.289 [b]	-0.051	0.290 [b]
ApoC-II (mg/L)	-0.231 [b]	-0.031	0.225 [b]
ApoC-III (mg/L)	-0.207 [b]	-0.020	0.194 [a]
ApoE (mg/L)	-0.156	-0.008	0.142
ApoB48 (mg/L)	-0.182 [a]	0.040	0.126

[a] $p<0.05$. [b] $p<0.01$

The change in percentage of large HDL correlated negatively with a change in proportion of SFA (Table 6-28). There were no other correlations between changes in proportions of large, intermediate or small HDL with any changes in groups of fatty acids.

Table 6-28: Correlations between the change of proportion of large, intermediate and small HDL particles with changes in proportions of fatty acids

Δ fatty acids	% of large HDL	% of intermediate HDL	% of small HDL
SFA as % of total FA	-0.183 [a]	0.081	0.098
MUFA as % of total FA	-0.148	0.104	0.049
n6-PUFA as % of total FA	-0.062	0.108	-0.029
n3-PUFA as % of total FA	0.075	0.111	-0.152

[a] $p<0.05$. [b] $p<0.01$

Table 6-29 shows the results of the correlations between changes in proportions of large, intermediate and small HDL with changes in anthropometric and fsIVGTT results. Somewhat surprisingly, it also correlated positively with insulin sensitivity (S_I) (r=0.193, p=0.014) and inversely with WHR ratio (r=-0.181, p=0.021). In contrast, the change in proportion of intermediate HDL correlated positively with WHR and BMI and inversely with S_I. The change in proportion of large HDL correlated negatively with body weight.

Table 6-29: Correlations between the change of proportion of large, intermediate and small HDL particles with changes in anthropometric and fsIVGTT variables

Δ Anthropometrics & IVGTT	% of large HDL	% of intermediate HDL	% of small HDL
Systolic BP (mmHg)	-0.107	-0.035	0.121
Diastolic BP (mmHg)	-0.029	0.018	0.011
Waist (cm)	-0.008	0.000	0.007
Hip (cm)	-0.043	-0.065	0.089
Waist : Hip	0.034	0.195 [a]	-0.181 [a]
Weight (kg)	-0.163 [a]	0.112	0.055
BMI (kg/m^2)	-0.145	0.184 [a]	-0.018
Body Fat (%)	-0.132	0.084	0.052
Lean body mass (%)	0.116	-0.055	-0.060
Body Water (%)	0.149	-0.099	-0.066
S_I ($x\ 10^4$ $(mU.L^{-1})^{-1}.min^{-1}$)	-0.017	-0.229 [b]	0.193 [a]
S_G (min^{-1})	-0.019	0.028	-0.006
AIR_G ($mU.L^{-1}.min$)	0.014	-0.072	0.044
DI	0.007	-0.130	0.096

[a] $p<0.05$. [b] $p<0.01$

The change in the proportion of small HDL correlated positively with adiponectin ($r=0.161$, $p=0.041$) but not with any other inflammatory markers or adipokines. Multiple regression analysis indicated that TG, ApoB, WHR, S_I, apoC-III, adiponectin and male gender were all predictors of changes in the proportion of small HDL, accounting for 29.4% of the variance.

Table 6-30: Correlations between the change of proportion of small HDL particles with changes in inflammatory markers

Δ Inflammatory markers	% of large HDL	% of intermediate HDL	% of small HDL
hsCRP (ng/mL)	-0.027	0.010	0.016
IL-6 (pg/mL)	-0.003	-0.116	0.093
TNF-α (pg/mL)	0.087	-0.145	0.038
PAI-1 (ng/mL)	-0.090	0.144	-0.034
sICAM (ng/mL)	-0.049	0.003	0.040
sVCAM (ng/mL)	-0.010	0.059	-0.038
Leptin (ng/mL)	-0.091	-0.049	0.117
Resistin (pg/mL)	0.011	0.078	-0.070
Adiponectin (μg/mL)	-0.069	-0.130	0.161 [a]

[a] $p<0.05$. [b] $p<0.01$

The following table is a summary of the positive findings of the delta-changes in sdLDL and the small HDL with anthropometric and inflammatory markers.

Table 6-31: Summary of significant correlations between changes in sdLDL or changes in small HDL with other anthropometrics and inflammatory markers

	Postive Correlation	Negative Correlation
sdLDL	sICAM	none
Small HDL	S_I, Adiponectin	WHR

6.6 DISCUSSION

Several studies have suggested that diet and lifestyle interventions may be more effective in preventing the development of DM than pharmacological agents (Knowler, Barrett-Connor et al. 2002; Mensink, Blaak et al. 2003; Orchard, Temprosa et al. 2005). However, the optimal dietary intervention has not yet been conclusively decided. On the other hand, energy-dense, high fat diets have been shown to promote obesity, IR and MetS (Feskens, Virtanen et al. 1995; Marshall, Bessesen et al. 1997; Storlien, Kriketos et al. 1997; Roche 2005).

This study showed that an isocaloric dietary intervention of different fatty acids over 12 weeks *per se* does not result in significant changes in anthropometrics, lipid profiles nor fsIVGTT parameters in subjects with MetS. Inflammatory markers also did not change post-dietary intervention and this has recently been reported by the original LIPGENE study group (Petersson, Riserus et al.). The lack of effect of n-3 PUFA on insulin sensitivity reported here is in agreement with a similar study involving 284 subjects between the ages of 45-70 (Griffin, Sanders et al. 2006) whose BMI were in the overweight category. The current study and the study by Griffin et al. differ from the results of the KANWU study which showed that HSFA diet reduced S_I (Vessby, Uusitupa et al. 2001). Other studies have shown that different FA compositions can affect insulin sensitivity (Vessby 2000) and even secretion (Rojo-Martinez, Esteva et al. 2006). One reason for this difference may be the fact that subjects in the current study were mainly weight-stable, obese subjects who may be resistant to dietary SFA modification than those who are slightly overweight. The other major difference is the dosage of n-3 PUFA used in this study – it is less than half of that used in the KANWU study (Vessby, Uusitupa et al. 2001). Another reason may be that a greater or more prolonged modification of dietary SFA is required to attenuate IR in already IR MetS subjects. Habitual intake is also like to be a factor.

The lack of changes of lipids by dietary intervention is not surprising. Individual studies often show a lack of effect of fatty acid intervention whilst studies that do report dietary effects often report contradictory findings. The commonest reasons cited include study duration, sample size, differing population groups and the usually small changes demonstrated. However, pooled results in the form of meta-analyses have often shown alterations in lipids. For example, in one meta-analysis of 10 studies, MUFA has been shown to reduce fasting plasma concentrations of TG and VLDL-chol by 19% and 22% respectively, an increased in HDL-chol by 10%, and no effects in LDL-chol (Garg 1998). Similarly a meta-analysis of sixty controlled trials reported that the replacement of carbohydrates with largely *n*-6 PUFA predicted the largest change in LDL-chol and total:HDL-chol ratio (Mensink, Zock et al. 2003). Another meta-analysis showed that T-chol is also reduced by PUFA (Clarke, Frost et al. 1997).

Changes in actual sdLDL by dietary intervention have also been previously reported. Whilst a high-MUFA diet has been shown to reduce the proportion of sdLDL (Zambon, Sartore et al. 1999) and possibly reduces the susceptibility of LDL to oxidation (Reaven and Witztum 1996), these results are conflicting (Ros 2003). On the other hand *n*-6 PUFA may directly exert a hypocholesterolaemic effect (Nichaman, Sweeley et al. 1967; Shepherd, Packard et al. 1980), but several studies have shown that diets supplemented by *n*-6 PUFA increase LDL oxidation in vitro, especially compared to MUFA (Berry, Eisenberg et al. 1991; Bonanome, Pagnan et al. 1992; Abbey, Belling et al. 1993). Therefore a quantitative reduction in LDL-chol may be accompanied by qualitative changes that are associated with increased atherogenicity, and these changes are often not assessed in the same studies. One of the main limitations of the current study was that it did not assess of oxidation susceptibility or atherogenicity of the measured particles, albeit being able to carefully assess multiple subfractions of LDL and HDL.

n-3 PUFA, including both EPA and DHA have been shown to reduce TG (Harris 1997; Sacks and Katan 2002; Lewis, Lookinland et al. 2004). The aforementioned study by Griffin et al. showed a reduction in the proportion of sdLDL by n-3 PUFA but the absolute differences were small (n-3 PUFA: 23.3 ± 12.1 (n=110) vs. without n-3 PUFA: 26.7 ± 10.1 (n=140), p=0.05) (Griffin, Sanders et al. 2006). The current study showed a correlation between sdLDL and TG, and similar to the study by Griffin et al, there were no correlations between sdLDL and n-3 or n-6 PUFAs. It is likely therefore that the findings in the current study are a confirmation of the already known association between TG and sdLDL and that dietary effects are minimal, if any, over a 12-week intervention period.

Apart from TG, lipids within the TRL fraction and $apoB_{48}$ also correlated strongly with sdLDL in this study. This would be consistent with evidence that the hypertriglyceridaemia that is prevalent in IR states results at least in part due to excess VLDL1 particles and possibly chylomicrons. LPL enzyme activity is found to be decreased, with concomitant increase in HL activity (Kasim, Tseng et al. 1987; Taskinen 1987; Baynes, Henderson et al. 1991; Tan, Shiu et al. 1999) which results in abnormal VLDL TG lipolysis into sdLDL (Packard, Demant et al. 2000). LPL activity correlates inversely with sdLDL, whereas HL activity correlates positively with sdLDL (Jansen, Verhoeven et al. 2002). Excess VLDL secretion also in turn delivers TG to muscle and other tissues, further exacerbating IR (Dresner, Laurent et al. 1999; Zammit, Waterman et al. 2001) setting up a vicious cycle.

The findings with regards to HDL are also noteworthy. Correlation analyses of pre-intervention data revealed that small HDL was found to correlate positively with TG, TRL-TG, TRL-chol and apoB. It also correlated positively with WHR as well as with % of lean body mass and body water, and even more unexpectedly with AIR_G and DI.

When results with regards to Δ changes pre- and post-dietary intervention were analysed, the proportion of small HDL correlated negatively with WHR and positively with S_I and adiponectin – suggesting an increase in the presence of small HDL with increased insulin sensitivity. This was despite an increase in small HDL with increases in most other lipids and lipoproteins including total-chol, LDL-chol, TG, TRL-TG, TRL-chol, apoA-I, apoB, apoC-II and apoC-III. One possible explanation is that the increase in lipids and lipoproteins, which increases the presence of sdLDL may also result in an increase in small HDL as an attempt to compensate for increased atherogenicity since small lipid-poor HDL particles are believed to be more efficient at reverse cholesterol transportation (Sviridov, Mukhamedova et al. 2008).

The exact role of small HDL though is still uncertain and still subject to much debate. Whilst reductions of very small lipid-poor HDL particles results in reduced cholesterol efflux (Miida, Miyazaki et al. 2003; Sviridov, Chin-Dusting et al. 2006), larger population based studies have suggested that large HDL particles are associated with protection against future CVD (Asztalos, Collins et al. 2005). Studies involving pharmacotherapies which change HDL size also yield conflicting results. Fibrates cause a shift from large to small HDL particles and promote cholesterol efflux (Guerin, Le Goff et al. 2003; Franceschini, Calabresi et al. 2007) but CETP inhibitors increase cholesterol efflux despite increasing the proportions of large HDL (Yvan-Charvet, Matsuura et al. 2007). Hence it is still unclear whether small or large HDL subtypes are more anti-atherogenic, and the current findings have led to the thinking that qualitative changes in HDL are just as, or perhaps even more important in **HDL's** ability to protect against atherosclerosis (Tsompanidi, Brinkmeier et al.).

Of all the cardiovascular markers and adipocytokines, only adiponectin was different between the 3 groups being lower in the HSFA group (-0.03 ± 0.11) compared to the LFHCC group (0.40 ± 0.11, p=0.007) and also LFHCC-n3 group (0.14 ± 0.10). This was not related to any significant changes in anthropometrics, insulin/glucose homeostasis parameters or changes in lipoprotein subfractions. Whilst long term

calorie restriction resulting in 8-9% weight loss has been shown to consistently increase adiponectin, this is not the same when isocaloric diets are used (Imbeault 2007). In rodents, n-3 supplementation in the form of fish oil EPA and DHA has been shown to increase adiponectin by up to two-fold (Rossi, Lombardo et al. 2005; Neschen, Morino et al. 2006). This has also been shown in human subjects although the magnitude is lower (Itoh, Suganami et al. 2007). In fact DHA levels have been shown to be positively correlated with adiponectin (Fernandez-Real, Vendrell et al. 2005). However, others have argues that this maybe due to weight loss rather than actual dietary supplementation (Lara, Economou et al. 2007). The mechanism for n-3 PUFA to be able to influence adiponectin is likely to be via PPAR-γ (He, Barak et al. 2003) and both fibrates and thiazolidinediones have been shown to increase adiponectin (Hirose, Kawai et al. 2002; Yilmaz, Sonmez et al. 2004; Hiuge, Tenenbaum et al. 2007). In our study, there were no significant changes in insulin sensitivity or anthropometrics, and no correlations between Δ-adiponectin and ΔS_I. Also, the differences in Δ-adiponectin were both for LHFCC without n-3 PUFA and LFHCC-n3 PUFA compared to HSFA. There is no published data on the effects of LFHCC alone without n-3 PUFA on adiponectin. Further studies in this area is warranted.

The limitations in this study include the fact that although this was a dietary supplementation interventional study, the lack of effects of dietary intervention on anthropometrics and fsIVGTT data means that further analyses of lipoproteins are by way of association rather than that of cause and effect. However, the analysis of the Δ changes sought to address this issue. Whilst the original LIPGENE study was one of the largest dietary intervention studies ever carried out, this current study was limited by the number of samples available for analysis. Whilst there were still a total of 160 samples available for investigation of lipoprotein subclasses, there it must be acknowledged that there is a possibility that the study is under powered especially in the context of a heterogenous dietary intake across Europe.

In conclusion, whilst the LIPGENE dietary intervention study did not show significant differences in markers of glucose / insulin homeostasis nor lipid profiles or subfractions of lipoproteins, the data has revealed important correlations between LDL and HDL subtypes and other factors, in particular TG. Although no direct correlation with S_I was found in this study, this may be due to the lack of change of SI in this particular study. The study also suggests that if dietary intervention were to be used to modify metabolic and lipid parameters to a meaningful degree, this would likely have to be over a much longer duration of time and probably with greater intensity or higher dosages of supplementation.

Chapter 7

General Discussion

"Don't use words too big for the subject. Don't say "infinitely" when you mean "very"; otherwise you'll have no word left when you want to talk about something really infinite."

C. S. Lewis

T2DM occurs when the degree of β-cell failure is unable to compensate for IR and maintain glucose levels within the normal range (Prentki and Nolan 2006) and its association with increased cardiovascular risk is already well established. However, prior to β-cell failure, IR individuals who are at risk of developing diabetes are also subject to increased cardiovascular risk. Evidence from various studies show that conventional cardiovascular risk factors do not fully account for the risk of development of CVD (Wilson, D'Agostino et al. 1998; D'Agostino, Grundy et al. 2001; Golden, Folsom et al. 2002). Large studies such as the San Antonio Heart Study (Hanley, Williams et al. 2002), Botnia Study (Isomaa, Almgren et al. 2001), Bruneck (Bonora, Kiechl et al. 2007), Verona Diabetes (Bonora, Formentini et al. 2002) and Insulin Resistance Atherosclerosis Study (IRAS) studies (Howard, O'Leary et al. 1996) have all shown a clear relationship between the presence of IR and future cardiovascular disease. Surrogate markers of CVD are also elevated in IR states, e.g. carotid IMT (Hedblad, Nilsson et al. 2000), and there is an association between IR and greater cardiovascular risk factor load (Ferrannini, Balkau et al. 2007).

This thesis explores some of the plausible mechanisms that might contribute to increased cardiovascular risk in IR individuals. 2 prevalent IR conditions – PCOS and MetS – were studied in detail with regards to postprandial lipids, adipokines, oxidative stress and inflammatory markers, and lipoprotein subfractions.

In the first study, postprandial lipid, glucose and insulin responses to a mixed meal were studied in obese PCOS women and compared with that of obese controls. Although some postprandial studies in PCOS women have been done previously, this is the first one to evaluate the contribution of the two main metabolic / endocrine disturbances in PCOS women – insulin resistance and hyperandrogenaemia – to postprandial metabolism in obese women with PCOS. Both IR and hyperandrogenaemia are implicated in accelerated atherosclerosis but the relative contribution of either is a subject of controversy (Dunaif 1997; Morin-Papunen, Vauhkonen et al. 2000; Coviello, Legro et al. 2006). The findings in this study showed

that compared to obese control subjects, obese women with PCOS had higher levels of AUC-TG, AUC-HDL-chol, AUC-glucose and AUC insulin. HDL-chol and insulin were different at most of the time points, but TG was only different at 2 hours. Independent predictors of AUC-TG included Si, AUC B48 and AUC-apoB, accounting for 55% of the variance. This suggests that postprandial $apoB_{48}$-containing chylomicron particles have an important role to play in postprandial hypertriglyceridaemia and is a novel finding.

The contribution of chylomicrons is a significant finding. Hypertriglyceridaemia signifies the presence of excess TRL which consists of hepatically-derived VLDL characterised by the presence of $apoB_{100}$, and intestinally-derived chylomicrons, which contain $apoB_{48}$. Both VLDL and chylomicrons share a common lipolytic pathway and are hydrolysed by lipoprotein lipase (LPL) and there are a number of described receptor mediated pathways through which these particles may eventually be cleared from the circulation (Mahley and Ji 1999). Excess production and / or a delay in clearance of postprandial lipoproteins of both TRL particles maybe implicated in atherosclerosis (Zilversmit 1979; Groot, van Stiphout et al. 1991) and add to further cardiovascular risk burden in IR individuals such as women with PCOS.

PCOS women are thought to be more insulin resistant than their BMI-matched counterparts (Dunaif, Segal et al. 1989; Carmina and Lobo 2004). This is represented by greater insulin levels at all time points and greater AUC- and iAUC-insulin in PCOS women compared to obese controls even following adjustment with BMI. In this study, IR was determined by the more convenient $HOMA_{IR}$ and insulin sensitivity by the more sophisticated FSIGTT derived S_I. After adjusting for $HOMA_{IR}$, insulins were still significantly higher in the PCOS group. However, these differences were largely no longer present when adjustments were made for S_I, which may be a better marker for insulin sensitivity than $HOMA_{IR}$ in obese women with PCOS.

The lack of clear correlations between androgens and TG, HDL-chol, B48, apoA-I, apoA-II and apoB suggest that they don't have a large role to play in lipid metabolism in PCOS, at least in obese subjects. However, very strong correlations between SHBG and TG, HDL-chol, AUC B48, AUC-apoB, and AUC-apoA-II, suggests that IR, which is strongly negatively correlated with SHBG, rather than androgens, may be the major determinant of postprandial lipids. In support of this is the finding that FAI, which is a quotient of testosterone and SHBG levels, but not testosterone *per se* is also strongly correlated with HDL at all time points. FAI also correlated with TG at 0 and 2 hours, and AUC of both HDL and TG.

Only 2 other previous studies have examined postprandial lipid metabolism in PCOS women. Neither study included obese control subjects, but only lean controls. The study by Valazquez et al. reported a strong correlation between postprandial triglycerides and WHR. Overweight (BMI: 27.41 ± 0.50 kg/m^2) PCOS women were compared to lean PCOS women and lean controls. Only free testosterone was measured, and there was no analysis between this and postprandial hypertriglyceridaemia (Velazquez, Bellabarba et al. 2000). A separate study by Bahceci et al. evaluated postprandial responses in lean PCOS women and lean controls (BMI: 23.5 ± 2.6 kg/m^2 vs. 23.1 ± 4.0 kg/m^2; $p > 0.05$). PCOS women had higher baseline insulin, $HOMA_{IR}$, AUC-TG, AUC-total-chol, AUC-VLDL cholesterol, and AUC-apoB. AUC-insulin did not differ between the two groups despite differences in $HOMA_{IR}$. As with the study by Valazquez et al., no analyses were reported between androgens and postprandial lipids (Bahceci, Aydemir et al. 2007).

In conclusion, the findings of the first study provide evidence that in obese women with PCOS, IR plays a more important role than hyperandrogenaemia in postprandial dyslipidaemia and cardiovascular risk. Targeting obesity and improving IR by lifestyle measures should be a priority in the treatment of obese PCOS women.

In the second study, adipokines, androgens, IR and β-cell function were studied in relation to each other and with anthropometric variables. Serum adiponectin levels were found to be associated with both IR and DI – the ability of β-cells to compensate for IR – in premenopausal women with and without PCOS. Hypoadiponectinaemia is now recognised as one of the most important variables associated with IR and risk of progression to diabetes (Lindsay, Funahashi et al. 2002; Krakoff, Funahashi et al. 2003; Snehalatha, Mukesh et al. 2003; Spranger, Kroke et al. 2003; Duncan, Schmidt et al. 2004; Daimon 2005; Kadowaki, Yamauchi et al. 2006; Mather, Funahashi et al. 2008). Among the oligomers of adiponectin, both the HMW- and MMW- forms were associated with IR while only the MMW- form was significantly associated with β-cell function. That both HMW- and MMW- oligomers was associated with IR is consistent with previous studies (Mather, Funahashi et al. 2008).

In contrast to the clear association of adiponectin levels with IR, data concerning the association of adiponectin with β-cell function are conflicting. Studies *in vitro* and *in vivo* animal models suggest a direct effect of adiponectin on β-cells (Kharroubi, Rasschaert et al. 2003; Winzell, Nogueiras et al. 2004; Staiger, Stefan et al. 2005; Liu, Michael et al. 2007), but *in vivo* studies in human subjects have reported apparently conflicting findings. Significant correlation between adiponectin and β-cell function has been reported in normal-weight and obese adolescents (estimated using fasting proinsulin-to-insulin ratio) (Hung, Chu et al. 2006), women in late pregnancy (using a model derived from OGTT) (Retnakaran, Hanley et al. 2005) and patients with non-alcoholic steatohepatitis (using fsIVGTT) (Musso, Gambino et al. 2005). However, one study in a group of normal subjects using insulin secretagogues in conjunction with glucose clamping did not find any difference (Staiger, Stefan et al. 2005). Differences between these findings might reflect differences in methodology or alternatively differences in patient groups. The current study confirms previous findings that adiponectin is related to β-cell function, but also demonstrates an association of MMW-adiponectin with β-cell function.

The presence of absence of a positive family history for diabetes may account for some of the conflicting difference between adipokines and β-cell function. In women with a family history of diabetes, no significant difference in first-phase insulin secretion relative to IR (a similar measure to DI) was observed between subjects with and without PCOS (Ehrmann, Sturis et al. 1995). However, when women with PCOS and a family history of diabetes were compared to a similar cohort with PCOS, but without a family history of diabetes, evidence of reduced first-phase insulin secretion in relation to IR was observed (Ehrmann, Sturis et al. 1995). Another study indicated reduced DI in both non-obese and obese women with PCOS compared respectively with non-obese and obese women without PCOS (Dunaif and Finegood 1996). However, within the obese cohort, subjects with PCOS were significantly heavier and were more likely to have a first-degree relative with diabetes than control subjects and statistical adjustments were not reported for these differences. Finally, in a group of normal weight women with PCOS, no difference in S_I or DI was observed compared to normal subjects (Gennarelli, Rovei et al. 2005). Taken together, the findings of these studies as well those of the current study confirm that IR is increased in women with PCOS, but provide little evidence that PCOS status or circulating androgens contribute independently to the ability of β-cells to compensate for increased IR.

Also described in this study is that circulating androgens, whilst greater in women with PCOS independent of other variables, did not appear to influence β-cell function. Despite evidence from animal models in which administration of testosterone has been shown to reduce HMW-adiponectin (Xu, Chan et al. 2005) and the observation that adiponectin levels are greater in women than in men, we found no association of circulating androgens with total adiponectin or any of its oligomers. This is consistent with most although not all studies in human subjects, which have suggested no effect, independent of body composition, of circulating androgens on adiponectin (Orio, Palomba et al. 2003; Panidis, Kourtis et al. 2003; Spranger, Mohlig et al. 2004; Ardawi and Rouzi 2005; Escobar-Morreale, Villuendas et al. 2006; Glintborg, Andersen et al. 2006; Aroda, Ciaraldi et al. 2007; Barber, Hazell et al. 2008). Studies, in which adiponectin oligomers have been specifically measured, however have included a

relatively small number of subjects and therefore it is possible that the inverse association between testosterone and HMW-adiponectin that would be predicted based on the mouse model would be observed in a larger cohort of subjects.

This study also showed that leptin levels were highest in the obese PCOS group compared to the obese control and lean control cohorts. Studies to date have yielded conflicting data regarding leptin levels in PCOS (Jacobs and Conway 1999). In a study by Lecke et al. comparing obese PCOS with obese BMI-matched controls, leptin was found to be non-significantly higher (Lecke, Mattei et al. 2011). However, other studies have found similar levels of plasma leptin between women with PCOS and weight-matched controls (Chapman, Wittert et al. 1997; Laughlin, Morales et al. 1997; Mantzoros, Dunaif et al. 1997). Other studies have also found that obesity, rather than PCOS appears to be the main determinant of leptin levels (Escobar-Morreale, Villuendas et al. 2006; Knerr, Herzog et al. 2006). Leptin plays an important role in obesity and is consistently found to be elevated in obesity. This is thought to be due to leptin resistance probably at the level of the hypothalamus where it acts as a satiety messenger (Sinha and Caro 1998). Our findings of higher leptin levels in the obese PCOS compared to obese controls maybe primarily due to the non-statistically significantly greater BMI in the PCOS cohort compared to controls (38.2 ± 1.1 kg/m^2 vs. 35.1 ± 0.9 kg/m^2). Differences in the criteria used for diagnosing PCOS and including such subjects in studies might account for differences in reported findings in leptin levels (Jacobs and Conway 1999). It has also been suggested that hyperinsulinaemia, rather than PCOS status *per se*, influences leptin levels (Krassas, Kaltsas et al. 1998).

In summary, PCOS status and adiponectin are independently associated with IR in pre-menopausal women. Furthermore, adiponectin is also associated with the ability of β-cells to compensate for increased insulin resistance. MMW adiponectin may be more strongly associated with IR and β-cell function than HMW adiponectin.

To further evaluate the differences between obesity, IR and PCOS status individually on cardiovascular risk of women with PCOS, a third study was done assessing markers of oxidative stress and low-grade inflammation. Both of these have been found to predict atherosclerotic risk in a range of inflammatory states, including MetS and T2DM (Haffner 2006). Specific to PCOS, evidence has accumulated over the last decade to support an association between PCOS, oxidative stress and low-grade chronic inflammation, in keeping with the observation that these subjects are at increased risk of cardiovascular complications. However, it is unclear whether PCOS is associated with inflammation and oxidative stress independent of obesity and IR. Therefore, this study has attempted to separate the influence of obesity and IR from PCOS status by examining two groups of PCOS subjects, one obese and one lean, and comparing them to control subjects matched for age, BMI and IR.

TOS levels were significantly higher in both PCOS groups, compared to their BMI and IR matched controls. They also show that TAC and the TAC:TOS ratio was significantly lower in the lean PCOS group, compared to their lean counterparts. It also found that lean PCOS subjects have similar oxidative stress levels as the obese control group as measured by TOS. This suggests that potentially PCOS subjects who, although lean in weight, present a similar risk of oxidative stress as normal obese women, and that this is independent of obesity or IR. The results confirmed the previously reported association between oxidative stress and obesity, and the parameters of the metabolic syndrome (Fenkci, Fenkci et al. 2003; Gonzalez, Rote et al. 2006; Verit and Erel 2008). Thus, they suggest that TOS and TAC, or their ratio, may be extremely useful biomarkers in predicting oxidative stress, not only in women with PCOS but also in obese individuals displaying markers of the metabolic syndrome.

The analyses on inflammatory markers found that obesity appears to be the main determinant of these markers in PCOS. When compared to control subjects who are BMI- and IR-matched, women with PCOS had similar levels of inflammatory markers

as the control subjects. hsCRP levels were not influenced by PCOS, but were influenced by obesity, confirming the findings of Kelly et al. (Kelly, Lyall et al. 2001). Although several investigators have suggested that hsCRP is raised in PCOS, once adjusted for BMI this difference was removed (Talbott, Zborowski et al. 2004; Diamanti-Kandarakis, Paterakis et al. 2006). The concept that obesity, rather than PCOS being the driving force behind this increased inflammation, was further confirmed by the positive correlation between hsCRP and systolic blood pressure, BMI and $HOMA_{IR}$. PAI-1also appeared to be influenced predominantly by obesity rather than PCOS status, in keeping with the findings of Anfosso et al. (Anfosso, Chomiki et al. 1993) and Atiomo et al. (Atiomo, Fox et al. 2000). However, it should be noted that these findings are in contrast to those of Orio et al. (Orio, Palomba et al. 2004), who reported that PAI-1 remained higher in PCOS subjects, even after adjustment for BMI, although their subjects were not match for IR, suggesting that IR, and potentially not obesity, may be the driving influence that increases PAI-1 in PCOS subjects.

There have not been any previous studies evaluating MPO and SAA in PCOS subjects. Previous studies have shown that MPO is associated with increasing adiposity and oxidative stress (Brennan and Hazen 2003), and with markers of the metabolic syndrome (Rector, Warner et al. 2007). The results presented here suggest that similar to hsCRP and PAI-1, obesity and/or IR affects MPO levels rather than PCOS status *per se*. Furthermore, MPO also correlated with aspects of the metabolic syndrome suggesting that MPO may be a useful predictor of inflammation in states of obesity and its comorbidities. The current study also suggests that obesity influences SAA levels but that PCOS may additionally influence its expression as there was a trend for SAA levels to increase across the groups in the following order: lean controls, lean PCOS, obese controls, obese PCOS. This is to be expected as SAA, as well as being released from the liver during episodes of acute inflammation, is also released in low-levels from subcutaneous adipose tissue, making it a useful tool in identifying adipose-derived inflammation (Poitou, Viguerie et al. 2005).

Information regarding the role of neopterin in PCOS is relatively limited, with one study reporting that it may be a useful predictor of inflammation in lean PCOS subjects (Barutcuoglu, Bozdemir et al. 2006), however, in this study subjects were not matched for IR, suggesting that it may be IR that was the driving force in raising neopterin. Measuring serum levels of neopterin can be regarded as an indirect estimation of oxidative stress during cell-mediated immune responses, and is also increased during periods of low-grade inflammation (Murr, Widner et al. 2002). We could not identify any differences in neopterin levels, relative to the presence of PCOS, and the only difference found was between the two control groups. Therefore, the results suggest little utility for the use of neopterin in identifying either PCOS or obesity driven oxidative stress/inflammation in PCOS subjects.

Thus, to summarise the findings of the third study, the results indicate that inflammation in PCOS is largely obesity driven, whereas PCOS status appears to independently influence oxidative stress. Overall, these results suggest that the regulation of the metabolic abnormalities of PCOS and the management of weight will help reduce both oxidative stress and chronic inflammation and, therefore, aid in lowering cardiovascular risk in these subjects.

Taking the findings of the first 3 studies together, it becomes clearer that obesity-related IR is a significant underlying cause of the multiple lipid, metabolic and inflammatory derangements which are associated with increased cardiovascular risks. The detrimental effects of obesity in young women with or without PCOS should be addressed at both an individual level and population wide.

MetS is another prevalent condition in which IR is a key underlying metabolic abnormality. The MetS phenotype includes central adiposity, hypertension, glucose intolerance, hypertriglyceridaemia and low HDL-chol. The latter two are in fact part of the ALP but as sdLDL is not routinely measured in clinical practice, only these two

are included in the criteria for diagnosis of MetS. However, numerous studies have shown that sdLDL is an integral feature of the ALP even in normal or subnormal LDL-chol concentrations (Tilly-Kiesi, Syvanne et al. 1992; Stewart, Laker et al. 1993; Lahdenpera, Syvanne et al. 1996; Gray, Robbins et al. 1997; Guerin, Le Goff et al. 2001; Sniderman, Scantlebury et al. 2001) and that its presence signifies a substantial increase in cardiovascular risk (Austin, Breslow et al. 1988; Coresh, Kwiterovich et al. 1993; Gardner, Fortmann et al. 1996; Lamarche, Tchernof et al. 1997). The ALP is closely related to IR, perhaps through its correlation with intracellular TG stores, and lipotoxicity, results in elevated TGs and TRLs with eventual development of the sdLDL phenotype (Ziegler, Quilliot et al. 2001).

It is not clear what the most appropriate dietary intervention for the treatment of atherogenic dyslipidaemia is. Regular consumption of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) of marine origin can improve serum lipid profile and reduce cardiovascular risk (Roche and Gibney 2000). Eicosapentaenoic acids (EPA) and docosahexaenoic acids (DHA) exert different effects on serum lipids and lipoproteins – the LDL particle size is increased after DHA but not after EPA supplementation (Mori, Burke et al. 2000). DHA supplementation lowered concentrations of TG and sdLDL particles (Kelley, Siegel et al. 2007).

The results of the LIPGENE showed that an isocaloric dietary intervention of 4 different types of fatty acids over 12 weeks *per se* does not result in significant changes in anthropometrics, lipid profiles nor fsIVGTT parameters in subjects with MetS. The lack of effect of n-3 PUFA on insulin sensitivity reported here is in agreement with a similar study involving 284 overweight 45-70 year olds (Griffin, Sanders et al. 2006). The current study and the study by Griffin et al. differ from the results of the KANWU study which showed that HSFA diet reduced SI (Vessby, Uusitupa et al. 2001). Other studies have shown that different FA compositions can affect insulin sensitivity (Vessby 2000) and even secretion (Rojo-Martinez, Esteva et al. 2006). One reason for this difference may be the fact that subjects in the current

study were mainly weight-stable, obese subjects who may be resistant to dietary SFA modification than those who are slightly overweight. Another reason may be that a greater or more prolonged modification of dietary SFA is required to attenuate IR in already IR MetS subjects. Habitual intake is also like to be a factor.

Whilst meta-analyses of dietary interventions have shown quantitative changes in lipid levels, individual studies often report conflicting results (Clarke, Frost et al. 1997; Garg 1998; Mensink, Zock et al. 2003). Qualitative changes have also been reported. The prevalence of sdLDL is reduced by a high MUFA diet (Zambon, Sartore et al. 1999). A high MUFA diet may also reduce the susceptibility of LDL to oxidation (Reaven and Witztum 1996), but these results are conflicting (Ros 2003). Whilst hypocholesterolaemia is one of the commonly reported findings of *n*-6 PUFA supplementation (Nichaman, Sweeley et al. 1967; Shepherd, Packard et al. 1980), but several studies have shown that diets supplemented by *n*-6 PUFA increase LDL oxidation in vitro, especially compared to MUFA (Berry, Eisenberg et al. 1991; Bonanome, Pagnan et al. 1992; Abbey, Belling et al. 1993). Therefore a quantitative reduction in LDL-chol may be accompanied by qualitative changes that are associated with increased atherogenicity, and these changes are often not assessed in the same studies.

The current study showed a correlation between sdLDL and TG, without correlation to any FA groups. This is similar to a study by Griffin et al. who showed that n-3 PUFA supplementation results in a reduction in the proportion of sdLDL but the absolute differences were small (Griffin, Sanders et al. 2006). It is likely therefore that the findings in the current study are a confirmation of the already known association between TG and sdLDL and that dietary effects are minimal if any.

Small dense LDL correlated strongly with TG and lipids within the TRL fraction as well as apo48. This would be consistent with findings that there is

hypertriglyceridaemia in IR states due to excess VLDL1 particles. LPL enzyme activity is found to be decreased, with concomitant increase in HL activity (Kasim, Tseng et al. 1987; Taskinen 1987; Baynes, Henderson et al. 1991; Tan, Shiu et al. 1999) which results in abnormal VLDL TG lipolysis into sdLDL (Packard, Demant et al. 2000). LPL activity correlates inversely with sdLDL, whereas HL activity correlates positively with sdLDL (Jansen, Verhoeven et al. 2002). Excess VLDL secretion also in turn delivers TG to muscle and other tissues, further exacerbating IR (Dresner, Laurent et al. 1999; Zammit, Waterman et al. 2001) setting up a vicious cycle. Hyperinsulinaemia, which occurs prior to the β-cell failure that eventually results in T2DM, is thought to promote atherogenesis (DeFronzo and Ferrannini 1991; Defronzo 2006; Kashyap and Defronzo 2007) by stimulating both *de novo* lipogenesis and hepatic VLDL synthesis (Tobey, Greenfield et al. 1981; Koopmans, Kushwaha et al. 1999) via its stimulation of sterol regulatory-element-binding protein-1c and its inhibition of acetyl-CoA carboxylase (Azzout-Marniche, Becard et al. 2000). LDL-cholesterol transport (Stout 1971) into arterial smooth muscle cell, as well as smooth muscle cell proliferation (Pfeifle and Ditschuneit 1981; Nakao, Ito et al. 1985) and both processes are augmented by insulin, which also serves as a growth factor, stimulating collagen synthesis. Insulin is also capable of turning on genes involved in inflammation (Coletta, Balas et al. 2008) which is a key process in atherogenesis. Animal (Koopmans, Kushwaha et al. 1999) and human (Del Prato, Leonetti et al. 1994) studies have shown that IR can be provoked by insulin infusions and impair glycogen synthesis, thus resulting in excess circulatory glucose (Iozzo, Pratipanawatr et al. 2001). It is also well recognised that insulin therapy is invariably associated with weight gain (Henry, Gumbiner et al. 1993; Holman, Thorne et al. 2007). In clinical practice, continual weight gain and exacerbation of IR is much more common than weight loss and improvement of insulin sensitivity.

In summary, whilst the LIPGENE dietary intervention study did not show significant differences in markers of glucose / insulin homeostasis nor lipid profiles or subfractions of lipoproteins, the data has revealed important correlations between LDL and HDL subtypes and other factors, in particular TG. Although no direct

correlation with SI was found in this study, this may be due to the lack of change of SI in this particular study. The study also suggests that if dietary intervention were to be used to modify metabolic and lipid parameters to a meaningful degree, this would likely have to be over a much longer duration of time and for a larger population for there to be a net benefit.

Future studies are warranted. These studies clearly show that IR is a major cardiovascular risk and it can be found in non-diabetes mellitus conditions such as PCOS and MetS. With regards to PCOS, whilst larger studies with weight-, BMI- and IR-matched controls will undoubtedly be useful, randomised controlled interventional trials involving weight loss or the use of medications such as metformin and thiazolidinediones to reduce IR should be conducted to see whether cardiovascular risk markers are altered post intervention. Longer longitudinal studies should be conducted to document whether such interventions will change the rates of cardiovascular outcomes or the onset of diabetes mellitus.

With regards to dietary supplementation, again a longer period of supplementation with dietary fatty acids that produce larger differences in fatty acid composition may prove to be useful. Studies involving larger populations will probably yield more useful **results and the inclusion of clinically relevant "hard" end**-points will assist in determining whether such interventions confer health benefits beyond that of surrogate markers of cardiovascular disease. In MetS studies, the duration of follow up will probably need to be at least 5 to 7 years to allow sufficient number of events for an adequately powered analysis. For studies in PCOS this is likely to be significantly longer as the subjects in question are premenopausal female. However, end points such as the development of T2DM might require shorter durations of study.

To conclude, IR is a common condition and plays a major role in cardiovascular risk. Not only does it precede T2DM, but is also considered to be the primary metabolic abnormality underlying other prevalent conditions such as PCOS and MetS, all of which are condition associated with increased cardiovascular risk. Closely linked with IR is the ever increasing prevalence of obesity, which further exacerbates both IR and cardiovascular risk. Whilst many risk markers have been assessed and new ones are being discovered to help better understand the pathogenesis of atherosclerosis, current measures to tackle the growing epidemic of obesity as a population, and in individuals will no doubt be a key element to success in reducing cardiovascular risk burden.

Chapter 8

Bibliography

"Do not go where the path may lead, go instead where there is no path and leave a trail."

Ralph Waldo Emerson

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Chapter 9

Appendix

"When it is obvious that the goals cannot be reached, don't adjust the goals, adjust the action steps."

Confucius

9.1 PCOS STUDIES

9.1.1 Exclusion criteria

Androgen excess from any other causes including:

Androgen producing neoplasm

Late- or adult-onset congenital hyperplasia

Hypothyroidism*

Hyperthyroidism

Cushing's syndrome

Addison's disease

Hyperprolactinaemia

Type 1 or Type 2 diabetes mellitus

Postmenopausal status

Any chronic inflammatory disease

Medications affecting hormones, lipids, glucose and/or insulin homeostasis

* Patients on a stable dose of levothyroxine replacement for > 1 year for treatment of primary hypothyroidism were permitted to be in the study.

9.1.2 Patient information leaflet

Patient Information and Consent

1. **Title of study:** Post-prandial lipid metabolism and cardiovascular risk in Polycystic Ovary Syndrome.

2. **Introduction:** Patients with diabetes, pre-diabetic conditions, polycystic ovary syndrome, hypothyroidism, growth hormone deficiency and patients who are receiving cortisol treatment are at increased risk of developing narrowing of the arteries. This might be caused by excessive levels of fat in the blood after eating. We have developed techniques to allow us to study why this occurs.

3. **Procedures:** Should you decide to take part, we would ask you to come to the diabetic day centre on two occasions. On one occasion, we will carry out a test called an intravenous glucose tolerance test. This involves having a cannula inserted into each arm through which glucose and insulin can be given and blood withdrawn. Blood is taken at the beginning of the test, following which glucose and insulin are given through the intravenous cannula. A total of 12 more blood tests are taken for three hours after this. On the second occasion, we will give you a standard breakfast. Blood samples would be taken through the intravenous cannula prior to the standard breakfast and 2, 4, 6 and 8 hours thereafter.

4. **Benefits:** The study will have no direct benefit to the individual participant but the results may benefit subsequent patients.

5. **Risks:** There are no risks associated with these tests. Having an intravenous cannula inserted can sometimes cause mild soreness, which quickly resolves.

During the intravenous glucose tolerance test, occasionally blood sugar levels can go low which can cause a sensation of weakness and nausea. If this happens, you will be given a glucose-containing drink which will relieve the symptoms immediately.

6. **Exclusion from participation:** Your doctor has told you that you cannot be in this study if any of the following are true:

    1. You are less than 18 or more than 45 years old
    2. You are pregnant
    3. You have another illness that might influence results
    4. You are on other medications that might influence results

7. **Alternative treatment:** You do not have to be part of this study to be treated. You can have other standard treatments which are suitable for your condition.

8. **Confidentiality:** Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone who is not involved in the evaluation/investigation.

9. **Compensation:** Your doctors are covered by standard medical malpractice insurance. Nothing in this document restricts or curtails your rights.

10. **Voluntary Participation:** You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.

11. **Stopping the study:** You understand that your doctor may stop your participation in the study at any time without your consent. However, you will be offered follow up in the clinics similar to other patients not in the study.

12. **Permission:** The trial has hospital Research Ethics Committee approval.

13. **Further information:** You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Dr. Tommy Kyaw Tun, who can be contacted at 01–414 3862 or 086-151 8380. If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.

9.1.3 Consent form for PCOS studies

Title of research study: Post-prandial lipid metabolism in PCOS

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date:

Date on which the participant was first furnished with this form:

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's signature:

Date:

(Keep the original of this form in the participant's medical record, give one copy to the participant, keep one copy in the investigator's records, and send one copy to the sponsor (if there is a sponsor).

9.1.4 Components of mixed meal (the "big breakfast")

3 x 28g sausages

1 x medium sized egg fried in 30mls of olive oil

2 x slices of white bread/toast

1 x 15g of butter

1 x 30g bowl of cornflakes

1 x 250mls of full fat milk

1 x medium sized fruit



Figure 9-1: Components of the mixed meal

9.2 LIPGENE STUDY

9.2.1 Recruitment posters



LIPGENE is the name of an exciting project involving a group of 25 partners across Europe including *Trinity College Dublin*. For more information see www.lipgene.tcd.ie

One aspect of **LIPGENE** sets out to examine and understand the Metabolic Syndrome, which is a common condition diagnosed as having at least 3 of the following:

1. **Being overweight or obese**
2. **Having high Blood Pressure**
3. **Having increased Triglyceride (a certain type of fat) levels of in your blood**
4. **Having a Glucose (sugar) intolerance**
5. **Having abnormal fat levels in your blood (Cholesterol)**

The main aim of the study is to alter the type and amount of dietary fat in the diet of people **aged 35 – 70 years** as studies have shown that this can influence the Metabolic Syndrome.

What will be involved in the study?

Volunteers will follow a type diet for 12-weeks. Clinical investigations including body measurements, blood samples and glucose tolerance tests will be completed before and after the 12-week dietary treatment. We are recruiting volunteers now and the study 12-week study begins at the end of January 2005.

If you meet the above criteria for the Metabolic Syndrome and are interested in whether altering the fat in your diet will have a positive effect for you, then we would be delighted to hear from you.

Contact: **Jolene McMonagle, Nutritionist (087) 9499581 or mcmonaj@tcd.ie**

Audrey Tierney, Dietitian (086) 8652977 or tierneac@tcd.ie

9.2.2 LIPGENE – Work Package 1.2 Human Dietary Intervention Participating EU partners

Partner No	Location	Abbreviation
P1	Trinity College Dublin, Ireland*§	TCD
P2	University of Reading, UK§	UoR
P3	University of Oslo, Norway	UO
P5	INSERM U476, Marseille, France §	INSERM
P8	University of Maastricht, The Netherlands	UM
P9	Hospital Universitario, Reina Sofia-University of Córdoba, Spain	HURS-UCO
P10	University of Krakow, Poland	JUMC
P11	Uppsala University, Sweden	UU
P12	Unilever Foods, The Netherlands **	UF

* Co-ordinating Centre

§ Core LIPGENE analysis was completed in these centres

** Sole purpose was to created the LIPGENE Dietary Intervention Foods

9.2.3 Inclusion and exclusion criteria for the MetS volunteers in the LIPGENE Human Dietary Intervention Study

Inclusion Criteria	Exclusion Criteria
Age: 35 -70years	Age: < 35 or >70years
Gender: males and females (not pregnant or lactating)	Diabetes or other endocrine disorders§
Body Mass Index (BMI) 20-40 kg/m^2	Kidney or liver dysfunction
Medications/nutritional supplements allowed, on condition that the subjects adhere to the same regimen during the intervention:anti-hypertensive medication (including beta-blockers), oral contraceptives, hormone replacement therapy, multi-vitamin supplements and other non-fatty acid based nutritional supplements (e.g. garlic, anti-oxidants, etc)	Medications affecting lipid metabolism and high consumers of oily fish (> 2 serving of oily fish per/wk of herring, mackerel, kippers, pilchards, sardines, salmon, trout, tuna (fresh), crabmeat or marlin). One portion is defined as a small herring or mackerel, one can of salmon or sardines or one salmon or tuna steak. Tinned tuna is permitted as it contains only minor amounts of long chain n-3 PUFA
Smokers and non-smokers	Iron deficiency anaemia (haemoglobin < 12g/dl men, < 11g/dl women)
Regular consumers of alcohol, which is not excessive as defined by elevated liver enzymes (AST and ALT)	Weight Loss Drugs (e.g. orlistat and sibutramine)

Ethnicity: Intention to include white Europeans	Fatty acid supplements including fish oils, evening primrose oil. Consumers of high doses of antioxidant vitamins (A, C, E, β-carotene) †
	Red rice yeast (Monascus purpureus) or supplement usage
	Highly trained or endurance athletes who train more than 3 times/wk
	Volunteers planning to begin a special diet to lose weight (e.g. Atkins Diet)
	Weight change >3kg within the last 3 months
	Drug or alcohol abuse
	Pregnant of lactating females, women planning an pregnancy in the next 12 months
	Chronic inflammatory conditions§

§ see Table 2.9 below for further information

† **All volunteers were instructed to bring medication and nutritional supplement** bottles, capsule packs, labels for inspection at screening. If individuals were willing to stop using fatty acid supplements, a wash-out period of 8 weeks (wks) was required, before participating in the study. Maximum permitted intake of vitamins were as **follows Vit A (800μg), Vit C (60mg), Vit E (10mg) and** β-carotene (400μg), equivalent to a single dose present in a multivitamin capsule.

9.2.4 Endocrine and inflammatory conditions excluded from the LIPGENE Human Dietary Intervention Study

Endocrine Disorders	Chronic Inflammatory Conditions
Diabetes (Type 1 and 2)	Inflammatory Bowel Diseases
Hypothyroidism *	Crohns' Disease
Hyperthyroidism	Ulcerative Colitis
Adrenal disorders	Rheumatoid Arthritis
Addison's Disease	Systemic Lupus Erythematosis
Cushing Disease	Atopic dermatitis
Phaeochromocytomas	Psoriasis, Coeliac disease
Multiple Endocrine Neoplasia (MEN)	Fibromyalgia
Other rare conditions encountered	Pelvic inflammatory Disease
	Endometriosis
	Severe Asthma

* Subjects diagnosed >1year and on regular thyroxine therapy were included

9.2.5 Lipid-modifying medications excluded from the LIPGENE Human Dietary Intervention Study

Lipid-modifying medications	
Fenofibrate	Atorvastatin
Gemfibrizol	Pravastatin
Ciprofibrate	Fluvastatin
Benzafibrate	Rosuvastatin
Nicotinic acid	Simvastatin
Isaghula	Ezetimibe/Simvastatin
Acipimox	Ezetimible
Red Rice Yeast Supplements (monoscus purpureus)	Chloestyramine

9.2.6 Anti-inflammatory medications excluded from the LIPGENE Human Dietary Intervention Study

Anti-inflammatory Medication*	NSAIDS†
Steroids	Ibuprofen (Brufen, Fenbid, Codfen)
Corticosteroids	Fenoprofen
Prednisolone	Acelofenac
	Aspirin**

* Anti-inflammatory medication must have been finished 3 months before the commencement of the dietary intervention study

† Subjects were provided with paracetamol before and during the study, however, each subject was instructed to consume for pain relief only.

** A standard prophylactic dose of 75mg was acceptable