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The interaction between dietary fatty acids and inflammation on insulin sensitivity

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A thesis submitted of the University of Dublin, Trinity College for the degree of Doctor of Philosophy (Ph.D)

by

Jolene Mc Monagle

2008

Nutrigenomics Research Group
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Declaration

I, the undersigned submit this thesis to the University of Dublin, Trinity College for the examination of the degree of Ph.D. This thesis has not been previously submitted as an exercise for a degree at this or any other University. The work herein is entirely my own except where acknowledged and has not been previously submitted for any other degree at this or any other University. This thesis may be made available from the library for lending or copying upon request.

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Jolene Mc Monagle

Jolene McMonagle

09-09-2008

Date

This thesis is dedicated to the loving memory of my mother,

Frances Mc Monagle

Summary

The metabolic syndrome (MetS) has been defined as a multi-component disorder consisting of a combination of metabolic abnormalities associated with an increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). These metabolic disorders include glucose intolerance, hyperinsulinemia, dyslipidemia and hypertension. Recent research has demonstrated that the MetS may also be characterised by elevated inflammatory levels, endothelial dysfunction hypercoagulability and oxidative stress.

An effective approach to alleviating the prevalence of the MetS may be to focus on the importance of dietary therapy, in particular the quality rather than the quantity of dietary fats being consumed. Early epidemiological studies suggest that saturated fatty acids (SFA) induces insulin resistance (IR) by altering insulin signalling, whereas, intake of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) can reverse this effect. The objective of this thesis was to investigate the association between dietary fatty acids and inflammation on insulin sensitivity and the possible involvement in attenuating other risk factors in CVD.

Conjugated linoleic acid (CLA) is a PUFA found naturally occurring in foods from ruminant sources. Several isomers of CLA exist with the *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) isomer being the most abundant, accounting for >90% of the total CLA produced. Chapter three examines the effects of naturally derived *c*9, *t*11-CLA, synthetically prepared *c*9, *t*11-CLA and *trans* vaccenic acid (TVA), the main precursor of *c*9, *t*11-CLA on metabolic and molecular markers related to IR. Results of this study demonstrated that male *ob/ob* mice fed the natural *c*9, *t*11-CLA diet showed significant reduction in body weight ($p<0.05$). Moreover, significant improvements in insulin sensitivity, inflammatory status and lipid metabolism were also demonstrated for the natural and synthetic CLA diets. Furthermore, at a cellular level, GLUT4 and IRS-1 mRNA expression in the white adipose tissue (WAT) was significantly increased by natural and synthetic *c*9, *t*11-CLA feeding ($p<0.01$) and at the protein level, GLUT4 and IRS-1 was also significantly increased by both CLA enriched diets in the WAT ($p<0.0005$). Plasma IL-6 concentrations were decreased and adiponectin concentrations were increased following the natural and synthetic *c*9, *t*11-CLA diets. Interestingly, both these diets showed improvement in lipoprotein status, wherein significant decreases in plasma TAG and NEFA concentrations were observed. These findings

suggest that feeding a diet enriched with natural and/or synthetic *c*9, *t*11-CLA beef sources may improve the diabetic phenotype and with further longer-term research *in vivo* CLA could be a possible functional food used to alleviate the ever increasing MetS.

The LIPGENE Human Dietary Intervention Study represents the largest pan-European Intervention Study designed to determine the effect of dietary fat modification on insulin sensitivity, inflammation, coagulation and lipid metabolism. Chapter four and chapter five highlight the results of the LIPGENE study using the same cohort of MetS volunteers. Firstly, the interaction between dietary fatty acids, inflammation and insulin sensitivity was determined in 483 MetS volunteers as detailed in chapter 4. Results of this chapter showed no significant associations between plasma fatty acids, inflammation and insulin sensitivity. However, an increase in plasma sICAM-1, PAI-1 and tPA concentrations as well as body weight and BMI (kg/m^2) were associated with lower insulin sensitivity and higher $HOMA_{IR}$. Interestingly, analysis demonstrated that an intake of dietary fatty acid (C16:1, palmitolic acid) was inversely associated with insulin sensitivity. Furthermore, females had higher CRP concentrations compared to males and those with BMI >30 have increased CRP concentrations compared to leaner MetS volunteers. Additionally, several markers of inflammation, endothelial dysfunction and oxidative stress were higher among the Irish, UK and Spanish cohort compared to the Scandavian volunteers.

The human dietary intervention study included 417 free-living volunteers who were randomly assigned to receive one of four dietary treatments for 12-weeks. The aim was to investigate the efficacy of reducing dietary SFA consumption, by altering the quality and reducing the quantity of dietary fats. Diet A: High-fat, SFA-rich diet, Diet B: High-fat, MUFA-rich diet, Diet C: Low-fat-high complex carbohydrate diet and Diet D: low-fat, high complex carbohydrate diet & 1.24g/d LC n-3 PUFA. An IVGTT was performed at pre- and post-intervention and samples were harvested for inflammatory and lipoprotein analysis. Results of the 12-week intervention demonstrated that dietary fat modification had no significant effects on markers of insulin sensitivity or inflammatory status. However, significant modifications were noted for lipoprotein concentrations, the majority of these being in Diet D. In regards to insulin sensitivity and inflammatory status, no one diet emerged as having advantageous effects over the others.

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Abbreviations

AA	Arachidonic acid
ABC	ATP-binding cassette
ABCA1	ATP-binding cassette transporter
ACC	Acetyl CoA cardoxylase
Acrp-30	adipocyte complement related protein of 30kDa/adiponectin
ADP	Adenosine diphosphate
AGE	Advanced glycation end products
AgRP	Agouti related peptide
AHA	Anthranilic hydroxamic acid
AIRg	Acute insulin response to glucose
ALA	Alpha-linoleic acid
AMNCH	Adelaide & Meath Hospital, Dublin, incorporating the National Children's Hospital
AMPk	AMP activated protein kinase
Ang II	Angiotension II
ANOVA	Analysis of Variance
AP-1	Activator protein-1
APAF-1	Apoptotic protein activating factor-1
Apo A1	Apolipoprotein A1
Apo B	Apolipoprotein B
ApoE	Apolipoprotein E
APPL	Adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper mofit
APR	Acute phase response
APS	Ammonium persulphate
Arg	Arginine
ASDF	Adipose tissue secretory factor
AT	Adipose tissue
ATP	Adenosine triphosphate
AT-R1	Angiotnesion II type I receptor
BAT	Brown adipose tissue
BHT	Butylated hydroxy toluene
BIA	Bioelectric impedance analysis
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
BW	Body weight
c9, t11-CLA	*cis*-9, *trans*-11 conjugated linoleic acid
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CE	Cholesterol esters
CEPA	Conjugated eicosapentaenoic acid
cDNA	Complementary deoxyribonucleic acid

CAMs	Cell adhesion molecules
CCR2	Chemokine receptor 2
C/EBPa	CAAT enhancer binding protein α
chREBP	Carbohydrate responsive-element binding protein
CH_3	Methyl group
CHD	Coronary heart disease
CHF	Coronary heart failure
CHO	Carbohydrate
CI	Confidence interval
CLA	Conjugated linoleic acid
CM	Chylomicrons
CNS	Central nervous system
CO_2	Carbon dioxide
COOH	Carboxyl acid group
COX	Cyclooxygenase
CV	Coefficient of variation
C-peptide	Connecting peptide
CRE	cAMP response element
CREB	cAMP response element binding protein
CRP	C-reactive protein
Cys	Cysteine
CVD	Cardiovascular disease
d	Day
db	Diabetes gene
DAB	Diaminobenzibine chromagen
DBP	Diasystolic blood pressure
DC	Dendritic cells
DHA	Docosahexaeoic acid
DI	Disposition Index ($AIRg*S_I$)
DIO	Diet induced obesity
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPA	Docopentaeoic acid
EC	Endothelial cells
EDTA	Ethylenediaminetetra-acetic acid
EETs	Epoxyeicosatrienoic acids
EFA	Essential fatty acids
EGF	Epidermal growth factor
EGIR	European group for the study of insulin resistance
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial NO synthase
EPA	Eicosapentaenoic Acid
ER	Endoplasmic reticulum
ET-1	Endothelial-1
EU	European Union

FA	Fatty acids
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FFA	Free fatty acids
Fig	Figure
FIZZ	Found in inflammatory zone
Foxo 1a, A2, 3a, 4	Forkhead transcription factor 1a, A2, 3a, 4
FPIR	First Phase Insulin Response
g/d	grams per day
G0	distribution glucose concentrations at time 0
GAPDH	Glyceraldehyde phosphate dehydrogenase
GBP28	Gelatin-binding protein-28
GC	Gas chromoatography
GLUT	Glucose transporter
GLUT4	Glucose transporter 4
GP	Glycoprotein
GSH	Glutathione
H	Hydrogen
Hr	hour
HBSS	Hanks balanced salt solution
HCL	Hydrochloric acid
HDL-c	High density lipoprotein cholesterol
HETEs	Hydroxyeicosatetraenoic acid
HGP	Hepatic glucose production
His	Histidine
HL	Hepatic lipase
HMUFA	Monounsaturated fatty acid rich diet
HMW	High molecular weight
$HOMA_{IR}$	Homeostasis model assessment-insulin resistance
HPLC	High performance liquid chromatography
HRE	Hormone response element
HRT	Hormone replacement therapy
HURS-UCO	LIPGNE Partner 9 - Córdoba, Spain
HsCRP	High sensitivity C-reactive protein
HSFA	High saturated fatty acid rich diet
ICAM	Intracellular adhesion molecule-1
IDF	International diabetes foundation
IDL	Intermediate density lipoproteins
Ig	Immunoglobulin
IGF-1	Insulin like growth factor-1
IGF-1	Insulin like growth factor-β
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
IkKb	I kappa B kinase beta
IL-6 (1,8)	Interleukin-6, (1,8)

INSERM	LIPGENE Partner 5 - U476, Marseille, France
IR	Insulin resistance
IRAS	Insulin resistance atherosclerosis study
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
IVGTT	Intravenous glucose tolerance test
JAKS	Janus kinases
Jnk1	C-jun N-terminal kinase 1
JUMC	LIPGENE Partner 10 - Krakow, Poland
kDa	Kilodaltons
Kg	Kilogram
LA	Linoleic acid
LC	Long chain
LC *n*-3 PUFA	long-chain *n*-3 polyunsaturated fatty acids
LDL-c	Low density lipoprotein cholesterol
LMW	Low molecular weight
LOG	Logarithmic
LFHCC	Low-fat, high complex carbohydrate diet
LFn-3PUFA	Low-fat, high complex carbohydrate diet & 1.24g per day LC n-3 PUFA
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	LDL-like receptor protein
LXR	Liver X receptor
MAPK	Ras-mitogen activated protein kinase
MCAF	Monocyte chemotatic & activating factor
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
MI	Myocardial infarction
MINMOD	Millennium software packaging to compute insulin sensitivity
MMPs	Matrix metalloproteins
MMW	Medium molecular weight
mmHG	millimetre of mercury
mmol/L	millimoles per litre
mRNA	Messenger ribonucleic acid
MSNA	Muscle sympathetic nervous system
MUFA	Monounsaturated fatty acids
m^2	Meter squared
NADH	Nicotinamide adenine dinucleotide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCD	Non communicable disease
NCEP-ATPIII	National cholesterol education program-adult treatment panel III
NDNS	National diet and nutrition survey
NEFA	Non-esterified fatty acids

NGT	Normal glucose tolerance
NHS	National health service
NIDDM	Non insulin dependent diabetes mellitus
NO	Nitric oxide
NOS	Nitric oxide synthase
NYP	Neuropeptide Y
ob	Obese gene
OD	optical density
OGTT	Oral glucose tolerance test
OR	Odds ratio
PAI-1	Plasminogen activated inhibitor-1
PBMC	Peripheral blood mononuclear cells
P2	Removal rate of insulin from the interstitial space
P3	Movement of circulating insulin to interstitial space
P13K-AKT	Phosphatidlylinositol-3-kinase
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
Pch	Phosphocholine
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEPCK	Phosphoenolpyruvate carboxykinase
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H synthase
PGI_2	Prostacyclin I_2
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbal 12-myristate 13-acetate
POX	Peroxidase
PPAR	Peroxisome proliferator activated receptor
PPARg	Peroxisome proliferator activated receptor γ
PPRE	Peroxisome proliferator response element
PUFA	Polyunsaturated fatty acid
QUICKI	Quantitative insulin sensitivity check index
RBC	Red blood cells
RCF	Revolutions per minute
RELM	Resistin like molecules
RM-ANOVA	Repeated measures analysis of variance
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RT	room temperature
RT-PCR	Reverse transcription polymerase chain reaction
Rx	Receiving treatment
RXR	Retinoid X receptor
ROS	Reactive oxygen species

SAA	Serum amyloid A
SAP	Serum amyloid P
SBP	Systolic blood pressure
SCYA2	Small inducible cytokine A2
SD	Standard deviation
SEM	Standard error of the mean
SFA	Saturated fatty acids
SDS	Sodium dodecyl sulphate
S_G	Glucose effectiveness
S_I	Insulin sensitivity
SMC	Smooth muscle cells
sICAM-1	soluble Intracellular adhesion molecule-1
Socs	Suppressor of cytokine signalling
SOD	Superoxide dismutase
SREBP-1c	Sterol regulator element binding protein-1c
SQRT	Square root transformation
STATS	Signal transducers activators of transcription
sTNFα	soluble tumour necrosis factor α
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAG	Triaclyglycerol
TAE	Tris acetate EDTA
TBM	Tetramethylbenzidine
TC	Total cholesterol
TCD	LIPGENE Partner 1 - Trinity College Dublin
TEMED	N,N,N',N'-tetramethyethylenediamine
TFA	Trans fatty acids
TLC	Thin layer chromatography
TNFα	Tumour necrosis factor α
TNFαR1	Tumour necrosis factor α Receptor 1
TNFαR2	Tumour necrosis factor α Receptor 2
Tx	Thromboxanes
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
tPA	Tissue type plasminogen activator
TPL	Triacylglycerol poor lipoprotein
***t*10, *c*12-CLA**	*trans* 10, *cis*-12 conjugated linoleic acid
TRL	Triacylglycerol rich lipoprotein
TVA	Trans Vaccenic Acid
TZD	Thiazolidinedione
TK	Thymidine kinase
UCP-2	Uncoupling protein-2
UF	Unilever Foods, The Netherlands
UO	LIPGENE Partner 3 - University of Oslo

UoR	LIPGENE Partner 2 - University of Reading
UM	LIPGENE Partner 8 - University of Maastricht
UU	LIPGENE Partner 11- Uppsala University
uPA	Urokinase plasminogen activators
VA	Vaccenic acid
VAT	Visceral adipose tissue
VCAM	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
VIF	Variance inflation factor
VLA-4	Very late antigen 4
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cells
WAT	White adipose tissue
WBC	White blood cells
WHO	World Health Organization
WHR	Waist to hip ratio
Wk(s)	week(s)
ZDF	Zucker Diabetic Fatty (*fa/fa*)
1/SQRT	Inverse square root
8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin-$F_{2\alpha}$
15-keto-hydro- PGF_{2a}	15-keto-13,14-dihydro-$PGF_{2\alpha}$

Publications and Presentations

Publications

McMonagle J, Toomey S, Reynolds C, Moloney AP, Roche HM (2008) Fatty acid modified beef improves insulin sensitivity – a case for functional food development? *In preparation.*

Tierney AC, **McMonagle J**, Shaw DI, Lovedal-Gulseth H, Helal O, vanHees A, Martin C, Partyka L, Karlstörm B, Vessby B, DEmbinska-Kiec A, López-Miranda J, Blaak E, Defoort C, Drevon CA, Lovegrove JA, Roche HM (2008) Effects of dietary fat modification on insulin sensitivity and metabolic markers of the metabolic syndrome. LIPGENE – a randomized European dietary intervention study. *In preparation.*

Hartwich J, Leszczynska-Golabek I, Partyka L, Siedlecka D, Pérez-Martinez P, Marin C, López-Miranda J, Tierney AC, **McMonagle J**, Roche HM, Defoort C, Wolkow P, Dembinska-Kiec A (2008). Lipoprotein profile, plasma Ischemia Modified Albumin and LDL density change in the course of postprandial lipemia. Insights from the LIPGENE study. *In preparation*

Toomey S, **McMonagle J**, Roche HM (2006) Conjugated Linoleic Acid: a functional nutrient in the different pathophysiological components of the metabolic syndrome. *Current Opinion in Clinical Nutrition and Metabolic Care* **9**, 740-747.

Presentations

Diabetes Mellitus, Insulin Action and Resistance. Keystone Symposia, Breckenridge, Colorado, USA. January 2008 **[Poster presentation]**

NuGO Workshop: Diabetes – from individual genes to the systems. Munich, Germany. July 2007 **[Poster presentation]**

Pre-diabetes and the Metabolic Syndrome. Barcelona, Spain. April 2007 **[Poster presentation]**

Royal Academy of Medicine in Ireland, Winter Meeting, Dublin City University, Dublin, Ireland. January 2007 **[Poster presentation]**

Teagasc Walsh Fellowship Seminar. RDS, Dublin, Ireland. November 2006 **[Oral communication]**

18th International Congress on Nutrition, Durban, South Africa. September 2005. **[Poster presentation]**

Chapter 1

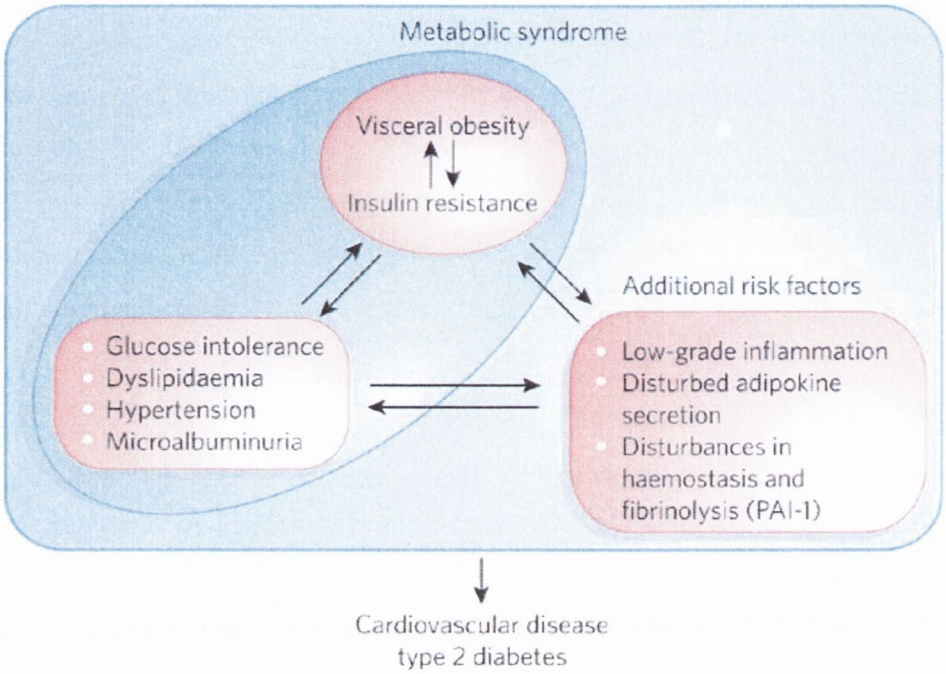
Introduction

1.1 The metabolic syndrome

Almost a century ago, one of the first studies was conducted to describe a positive relationship between non-communicable diseases (NCD) such as type 2 diabetes mellitus (T2DM) and hypertension and their link to obesity (Maranon, 1922). This was followed some years later when the initial proposal for a constellation of metabolic irregularities came from a French study that investigated the factors determining the predisposition to T2DM (Vague, 1956). Subsequently, a study investigating the importance of body fat distribution concluded that the sites of fat predominance offer an important prognostic marker for glucose intolerance, hyperinsulinemia, and hypertriglyceridemia (Kissebah *et al*, 1982). More recently 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, hyperinsulinemia, dyslipidemia and hypertension (Reaven, 1988). This saw the birth of a condition coined Syndrome X, an unknown phenomenon of heart threatening abnormalities occurring in insulin resistant (IR) individuals, who interestingly, did not necessarily proceed to develop T2DM. Over the years several definitions and explanations of the syndrome have emerged with IR, dyslipidemia and hypertension being the fundamental basis of this syndrome. These different definitions resulted in various names to describe the same morbid state such as the Insulin Resistance Syndrome, Plurimetabolic Syndrome and Dysmetabolic Syndrome (Sarafidis & Nilsson, 2006; Reaven, 2004). Some years after the naming of Syndrome X, Björntrop (1993) presented strong evidence where he powerfully demonstrated that abdominal or central obesity is an integral characteristic of this disorder. It was recommended that this syndrome be titled the metabolic syndrome (MetS) due to its metabolic nature. At present, the MetS is the most frequent name used to describe this multi-component disorder thought to originate from an obese, IR state and can progress to develop T2DM (Assmann *et al*, 2007). However, there still remains no universally accepted definition for this disorder, which is merely due to current research remaining illusive on the whether IR is the core explanation for this detrimental disorder (Bosello & Zamboni, 2000).

More recently, it has been hypothesis that the MetS may also be characterised by the presence of several other metabolic instabilities or pro-inflammatory and pro-thrombotic state. This state may include elevated levels of C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and other inflammation mediators as illustrated in *Figure 1.1* (Novo *et al*, 2007; Gannage-Yared *et al*, 2006), endothelial dysfunction hypercoagulability and oxidative stress (Nieuwdorp *et al*, 2005; Hansel *et al*, 2004).

Figure 1.1 The inter-relationship between classical and novel components related to the causes and consequences of the metabolic syndrome.



1.1.1 Metabolic syndrome criteria according to the expert panels

The large variety of signs and symptoms associated with the MetS has made it difficult to define the clinical syndrome with precision (Bray *et al*, 2004). In 1999, researchers from a specialist group of the World Health Organisation (WHO) presented criteria for the MetS (Alberti & Zimmet, 1998; WHO, 1999). Their classification was based on an oral glucose tolerance test (OGTT) and did not account for IR in subjects with normal glucose tolerance (NGT). Nevertheless, this was thought to be the most sensitive method of defining the MetS and could be applicable to both diabetic and non-diabetic patients (Alberti *et al*, 1998). According to the WHO, impaired glucose tolerance (IGT) or IR is the central criteria for diagnosis of the MetS combined with two of the following conditions: obesity, dyslipidaemia, hypertension and microalbuminuria (Alberti *et al*, 2006). Subsequently, in 1999 the European Group for the study of Insulin Resistance (EGIR) presented their definition of the MetS. However, unlike the WHO definition, this was defined only for the diabetic population (Balkau & Charles, 1999). The ERIG definition relies on waist circumference as a measure of obesity and did not account for microalbuminuria, an independent risk factor for

cardiovascular disease (CVD). Again, in the ERIG definition IR is the hallmark to what they labelled the MetS.

A more recent clinical identification of the MetS was presented in the summary of the Third Report of the National Cholesterol Education Programme Adult Treatment Panel III (NCEP-ATPIII) in 2001. This provided a detailed approach to identify and diagnose the MetS, and it recognized the MetS as an independent risk factor for CVD and considered it as an indication for intensive lifestyle transformation (Alizadeh-Dehnavi *et al*, 2008). The NCEP proposed that IR was not central to their criterion of the MetS due to the invasive procedure required to routinely measuring insulin sensitivity (Jolliffe & Janssen, 2007). The NCEP-ATPIII definition concentrates primarily on laboratory analysis and requires examine from an OGTT. They recommended that great focus must be placed on large randomized controlled clinical trial (Grundy *et al*, 2004).

Finally, the International Diabetes Foundation (IDF) put forward the latest definition describing the MetS, which placed greater emphasis on central obesity defined as waist circumference rather than IR and accounts for sex and ethnic group specificities (Fulop *et al*, 2006). In a recent perspective review, Reaven (2006) summarised the similarities and differences of the WHO, NCEP-ATPIII and IDF definitions of the MetS, but categorically reported that the components clustered in the syndrome occur only in IR individuals. This is supported by results from animal studies (Shafrir, 1996; Zimmet *et al,* 1999), and from human metabolic studies (Hanley *et al*, 2003*a*; Karter *et al*, 1996; Howard *et al*, 1998). The IDF definition was found to have a tendency towards higher prevalence rates of the MetS (Athyros *et al*, 2005; Lawlor *et al*, 2006). However, a recent study reported 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 *et al*, 2007). This may be due to waist circumference as this criterion seems to be less specific compared with waist to hip ratio (WHR), which defines the risk more specifically and is also a better indicator of IR (Pavlić-Renar *et al*, 2007).

All definitions vary in their estimates of the prevalence of the MetS, although the WHO and the NCEP-ATPIII were shown to correlate strongly in identifying people at risk of CVD (Eckle *et al*, 2005). These classifications of diverse, genetically heterogeneous clinical criteria that define MetS give an indication of the complexity of this disorder, which involves the deregulation of multiple metabolic pathways (Moebus & Stang, 2007). *Table 1.1* contains the required criteria for diagnosis of the MetS according to each panel.

Table 1.1: Diagnostic criteria for the Metabolic Syndrome

	WHO	**ERIG**	**NCEP-ATPIII**	**IDF**
IR	Present	Present		
Hyperglycaemia (mmol/L)	IFG ≥ 6.1, IGT ≥ 7.8 or T2DM	IFG ≥ 6.1	IFG ≥ 6.1	IFG ≥ 5.6 or Rx
Hypertension (mmHG)	≥ 140/90	≥ 140/90	≥ 130/85	≥ 130/85 or Rx
TAG (mmol/L)	≥1.7	>2.0	≥1.7	≥1.7 or RX
HDL-c (mmol/L)	<0.9(Male),<1.0 (Female)	<1.0	<1.04 (Male), <1.29 (Female)	<1.03 (Male), <1.29 (Female) or Rx
Waist circumference or WHR	WHR >0.90 (Male),>0.85 (Female) BMI >30kg/m2	Waist (cm) ≥ 0.94 (Male), ≥ 0.80 (Female)	Waist (cm) >102 (Male), >88 (Female)	Waist (cm) ≥ 94 (Male), ≥ 80 (Female)
No of components for diagnosis	***IR, IFG or IGT*** Plus ≥ 2 others from central obesity, hypertension, dyslipidemia or microalbuminuria	***IR*** plus ≥ 2 others from central obesity, IFG, hypertension, dyslipidemia	**≥3 of the above components**	***Central Obesity*** plus ≥ 2 others from the criteria above. Waist circumference define for different ethnic groups

WHO= World Health Organisation; EGIR=European Group for the Study of Insulin Resistance; NCEP (ATPIII)=National Cholesterol Education Program (Adult Treatment Panel III); IDF=International Diabetes Feredation; IR= Insulin Resistance; IFG=Impaired Fasting Glucose; IGT=Impaired Glucose Tolerance; BMI=Body Mass Index; WHR=Waist:Hip ratio; TAG=Triglycerides; HDL-c=High Density Lipoprotein Cholesterol; Rx=Receiving Treatment.

1.1.2 Prevalence and consequences of the metabolic syndrome

Naturally, the prevalence of the MetS will depend on the definition applied and evidently with the lack of one standard classification this leads to anomalies when comparing results of similar studies (Meigs *et al*, 2003). Additionally, as there is no universal definition, this hinders the determination of the true global prevalence of the MetS. As reported previously, the definitions vary considerably and not all include IR as a core criterion. Nevertheless, even with varying definitions it is evident that the incidence of this disease pattern is rising at a startling pace for both genders and all ages of the global population (Ford *et al*, 2002). Since, 1996 the number of individuals diagnosed with T2DM in the UK has increased from 1.4 to 1.8 million in 2004 and it has been already suggested that 25% of the population show clear signs of the MetS (Tonkin *et al*, 2003). By 2010, approximately 31 million of the European population will require treatment for T2DM and its related complications (Buttriss & Nugent, 2005; Zimmet *et al*, 2001). In the USA, 47 million adults meet the NCEP-ATP III criteria for the MetS and approximately 2500 Americans die from CVD each day (Ford *et al*, 2002; Spinler, 2006). Globally, diabetes currently affects 190 million people, approximately 2% of the world's population and this figure is set to escalate to 324 million by 2025, unless effective treatment is enforced (Zimmet *et al*, 2005). Interestingly, it has been reported that 90% of diabetes can be attributed to T2DM (Zimmet *et al*, 2001). Non insulin dependent diabetes mellitus (NIDDM) or T2DM is a heterogeneous condition characterised by chronic hyperglycaemia caused by variable degrees of IR and β-cell dysfunction. It has been approximated that 40% of people with IGT progress to develop T2DM over a period of 5 – 10 years (Zimmet *et al*, 2001). T2DM is characterised on the basis of elevated fasting glucose and it generally presents itself in individuals over the age of 30 years and statistics show that nearly half of all people with T2DM are obese (Body Mass Index (BMI) $\geq$30 kg/m^2), and two-thirds are overweight (BMI $\geq$27 kg/m^2) (Lustman *et al*, 2000).

In western countries, the development of CVD as a leading cause of morbidity and mortality has been seen for decades and is associated with substantial costs and suffering. Epidemiological studies have shown that individuals with this syndrome have a 3-5 fold increase in cardiovascular mortality as well as risks of T2DM, premature death and atherosclerosis (Isomaa *et al*, 2001; Assmann *et al*, 2007). The grim reality is that this condition is having a detrimental impact on healthcare and social welfare costs across all countries and effective public health strategies must be enforced in order to tame this epidemic. Internationally, the high prevalence of CVD is associated with an immensely high

cost. Around 10% of National Health Service (NHS) spending per year is on treating T2DM complications and this is set to increase to 25% by 2040 (WHO, 2004). The total cost of obesity for European Union (EU) was estimated to be approximately €32,800 x 10^6 per year (Fry & Finlay, 2005). Moreover, in USA, the direct and indirect costs of CVD were estimated to be $403.1 billion in 2006 (Spinler, 2006). This constitutes an increase in worldwide prevalence from 4.0% of the population in 1995 to 5.4% by the year 2025 (King *et al*, 1998).

Many factors such as environment, genetic predisposition and the 'thrifty phenotype' have been suggested to be involved in the etiology of MetS, making it a polygenic, multi-factorial and complex disease (Isomaa, 2003; Roche *et al*, 2005). As the human genome is known to be relatively stable, environmental factors, such as diet and physical activity, both of which are variable become interesting to investigate. This may imply that diet, nutrition and one's lifestyle habits may play an integral role in the vast rise in the occurrence of T2DM (Roche, 2006; Weisberg *et al*, 2003).

1.2 Criterion for the metabolic syndrome

It is widely known that the prevalence of the MetS is increasing throughout the world and risks increase with age and are higher in certain ethnic groups such as Pima Indians (Royer *et al*, 2007; Leslie *et al*, 2007). The MetS is characterised by an ongoing positive energy balance (decrease in physical activity and increase in energy intake), cigarette smoking, alcohol intake and stress (Pietrobelli *et al*, 2008). Central distributed adiposity and obesity are associated with a development of T2DM (Autuna-Puente *et al*, 2007). However, increasing physical activity has been reported to be efficient in reducing the incidence of IR, T2DM and CVD (Knowler *et al*, 2002; Byberg *et al*, 2001*b*). Each component of the MetS is independently associated with an increased risk of CVD. However, all criteria of this syndrome are potentially modifiable with modifications to one's diet and lifestyle (Case *et al*, 2002).

1.2.1 Obesity and the metabolic syndrome

Obesity is defined as an increase in body weight beyond the limitations of skeletal and physical requirements, as a result of an excessive accumulation of fat in the body. Although obesity *per se* is not a required element for the diagnosis of the MetS, there is considerable evidence that visceral intra-abdominal fat increases with age and is closely linked to the development of IR (Moller & Kaufman, 2005). Obesity is determine as a ratio between weight (kg) and height square (m^2), referred to as BMI. Overweight is the borderline condition

between normal weight and obesity and is classified as a BMI 25-30kg/m^2, whereas obesity is defined as >30kg/m^2 (WHO, 2000). Interestingly, a report published in 2005 found that WHR and waist circumference are much better indicators of CVD risk than BMI (Yusuf *et al*, 2005). Waist circumference is an anthropometric index abdominal visceral adipose tissue (AT) and a strong predictor of the lipid and non-lipid abnormalities associated with the MetS (Case *et al*, 2002).

Obesity is associated with a state of chronic low-level inflammation and with increasing obesity, fat is not only deposited in adipose depots (subcutaneous fat stores) but also in smaller deposits within the abdominal cavity, surrounding the heart and lungs and in non-adipose sites such as skeletal muscle. Recent clinical research demonstrated that the distribution of body fat rather that the total amount of fat is related to obesity-linked disorders (Funahasti & Matsuzawa, 2007). Therefore, excess fat storage on the trunk is metabolically more damaging than the same amount of fat stored on the limbs. Central obesity/visceral/android or upper-body obesity are terms used to describe fat stored on the trunk. It has been known for many years that android obesity poses a higher risk for T2DM than gynoid-type obesity (Vague, 1956). Additionally, excess visceral AT became associated with a more detrimental profile than excess subcutaneous AT (Wajchenberg, 2000). This observation has led to the widespread use of waist circumference in epidemiological analyses as a surrogate measure of visceral AT (Lemieux *et al*, 2000; Gasteyger & Tremblay, 2002).

The pathophysiology behind the risk factor clustering is complex and unlikely to be caused by one single underlying factor. Yet, increased AT particularly abdominal obesity with subsequent IR may be the primary mechanisms to commence the development of the MetS (Bjorntorp, 1993; Moller & Kaufman, 2005). This may be due to an increase in the concentration of non-esterified fatty acids (NEFA) in the portal vein and subsequently increasing the secretion of very low density lipoproteins (VLDL) and decreasing the clearance of insulin by the liver as a result of the an obese state (Arner, 2002).

Currently, 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 the integration of social, behavioural, cultural, physiological, metabolic and genetic factors (Smiley, 2006). In a study conducted in 2002, 185 individuals were placed on a protein-sparing, very low-calorie, weight-loss diet. A maximum total intake of 600-800kcal per day (/d) for 4 weeks was consumed. At baseline, 125 (68%) of the volunteers had 3 out of the 5

defined MetS criteria, 23% had 4 characteristics and 12% had 5 components of the MetS. Results showed that after 4 weeks weight-loss that the average BMI declined from 40.7kg/m^2 +/-9.7 to 34.6kg/m^2 +/-7.8, blood pressure (BP), total cholesterol (TC), tricylglycerol (TAG) and glucose concentrations were all significantly reduced at post-intervention (Case *et al*, 2002). There is indeed an urgent requirement to devise a public health strategy for early identification of abdominal obesity and to adequately enhance behavioural transformation necessary for total and abdominal obesity reduction (Okosun *et al*, 2004).

1.2.2 Dyslipidemia and the metabolic syndrome

The most common lipid disorder in subjects with the MetS is raised TAG levels and lowered high density lipoprotein cholesterol (HDL-c) concentrations, while low density lipoproteins cholesterol (LDL-c) concentrations may be elevated or at normal levels. However, these subjects often have increased circulating LDL-c particles that are smaller and more dense as compared to LDL-c in healthier subjects that appear to be larger and more buoyant constituents (Festa *et al*, 1999). Such small, dense LDL-c particles are associated with increased risks for coronary heart disease (CHD) and arthrosclerosis. LDL particles enter the sub-endothelial space and are oxidized with subsequent uptake by macrophages, leading to their activation, thereby beginning the process of atherosclerotic plaque development. Each LDL and VLDL particle has one apolipoprotein B (ApoB) molecule and on average there are nine LDL particles for every circulating VLDL particle. In a Swedish study, ApoB and apolipoprotein A1 (ApoA1) and the ratio between ApoB/ApoA1 were highly predictive in the evaluation of cardiac risk. Interestingly, ApoB was a stronger predictor of risk than LDL-c (Walldius *et al*, 2001). An explanation might be that ApoB is present not only in LDL particles, but also in the atherogenic VLDL cholesterol subunits (Sacks *et al*, 2000). ApoA1 has been shown to modify the ability of HDL particles to remove cholesterol from cells (Jackson *et al*, 1975), therefore it may be the content of ApoA1 rather than the cholesterol in HDL particles that represents the atheroprotective capacity (Francis & Frohlich, 2001).

1.2.3 Hypertension and the metabolic syndrome

During the last decade several studies have shown that patients with hypertension are resistant to insulin-stimulated glucose uptake and are hyperinsulinemic compared to normotensive controls, independently of obesity (Pollare *et al*, 1990; Reaven, 1991). In addition, it has been shown that the prevalence of hypertriglyceridemia and low levels of HDL-c is increased in

hypertensive subjects (Pollare *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 *et al,* 1996). Consistent with this, insulin does exert an anti-natriuretic effect and increased total body exchangeable sodium is found in T2DM (Reaven, 1988; Rocchini, 1991). Moreover, hyperinsulinaemia during euglycemic clamp studies acutely stimulates both norepinephrine release and increases in directly measured sympathetic neural activity (Anderson *et al*, 1991; Anderson *et al*, 1992). Nonetheless, an accumulating body of evidence suggests that despite this potential for hyperinsulinemia to increase peripheral vascular resistance, insulin exerts a direct vasodilatory role. Accordingly, vascular smooth muscle manifestations of IR, rather than hyperinsulinaemia, may result in hypertension. However, not every patient with hypertension is IR (Reaven *et al*, 1996). IR has also been found in normotensive first-degree relatives of hypertensive patients, which might imply a genetic predisposition to IR in subjects prone to a hypertension.

1.3 Insulin synthesis and secretion

Insulin is a pleiotropic or anabolic hormone that affects a range of biological functions depending on its target organ or tissue such as muscle, AT and inflammatory cells (Pessin & Saltiel, 2000). Most of the carbohydrates in food are rapidly converted to glucose, the primary sugar in blood. Insulin is produced by β-cells of the pancreas in response to rising levels of plasma glucose levels which occur in the postprandial state. Insulin makes it possible for most body tissues to remove glucose from the blood for its use as fuel and for the conversion of glucose to glycogen for storage in liver and muscle cells. Insulin directs energy metabolism during the postprandial phase and in doing so is the only hormone committed to the prevention of hyperglycaemia (Bailey, 1999). 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 of peripheral tissues (Saltiel & Pessin, 2002).

Lowered insulin levels result in the reverse conversion of glycogen to glucose when glucose levels fall, though only in the liver not in muscle tissue. Whereas, higher insulin levels may increase many anabolic processes such as cell growth, cellular protein synthesis, and fat storage (Gonzalez & Serrano, 2007). It is well accepted that metabolism of glucose through pyruvate to acetly-CoA with subsequent mitrochondrial oxidative increases the adenosine triphosphate /adenosine diphosphate (ATP/ADP) ratio, which results in the closure of ATP-sensitive K^+ channels, depolarisaion of plasma membranes, opening of voltage-dependent Ca^{2+} channels and Ca^{2+} triggering of insulin granule exocytosis (Prentki, 1996). Firstly, this occurs through glucose stimulating insulin release by the pancreatic β-cells through ATP-sensitive K^+ channels, dependent and independent pathways, which result in a biphase insulin release in response to a square glucose load (Komatsu *et al*, 2002). Weyer *et al* (1999) demonstrated a secretory failure of insulin when comparing people with NGT to those with IGT and T2DM. The experimental design as shown in *Figure 1.2* initially measures the first phase insulin response (FPIR) also known as the acute insulin response to glucose (AIRg), which is a small but rapid spike of insulin secretion that occurs within minutes after a glucose challenge. 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 *et al*, 1994). Similarly, a study in Caucasian individuals ~60years of age showed impaired first-phase insulin secretion after intravenous glucose in IGT (Davies *et al*, 1993). Therefore, these results show that FPIR may be linked with IR and the development of T2DM.

The FPIR is triggered by glucose via ATP-sensitivity K^+ channel-dependent ionic events, elevation of sub-membrane free Ca^{2+} concentration. This early insulin production then declines to baseline levels and is soon followed by a second phase of insulin secretion that is sustained during hyperglycemia. In the second phase insulin release, the mechanism above is augmented by ATP sensitive K^+ channel-independent, non-ionic signals (Komatsu *et al*, 2002). The magnitude of the second phase is determined by the elevation of Ca^{2+} (Henquin *et al*, 2002; Bratanova-Tochkova *et al*, 2002). Defects in the second phase insulin release have not been widely considered as phenomena in the development of T2DM. However, it may be more important in genetically predisposed subjects than previously recognized (Gerich, 2002).

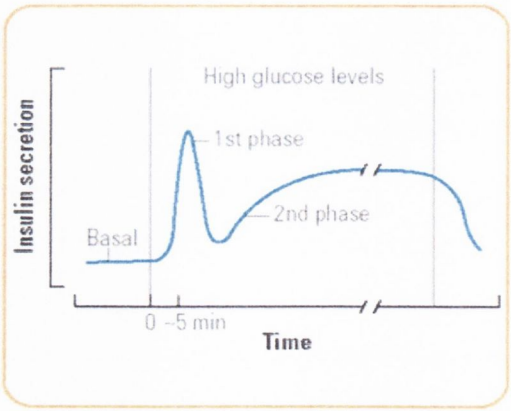


Figure 1.2: The diphasic pattern of glucose stimulated insulin secretion from the pancreatic ß-cell (Persaud & Howell, 2003).

Individuals with T2DM have essentially no acute insulin response to a glucose challenge. Thus, it is not surprising that postprandial glucose levels can increase to between 200mg/dL and 400mg/dL in individuals with T2DM (Spellman, 2007). A specific alteration of FPIR after a glucose challenge is an early and progressive defect of β-cell failure and their crucial physiological role in postprandial glucose homeostatis. The effects of FPIR release is primarily achieved in the liver, allowing a prompt inhibition of postprandial hepatic glucose production (del Prato *et al*, 2002). The likely mechanisms of early β-cell demise includes oxidative stress, endoplasmic reticulum stress, increased TAG and NEFA and glucolipotoxicity (Pretki & Nolan, 2006).

The dynamic regulation of insulin secretion is very complex. There is a time dependent inhibition of insulin release after short glucose stimulation, which makes the β-cells refractious (Nescher & Cerasi, 2002). Furthermore, there is a 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 *et al*, 1997).

Insulin release is pulsatile *in vivo*, in periods of 4-13 mins, 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 *et al*, 1979). This effect may be explained by the dose-response curve of the insulin receptor and of prevention of receptor desensitisation (Kennedy *et al*, 2002). If the amount of insulin produced is insufficient, if cells respond poorly to the effects of insulin or if the insulin itself is defective, plasma glucose

clearance is not as efficient from body cell and this may result in insulin insensitivity or IR. Moreover, loss of regular pulsatile insulin release is an early symptom of T2DM (Polonsky *et al*, 1988).

1.3.1 Insulin Resistance and the metabolic syndrome

The MetS represents a heterogeneous group of metabolic disorders and IR appears to be a frequent antecedent behind these aberrations according to the WHO and ERIG definitions (Laaksonen *et al*, 2005). In this regard, clinical studies have been important in highlighting the pathogenesis of T2DM. Studies show that IR precedes the development of hyperglycaemia in subjects that eventually develop T2DM (Giorgino *et al*, 2005). IR can be defined as a state in which normal levels of insulin produce a subnormal biological response (Berson & Yallow, 1970). The tissues of the body have a reduced sensitivity to the actions of insulin and therefore greater than normal amounts of insulin are required to elicit a quantitatively normal response. As a result, hyperinsulinemia develops, which can be accompanied by either normoglycaemia or hyperglycaemia, the β-cell function eventually subsides and T2DM develops (Prentki & Nolan, 2006). Thus, IR is an early precursor of DM, with sub-clinical dysregulation of glucose metabolism. It is a central feature in the natural history of T2DM and is emerging as a possibly important metabolic disorder in the aetiology of a number of other highly prevalent chronic diseases, including CHD (Hanley *et al*, 2003*b*). The molecular basis of IR is quite complex and appears to be multifactorial. Insulin action is mediated by signal transduction over the insulin receptor. The insulin receptor belongs to the superfamily of receptor tyrosine kinases 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 (de Luca & Olefsky, 2008). The signalling cascade branches into two main pathways. The first is the phosphatidlylinositol-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 *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 *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 *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 *et al*, 2000; Sun *et al*, 2002). Glucose enters the cell by facilitated diffusion mediated by a group of structurally related glucose transporter (GLUT) proteins. To date, at least 12 GLUT's have been described (Joost *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 *et al*, 1989). In T2DM, defects in the GLUT4 and glycogen synthesis in skeletal muscle have been identified (Vestergaard *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 *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 *et al*, 1995).

1.4 *In vivo* measures of insulin sensitivity

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 euglycemic-hyperinsulinemic glucose clamp is considered the reference method for evaluating insulin sensitivity and the intravenous glucose tolerance test (IVGTT) with MINMOD modelling assessment is an alternative reference method (DeFronzo *et al*, 1979; Monzillo & Hamdy, 2003). However, both tests are difficult to accomplish, time-consuming and expensive and they have been criticised as they assess insulin sensitivity under non-physiological conditions

(Breda *et al*, 2001). 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 hyperglycemia is present. Among these simple indexes are the homeostasis model assessment-inulin resistance ($HOMA_{IR}$), the reciprocal of $HOMA_{IR}$ and the quantitative insulin sensitivity check index (QUICKI) as described below.

1.4.1 Fasting insulin

It still remains illusive as to whether hyperinsulinemia *per se* contributes to the MetS or is a physiological consequence of diminished insulin-mediated glucose uptake in skeletal muscle and hence, is only a marker of IR (Lerman *et al*, 2003). However, as a result fasting insulin is frequently used as a surrogate marker of IR in clinical studies (Mannucci *et al*, 2003). Because direct insulin sensitivity measurement by euglycemic clamp or minimal model 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 *et al*, 1990; Skarfors *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* (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 *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 insulinemia (Tillil *et al*, 1988). C-peptide is formed when proinsulin, the presuror 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 type 1 diabetes mellitus (T1DM) (Walenciak *et al*, 2007).

1.4.2 Homeostatis Model Assessment – Insulin Resistance ($HOMA_{IR}$)

$HOMA_{IR}$ is a model of interactions between glucose and insulin dynamics that is used to predict fasting steady-state glucose, insulin to c-peptide concentrations and for a wide range of possible combinations of IR and β-cell function (Matthews *et al*, 1985; Wallace *et al*, 2004). $HOMA_{IR}$ is calculated as [fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml)] divided by 22.5 (Matthews *et al*, 1985). The denominator of 22.5 is a normalizing factor. The product of normal fasting plasma insulin is 5μU/mL multiplied by the typical plasma glucose of a healthy individual which is 4.5mmol/L equal 22.5. Therefore, for an individual with normal insulin sensitivity $HOMA_{IR}$ equals 1 or low $HOMA_{IR}$ values indicate high S_I, whereas high $HOMA_{IR}$ values indicate low S_I or IR (Ura *et al*, 2006).

Bonora *et al* (2002) concluded that $HOMA_{IR}$ 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. The coefficience of variance (CV) for HOMA varies considerably depending on the number of fasting samples obtained and the type of insulin assay used (Bonora *et al*, 2002; Matthews *et al*, 1985). A study was performed to determine the association between $HOMA_{IR}$ and glucose clamp IR in 80 subjects with T2DM either treated with sulponylureas medication or dietary therapy alone. Results from the study showed that the $HOMA_{IR}$ strongly correlated with the glucose clamp in both treated groups (Emoto *et al*, 1999). $HOMA_{IR}$ has been validated against and compares favourably with a variety of physiological methods and has the advantage of requiring only a single plasma sample assayed for insulin and glucose. $HOMA_{IR}$ model has become a widely used clinical and epidemiological tool and when used appropriately can yield valuable data. Yet, as with all models, the primary input data need to be robust and the data need to be interpreted carefully (Wallace *et al*, 2004).

1.4.3 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 *et al*, 2000). QUICKI is calculated as 1/[log(fasting insulin) + log (fasting glucose)]. Previously, it has been reported to be a reliable, reproducible and accurate index of insulin sensitivity with excellent positive predictive power (Cho *et al*, 2001; Chen *et al*, 2003; Chen *et al*, 2005*b*; Hanley *et al*, 2003*b*). However, QUICKI is less correlated to the glucose clamp in non-obese, non-diabetic control subjects

compared to obese, T2DM patients (Katz *et al*, 2000). The traditional mathematical QUICKI calculation underwent revision to incorporate plasma free fatty acids (FFA) in particular, NEFA. The revised QUICKI mathematical formula is [1/(log $insulin_0$ + log $glucose_0$ + log $NEFA_0$)] (Roche, 2004). Perseghin *et al* (2001) aimed to ascertain whether 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 indexes that offer correlations similar to or higher than the $HOMA_{IR}$ method (Rabasa-Lhoret *et al*, 2007). Contrary to this, significant increasing in insulin sensitivity induced by weight-loss was detected by QUICKI, but not by revised QUICKI (Bastard *et al*, 2002*a*). Although, another study found that the revised QUICKI demonstrated a statistically strong relationship with the IVGTT model, it is however, differentiate between insulin-sensitive and resistance groups in the study (Brady *et al*, 2004*a*). QUICKI and $HOMA_{IR}$ were derived in a completely different conceptual fashion. Nevertheless, these two surrogate markers are mathematically related, wherein, QUICKI is very comparable to the log[$HOMA_{IR}$] and QUICKI is directly proportional to the 1/ log[$HOMA_{IR}$].

QUICKI is among the most thoroughly evaluated and validated surrogate index of insulin sensitivity. As a simple, useful, inexpensive and minimally invasive surrogate for glucose clamp derived measures of insulin sensitivity, QUICKI is appropriate and effective for use in large epidemiological and/or clinical research studies (Muniyappa & Quon, 2007).

1.4.4 Hyperinsulineamic euglycemic glucose clamp

The hyperinsulineamic euglycemic clamp method is a widely accepted reference method for quantifying insulin sensitivity in humans because it directly measures effects of insulin to promote glucose utilisation under steady-state conditions *in vivo* (DeFronzo *et al*, 1979). The validity of this glucose clamp measurements of insulin sensitivity depends on achieving steady-state conditions, it is assumed that the 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 mins (at least 2 hour (hr) after initiation of insulin infusion) during which CV for blood glucose, plasma insulin and glucose infusion rate are less that 5% is a more rigorous approach that may determined more values for glucose disposal rate and insulin sensitivity

(Chen *et al*, 2003; Katz *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 as a dynamic test such as an oral meal or oral glucose load may determine (Mauiyappa & Quon, 2007).

Although the hyperinsulineamic euglycemic clamp is considered the '*gold-standard*' for technique for measuring insulin sensitivity the main limitations with this procedure, whereby 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.

1.4.5 Intravenous Glucose Tolerance Test (IVGTT)

The minimal model IVGTT provides an indirect measure of metabolic insulin sensitivity/resistance based on glucose and insulin data (Bergman *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 & Bergman, 1986). Beard *et al* (1986) proposed a modified protocol which requires an infusion of exogenous insulin or tolbutamide, 20 mins after the infusion of glucose at baseline (Saad *et al*, 1997; Yang *et al*, 1987). This is to ensure an adequate endogenous plasma insulin response which is critical for determining insulin sensitivity (S_I) using minimal MINMOD technique. The MINMOD Millennium® is a soft-ware package that computes estimates of S_I, glucose effectiveness (S_G) and β-cell function for each individual profile by curve fitting techniques (Bergman *et al*, 1979; Pacini & Bergman, 1986; Boston *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 equation 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 IVGTT. S_I is calculated from two of these model parameters and is defined as fractional glucose disappearance per insulin concentrations unit.

Unlike the hyperinsulineamic euglycemic clamp method which depends on steady-state conditions, the IVGTT technique used dynamic data. Valid minimal model analysis of the modified IVGTT 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 IVGTT 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 IVGTT is above a certain threshold (Yang *et al*, 1987).

However, the IVGTT method is more advantageous compared to the glucose clamp as it is slightly less labour intensive, steady-state conditions are not obligatory and there are no intravenous infusions that require constant adjustments. Furthermore, information about S_I, S_G and β-cell function can be obtained from a single dynamic test. The IVGTT model has been reported to generate excellent predictions of glucose disappearance and the CV for S_I is comparable to glucose clamp estimates insulin sensitivity (Ferrari *et al*, 1991; Steil *et al*, 1994).

1.5 Inflammation and the inflammatory response

Survival of multicellular organisms depends on the ability to fight infection, heal damage and the ability to store energy for times of low nutrient availability or high energy needs. Cells are regularly exposed to stress, which mainly consists of inflammatory stress and/or metabolic stress. The immune system offers two lines of defence; the innate and the adaptive (acquired) immune system, and both are thought to be profoundly involved in the development of diabetes mellitus. T1DM is more frequently associated with the classical adaptive (acquired) immunity due to the complete autoimmune destruction of the islet cells. Adaptive immunity is the response of antigen-specific T and B lymphocytes to antigens, including the development of immunological memory. On the other hand, T2DM is more associated with innate immunity. The innate immune system is the body's first line of defence against microbial, chemical, physical and psychological injury, whereby various reactions repair damage, avoid or isolate threats and restore homeostasis. The systemic component of innate immunity or systemic inflammation is called the acute-phase response (APR). Here, sentinel trouble-shooting cells throughout the body, such as macrophages, adipocytes and endothelial cells (EC), detect environmental threats and release pro-inflammatory cytokines, such as interleukin

(IL)-6 and tumour necrosis factor (TNFα) as depicted in *Figure 1.3*. Generally, cytokines stimulate the production of APR from the liver, such as CRP, serum amyloid A, 1-acid glycoprotein, complement and fibrinogen. Acute-phase proteins are a diverse group of proteins whose plasma concentration increases or decreases in response to injury and inflammation and have therefore come to be known also as inflammatory markers. Many APR's are known to be increased in T2DM and show a graded increase with increasing features of the MetS.

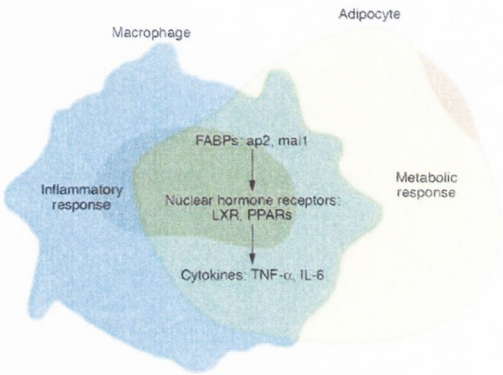


Figure 1.3: Lipids and inflammatory mediators: integration of metabolic and immune responses in adipocytes and macrophages through shared mechanisms (Wellen & Hotamisligil, 2005). **aP2** (adipocyte FABP); **FABP** (Fatty acid binding proteins); **Mal1** (FABP5); **IL-6** (Interleukin-6); **LXR** (liver X receptor); **PPARγ** (Peroxisone proliferator activated receptor γ); **TNFα** (Tumour Necrosis Factor α).

Inflammatory stress is exerted by adipokines or cytokines that are released in large quantities by immune cells in response to invading micro-organisms. Expression of cell adhesion molecules (CAMs) by EC (intercellular adhesion molecule; ICAM and vascular adhesion molecule (VCAM)) draws circulating monocytes to the arterial site of injury. In addition, a gradient of monocyte chemoattractant such as monocyte chemoattractant protein-1 (MCP-1) and TNFα are expressed by ECs, smooth muscle cells and already resident monocytes/macrophages. Monocyte to macrophages differentiation is complex and involves finely tuned alterations in gene expression regulated by several transcription factors (Valledor *et al,* 1998). The main function of macrophages is to phagocytose and destroy particulate material and to present antigens to lymphcytes. The expression of inflammatory mediators such as interleukin (IL)-6 is increased in adipose tissue macrophages in obesity (Lumeng *et al,*

2007). Macrophages of obese mice show an increase in oxidative stress (Li *et al*, 2006; Lee *et al*, 1999). In addition, monocytes from diabetic subjects exhibit enhanced adherence to the endothelium, suggesting an increased ability of tissue infiltration under diabetic conditions (Bouma *et al*, 2004). These abnormalities therefore may contribute to the development of systemic inflammation and IR associated with obesity and T2DM. Monocyte-macrophages isolated from diabetic subjects and animal models have shown decreased surface expression and tyrosine kinase activity of IR (Liang *et al*, 2004; Accili *et al*, 1996) and diminished insulin-stimulated signaling of insulin receptor substrate-2 (IRS2), PI3K-AKT (Liang *et al*, 2004. Thus macrophages in IR and T2DM may be resistant to circulating insulin at the cellular level.

1.6 Adipose tissue biology

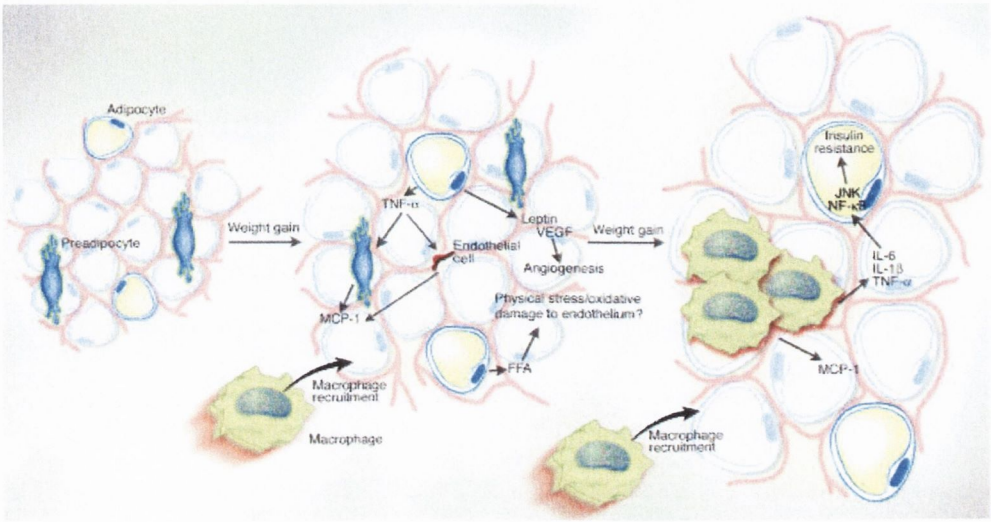
AT is a loose connective tissue that consists of a mass of adipocytes or fat cell loosely held together by collagen fibres (Trayhurn, 2007). In normal, lean individuals AT makes up a substantial proportion of total tissue mass. Typical fat mass for a male is between 9-18% body weight, and for a female between 14-28% body weight (Hausman *et al*, 2001). Recent research has noted that an adult male with a BMI=22, approximately 20% of total tissue is white fat, whereas in an obese individual BMI=30, up to one half of total body tissue mass may be due to white fat (Trayhurn & Bing, 2006). AT can be classified in several ways, white adipose tissue (WAT) or brown adipose tissue (BAT), as subcutaneous fat or visceral fat, as android fat or gynoid fat, and as hypertrophic fat or hyperplastic fat. This thesis will focus on WAT, which is white or yellow in colour and contains few nerves and blood vessels. At a histological level, WAT appears remarkably simple in structure, there appears to be little other that a mature adipocytes, filled with a large uniocular droplet of TAG that is coated by the perilipin phospholipoproteins (Granneman & Moore, 2008). Despite superficial appearances, there is in practice considerable cellular heterogeneity, with mature adipoytes constituting no more than 50% of the total cell content of the tissue (Hausman, 1985). This apparent simplicity of the WAT is partly why it is only recently that the complexity of the tissue has become recognised (Trayhurn & Bing, 2006). Although the distinctive feature of WAT is the adipocytes comprising 30-60 % of AT, several other cell types including pericytes, preadipocytes white and brown adipocytes, fibroblasts, mast cells, lymphocytes, vascular and dendritic cells and macrophages (Haugen & Drevon, 2007). Enlargement of adiocytes is frequently observed in obesity and has been demonstrated in pre-diabetic individuals and

T2DM (Weyer *et al*, 2001*b*). This increased adipocyte size represents a failure in the recruitment of new adipocytes due to impaired differentiation, which appears to be a precipitating factor in the development of T2DM (Goossen, 2007). Interestingly, some macrophages found in the AT of obese rodents were large and multinucleated (Sartipy & Loskutoff, 2003; Weisberg *et al*, 2003). Such multinucleated giant cells are often found at sites of chronic inflammation and result from the infusion or engulfment of macrophages by each other, and are thought to aggregate at sites of adipocyte necrosis (Kolonin *et al*, 2004). Under normal conditions, adipocytes store lipids and regulate metabolic homeostasis, and macrophages function in the inflammatory response, although each cell type has the capacity to perform both functions. In obesity, AT becomes inflamed, both via infiltration of AT by macrophages and as a result of adipocytes themselves becoming producers of inflammatory cytokines as illustrated in *Figure 1.4*.

Adipocytes and macrophages share common features such as expression of cytokines, fatty acid binding protein (FABP), nuclear hormone receptors, and many other factors. As evidenced by genetic loss-of-function models, adipocyte/macrophage FABPs modulate both lipid accumulation in adipocytes and cholesterol accumulation in macrophages, as well as the development of IR and atherosclerosis. Peroxisone proliferator activated receptor gamma (PPARγ) and liver X receptor (LXR) pathways oppose inflammation and promote cholesterol efflux from macrophages and lipid storage in adipocytes.

In obesity, adipocytes secrete low levels of TNFα, which stimulates preadipocytes to produce monocyte chemoattractant protein-1 (MCP-1) (Xu *et al*, 2003). Similarly, EC also secrete MCP-1 in response to cytokines. Thus, either preadipocytes or EC could be responsible for attracting macrophages to WAT. In addition, increased secretion of leptin and/or decreased production of adiponectin by adipocytes may also contribute to macrophage accumulation by stimulating transport of macrophages to WAT and promoting adhesion of macrophages to EC as shown in *Figure 1.4* (Sierra-Honigmann *et al*, 1998; Maeda *et al*, 2002).

Figure 1.4: Obesity is a pro-inflammatory state & macrophage infiltration in adipose tissue (Wellen & Hotamisligil, 2003).



FFA (Free Fatty Acids), **IL-1β, 6** (Interleukin 1β, 6), **JNK** (Jun Kinase), **MCP-1** (Monocyte Chemoattractant Protein-1), NF-κB (Nuclear factor-κB), **TNFα** (Tumour Necrosis Factorα); **VEGF** (Vascular Endotelial Growth Factor).

1.7 The role of adipose tissue in the pathogenesis of insulin resistance

1.7.1 Non-esterified fatty acids

There are at least two hypotheses proposed to explain how AT may influence glucose homeostatis and act as a key aetiological factor in the development of IR and the MetS. Firstly, expanded AT leads of an increased in NEFA to the peripheral tissues (Kahn & Flier, 2000). NEFA are FFA that are found in circulation and account for about 10% of the total blood FAs, typically 0.5-2.0 μmmol/L (Leaf, 2000). IR is a condition in which defects in the action of insulin are such that normal levels of insulin do not trigger the signal for glucose absorption. Elevated plasma NEFA concentrations are implicated as an important link between IR and the lipid profile reported in the MetS (Jellinger, 2007).

Insulin normally suppresses the activity of hormone-sensitive lipase, the enzyme that hydrolyses intracellular TAG in the AT, leading to sustained release of FAs into the circulation and raised circulating NEFA levels. Hormone-sensitive lipase is elevated under fasted conditions and reduced under fed conditions as a result of inhibition by insulin. However, in IR subjects hormone-sensitive lipase activity is not fully suppressed in the fed state and NEFA levels are constantly elevated (Shaw *et al*, 2005). An expanded AT mass, as

seen in the obese state, delivers more NEFA to the systemic circulation which results in IR in human skeletal muscle by interfering with the effect of insulin in increasing GLUT4 mediated glucose transport across the plasma membrane (Cline *et al*, 1991). The molecular mechanism for this interference in insulin action appears to be the consequence of a reduction in IRS-1 expression (Dresner *et al*, 1999). This increases blood glucose concentration and provides the stimulus for increased insulin secretion and hyperinsulinaemia develops which is a key feature of the MetS (Karpe & Tan, 2005).

Elevated NEFAs have various deleterious effects, including stimulation of hepatic TAG and VLDL synthesis and competitive inhibition of peripheral tissue uptake of glucose that further exacerbate IR related glucose intolerance (Lebovitz & Banerji, 2001). Longitudinal data from humans indicate that elevated NEFA precedes a demonstrated β-cell defect (Bergman & Alder, 2000). Whilst, in the liver, increased NEFAs impair the ability of insulin to suppress hepatic glucose output (Kahn & Flier, 2000). Both NEFA and TNFα have been shown to cause peripheral IR. Despite the putative importance of NEFAs in the MetS, few studies have been performed to investigate the effects of fish oil supplements on NEFA production in the MetS (Brady *et al*, 2004*b*).

1.7.2 Secretory capacity of white adipose tissue

The second hypothesis proposed to explain how AT may influence glucose homeostasis is its secretory ability. AT is now regarded as the largest endocrine organ in most humans and above all in obese individuals. With increased body weight, AT undergoes molecular and cellular alterations affecting systemic metabolism, the number of adipocytes increases and enlarges in size and results in the release of fasting FFA and glycerol into the bloodstream, control mechanisms become dysregulated, macrophages accumulate in the AT and inflammation ensues (Greenberg & Obin, 2006).

IR is an important contributor to the pathogenesis of obesity and T2DM, partly due to AT secreting several proteins called adipokines that may influence insulin sensitivity. Therefore, it is now evident that WAT is a secretory organ with potent autocrine, paracrine and endocrine functions, allowing it to maintain a complex communication with many other organs, far beyond what its role in fuel storage would imply (Trayhurn, 2007; Hauner, 2007). The key features of adipokines include lipid storage and a rapid response to insulin stimulation that involves activation of a number of signalling pathways leading to diverse metabolic

changes (Morganstein *et al*, 2007). Recent evidence has also shown that adipokines are an important source of inflammatory cytokines (Xu *et al*, 2003). Cytokines have been described as a vast array of relatively low molecular weight, pharmacologically active proteins that are secreted by a single cell for the purpose of altering their own function (autocrine effect) or those of adjacent cells (paracrine effect) (Coppack, 2001). Yet, the secretory products also exert systemic effects, suggesting a possible link between WAT and IR (Hauner, 2007). Several *in vitro* studies have shown that the AT becomes inflamed in obesity as a consequence of an increased infiltration of inflammatory cells (Permana *et al*, 2006; Yamashita *et al*, 2007; Rotter *et al*, 2003). This is due to the increased abundance of macrophages in AT, which increases the expression of pro-inflammatory cytokines such as IL-6, CRP and TNFα as illustrated in *Figure 1.5*, that interfere with adipocyte function and reduce insulin responsiveness in adipocytes. Hammarstedt *et al* (2005) reported a clear association between IR, T2DM and AT dysfunction as several molecules for insulin signalling and action, such as IRS-1 and GLUT4, are reduced in IR adipose cells with or without T2DM. Adiposity contributes to a pro-inflammatory milieu and a reduction of fat mass correlates with a decrease in serum levels of many adipokines, therefore fat loss approaches are useful in attenuating the pro-inflammatory status associated with obesity (Lyon *et al*, 2003). The WAT used to be regarded as simply an energy store which also provided protection to internal organs. However, in recent years, this concept has been turned on its head and it is now recognized that WAT is highly dynamic, involved in a diverse range of physiological and metabolic processes (Trayhurn & Wood, 2004).

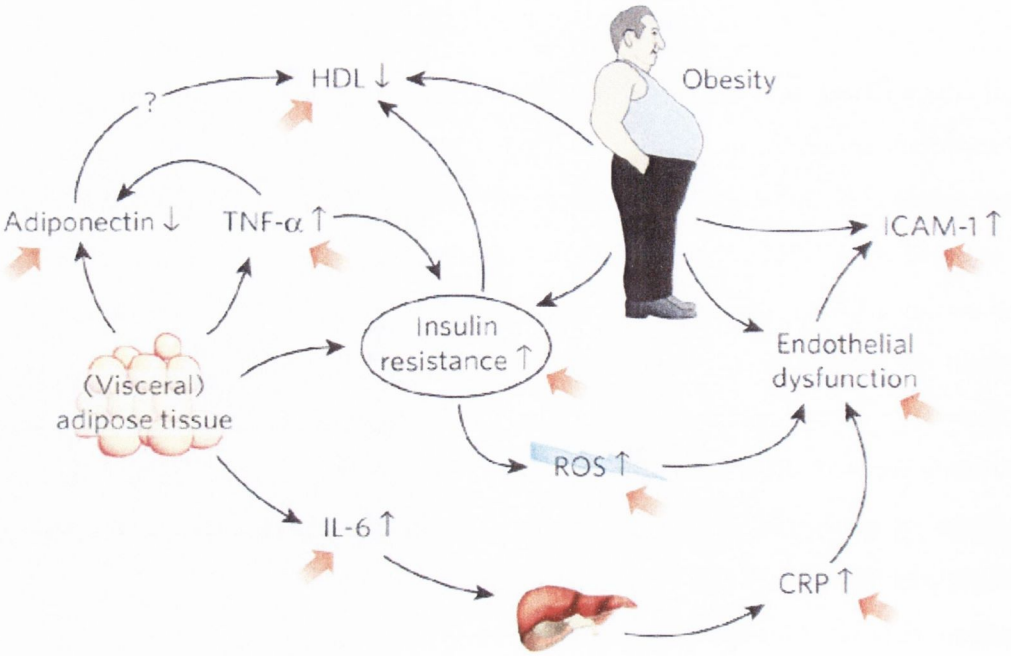


Figure 1.5: Schematic representation of how obesity might add to several mechanisms linking insulin resistance to cardiovascular disease. **CRP**: C-reactive protein; **HDL**: High density lipoproteins, **IL-6**: Interleukin-6; **TNFα**: Tumour Necrosis Factor-α, **ICAM**: Intercellular Adhesion Molecule, **ROS**: Reactive Oxygen Species.

1.7.3 Adipocyte biology

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 *et al*, 2003). In addition to their storage function, adipocytes have been shown to be dynamic endocrine cells that produce and secrete various biological molecules known as adipokines or adipocytokines (Kanda *et al*, 2006). Cytokines for short, comprise a variety of proteins with signaling properties produced in body fat. They are humoral immunoregulatory protein/glycoproteins, which have the ability to regulate local and/or systemic inflammation or are small soluble polypeptides that function as intercellular signal mediators acting in an autocrine, paracrine or even endocrine manner (Sano *et al*, 2000). They differ from classical hormones in that they are produced by a number of tissue or cell types rather than by specialized glands. Their molecular weight can range from 15kDa to 30kDa. At concentrations as low as 10^{-10} to 10^{-15} mmol/L these molecules have the ability to bind to cytokine specific receptors and induce downstream signalling events (Schindler *et al*, 2002).

To date, over 50 cytokines have already been identified, with diverse structures and can be classified according to their functional role; appetite and energy balance, insulin

sensitivity, immunity, angiogenesis, blood pressure, lipid metabolism and homeostasis (Lago *et al*, 2007*a*). 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 & Wood, 2004). Leptin signals the status of energy stores and its secretion can reduce appetite and increase energy expenditure.

Cytokines act as communicators between other cells of the immune system as well as non-lymphoid tissues. Cytokines such as TNFα and IL-6 are classified as pro-inflammatory and can induce the hepatic APR, which consists of local and systemic reactions and is accompanied by upregulated and downregulated synthesis and/or activation of liver-enriched transcription factors (Rai *et al*, 1998). The APR is critical to promote clearance of damaged muscle tissue, as well as to induce muscle repair and growth, it includes the activation of the complement system, neutrophils and monocytes. The magnitude of the APR is dependent on muscle-cell distribution. Within the tissues, monocytes undergo morphological and functional differentiation, inducing macrophage formation. Excessive cytokine production has the potential for tissue damage, hence cytokine production is strictly controlled and the cytokine profile of healthy populations and individuals mounting an immune response will be different (Kelly *et al*, 2001). Macrophage infiltration into the muscle peaks 24-48h after muscle damage, subsequently producing cytokines such as TNFα (Mukhopadhyay *et al*, 2006). There appears to be a hierarchy of cytokines, such as TNFα, which orchestrates the synthesis, secretion and activity of other cytokines (Coppack, 2001).

1.8 Adipocytokines

1.8.1 Structure and function of Tumour Necrosis Factor-α

In 1975, the first molecular link between inflammation and obesity was described by Carswell *et al* (1975). Thus, this saw the discovery of TNFα, a 185 amino acid glycoprotein peptide, expressed as a 26-kDa cell surface transmembrane protein and cleavage by TNFα converting enzyme generates a 17-kDa soluble form of TNFα (sTNFα) (Moller, 2000). Both TNFα and sTNFα can affect both biological and metabolic responses (Perez *et al*, 1990; Xu *et al*, 1999). However, TNFα may mediate paracrine and autocrine signals, leaving sTNFα to mediate endocrine effects (Grell, 1995). TNF was originally described as a circulating factor that can cause necrosis of tumours, but has since been identified as a key regulator of the inflammatory

response (Bradley, 2008). It is now well established that TNFα is a pro-inflammatory cytokine with dual roles in metabolism and immune function (Cawthron & Sethi, 2008).

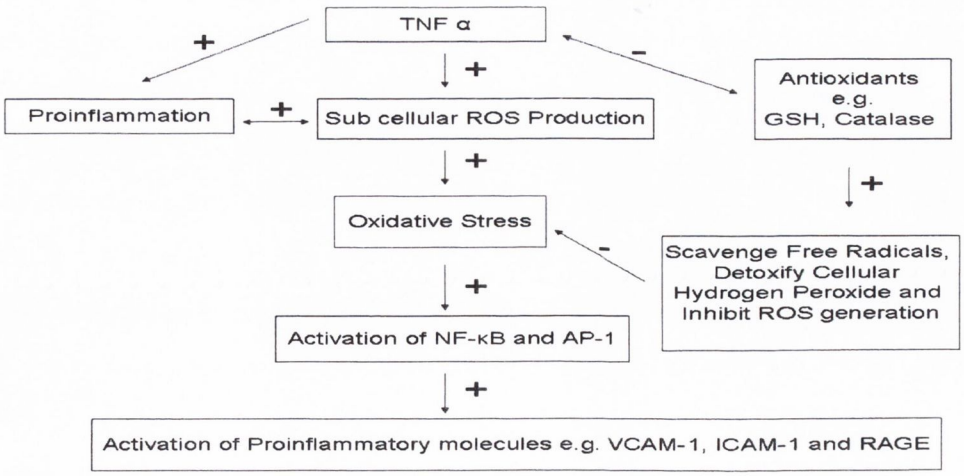
TNFα exerts its effects by binding to two different cell-surface receptors that have been identified, Tumour Necrosis Factor receptor 1 (TNFR-1) and Tumour Necrosis Factor receptor 1 (TNFR-2), which are present on virtually all cells except for erythrocytes and these can initiate both distinct and overlapping signal transduction pathways. In humans these are known as gp/60/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 TNFR1 and TNFR2 from the extracellular domain (Loetscher *et al*, 1990; Steppan *et al*, 2001) and thus releasing the soluble fraction of these receptors, sTNFR1 and sTNFR2 (Reddy *et al*, 2000). Hotamisligil *et al* (1997) found circulating levels and messenger ribonucleic acid (mRNA) levels of sTNFR2 to be significantly elevated in obese females subjects compared with lean controls. This study also found that mRNA levels of sTNFR2 were strongly correlated with BMI and degree of hyperinsuliemia. 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 TNFα receptors and insulin levels (Lo *et al*, 2007).

In AT, TNFα expression and secretion is primarily from the stromal vascular fraction and to a lower extent from adipocytes (Fain *et al*, 2004). It is highly expressed in the AT of obese subjects and circulating sTNFα seems to be strongly correlated to BMI and WHR (Hotamisligil *et al*, 1995; Zahorska-Markiewicz *et al*, 2000). This is believed to reflect the increased macrophage accumulation and low-grade inflammation that occurs in AT with obesity (Cinti *et al*, 2005; Weisberg *et al*, 2003). The consistent correlation between severe obesity and elevated TNFα expression suggests that adipose-derived TNFα may contribute to the pathogenic effects of obesity including IR, diabetes and CVD. TNFα has potent effect on adipocyte metabolism including inhibition of adipogenesis and fat-producing lipogenic enzymes as well as activation of lipolytic activity (Beutler *et al*, 1985; Cornelius *et al*, 1988). TNFα has been shown to increase plasma TAG and VLDL as well as lipolysis in rodent and human fat cells. A possible involvement of TNFα in IR has been suggested as TNFα reduces insulin stimulated receptor tyrosine kinase activity at low concentrations and can also decrease the expression of the insulin receptor, IRS-1 and GLUT4 at higher concentrations as well as

increase the phosphorylation of IRS-1 serine 307 and activation of NF-κB, thus impairing its ability to bind to the insulin receptor and initiate downstream signalling (*Figure 1.6*) (Hotamisligil *et al*, 1996). These combined actions of TNFα result in a reduction in AT mass and could be the physiological mechanism producing cachexia and hyperlipidemia that occurs with certain infections and malignancies that increase serum levels of this cytokine (Goralski & Sinal, 2007).

Under normal healthy circumstances TNFα circulates in low concentrations, reported to be in the ranges of 10-80pg/mL (Yudkin, 2007; Spengler *et al*, 1996). However, obese women express 2-fold more TNFR2 mRNA in AT and 6-fold more sTNFR2 in circulation relative to lean controls (Hotamisligil *et al*, 1997). Interestingly, concentrations of TNFα have been reported to decrease with weight-loss and improvements in insulin sensitivity (Bruun *et al*, 2003*b*; Guerro-Millo, 2003; Moller, 2000). More recently, it has been shown that TNFα is also expressed in skeletal muscle and that levels are 4-fold higher in IR, obese humans than in healthy humans (Ferrier *et al*, 2004). Anti-TNFα antibodies improve insulin sensitivity in obese rodents and TNFα 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 TNFα activity have emerged as novel strategies for the treatment of obesity-related diseases.

Figure 1.6: Schematic diagram of the mechanism of action of TNFα



1.8.2 Structure and function of 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* (1981), IL-6 has been alternatively named IFNβ2, IL-1-inducible protein, hepatocyte-stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor and/or B cell stimulatory factor 2. IL-6 is produced by numerous cell types including fibroblasts, endothelial cells, monocytes/macrophages and T-cells (Van Snick, 1990; Akira *et al*, 1990). Human IL-6 is a variable 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 *et al*, 1997). The production of IL-6 from these cells acts as a pro and/or anti-inflammatory agent. IL-6 expression and secretion is induced by TNFα and IL-1 (Wilbrink *et al*, 1991; Delneste *et al*, 1994). Subsequently, IL-6 regulates these cytokines by directly influencing TNFα and promoting the release of IL-1 from the IL-1 receptor activity. IL-6 is unusual among cytokines as most of the cytokines identified to date function via autocrine-paracrine manner, whereas with IL-6 the majority of effects takes place at sites distinct from its origin (Yudkin *et al*, 2000). 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 (STATS). Soluble forms of the IL-6 receptor have been identified, but neither their functional significance nor the regulation of their production is understood (Fruhbeck *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 (Rotter *et al*, 2003; Aldhahi & Hamdy, 2003). Omental or visceral AT produces 3-fold more IL-6 compared to subcutaneous AT (Fried *et al*, 1998). Circulating IL-6 concentrations of normal healthy individuals tend to approximately 1pg/mL (Yamamura *et al*, 1998) slight elevations have been seen during the menstrual cycle (Angstwurn *et al*, 1997) modest elevations in certain cancers such as melconoma (10pg/mL) and a possible large elevation following surgical treatment (30-430pg/mL) (Mouawad *et al*, 1996). To date, all

proinflammatory cytokines identified have been shown to be elevated in T2DM (Pradhan *et al*, 2001) suggesting that adipokines might be a possible link between IR, diabetes and obesity (Grimble, 2002). However, among all the cytokines, IL-6 presents the strongest correlation with IR and T2DM in humans (Pradhan *et al*, 2001; Bastard *et al*, 2002*b*). A study was conducted to investigate the predictive value of IL-6, TNFα and CRP for the incidence of CHD, stroke and coronary heart failure (CHF) in older adults without evidence of CVD at baseline. Results showed that all 3 markers predicted the onset of CVD, however IL-6 levels appeared to be the strongest and most consistent risk factor for CVD events compared to TNFα and CRP levels (Cesari *et al*, 2003).

Dynamic studies of IL-6 concentrations in humans showed that IL-6 increases postprandially, in parallel to glucose and insulin levels in the interestitual fluid of subcutaneous AT (Orban *et al*, 1999). These findings suggest that IL-6 might modulate adipose glucose metabolism in the fed state. In obese humans IL-6 expression and secretion from AT increases as BMI and body fat increase. Additionally, exercise has also been found to increase circulating levels of IL-6, occurring through increased production by the muscles (del Aguila *et al*, 2000; Pedersen *et al*, 2001). However, a weight-loss program through increased physical activity and dietary intervention trial for 2 years in premenopausal obese females saw a significant decline in IL-6 and CRP concentrations (Esposito *et al*, 2003). The primary role of systemic IL-6 is the activation of hepatic acute phase proteins including CRP, fibrinogen, serum amyloid A (SAA) and a1-antitrypsin (Harris *et al*, 1999).

1.8.3 Structure and function of 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 in 1930 at the Rockefeller Institute of Medical Research; it has a half-life of about 19 hrs and is regarded as a stable blood protein (Tillet & Francis, 1930; Ford *et al*, 2005). It has Ca^{2+} dependent binding specificity for phosphocholine (PCh), a constitution of many bacterial and fungal polysaccharides and for most biological cell membranes (Volanakis & Kaplan, 1971). Subsequently, ligand-complexed CRP is recognised by C1q and efficiently activates the classical pathway of human complement. The major site for CRP synthesis is

from the liver hepatocytes after stimulation by IL-6 in an acute-phase response (Gortney & Sanders, 2007). *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 certain other factors including complement activation products (Toniatti *et al*, 1990; Szalai *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 (Ablij & Meinders, 2002). Under physiological circumstances, human CRP is a protein with a medium serum concentration of 0.8mg/L. Circulating plasma CRP concentrations between 1-3mg/L has been regarded as average risk for CHD, whereas concentrations >3mg/L is deemed as high risk for CHD (Mackey *et al*, 2003).

The ability of CRP to recognize pathogens and to mediate their elimination by recruitment of the complement system and phagocytic cells makes CRP an important constituent of the first line of innate 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 *et al*, 2001). Additionally, CRP can also induce the expression of cell adhesion molecules (CAM's) in EC, thereby propagating antigens and stimulating monocyte recruitment (Labarrere & Zaloga, 2004).

High concentrations of CRP correlate with the adiposity, the presence of CVD and risk of acute CVD events (Jovelic *et al*, 2005). Furthermore, increased plasma CRP levels are strong independent predictors of future MI, stroke and pulmonary vascular disease, retinopathy and subsequent development of microalbumuria, a marker of impaired kidney function. It has been proposed that CRP is actually a stronger overall predictor of heart disease and stroke than is LDL-c. Thus, recent practice recommendations have been to measure cholesterol levels and CRP combined (Ridker *et al*, 2002).

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 *et al*, 2007; Yoshikane *et al*, 2007; Pradhan *et al*, 2003). Moreover, elevated levels of CRP are known to predict the development of T2DM and CVD and in fact, all 5

characteristics of the MetS are associated with increased CRP levels (Ridker *et al*, 2003; Nakamura *et al*, 2007). In 2004, a cross-sectional study provided a detail analysis of the distribution of CRP concentrations among US women. Results showed that CRP concentrations varied according to race or ethnicity, whereby Mexican-American women had higher levels of CRP compared to white women. Additionally, use of hormone replacement therapy (HRT) significantly increased circulating CRP concentrations (Ford *et al*, 2004).

1.8.4 Structure and function of adiponectin

Adiponectin was discovered during gene-expression profiling of human AT conducted by the human complementary deoxyribonucleic acid (cDNA) project, which analysed visceral and subcutaneous AT to elucidate the molecular mechanism of obesity-related diseases (Maeda *et al*, 1996). Adiponectin has also been termed Acrp30 due to adipocytes complement related protein of 30-kDa (Scherer *et al*, 1995), AdipoQ (Hu *et al*, 1996), apM1 as it is a product of the adipose most abundant gene transcript 1 (Maeda *et al*, 1996) and GBP-28 (Nakano *et al*, 1996). Located on the chromosome 3q27, a locus for diabetes and MetS susceptibility (Mori *et al*, 2002; Vasseur *et al*, 2002), adiponectin encodes a secretory protein apparently expressed exclusively in white and brown AT (Kadowaki *et al*, 2006).

Adiponectin is a signal peptide containing 244 amino acids, it is 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 the absence of primary sequence similarities, crystal structures of the C-terminal globular domain resembles that of TNFα (Shapiro *et al*, 1998). Until recently, it was thought that adiponectin was solely synthesized and secreted by mature adipocytes from both WAT and BAT. However, recent evidence indicates that adiponectin can be expressed from the bone marrow (Yokota *et al*, 2002), fetal tissue (Berner *et al*, 2004), myocytes and cardiomyocytes (Pineiro *et al*, 2005). It is known is that adiponectin is present in plasma in copious amounts (500-30,000μg/L), accounting for 0.01-0.03% of total circulating plasma proteins (Kim *et al*, 2006*c*).

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: trimer (low-molecular weight: LMW), hexamer (medium-molecular weight: MMW) and (high-molecular weight: HMW) 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 and in

protecting against diabetes. It is noteworthy that the ratio of HMW adiponectin to total adiponectin correlated more significantly with glucose and insulin levels than did total adiponectin levels alone (Lara-Castro *et al*, 2006). This suggests that alterations in plasma HMW adiponectin levels may be more relevant to the prediction of IR than are total plasma adiponectin alterations. Kadowaki *et al* (2006) demonstrated that full-length adiponectin stimulates AMP-activated protein kinase (AMPK) in the liver while globular adiponectin exerts its effects in both muscles and in the liver, resulting in increased FA combustion. In addition, adiponectin enhances glucose transport in the muscles and inhibits hepatic glucose production (HGP) by decreasing the expression of gluconeogenic enzymes via AMPK activation (Combs *et al*, 2001; Tomas *et al*, 2002). Furthermore, adiponectin also activates peroxisome proliferators-activated receptor-α (PPARα), 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. PPARα is mainly expressed in the liver, BAT, small intestine, skeletal muscle and heart. The level of PPARα in the WAT and in adipocyte cell lines is very low suggesting a limited role for this isoform during adipogenesis (Grimaldi, 2001).

In 2003, two adiponectin receptors, belonging to a novel class of seven transmembrane domain cell surface receptors were identified (Yamauchi *et al*, 2003*a*). AdipoR1 is found predominantly expressed in the skeletal muscle, while AdipoR2 is found primarily in the liver (Lago *et al*, 2007*b*). Adiponectin binds to the C-terminal extracellular domain of AdipoR1, whereas the N-terminal cytoplasmic domain interacts with an adaptor protein APPL (adaptor protein containing pleckstrin homology domain, phosphotyrosine-binding domain and a leucine zipper mofit). Transduction of the adiponectin signal by AdipoR1 and AdipoR2 involves the activation of mainly AMPK and PPARα. However, the precise pathways leading to the activation of AMPK and PPARα still remain unknown (Capeau, 2007).

Both TNFα and IL-6 are potent inhibitors of adiponectin expression and secretion from human WAT and cultured adipose cells, suggesting that TNFα and IL-6 induced IR may reply on an autocrine-paracrine inhibition of adiponectin release (Fasshauer *et al*, 2002; Fasshauer *et al*, 2003; Bruun *et al*, 2003*b*). Evidence also suggests that adiponectin regulates TNFα metabolism, as increased TNFα has been reported in adiponectin knockout mice (Yamauchi *et al*, 2003*b*).

Adiponectin is known to increases FA oxidation and reduces the synthesis of glucose in the liver. Unlike many other adipokines, circulating levels have been noted to be low in obesity and diabetic status of humans and animals. Plasma adiponectin levels decreased in rhesus monkey, which developed obesity spontaneously (Hotta *et al*, 2001). In addition, plasma adiponectin was decreased in the MetS, paralleling diminished insulin sensitivity (Bahia *et al*, 2006). A retrospective study on Pima Indians, a unique population with high propensity of obesity and T2DM, confirmed these results (Lindsay *et al*, 2002). Interestingly, the expression of both AdipoR1 and AdipoR2 was significantly decreased in muscle and AT of IR *ob/ob* mice (Tsuchida *et al*, 2004). A recent diet and exercise intervention performed on 188 men at risk of diabetes and CVD showed no significant improvements in plasma adiponectin levels after 1 year (Rokling-Anderson *et al*, 2007).

Despite these findings, nutritional and therapeutic manipulations including weight-loss, caloric restriction and pharmacotherapy with thiazolidinedione (TZD's), are known to ameliorate insulin sensitivity and subsequently increase adiponectin gene expression in the WAT and in circulation (Bruun *et al*, 2003*a*).

1.8.5 Structure and function of resistin

Resistin is a novel peptide hormone 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 *et al*, 2000). Resistin is also known as adipose-tissue secretory factor (ADSF). This 12.5kDa protein was discovered by Steppan & Lazar (2002) when identifying potential targets of the TZD class of insulin sensitizers in 3T3-L1 adipocytes. Subsequently, additional members of this family resistin-like molecule-α and β (RELMα and RELMβ, respectively) and alternative biological activities were reported in humans (Steppan *et al*, 2001). Resistin mRNA encodes a 114 amino acid polypeptide containing a 20 amino acid signal sequence (Shuldiner *et al*, 2001). Resistin is encoded by the Retn gene and is secreted as a disulfide-linked dimmer (Kougias *et al*, 2005). In mice, the Retn gene is expressed almost exclusively in WAT and the protein is detectable in adipocytes and in the circulation. This observation suggests that resistin is produced primarily by AT and may act at sites distant from where it is produced (Steppan *et al*, 2001). Interestingly, the highest levels of resistin are observed in female gonadal AT (Degawa-Yamauchi *et al*, 2003). Unlike

murine resistin, human resistin is expressed at low levels in adipocytes but is readily detectable in mononuclear blood cells (Banerjee & Lazar, 2003; Savage *et al*, 2001). The original report by Steppan *et al* (2001) showed that levels of this protein in the blood of obese mice are increased and that treatment with TZDs acted to suppress this obesity-related increase in resistin expression. Moreover, *in vivo* 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 *et al*, 2001). Resistin expression in human monocytes was found to be markedly increased by treatment with endotoxin and pro-inflammatory cytokines such as CRP levels (Kunnari *et al*, 2006; Kaser *et al*, 2003; Lu *et al*, 2002; McTernan *et al*, 2002). Recombinant resistin upregulates cytokines and CAM expression on human ECs, suggesting a potential role in atherosclerosis (Verma *et al*, 2003; Kawanami *et al*, 2004). However, the relationship of resistin to inflammation and IR in humans remains largely unexplored (Reilly *et al*, 2005; Hotamisligil, 2003).

The adipocyte specificity of resistin gene expression is thought to be caused by CCAAT/enhancer-binding protein-α (C/EBPα) binding (Hartman *et al*, 2002). C/EBP is a family of transcriptional factors that like the (peroxisome proliferators-activated receptor) PPAR system, have been shown to be essential in the regulation of adipocyte differentiation (Darlington *et al*, 1998). Resistin appears to be an important regulator of glucose homeostatis, adipogenesis and inflammation. However, the role of resistin in human obesity remains controversial as previous resistin mRNA expression studies have produced conflicting reports about the levels of expression of this gene in human AT (Janke *et al*, 2002; McTernan *et al*, 2002). A study in T2DM subjects reported increased concentrations of resistin compared to non-diabetic controls, however this increase was not found to be linked to markers of IR or adiposity (Hasegawa *et al*, 2005). Their results are in line with several other studies (Utzschneider *et al*, 2005; Iqbal *et al*, 2005). On the other hand, results indicate higher levels of resistin gene and protein expression in central AT depots and in diabetes (Azuma *et al*, 2003; Vorarova *et al*, 2004). Therefore, it is thought that resistin may play a role in linking obesity with diabetes as well as having an important function in the pathogenesis of IR (Kowalska, 2007).

1.8.6 Structure and function of leptin

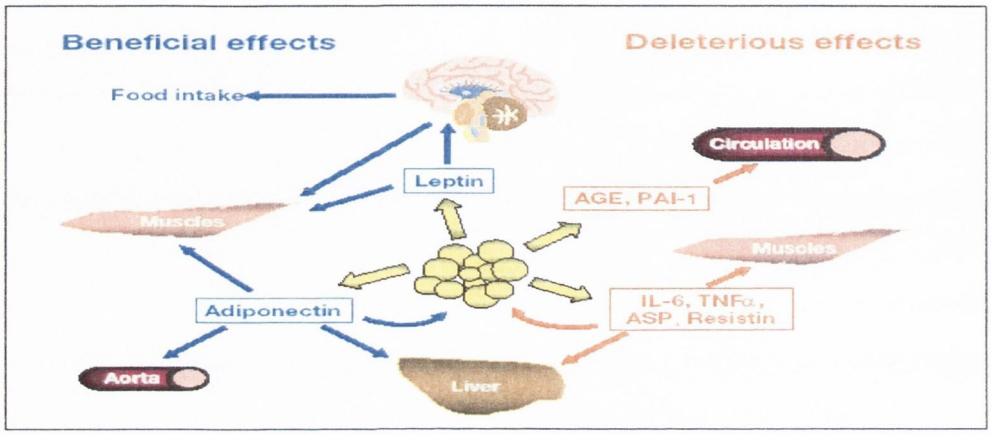
Leptin is a non-glycosylated peptide hormone that is synthesized and released from fat cells in response to changes in body fat. The discovery of leptin in 1994 was very promising for

understanding the pathophysiology of human obesity. Leptin is 167 amino acid encoded by the gene obese (*ob*), the murine homologue of the human gene LEP (Zhang *et al*, 1994b). Structurally, it belongs to the class I cytokine superfamily, consisting of a bundle of four α-helices and 2 short β-strands, containing an intrachain disulfide bond necessary for its biological activity (Zhang *et al*, 1997). It is produced by adipocytes and plasma levels correlate with WAT mass (Peelman *et al*, 2004). Adipocyte leptin expression is transcriptionally regulated with the status of the energy stores in WAT and the adipocyte size as major determinants (Kougias *et al*, 2005). 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 (Shina & Caro, 1998). Leptin circulates partially bound to plasma proteins and enters the central nervous system (CNS) by diffusion through capillary junctions in the median eminence and by saturable receptor transport in the choroids plexus (Ronti *et al*, 2006).

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 the signal of the state of body energy balance, which helps to control the appetite and food intake and maintain a stable body weight (Kowalska, 2007). Leptin expression and serum levels increase after food intake. In contrast, leptin expression is rapidly suppressed with food restriction exceeding the rates at which fat mass and adipocyte size is reduced (Peelman *et al*, 2004). It has been reported that within 24 hrs of fasting leptin levels decline to approximately 30% of initial basal levels, whereas massive overfeeding over a 12 hrs period increases leptin levels by approximately 50% of initial basal levels (Sinha & Caro, 1998). Leptin's main site of action is the hypothalamus (Flier, 1998). Therefore, leptin decreases the desire for food intake and increases energy consumption by acting on the hypothalamic cell populations (Chan *et al*, 2003). These include anorexigenic factors (CART, POMC), inhibition of orexigenic neuropeptide Y (NPY), agouti-related peptide (AgRP) and orein. In addition, leptin levels correlate negatively with glucocorticoids (Zakrzewska *et al*, 1997) yet, correlate positively with insulin concentrations (Boden *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 a wide range of pro-inflammatory mediators as shown in *Figure 1.7* (Sarraf *et al*, 1997; Gualillo *et al*, 2000). As a result of the sex hormones, leptin levels are higher in women than in men even when adjusted for BMI, which may be important in the development of

chronic diseases such as osteoarthritis (Teichtahl *et al*, 2005). Interestingly, age, basal glucose levels and ethnicity do not influence circulating leptin levels (Sinha & Caro, 1998).

Figure 1.7*:* Secretory factors from adipose tissue implicated in insulin sensitivity (Guerre-Millo, 2003)



IL-6: interleukin; **TNFα:** tumour necrosis factor α; **PAI-1**: Plasminogen Activator Inhibitor-1

Blue arrows indicate an increase in insulin sensitivity

Red arrows indicate a decrease in insulin sensitivity or an increase in Insulin resistance

Leptin exerts its biological effects by binding to its receptors. These are encoded by the gene diabetes (*db*) and belong to the class I cytokine receptor superfamily, which also includes IL-6 and gp130. Six isoforms of the leptin receptor exists, the soluble form Ob-Re which lacks a cytoplasmic domain, four other forms with short cytoplasmic domains (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf) and the long form Ob-Rb which is expressed throughout the body and has been located in the hypothalamus, monocytes, natural killer cells, CD4 and Cdd8 lymphocytes, pancreatic β-cells, enterocytes and endothelial cells (Fruhbeck, 1999). It is generally thought that leptin enters the brain at the choroid plexus. As in the case of other class I cytokine, the main routes by which Ob-Rb appears to transmit the extracellular signal it receives are JAK-STAT pathways, which involves JAK2 phosphorylation tyrosines in the cytoplasmic domain of the receptor (Fruhbeck, 2006). Mutations of the intracellular tyrosine Y1138 of murine Ob-Rb prevents STAT3 activation and results in hyperphagia, obesity and impaired

thermoregulation and replacing Y1138 with a serine residue likewise causes pronounced obesity in knockout mice (Bates *et al*, 2003)

To date, the role of leptin in regulating insulin sensitivity is not precisely defined. The *ob* gene product, leptin, circulates as a 16 kDa protein in both mouse and human plasma but is undetectable in plasma from C57BL/6J *ob/ob* mice (Halaas & Friedman, 1997). Therefore, a deficiency in leptin in the *ob/ob* mouse strain or its receptor as occurs in the *db/db* mouse strain, results in severe IR (Kahn & Flier, 2000). Several studies have demonstrated that leptin concentrations are modulated through the actions on the muscle sympathetic nerve activity (MSNA) (Lambert *et al*, 2007; Shen *et al*, 2007). A consequence of increased MSNA activity is a decrease in femoral blood flow and conductance, an increase in femoral vascular resistance and a lower oxygen demand (Dinenno *et al*, 1999). The sympathetic nerve is known to regulate energy balance by decreasing food intake and increasing resting metabolic rate (RMR) and has been reported to be possibly conducive to the development of a MetS (Tentolouris *et al*, 2006). Additionally, it has also been postulated that an increase in the sympathetic vasoconstrictor activity and a reduction in skeletal muscular blood flow could contribute to the development of IR (Julius *et al*, 1992). A low-fat diet and exercise trial for 4 months on obese women, showed significant reduction in body weight and subsequently observed a significant decrease in MSNA activity. This study concluded that this provides convincing evidence for a low-fat diet and weight-loss, a non-pharmological therapy for human obesity (Tonacio *et al*, 2006). Another weight-loss study performed on MetS subjects, feed a hypocaloric diet (26% fat, 22% protein, 51% CHO and 100mmol/d sodium) for 3 months, demonstrated significant body weight loss and subsequent improvements in all MetS criteria, as well as significant reduction in MSNA activity (Straznicky *et al*, 2005). This effect may be mediated through the activation of AMPK by an early direct effect of leptin on certain skeletal muscles, followed by a later indirect effect of AMPK activation by leptin through the hypothalamic-sympathetic nervous axis (Minokoshi *et al*, 2002). Then activated AMPK phosphorlyates Ser-221 of acetyl-CoA cardoxylse (ACC) which suppresses the activity of the enzyme (Rutter *et al*, 2003). ACC catalyses the synthesis of malonly-CoA (Ruderman *et al*, 2003). Consequently there is a decrease in the concentration of malonyl CoA. Malonly CoA is a allosteric inhibitor of carnitine palmitoyltransferase 1, the enzyme which regulates the rate at which long-chain fatty acyl-CoA are transferred from the cytosol into mitrochondria where they are oxidized (Ruderman *et al*, 2003). Thus, phosphorylation of ACC by AMPK leads to inhibition of ACC activity, a fall in malonly-CoA content, and a subsequent increase in fatty-

acid oxidation of long-chain acyl-CoA's by mitrochondria via dis-inhibition of carnitine palmitoyltransferase in skeletal muscle (Neilsen *et al*, 2003).

A study set out to examine if changes in dietary sources and exercise-mediated energy expenditure, alone or in combination would affect plasma leptin concentrations. The randomized intervention included 186 men with the MetS and lasted for 1-year. Results showed that both diet and exercise combined therapy decreased concentrations of leptin, as well as BMI and body fat mass (Reseland *et al*, 2001*a*). Within the same research group, supplementation with n-3 polyunsaturated fatty acids (PUFA) for 6 weeks did not alter plasma leptin concentration in male smokers. However, dietary n-3 PUFA reduced human leptin mRNA expression both *in vivo* and *in vitro*. The study concluded that direct effects of PUFA on leptin promoter activity indicate a specific regulatory action of FAs on leptin expression (Reseland *et al*, 2001*b*).

1.9 Endothelial dysfunction and insulin resistance

The endothelium is a monolayer of cells that line the interior surface of the blood vessels walls, forming an interface between circulating blood in the lumen and the rest of the vessel wall. The entire vascular system is made up of approximately $1x10^{13}$ cells, weighting 1 to 1.5kg and covering $700m^2$ (Sumpio *et al*, 2002; Laroia *et al*, 2003). Normal functions of EC include mediators of regulation of vascular tone of the underlying vascular smooth muscle mainly through the production of vasodilator mediators as well as platelet adhesion, coagulation and fibrinolysis (Mombouli & Vanhoutte, 1999). It senses mechanical stimuli, such as pressure and/or shear stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes and affect homeostasis. Among the vasodilatory substances produced by the endothelium are nitric oxide (NO), prostacyclin 1_2 (PGI_2), different endothelium-derived hyperpolarizing factors, and C-type natriuretic peptide. NO is an endogenous signalling molecule produced by nitric oxide synthase (NOS). NO acts as a signal transduction molecule for a number of physiological process, such as vasodilation and are involved in IR (de Luca & Olefsky, 2008). Vasoconstrictors include endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A_2, and reactive oxygen species (ROS) (Verma & Anderson, 2002; Schiffrin, 2001).

When the endothelium is functioning normally, all these functions are tightly balanced, whereas major imbalances in these processes arise during endothelial dysfunction (Hartge *et al*, 2007). Endothelial dysfunction is defined as a physiological dysfunction of normal

biochemical processes carried out by the endothelium. The pro-adhesive quality of dysfunctional endothelium involves the recruitment of leukocytes and platelets from the circulation. ICAM-1 is thought to be the key factor when circulating monocytes adhere to the endothelium and subsequently transmigrate into the intima (Blanco-Colio *et al*, 2007). The leukocyte adhesion cascade is a sequence of adhesion and activation events that ends with extravasation of the leukocyte, whereby the cell exerts its effects on the inflamed site. At least five steps of the adhesion cascade are capture, rolling, slow rolling, firm adhesion and transmigration as depicted in *Figure 1.8*. These steps are not phases of inflammation, but represent the sequence of events from the perspective of each leukocyte. At any given moment, capture, rolling, slow rolling, firm adhesion and transmigration all happen in parallel, involving different leukocytes in the same microvessels. Dysfunction of the endothelium has been implicated in the pathophysiology of different forms of CVD, including hypertension, coronary artery disease (CAD), CHD, peripheral artery disease, T2DM, and chronic renal failure (Endemann & Schiffrin, 2004). It can arise from insults such as elevations and modifications of LDL-c (Engler *et al*, 2003), IR (Kim *et al*, 2006*b*), obesity (Raitakari *et al*, 2004), free radicals from cigarette smoking (Oida *et al*, 2003), hyperhomocysteinemia (Virdis *et al*, 2001) and a sedentary lifestyle (Green *et al*, 2003).

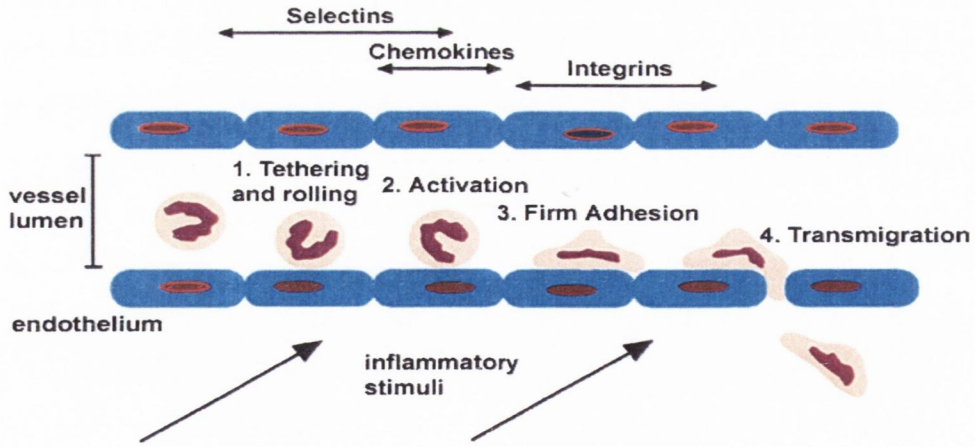


Figure 1.8: Cell adhesion molecule mediation of leukocyte rolling, tethering, arrest and subsequent transmigration of endothelial cells (Adapted from Blankenberg *et al*, 2003).

It is thought the endothelial dysfunction is the site where the processes of inflammation in obesity and T2DM may commence (Hartge *et al*, 2007). Endothelial dysfunction may alter the presentation and function of insulin receptors (Kearney *et al*, 2008). In states of IR, such as in T2DM, insulin signalling is altered differently, affecting the two major pathways emerging from the insulin receptor. Firstly, the pathway leading via the (P13K)-AKT pathway, phosphoinositide-dependent kinase-1, and Akt/protein kinase B to phosphorylation and activation of endothelial NO synthase (eNOS) is dramatically downregulated, whereas the pathway leading via mitogen activated protein kinases to mitogenic effects and growth remains unaffected (Montagnani *et al*, 2002; Osman *et al*, 2000). Moreover, hyperglycemia leads to advanced glycation end products (AGE), which were shown to quench NO and impair endothelial function, as evidenced by inhibition of advanced glycosylation with aminoguanidine (Bucala *et al*, 1991). AGE induce ROS and promote vascular inflammation, with enhanced expression of IL-6, adhesion molecules such as VCAM-1 and MCP-1 (Zhang *et al*, 2003). Additionally, acute hyperglycemia itself can reduce NO and attenuate endothelium-dependent vasodilation in human's *in vivo* (Williams *et al*, 1998). Inhibition of AGE formation with aminoguanidine prevents NO depletion and sustains endothelial function (Bucala *et al*, 1991).

1.9.1 Structure and function of soluble Intercellular Adhesion Molecule-1 (sICAM-1)

ICAM-1/CD54 is an 80-110-kD glycoprotein and a member of an immunoglobulin (Ig) supergene family and is expressed on a wide variety of cells constitutively or under conditions of inflammation (Simmons *et al*, 1988; Stuanton *et al*, 1988). A strong attachment of leukocytes to endothelial cells is mediated by both ICAM-1 and VCAM-1 in response to inflammatory cytokines (Malik *et al*, 2001). ICAM-1 is the most widely expressed cell adhesion molecule and is found on leukocytes, fibroblasts, epithelial and EC's. Its expression is upregulated in response to lipopolysaccaride (LPS), phorbol esters, pro-inflammatory cytokines and LDL exposure (Yuan *et al*, 2001). Soluble forms of these adhesion molecules are found to enter the circulation as a result of proteolytic cleavage at the cell surface, releasing the extracellular domain, they are shredded cell membrane ligands and may therefore be used as a monitor of adhesion molecule expression (Tsakadze *et al*, 2004). Previous studies have shown an increased cellular adhesion molecule expression in several components of the atherosclerotic plaque and a positive association of increase plasma concentrations of soluble ICAM-1 (sICAM-1) (Enders *et al*, 1997; Ridker *et al*, 1998). Levels of sICAM-1 were shown

to be higher in T2DM patients compared to non-diabetic controls (Fasching *et al*, 1996; Blüher *et al* 2002). These results are in line with a study carried out on 631 subjects, interestingly it was reported that those subjects who had sICAM-1 concentrations higher than 550ng/mL had a 2-fold increase in CVD mortality compared to individuals with lower sICAM-1 concentrations (Becker *et al*, 2002). sICAM-1 levels have also been reported to increase in response to the *western-style* diet, this is a diet that is high in atherogenic lipids or is hypercholesterolemic (Blackenberg *et al*, 2003; Kvasnicka *et al*, 2003). Brake *et al* (2006) reported that plasma sICAM-1 expression is increased in mice following a high-fat diet for 6 months, in fact significant increases were seen in sICAM-1, IL-6 and MCP-1 concentrations after 3 weeks of feeding.

1.9.2 Structure and function of soluble Vascular Adhesion Molecule-1 (sVCAM-1)

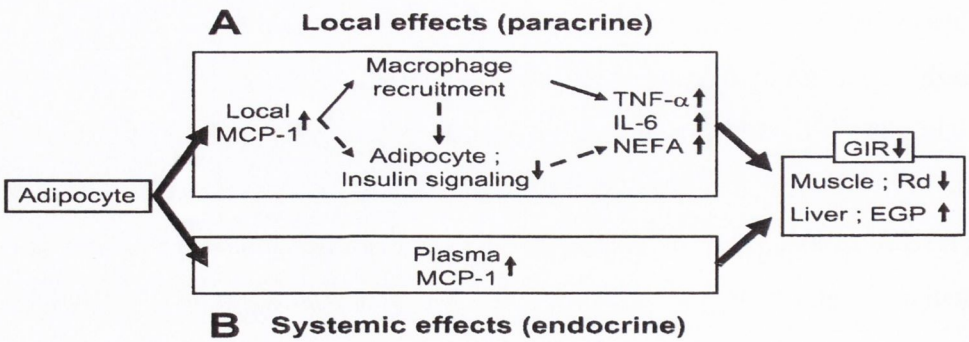
VCAM-1 also known as CD106 is also a member of the Ig superfamily, it is a glycoprotein that contains six or seven Ig domains and is expressed on both large and small vessels only after the endothelial cells are stimulated by cytokines including TNFα, IL-1 and IL-4. It is expressed on the membrane surfaces of cells including endothelial cells, 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 *et al*, 1990; Zhang *et al*, 2004). VLA-4 is composed of α4 and β1 subunits and is expressed on the surface of lymphocytes, monocytes and eosinophils (Kartikasari *et al*, 2004). VCAM-1 also participates in the adhesion of circulating leukocytes to endothelial cells and allows subsequent transmigration to the arterial intima (Munro & Cotran, 1988). The soluble form of VCAM-1 (sVCAM-1) is shed following the proteolytic cleavage of the membrane-bound form of VCAM-1 on cell surfaces (Singh *et al*, 2005; Cernuda-Morollon & Ridley, 2006). sVCAM-1 has a lower molecular weight than VCAM-1 in endothelial cells, resulting from the absence of the cytoplasmic domain, which remains in the cell of origin (Pigott *et al*, 1992). It has been reported that the expression of sVCAM-1 is upregulated by the proinflammatory cytokines IL-1 and TNFα in vascular EC and SMC (Singh *et al*, 2005; Mackenzie *et al*, 2007). sVCAM-1 in the blood of T2DM was found at a higher level than in non-diabetic subjects (Wautier & Wautier, 2001). Moreover, the serum level of sVCAM-1 is increased in atherosclerosis and hypertension (Blankerberg *et al*, 2003; Parissis *et al*, 2001). These findings imply that sVCAM-1 may play

an important role in vascular disorders, including atherosclerosis and angiogenesis (Berliner *et al*, 1995; Byrne *et al*, 2000).

A study on MetS patients with familial low HDL-c were found to have significantly higher levels of sICAM-1 and sVCAM-1 as well as insulin and TAG concentrations compared to normolipidemic controls. In addition, concentrations of adhesion molecules and CRP increased in accordance with the number of features of the MetS (Soro-Paavonen *et al*, 2006). The Hoorn study found sVCAM-1 levels to be significantly associated with the risk of CV mortality, particularly among T2DM subjects. It also reported sVCAM-1 levels to be significantly associated with males, age, fasting insulin levels and hypertension (Jager *et al*, 2000). Interestingly, a dietary study on 90 males with dyslipidemia supplemented with α-linolenic acid (ALA, C18:3n-3) for 12 weeks showed significant decreases in sVCAM-1 levels. Thus, this effect may present a potential mechanism for the beneficial effect of plant n-3 PUFA in the prevention of CAD (Rallidis *et al*, 2004).

1.9.3 Structure and function of Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1/CCL2 is a monomeric polypeptide and member of the CC chemokine family that exhibits potent chemotactic activity for monocytes/macrophages. MCP1 has also been called small inducible cytokine A2 (SCYA2) and monocyte chemotactic and activating factor (MCAF). MCP-1 is the main chemokine responsible for the recruitment of monocytes across EC's, as well as epithelial cells both *in vivo* and *in vitro* to sites of active inflammatory cytokine such as TNFα, IL-1 and IL-6 (Gunn *et al*, 1997; Maus *et al*, 2001).



GIR = Glucose infusion rates

Figure 1.9: Overexpression of MCP-1 in adipose tissues causes insulin resistance in both a paracrine and an endocrine manner.

Kamei *et al* (2006) proposed that both increased accumulation of macrophages in AT **(A)** and higher plasma levels of MCP-1 **(B)** contributed to the development of IR as illustrated in *Figure 1.9*. This study was carried out on aP2-MCP-1 transgenic mice. MCP-1 is produced by adipocytes and induces recruitment of macrophages to AT, thereby causing inflammation and increased gene expression of inflammatory secretory molecules such as TNFα and IL-6. In addition, plasma NEFA levels were increased in transgenic mice. Increased NEFA levels as well as TNFα and IL-6 gene expressions may contribute to the development of IR, and circulating plasma MCP-1 may also contribute to the development of systemic insulin resistance in aP2-MCP-1 mice. This study also demonstrated that MCP-1 blunted insulin signalling and insulin-stimulated glucose uptake in myotube cells and isolated skeletal muscle, Alternation of these two pathways may cause IR.

The main receptor *in vivo* for MCP-1 is chemokine receptor-2 (CCR2), although other receptors capable of interacting with MCP-1 have been identified (Kunkel, 1999). Several different cell types including adipocytes have been reported to express CCR2. Importantly, long-term incubation of adipocytes with exogenous chemokines suppressed lipid accumulation and PPARγ expression and chemokines acutely increased leptin secretion from mature adipocytes (Gerhardt *et al*, 2001). These observations suggest that chemokines may have important biological effects on adipocytes.

The expression of MCP-1 has been found to be elevated in the WAT of *ob/ob* mice and in diet-induced obese (DIO) mice (Sartipy & Loskutoff, 2003; Takahasti *et al,* 2003). Interestingly, the AT from the *ob/ob* mice expressed 10 to 100-fold more MCP-1 mRNA than the liver, kidneys and lungs, suggesting that the AT may be a major source of the increased plasma levels of MCP-1 observed in these animals (Sartipy & Loskutoff, 2003). Furthermore, genetic ablation of either MCP-1 or CCR2 substantially attenuated both monocyte infiltration into atherogenic lesions and the development of atherosclerosis (Boring *et al,* 1998; Gu *et al*, 1998; Gosling *et al*, 1999). Thus, obesity-induced elevation of adipose MCP-1 expression may contribute to atherosclerosis. In addition, MCP-1 impairs insulin stimulated glucose uptake by cultured adipocytes *in vitro*, therefore an increase in adipose MCP-1 mRNA expression may increase the risk of IR via adipose macrophage accumulation and inflammation (Sartipy & Loskutoff, 2003).

Kanda *et al* (2006) demonstrated that the expression of an MCP-1 transgene in AT under the control of aP2 gene promoter was sufficient to induce macrophage infiltration into

AT. In another study, the aP2-MCP-1 mice had an increased number of AT macrophages with higher plasma MCP-1 levels. It was assumed that both the paracrine and endocrine effects of MCP-1 contributed to the development of IR in aP2-MCP-1 mice. Furthermore, modifications in pro-inflammatory adipokines may contribute to the development of IR. Elevations of TNF-α and IL-6 mRNA in WAT were noted in the aP2-MCP-1 mice can this may be attributable to the increased macrophage accumulation (Kamei *et al*, 2006). A human drug study using atorvastatin for 12 weeks was found to decrease the concentrations of ICAM-1 and MCP-1 expression through the inhibition of transcription factor activation mainly NF-κB (Blanco-Colio *et al*, 2007). This transcription factor is the main nuclear factor implicated in the activation of the transcription of several pro-inflammatory proteins including TNFα as well as ICAM-1 and MCP-1. Additionally, the diminution of NF-κB activation has been associated with the reduction of macrophage infiltration in human atherosclerotic plaques.

1.10 Coagulation, inflammation and insulin resistance

The MetS includes metabolic abnormalities such as dyslipidemia, hypertension and hyperglycaemia. In addition to these disorders, alterations in coagulation and fibrinolytic factors resulting in a pro-thrombotic state are now considered to be an additional aspect of the MetS. In the human body, blood clots are constantly formed and dissolved as regulated by the coagulation and fibrinolytic systems. Fibrinolysis results in the breakdown of solid fibrin filaments that consitiute the blood clot. For the activation of this system, tissue-type plasminogen activator (tPA) binds to the fibrin surface wheFre plasminogen is cleaved by tPA into its active form, plasmin. Plasmin then cleaves the fibrin filaments. The plasminogen activators, tPA and urokinase (uPA) in turn, are regulated by plasminogen activator inhibitor (PAI-1). Under normal circumstances these systems are in a tightly controlled balance. However, when this balance is disturbed, a tendency for bleeding or thrombosis occurs. Thrombotic events are the major cause for CVD and PAI-1 levels have been demonstrated to predict CHD (Kohler & Grant, 2000).

With regard to the fibrinoytic system, plasma concentrations of tPA and PAI-1 have been shown to be elevated with the MetS suggesting a reduced endogenous fibrinolytic activity (Alessi & Juhan-Vague, 2006; Trost *et al*, 2006). Interestingly, Takeshita *et al* (2008) found PAI-1 concentrations to be upregulated in the liver of patients with obesity and T2DM patients.

1.10.1 Structure and function of Plasminogen Activator Inhibitor (PAI-1)

PAI-1 is a 50 kDa glycoprotein of a 379 amino acid and belongs to the serine protease inhibitor (serpin) family (Gils & Declersk, 2004). A number of cell types produce PAI-1, including EC, hepatocytes, adipocytes and AT stromal cells (Samad & Luskutoff, 1996; Vidal, 2001; Bastelica *et al*, 2002). PAI-1 is a fast acting inhibitor of fibrinolysis in the blood that was first described in the early 1980's as a rapid inhibitor of tPA (Wiman *et al*, 1984). A number of growth factors increase the production of PAI-1, although the mechanisms remain largely unknown. However, the mechanism of action for the effects of transforming growth factor-β (TGF-β) on PAI-1 production is well established and includes both activation of gene transcription and increased stability of PAI-1 mRNA (Gabriel *et al*, 2003). The tumour protein p53 binds the PAI-1 promoter and stimulates gene transcription when it is over-expressed, suggesting a possible mechanism whereby tumours regulate PAI-1 expression when required for metastasis. Glucose increases the gene expression of PAI-1, whereas insulin and insulin-like growth factor (IGF-1) increase the production of PAI-1 only by increasing the stability of PAI-1 mRNA (Suzuki *et al*, 2002). The mechanism of activation of PAI-1 production by glucocorticoids is suggested to be indirect. It has been hypothesised that the association between insulin sensitivity and PAI-1 is mediated though serum lipids. Many studies have demonstrated to increase the production of PAI-1 after stimulation by TAG-rich lipoproteins (TRL) particles. Additionally, FAs have been shown to increase the production of PAI-1 via a VLDL responsive element, which is also activated by TRL particles (Dichtl *et al*, 2000). Furthermore, insulin increases PAI-1 activity by decreasing the degradation of PAI-1 mRNA and has no effect on the transcription of the PAI-1 gene (Fattal *et al*, 1992). In addition, angiotension II, a well-known vasoconstrictor, also increases the production of PAI-1, probably by acting via the angiotensin II type 1 receptor (AT-R1) (Vaughan *et al,* 1995; Vaughan, 2002). PPARγ activators, both TZDs and 15d-PGJ_2 suppress the expression of AT-R1 at the level of transcription in vascular SMC (Takeda *et al*, 2000). This offers a potential means by which to intervene in the atherosclerotic processes, because it is SMC that are largely responsible for the increase in PAI-1 in diabetes (Boyle, 2007). Atherosclerotic plaques are stabilized by the elaboration of the extracellular matrix by proliferating vascular SMC. However, plaques are destabilized by the matrix metalloproteinases (MMPs) released by macrophages, whereby these enzymes degrade the cross-linking collagen fibrils and therefore promote plaque rupture. *In vitro* studies have shown that the activation of PPAR in

human monocyte-derived macrophages decreases levels and activity of MMP9 (the main metalloproteinase secreted by macrophages *in vitro*) (Marx *et al*, 1998).

It is well established that TNFα is involved in IR. The group of Loskutoff was the first to emphasize the contribution of TNFα in PAI-1 regulation during obesity. In *ob/ob* mice, deletion of both TNF receptors (TNF RI and RII) significantly reduced the plasma PAI-1 levels as well as the AT PAI-1 mRNA levels. TNFα-neutralizing antibodies decreased plasma PAI-1 levels, proving a direct link between TNFα and PAI-1 during obesity (Kohler & Grant, 2000).

1.10.2 Structure and function of tissue-type Plasminogen Activator (tPA)

tPA is a serine protease normally found on the surface of EC of veins, capillaries and the pulmonary artery. This enzyme plays a role in cell migration and tissue remodelling. Increased enzymatic activity causes hyperfibronolysis, which manifest as excessive bleeding, whereas decreased activity leads to hypofibrinolysis which can result in thrombosis or embolism. The capacity of the endothelium to release tPA is an important endogenous defence mechanism against intravascular fibrin deposition and thrombosis (Muldowney & Vaughan, 2002). The thrombotic potential for tPA is greatest if it is locally released in large quantities to be incorporated during thrombosis formation (Brommer, 1984). Animal studies have shown that the ability of the endothelium to locally and rapidly release tPA in response to a thrombogenic stimulus results in fibrin degradation and clot resolution (Giles *et al*, 1990; Kruithof *et al*, 1997). On the other hand, deficiencies in tPA is associated with accelerated atherogenesis with uncontrolled luminal fibrin deposition and severe myocardial tissue necrosis (Carmeliet *et al*, 1994; Christie *et al*, 1999). Obesity and IR, which often occur together, are considered to be the major underlying risk factors for the MetS (Dandona *et al*, 2005; Grundy *et al,* 2004). Van Guilder *et al* (2008) demonstrated that the capacity of the endothelium to release tPA is affected by adiposity than the presence of IR or the MetS. The same research group demonstrated that regular aerobic exercise 5-7 days per week, 40-50 minutes per day for 3 months can increase the capacity of the endothelium to release tPA in obese adults (Van Guilder *et al*, 2005).

1.11 Lipid peroxidation and oxidative stress

When lipids are oxidised without the release of energy the reaction is called lipid peroxidation. PUFA are more prone to lipid peroxidation that saturated fatty acids (SFA) and MUFA. The lipid peroxidation reactions occur rapidly either through catalysation by free radicals (non-enzymatic lipid peroxidation) or by enzymes (enzymatic lipid peroxidation) as illustrated in *Figure 1.10* (Basu, 1999).

The non-enzymatic lipid peroxidation process involves oxidative stress. Oxidative stress which is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ, caused by the ROS. Cell damage is induced by ROS, which are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. The main source of ROS *in vivo* is aerobic respiration, although ROS are also produced by peroxisomal β-oxidation of FAs, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens or lipopolysaccharides, arginine metabolism, and tissue specific enzymes. Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalyse, or glutathione peroxidase (GSH). The main damage to cells results from the ROS-induced alteration of macromolecules such as PUFA in membrane lipids, essential proteins, and DNA. Several *in vitro* markers of oxidative stress are available, but most are of limited value *in vivo* because they lack sensitivity and/or specificity or require invasive methods.

Isoprostanes are prostaglandin-like compounds that are produced *in vivo* independently of cyclooxygenase (COX) enzymes, primarily by free radical-induced peroxidation of AA. (Montuschi *et al*, 2007). The formation of PG-like compounds during auto-oxidation of PUFA was first reported in the mid-1970's (Pryor *et al,* 1976), but isoprostanes were not discovered to be formed *in vivo* in humans until 1990 (Morrow *et al*, 1990). F_2-isoprostanes are a group of 64 compounds isomeric in structure to COX-derived $PGF_{2\alpha}$. They are formed *in vivo* by peroxidation of AA and are rapidly metabolised and excreted in the urine (Basu, 2004). Oxidative stress is now recognized to be a prominent feature of many acute and chronic diseases including cancer, CVD, neurodegenerative disease, lung disease and even the normal aging process. In addition, oxidative stress has been associated with all the individual components and with the onset of CVD complications in MetS subjects (Wellen & Hotamisligil, 2005).

1.11.1 Isoprostanes

It has now been established that measurement of F_2-isoprostanes is the most reliable approach to assess oxidative stress status *in vivo*, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease. Isoprostanes are eicosanoids generated via a free-radical catalysed mechanism. Isoprostanes from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been identified *in vivo* (Roberts *et al*, 1998). Most interest has focused on isoprostanes derived from AA due to their greater abundance. These compounds were first shown to be produced in humans when they reported the discovery of prostaglandin F_2-like compounds, termed F_2-isoprostanes, generated by free-radical induced peroxidation of AA. After AA peroxidation, three arachidonyl radicals are produced and undergo endocyclization to form four prostaglandin H_2 (PGH_2) bicyclic endoperoxide intermediate regioisomers (Taber *et al*, 1997). These PGH_2 isomers can be fully reduced to form four esterified prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) isomers or partially reduced to form four esterified prostaglandin E_2 (PGE_2) isomers. The isoprostanes are subsequently released in free form by the action of phospholipases (Cracowski *et al*, 2001).

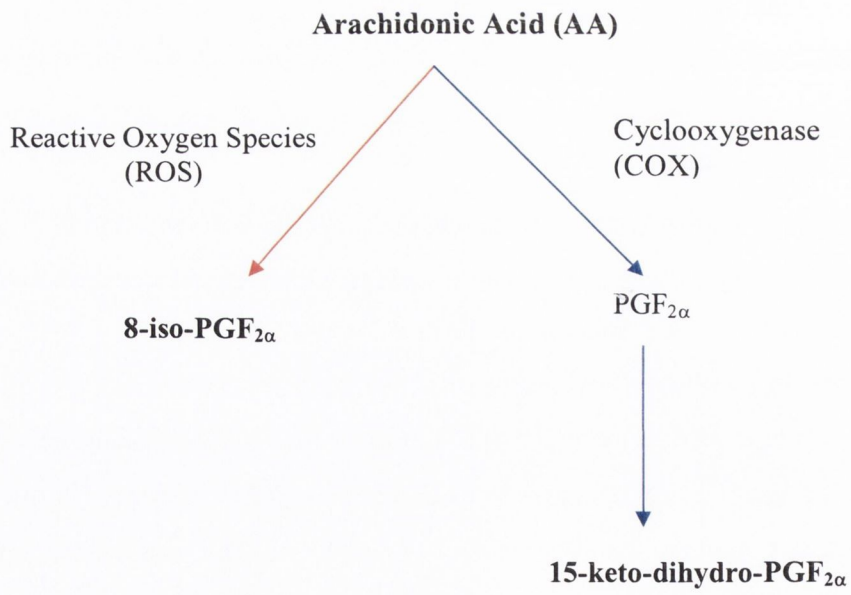


Figure 1.10: ⟶ Non-enzymatic lipid peroxidation of arachidonic acid to form isoprostane 8-iso-$PGF_{2\alpha}$ and ⟶ enzymatic lipid peroxidation of arachidonic acid to form prostanglandin 15-keto-dihydro-$PGF_{2\alpha}$

Isoprostane endoperoxides may undergo rearrangement in vivo to form an E- or D-ring and thromboxane-ring compounds. E2- and D2-isoprostanes undergo dehydration *in vivo* to form reactive cyclopentenone A2- and J2-isoprostanes (Morrow & Roberts, 1997). Isoprostaglandin H_2 may rearrange to form isolevuglandins, which are suspected to have potent biological effects due to their ability to adduct to proteins (Brame *et al*, 1999). To date 64 different F_2 and 64 different E_2 isomers have been identified and currently two different nomenclatures are used to identify these compounds (Taber *et al*, 1997; Rokach *et al,* 1997).

Isoprostaglandin $F_{2\alpha}$ type III (8-iso-$PGF_{2\alpha}$ or 8-epi-$PGF_{2\alpha}$) and isoprostaglandin F_2 type VI (5-F_{2t}-isoP) are the two most widely studied isoprostanes. This may be because the metabolic fate of isoprostanes is mostly unknown except for 8-iso-$PGF_{2\alpha}$ isoprostane (Montuschi *et al*, 2004). Indeed, clinical studies have suggested that F_2-isoprostanes are formed via a non-COX dependent mechanism *in vivo*, as COX inhibitors were unable to decrease the formation of isoprostanes in healthy subjects or in patients with elevated isoprostanes (Dworski *et al*, 1999). This is consistent with conditions of increased COX-2 expression where the formation of 8-iso-$PGF_{2\alpha}$ was not altered by COX inhibitors despite decreased prostanoid production (Mc Adam *et al*, 2000). COX-2, an isoform of COX, has been shown to be expressed in macrophages, epithelial cells, and fibroblasts after exposure to several pro-inflammatory stimuli such as cytokines, growth factors, leading to the release of prostaglandins (PG's) (Vane & Blotting, 1995).

1.11.2 Prostaglandins

Lipid peroxidation may also occur via enzymatically catalysed pathways. PGs are derived from AA through the COX pathway (*Figure 1.10*). They were first reported in the 1930s as eicosanoids as they consisted of a 20 carbon skeleton that also contained a four-five member ring (Euler, 1936; Goldblatt, 1933). Subsequently, two independent research groups successfully synthesised PGE_2 from AA using enzyme preparations from sheep seminal vesicles (Bergstroem *et al*, 1964; Van *et al*, 1964). Following this, the elucidation of the structure of thromboxane A_2 (TXA_2) and that of its degradation product thromboxane B_2 (TXB_2) (Hamberg *et al*, 1975) and subsequently discovered prostacyclin (PGI_2) (Moncada *et al*, 1976). Later, two unstable cyclic endoperoxidases, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2) were isolated and identified (Flower, 1978). 15-keto-13,14-dihydro-

$PGF_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$) is the major metabolite of prostaglandin $F_{2\alpha}$ and is increased as a result of the inflammatory response (Basu, 1998*b*; Basu, 1999).

AA is an important PUFA of the (n-6) series and can be converted enzymatically through the COX pathway to bioactive PGs ($PGF_{2\alpha}$, PGE_2 and PGI_2) and thromboxanes (TX) (Samuelsson *et al*, 1975). It is the most abundant precursor of eicosanoids in humans, and is either derived from linoleic acid (LA) or ingested in the diet. LA is a PUFA used in the biosynthesis of PGs. It is found in the lipids of cell membranes. It is abundant in many vegetable oils, especially safflower and sunflower oils. The concentration of free AA is low as it is usually stored esterified in the lipid membrane. Therefore, the biosynthesis of the eicosanoids depends primarily on the release of AA from cellular stores. Once released AA is rapidly metabolised to oxygenated products. Products that contain ring structures, such as the prostaglandins, thromboxanes and prostacyclin, result from the action of COX, whereas derivatives of straight chain FAs, such as the leukotrienes, result from the action of the lipoxygenases (Smith *et al*, 2000). The enzymatic pathway for the formation of prostaglandins from AA can be divided into three stages: mobilisation of arachidonate from precursor lipids through the action of lipases, conversion of arachidonate to the unstable prostaglandin endoperoxide PGG_2, and subsequently to PGH_2 and the cell specific isomerisation or reduction of PGH_2 to the biologically active prostaglandins.

The KANWU study was the first study using a sufficient sample size to investigate the effect of dietary fat quality of insulin sensitivity. It involved 162 healthy individuals from 5 different countries, randomly assigned to consume a SFA-rich diet or MUFA-rich diet, without any changes in other dietary constituents. Subsequently, a randomly selected sub-sample within each group was also given n-3 PUFA supplementation or placebo. Results showed that the SFA-rich diet significantly impaired insulin sensitivity (-10%, $p=0.05$), but insulin sensitivity remained unchanged in the MUFA diet. Whereas, the n-3 PUFA (EPA & DHA) supplementation did not influence insulin sensitivity within the whole study group (Vessby *et al*, 2001). Another aspect of this study was to investigate whether n-3 PUFA affects non-enzymatic and enzymatic lipid peroxidation and antioxidant status. Interestingly, results revealed that supplementation with n-3 PUFA significantly decreased plasma 8-iso-$PGF_{2\alpha}$ concentrations decreased at 3 months supplementation, whereas no effect was noted for 15-keto-dihydro-$PGF_{2\alpha}$ at post-intervention (Nälsén *et al*, 2006).

1.12 Effects of dietary fatty acids on inflammation and insulin sensitivity

FAs are organic acids which can be SFA or unsaturated FAs for examples MUFA and PUFA. There are two essential FA, LA and α-linoleic acid (ALA), these cannot be synthesized *de novo* in animal cells and therefore must be obtained from the diet. Linoleic acid is the predominant dietary n-6 PUFA which is derived from seed oils, whereas ALA is the predominant dietary n-3 PUFA derived from linseed oil products. ALA is converted to long chain (LC) n-3 PUFA EPA and DHA. The metabolism of the n-6 and n-3 FA is competitive, since both pathways employ the same set of enzymes. The major end product of the n-6 pathway is AA. The steps involved in both n-3 and n-6 FA pathways are presented in *Figure 1.11.* FAs represent one of the most important energy sources for the human body. They circulate in the blood predominantly as TAG within lipoproteins or as NEFA or FFA (Bosello & Zamboni, 2000).

FFA activates the pro-inflammatory transcription factor NF-κB which has been linked to FA induced impairment of insulin action in skeletal muscle of rodents (Jové *et al,* 2005). FA catabolism in skeletal muscle is mainly regulated by PPARs, ligand transcription factors that regulate the expression of genes involved in FA uptake and oxidation, lipid metabolism and inflammation. It has been reported that n-3 and n-6 FA are ligands or activators for the nuclear receptors NF-κB, PPARs and SREBP-1c (Schmitz & Ecker, 2007). Increased FFA turnover and increased levels of circulating FFAs as found in the obese state are well known to promote IR in tissues such as skeletal muscle (Moller & Kaufman, 2005). One underlying cause for the increased release of FFAs is secondary to alterations in perilipin expression. Perilipins are phosphoproteins found in adipocytes on the surface of TAG droplets that acts are gatekeepers, preventing lipases from hydrolyzing TAG to facilitate the release of FFAs. Obese individuals have a deficiency in perilipins even if their fat cells are larger, hence increased basal rate of lipolysis (Tansey *et al*, 2001; Rebrin *et al*, 1995).

There is increasing evidence that the composition of the diet in terms of quality and quantity of fat plays an important role in glucose homeostasis and insulin sensitivity. It is generally agreed that SFA have a detrimental effect on lipoproteins and on insulin sensitivity whilst unsaturated FA have a more beneficial outcome. Animal and human studies have demonstrated that SFA intake increases IR (Taousis *et al*, 2002; Lou *et al*, 1996; Hu *et al*, 2001*b*). As far back as 1979, a study carried out with female Sprague-Dawley rats fed either LA or 20% SFA for 14-days, showed that those rats fed the LA diet had significantly lower

incidence of carcinogenic tumours compared to those on the SFA diet ($p<0.05$) (Kollmorgen *et al*, 1979). Additionally, a positive association between a SFA and CHD has been reported, whereas a negative relationship between unsaturated fat and CHD has also been reported (Willett *et al*, 1993: Keys *et al*, 1986). In prospective studies, it has often been difficult to demonstrate this positive relationship between intakes of dietary SFA and CHD (Renaud & Lanzmann-Petitory, 2001; Hu *et al*, 2001*a*; Pietinen *et al*, 1997). Whereas, numerous cross-sectional trials have found strong associations between FA composition and markers of IR. For example, Warensjö *et al* (2006*b*) found cross-sectional FA composition to be significantly different between subjects with or without MetS, whereby the proportions of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:1), γ-LA (C18:3:6), dihomo-γ-LA (C20:3:6) and Δ6 desaturase activity was significantly higher, on the other hand LA (C18:2:6) and Δ5 desaturase activity was significantly lower in subjects with MetS at the ages of 50 and 70years. Furthermore the study showed that the generated FAs factors, which represent dietary fat quality and endogenous FA metabolism, may be important in the development of MetS, wherein a high factor (low LA) and a low factor (n-3 PUFA) significantly predicted the development of MetS over 20 years, independent of lifestyle factors. The study suggestes that changes in FA composition and desaturase activities may appear long before the onset of MetS. Another recent cross-sectional study set out to determine how dietary FA composition could be used to identify Asian Indian men and women who are at greater risk of obesity and dyslipidemia. Results showed that for men the lean controls had lower waist circumference, WHR, TC, TAG, LDL-c, fasting plasma glucose and intakes of TFA. This study concluded that clinicians should consider obesity measures, metabolic profiles and dietary FA simultaneously to better comprehend the existent of the risk factors associated with the MetS (Ghosh, 2007*a*). Similar results were noted for the female cohort of this study and concluded that dietary FA composition is related to indicators of obesity and the recent shift in dietary habits may have caused an increase in the prevalence of obesity and dyslipidemia (Ghosh, 2007*b*). Moreover, a cross-sectional, population-based Spanish study found a favourable relationship between intakes of MUFA and β-cell insulin secretion (Rojo-Martínez *et al*, 2006). Yet, another cross-sectional Spanish study found that oleic acid (C18:1) levels decreased with age, whereas LA proportion increased significantly with age in the skeletal muscle and AA proportion increase with age in the AT. It was suggested that these findings may be explained on the basis of the changing dietary habits of children, who are

progressively abandoning the habitual *Mediterranean diet* and favouring the high SFA typical *western diet*. Although this study was carried out on children, it also noted an age-independent positive correlation between insulinemia/$HOMA_{IR}$ index and AA content in AT TAG ($r = 0.47, p<0.001$) (Aldámiz-Echevarría *et al*, 2007).

The mechanisms that link dietary FA with CVD have been related to effects in blood cholesterol levels (Schaefer, 2002; Katan *et al,* 1994), IR (Vessby, 2000), inflammation and endothelial dysfunction (De Caterina *et al,* 2006). SFA are known to elevate LDL-c compared to carbohydrates, mysteric acid (C14:0) is the most potent cholesterol elevating FA, followed by lauric acid (C12:0) and palmitic acid (C16:0). However, stearic acid has been reported to remain neutral with regards to blood cholesterol concentrations (Kris-Etherton *et al*, 2005; Katan *et al*, 1994). In contrast, LA is the most promising cholesterol lowering FA to exist (Wijendran & Hayes, 2004) and MUFA oleic acid (C18:1) have also been found to lower plasma LDL-c concentrations (Renaud & Lanzmann-Petithory, 2001). In addition, intake of oleic acid has been shown to lower oxidative stress compared to LA consumption (Kratz *et al*, 2002; Svegliati-Baroni *et al*, 1999).

The role of n-6 and n-3 PUFA on insulin sensitivity remains controversial (Berry, 2001; Roche *et al*, 2005). Studies determining the effect of MUFA on IR have demonstrated improved peripheral insulin sensitivity following MUFA rich diets in both healthy (Vessby *et al*, 2001; Salas *et al*, 1999) and diabetic cohorts (Parillo *et al*, 1992). However, the effect of MUFA on IR is still somewhat controversial since it has also been suggested that dietary oleic acid influences FA oxidation (Lovejoy *et al*, 2002) which may in turn have a negative effect on insulin sensitivity. However, it was recently reported that the intake of n-6 FAs doubled, whereas CHD mortality fell by 50% over a period of several decades in the USA (Willet, 2007). Additionally, fish-oils containing n-3 FA have been demonstrated to inhibit the production of inflammatory cytokines and to decrease the expression of CAM's, hence decreasing the adhesion of lymphocytes to macrophage monolayers (Lo *et al*, 2000; Yaqoob, 2004). A recent study investigated the effects of supplementing 1.8 grams per day (g/d) n-3 PUFA for 2 months to T2DM females. At post-intervention, results revealed that although body weight remained unchanged and no significance change was found for insulin sensitivity, subcutaneous adipocyte mass was reduced, plasma TAG and the ratio of TAG:HDL-c concentrations were significantly decreased as well as a reduction in PAI-1 concentrations compared to those supplemented with placebo (Kabir *et al*, 2007). Several other studies on T2DM subjects witnessed similar results by showing a significant decrease in

plasma TAG concentrations after supplementation with varying doses of n-3 PUFA (Ouguerram *et al,* 2006; Rivellese *et al*, 1996; Annuzzi *et al*, 1991).

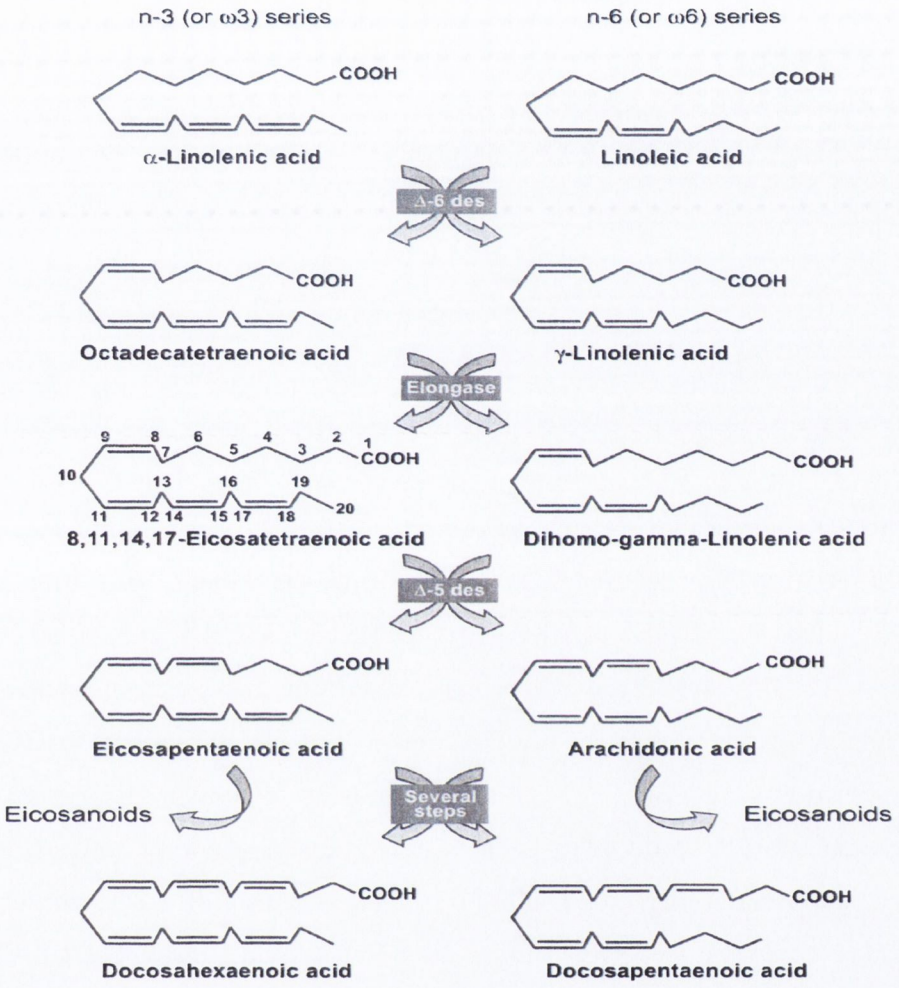
Healthful diets that avoid excess energy intake and reduce SFA consumption may be instrumental in alleviating the current worldwide obesity epidemic (Stone & Schmeltz, 2007; Hu *et al*, 2007). An effective approach to alleviate the prevalence of the MetS may be to focus on the importance of dietary therapy, and in particular the quality of dietary fats being consumed. The KANWU study as reported above included 162 healthy individuals who participated for 3 months and results of this study indicated that insulin sensitivity was significantly impaired by the high SFA-rich diet, yet remained unaffected by the MUFA-rich diet (Vessby *et al*, 2001). Similar results have been reported by Summers *et al* (2002) where a PUFA-rich diet increased insulin sensitivity compared to a diet rich in SFA. Furthermore, Pérez-Jiménez *et al* (2001) conducted a cross-over study in healthy young individuals and found that substituting carbohydrates and MUFA for SFA improved insulin sensitivity. Additionally, CVD mortality was reported to be significantly reduced when a high intake of PUFA and LA were consumed for 15 years (Laaksonen *et al*, 2005).

1.12.1 Long Chain n-3 PUFA on inflammation and insulin sensitivity

The therapeutic and preventative benefits of diets enriched in LC n-3 PUFA on CVD has been well documented. Since the mid-1970s, epidemiologic studies have supported the idea that people who eat fish containing LC n-3 PUFA are at lower risk for several CVD end points than those consuming little or no fish (Kromhout *et al*, 1985).

Recent analyses in the GISSI-Prevenzione study indicated that patients surviving a recent MI had a significantly lower risk of CV death if their diets were supplemented with 1 g/d of LC n-3 PUFA (Investigators GISSI-Prevenzione, 1999). Additionally, in the Nurses' Health Study, women without prior CVD had a lower risk of CHD, including fatal CHD and nonfatal myocardial infarction, with the intake of fish or LC (n-3) PUFA (Hu *et al,* 2002). These CVD benefits were largely associated with the EPA and DHA, whose presence in tissues is directly related to their dietary intake. In a study where rats were fed LC n-3 PUFA rich in EPA and DHA, IR was prevented in muscle by decreasing SFA content and maintaining expression and translocation of GLUT4 receptors in the liver by sustaining the inhibition of hepatic glucose production (Carpentier *et al*, 2006*a*). Laaksonen *et al* (2002) showed that individuals with a high proportion of plasma LA (C18:2:6) had a lower risk for developing T2DM.

Figure 1.11: The n-3 and n-6 PUFA biosynthetic pathway. EPA, DHA and arachidonic acid may be biosynthesized from the essential α-linolenic and linoleic acids (Yaqoob & Calder, 2003).



These findings are in line with dietary epidemiology studies which indicate that individuals with a low proportion of LA or vegetable oil in their diet have an increased risk of developing T2DM (Feskens, 2001).

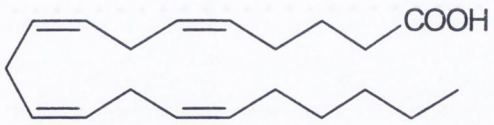
Experimental data at the cellular level, in animal studies and in human studies, suggest that these dietary PUFA have anti-inflammatory, anti-thrombotic, and anti-arrhythmic properties (Calder & Grimble 2002; Harris *et al*, 2003). LC n-3 PUFA are known to lower fasting plasma TAG concentrations and to reduce the postprandial lipidemic response as well as increasing HDL-c concentrations. On the other hand, LC n-3 PUFA are also known to increase plasma LDL-c concentrations (Brouwer *et al*, 2006; Seo *et al*, 2005; Von Schacky & Harris, 2007).

1.13 Conjugated Linoleic Acid (CLA)

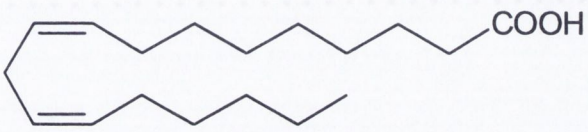
CLA is a generic term for a family of positional and geometric isomers of LA. LA contains two double bonds in positions 9 and 11 or 10 and 12 and they may be in *cis* or *trans* configuration. To date, eighteen isomers of CLA have been identified, however the *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) isomer is the most abundant, accounting for 90%, whereas its counterpart *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA) makes up <10% in natural food products (Choi *et al*, 2005; Chin *et al*, 1994). Typical CLA content in total fat of meat, dairy foods and oils are presented in *Table 1.2*.

The two most common of which are *c*9, *t*11-CLA and *t*10, *c*12-CLA are also shown in *Figure 1.12*. CLA is formed when reactions shift the location of one or both of the double bonds of LA, in such a manner that the two double bonds are no longer separated by two single bonds. Bacterial synthesis (Butyrivibrio fibrisolvens) of CLA occurs through the microbial isomerisation of dietary LA in the digestive tracts of ruminant animals (Pariza *et al*, 2001) and for this reason at least eighteen different isomers have been identified and these differ in the relative position of the two double bonds (Eulitz *et al*, 1999). Smaller amounts of the *c*9, *t*11-CLA isomer can also be synthesised from *trans* vaccenic acid (TVA) by the action of Δ-9 desaturase (Steroyl CoA desaturase) in cows and in humans (Griinari *et al*, 2000; Turpeinen *et al*, 2002). TVA (11-*trans* octadecenoic acid), a major *trans* monoene found in ruminant fat, it is produced in the rumen can be endogenously converted to *c*9, *t*11-CLA by Δ9-desaturase (Griinari *et al*, 2000). In fact, synthesis of *cis*-9, *trans*-11CLA via the Δ9-desaturase enzyme was shown to be the primary source of *c*9, *t*11 CLA in bovine milk fat. Additionally, the desaturation of TVA to *c*9, *t*11-CLA was also shown to occur in humans (Santora *et al,* 2000) and rodents (Salminen *et al*, 1998; Basu *et al*, 2000)

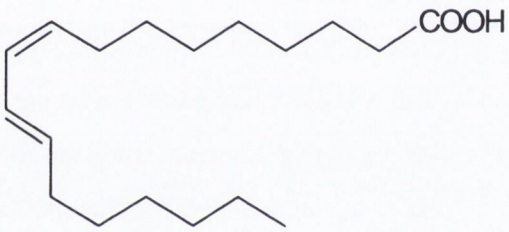
The major sources of CLA in the human diet is fat from ruminants, thus CLA is often considered to be a functional food as it can be consumed as part of a daily diet. CLA is primarily from fat from milk products but also can be obtained from fat within beef and mutton produce. A cow's diet has a major influence on the final concentration of VA and CLA in its milk fat. The highest natural levels of these fatty acids are obtained from cows grazing on fresh pasture rather than being fed concentrate diets, and alpine pastures yield greater concentrations compared to lowland pastures. The CLA content of other diary products like cheese and yoghurt is largely dependent on the CLA content of the milk they are derived from since processing appears to have little or no affect on the final content of the CLA in these products. In recent years the biological activities of CLA have been reported to



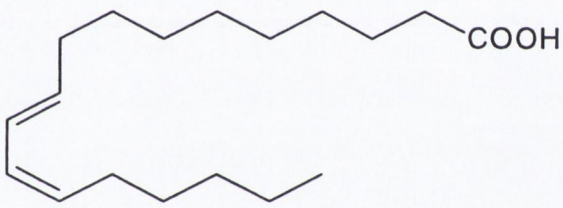
Arachidonic acid



Linoleic acid



cis-9, trans-11 CLA



trans-10, cis-12 CLA

Figure 1.12: Structures of linoleic acid and conjugated linoleic acid isomers examined.

have anti-carcinogenic (Beppu *et al*, 2006), anti-artherogenic (Toomey *et al*, 2005; Arbonés-Mainar *et al*, 2006), anti-obesity (Park *et al*, 2007), anti-adipogenic (Brandebourg & Hu, 2005) and anti-inflammatory effects (Loscher *et al*, 2005). All of these potentially positive target functions highlight the importance of CLA at a time when the increasing prevalence of obesity and the MetS affects not only adults but also a worrisome proportion of adolescents and children, globally (Klein-Platat *et al*, 2005).

1.13.1 Effects of CLA on body composition

CLA has attracted considerable attention because of its reported beneficial effects on body composition and obesity, by decreasing body fat and increasing lean body mass in both *in vivo* and *in vitro* (Simon *et al*, 2006; Kim *et al*, 2006*a*; Gaullier *et al*, 2005; Hargrave *et al*, 2005). To date, clarity still remains illusive on the mechanistic effects of CLA on body composition, although several reports have endeavoured to describe this phenomenon as presented in *Table 1.3* (House *et al*, 2005; Corino *et al*, 2005; Kovacs & Mela, 2006).

The first report to suggest that CLA may have potential as an anti-obesity nutrient was published in 1997, when mice supplemented with 0.5% CLA had a 60% decrease in body fat after 4-5 weeks (Park *et al*, 1997). Accumulating evidence suggests that the *t*10, *c*12-CLA isomer appears to be responsible for the reduction of fat pad weight due to its ability to suppress body fat accumulation (Wargent *et al*, 2005; Evans *et al*, 2002). Therefore, this isomer may have potential in the treatment and management of obesity. Recent literature on CLA shows that body fat accumulation in both human and animal appears to be isomer specific, dose responsive and independent of dietary fat content as well as being influenced by study duration (Mensink, 2005; House *et al*, 2005; Kovacs & Mela, 2006; Wahle & Heys, 2004). In terms of the effect of CLA on obesity in man, the majority of recent studies have investigated the effect of CLA supplementation for short time periods. Gaullier *et al* (2004) supplemented moderately obese subjects with 3.4g/d CLA for 1 year and demonstrated significant weight and body fat losses of 2.0 and 2.2kg, respectively. Contrary to this, no significant differences in body weight or composition were shown when the same amount of CLA was consumed by overweight and obese subjects for the same time period (Larsen *et al*, 2006). Another human study on 81 healthy overweight male and female subjects showed that the consumption of up to 3g/d *c*9, *t*11-CLA or *t*10, *c*12-CLA had no significant effect on body fat mass after 18 weeks (Malpuech-Brugere *et al*, 2004). These results concur with a recent

long term animal study, wherein feeding 344 Fischer rats 1% CLA or control diet for 18 months

Table 1.2: Dietary sources of CLA and content of CLA relative to total fat (Data from Chin *et al*, 1992; Evans *et al*, 2002)

Food Source	g/100g fat	Food Source	g/100g fat
Dairy Products		***Meat/Poultry/Fish***	
Whole Milk	0.55	Lamb	0.58
Low fat milk	0.41	Fresh Ground Beef	0.43
Condensed Milk	0.70	Veal	0.27
Cultured buttermilk	0.54	Fresh Ground Turkey	0.26
		Chicken	0.09
Butter	0.47	Pork	0.06
		Egg Yolk	0.06
Plain Yoghurt	0.48	Salmon	0.03
Low-fat yoghurt	0.44		
Custard style yoghurt	0.48	***Vegetable Oils***	
Frozen yoghurt	0.28		0.07
		Safflower Oil	0.04
Cheddar Cheese	0.41	Sunflower Oil	0.02
Processed Cheese	0.50	Peanut	0.00
Ricotta	0.56	Olive	
Mozarella	0.49		
Cottage	0.45		
Parmesan	0.30		
Ice-cream	0.36		
Sour cream	0.46		

Table 1.3: Potential Mechanisms of Action of CLA to Alter Body Composition

1	Alters membrane structure and function
2	Alters eicosanoid, arachidonic acid and/or prostaglandin metabolism
3	Alters cytokine production or response
4	Alters peroxisone proliferators- activator receptor PPAR activity
5	Alters sympathetic nervous system activity
6	Alters growth hormones and growth factors

did not significantly reduce body fat as compared to controls, but induced chronic renal failure, the severity of which was attenuated by CLA (Park *et al*, 2005*b*). Tsuzuki *et al* (2005) showed that 200mg/d FA supplement providing LA, EPA, CLA or conjugated EPA (CEPA) showed significant weight-loss in male Sprague-Dawley rat's epididyml AT and significant decreases in levels of liver TAG and TC in the group fed the CEPA. There is accumulating evidence to suggest that the *t*10, *c*12-CLA isomer may adversely influence human health by promoting lipodystrophy, which may lead to IR associated lipodystrophy (Larsen *et al*, 2003), which might explain the decrease in AT seen in the Sprague-Dawley rats above.

It is clear that rodents, most especially mice are particularly sensitive to CLA (Kovacs & Mela, 2006). In a study to investigate the combined effects of coconut oil with CLA (7% CO + 0.5% CLA) and soy oil with CLA (7% SO and 0.5% CLA) on body mass showed that mice fed dietary CO + CLA were 11% leaner than controls (Hargrave *et al*, 2005). In KK and KKAy, mildly and moderately obese/diabetic mice respectively, feeding a diet supplemented with 0.5% CLA, consisting of 30.5% *c*9, *t*10-CLA and 28.9% *t*10, *c*12-CLA, for 4 weeks decreased WAT mass (Ohashi *et al*, 2004).

1.13.2 Effects of CLA on insulin sensitivity

IR is the primary hallmark of the MetS, it occurs as result of excess AT and it is associated with disturbances in carbohydrate and lipid metabolism (Shaw *et al*, 2005; Bray & Champagne, 2004). Inconsistencies exist in the studies which examined the isomer specific effects of CLA supplementation on insulin sensitivity between some animal studies which showed a positive effect of CLA (Simon *et al*, 2006) yet, some human studies did not (Moloney *et al*, 2004; Risérus *et al*, 2002). A study investigating the effect of a blend of *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers (3.0g/d CLA) for 8 weeks in healthy subjects demonstrated no significant effects on fasting glucose or insulin concentrations, yet significant improvements were observed for plasma TAG and VLDL metabolism (Noone *et al*, 2002). Another study examining the effects of the *c*9, *t*11-CLA isomer alone showed decreased insulin sensitivity and increased lipid perioxidation in obese middle aged men at high risk for CVD (Risérus *et al*, 2004*c*). In a group of diet-controlled T2DM subjects, CLA supplementation had a negative effect on fasting glucose concentrations and insulin sensitivity when humans were supplemented with 3.0g/d CLA for 8 weeks (Moloney *et al*, 2004). In a recent crossover study determining the health benefits of CLA enriched dairy products (1.421g/d *c*9, *t*11-CLA) versus control dairy products (0.151d/g *c*9, *t*11-CLA) for 6 weeks

showed that consumption of *c*9,*t*11-CLA fortified foods had no significant impact on insulin, glucose or lipid metabolism in healthy middle aged men (Tricon *et al*, 2006). In contrast, young adults with a sedentary lifestyle, supplemented with 4g/d CLA (35.5% *c*9, *t*11-CLA and 36.8% *t*10, *c*12-CLA) for 8 weeks showed reduced fasting insulin concentrations and improved insulin sensitivity compared to placebo (Eyjolfson *et al*, 2004). The whole body effects of CLA on insulin sensitivity appear to be isomer specific, and the human studies carried out would suggest that the effect of CLA supplementation may be dependent upon the age and background insulin sensitivity of the individual.

The data generated from animal studies have been important in defining the potentially diverse effect of CLA isomers. Roche *et al* (2002) has shown that supplementing a high-fat diet with *t*10, *c*12-CLA prevented obesity but induced IR in male *ob/ob* mice. IR was also promoted in mice fed 0.5% CLA oil (30.5% *c*9, *t*10-CLA and 28.9% *t*10, *c*12-CLA) for 4 weeks (Ohasti *et al*, 2004). It has been suggested that leptin deficiency and reduced GLUT4 protein concentrations in adipocytes are key molecular events that led to marked IR in CLA fed mice (Tsuboyama-Kasaoka, 2000; Chung *et al*, 2005). de Roos *et al* (2005) determined the isomer specific effect of CLA in $ApoE^{-/-}$ mice, *c*9, *t*10-CLA reduced glucose and insulin concentrations, improved the indexes of insulin sensitivity (revised QUICKI) and IR ($HOMA_{IR}$). In contrast, *t*10, *c*12-CLA enriched diet increased glucose and insulin concentrations, had a negative impact on the revised QUICKI index of insulin sensitivity and $HOMA_{IR}$ measure of IR (de Roos *et al*, 2005). Choi *et al* (2005) also demonstrated that *c*9, *t*11-CLA was the more effective isomer in the treatment of IR and this was associated with upregulated acyl-coenzyme A oxidase and uncoupling protein-2 (UCP-2) mRNA expression in liver and skeletal muscle.

1.13.3 Effects of CLA on inflammatory status

The MetS is often associated with a sub-acute pro-inflammatory state (Ghanin *et al*, 2004; Dandona *et al*, 2004; Roche *et al*, 2005). Therefore, the effects of CLA on markers of the inflammatory response are important to investigate. The pro-inflammatory, insulin de-sensitising effects of TNFα within the context of the MetS is best characterised as TNFα impedes insulin signalling and has been suggested to be involved in the pathogenesis of T2DM (Roche, 2004). Human obesity is positively related to TNFα expression in WAT and muscle, and plasma levels of TNFα are positively associated with BMI, fasting glucose and TAG concentrations, and inversely associated with HDL-c (Ohya *et al*, 2005). Overall, the

human studies examining the effect of dietary CLA supplementation on markers of inflammation have shown conflicting results. Another pro-inflammatory marker is CRP, which is a major downstream regulator of the acute phase response (APR) and an indicator of inflammation that is often elevated in the MetS and may predict future CVD events (Smedman *et al*, 2005). Another study showed elevated CRP concentration in subjects with the MetS after receiving 3.4g *t*10, *c*12-CLA per day for 12 weeks, however no changes were observed for circulating IL-6 concentrations (Risérus *et al*, 2004*b*). A randomised, double-blind placebo controlled study demonstrated that CLA supplementation (4.2g/d; 75.9% CLA, providing equal proportions of *c*9, *t*11-CLA and *t*10, *c*12-CLA) increased CRP levels compared to placebo (Smedman *et al*, 2005). These findings are important given that elevated CRP concentrations, even at sub clinical levels, is associated with the MetS (Gonzalez *et al*, 2006). Indeed, it has also been demonstrated that supplementation with 3g/d *c*9, *t*11-CLA showed a 15% reduction in insulin sensitivity and this was associated with increased lipid peroxidation in obese men, whereby urinary 8-iso-$PGF_{2\alpha}$ levels were increased by 50% and urinary 15-keto- $PGF_{2\alpha}$ levels were increased by 15% (Risérus *et al*, 2004*c*).

The immune modulating effects of CLA were also evaluated in a population with high risk of CHD (Ramakers *et al*, 2005). Subjects were characterised by LDL phenotype B, which is typified by the presence of increased proportions of the highly atherogenic, small dense LDL particles (Garin *et al*, 2005). Supplementation with 3g/d of *c*9, *t*11-CLA or *t*10, *c*12-CLA for 13 weeks had no significant effect on plasma CRP, TNFα, IL-6 or IL-8 concentrations (Ramakers *et al*, 2005). Peripheral blood mononuclear cell (PBMC) cytokine expression was predominantly enhanced by both CLA isomers as assessed using an antibody array, which may indicate enhanced immune function, however these changes were based on pooled sample analysis and were not significant. Furthermore, Smedman *et al* (2005) also demonstrated no significant effect of either CLA isomer on plasma / PBMC TNFα concentration / expression (Smedman *et al*, 2005) and 3g/d supplementation of an 80:20 blend of *c*9, *t*11-CLA and *t*10, *c*12-CLA had a minimal effect on markers of immune function in PBMC isolated from healthy subjects. Furthermore, our group have shown that supplementation with CLA (3g/d of either a 50:50 or an 80:20 blend of the *c*9, *t*11-CLA and the *t*10, *c*12-CLA isomers for 8 weeks) had no immunological benefit compared to LA (Nugent *et al*, 2005).

CLA is a known agonist of PPARγ (Cimini *et al*, 2005) and the anti-inflammatory effects of CLA may be mediated at least in part by PPARγ dependent inhibition of NF-κB activation (Zhao *et al*, 2005). Activation of NF-κB is critical for the induction of several cytokine and CAM expression. Inhibition of the NF-κB mediated inflammatory response may therefore attenuate pro-atherogenic processes such as CAM expression and chemokine release in the endothelium. It has also been demonstrated that TNFα expression is regulated by PPARγ agonists (Choi *et al*, 2005). CAM expression, monocyte adhesion and chemokine release are strongly induced in response to TNFα which is secreted during acute inflammation and the inflammatory process associated with the development of atherosclerosis (Silswal *et al*, 2005). Again the isomer specific effects of CLA are important determinants of the nature of the inflammatory response. It has been demonstrated that *t*10, *c*12-CLA induces cytokine expression in primary human adipocytes through NF-κB activation (Chung *et al*, 2005). In contrast, physiological concentrations (5-50μM) of *c*9, *t*11-CLA and *t*10, *c*12 CLA that could be achieved by dietary CLA supplementation had no effect on cytokine stimulated CAM expression, monocyte adhesion or chemokine release in human aortic endothelial cells (HAEC) (Schleser *et al*, 2005). In contrast, *t*10, *c*12-CLA inhibited COX-2 protein expression and activity, disrupted NF-κB activation and decreased cytokine gene expression in RAW264.7 macrophages (Li *et al*, 2005). Several other studies have also shown that the immuno-modulatory effects of CLA in various cell culture models are inconsistent (Loscher *et al*, 2005; Luongo *et al*, 2003). In essence, it is unclear from the *in vitro* and *in vivo* studies whether the presence of even small amounts of the *t*10, *c*12-CLA isomer exclusively has deleterious pro-inflammatory effects or whether these effects are due to both the *c*9, *t*11-CLA and *t*11-*c*12-CLA isomers combined.

1.14 Research objectives

Previous work has demonstrated that CLA possesses the ability to modulate insulin sensitivity and risk factors associated with it. At the initiation of this post-graduate doctorate a limited number of studies were conducted to examine the effects of natural and synthetic sources of CLA on markers of inflammation in the IR state. In addition, a limited number of large human dietary intervention studies have been performed to determine the effects of dietary quantity and quality on risk factors associated with IR, T2DM and CVD, with the exception of the KANWU study (Vessby *et al*, 2001). However, this study determined whether a change in dietary fat quality alone alters insulin action in healthy humans. Therefore, the beneficial effect of substituting saturated fat with unsaturated fat on insulin sensitivity remains illusive in in MetS individuals and requires investigation. In addition, in the current literature a gap exists in determining whether the natural and/or synthetic *c*9, *t*11-CLA isomer alone can modulate insulin sensitivity in mice with severe metabolic derangements. Therefore the overall aim of this thesis is to fully understand the concept of the MetS and to investigate if manipulation of dietary fatty acid composition is an appropriate strategy through which IR and the MetS can be treated *in vivo*.

The aims of this thesis are to:

- To elucidate the relationship between natural *c*9, *t*11-CLA, synthetically prepared *c*9, *t*11-CLA and synthetically prepared TVA on metabolic and molecular risk factors associated with the MetS, namely IR, dyslipidaemia, adiposity and inflammation, by conducting an animal experimentation on four week old male, *ob/ob* (C57BL/6J) mice.

- To investigate the interaction between plasma and dietary FAs, inflammation and insulin sensitivity in a trans-EU cross-sectional dataset.

- To determine the efficacy of reducing dietary SFA, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS in the LIPGENE 6th Framework WP1.2 human dietary intervention cohort.

Chapter 2

Materials and Methods

2.1 Animal experimentation

The animal feeding experiment was conducted at the BioResources Unit, Trinity College Dublin (TCD) Ireland according to European Union (EU) animal research welfare protocol (Roche *et al.* 2002). Ethical approval for this study was granted by the Ethical Committee for Animal Studies at TCD under the Protection of Animals used for Scientific / Medical Experiments, Cruelty to Animals Act 1876 as amended by the European Communities (Amendment of Cruelty to Animals Act 1876) regulations 2002.

Thirty, 4 week old male, *ob/ob* (C57BL/6J) mice were purchased from Harlan, UK. The mice were acclimatised for 7 days during which time they received a purified control diet, before, being assigned to one of four treatment groups as described in *Table 2.1* for a 28 day period. During the intervention period, the animals were exposed to 12 hrs light/ 12 hrs dark cycles, maintained at a constant temperature of 22°C. The *ob/ob* mouse model is a well characteristised model for obesity and IR. It has been reported that at 28 days of age, the *ob/ob* mouse contains 3 times as much fat compared to a lean mouse of same age (Thurlby & Trayhurn, 1978).

Table 2.1: Description of the dietary treatments fed to the *ob/ob* mice

	Diet Description	n
Diet A	*Control Diet* Low CLA Beef & Linoleic Acid (LA)	8
Diet B	*CLA Beef Diet* Meat derived *c9,t11*-CLA (High Natural CLA beef)	7
Diet C	*Synthetic Diet* Low CLA & Synthetic derived *c*9,*t*11-CLA	8
Diet D	*TVA Diet* Low CLA & Synthetically derived TVA	7

LA : (Linoleic Acid); **CLA** : (Conjugated Linoleic Acid); *c*9, *t*11-CLA : (*cis*-9, *trans*-11-CLA) ; **TVA** : (*trans*Vaccenic Acid); **n** : (number).

2.1.1 Dietary composition of the animal feeds

Diets were produced by Special Diets Service, Essex, UK and were received as 1kg vacuum packed, heat sealed plastic bags. The synthetic conjugated linoleic acid (CLA) was produced by Lipid Nutrition (Looders Crooklan, The Netherlands), all other CLA test material was

provided by Teagasc (Grange Research Centre, Dunsany, Co. Meath). The control diet, linoleic acid (LA) (Diet A) was composed of freeze dried low CLA beef at a 36% inclusion rate blended to a homogenous mixture with (18% / final feed) wheat feed and (18% / final feed) of maize feed diet. The high natural *c*9, *t*11-CLA beef (Diet B) was prepared by the addition of high CLA beef to a supplementation level of 36% (2.3kg/ 6.3kg feed), which corresponds to 600mg/100g final feed. It was composed of 36% freeze dried beef blended to a uniform mixture with (18% / final feed) of wheat feed and (18% / final feed) of maize.

The synthetic *c*9, *t*11-CLA (Diet C) and synthetic trans vaccenic acid (TVA; Diet D) was composed of freeze dried low CLA beef at a 36% inclusion rate blended to a homogenous mixture with (18% / final feed) wheat feed and (18% / final feed) of maize feed diet. The synthetic CLA (Diet C) mixture was supplemented with 44.16g of synthetic CLA / 6.3kg feed, corresponding to 700mg/100g supplementation level. Whereas, the synthetic TVA (Diet D) mixture was supplemented with 68g of a combined mixture of TVA and vaccenic acid (VA) per 6.3kg of feed. The w/w% composition of the diets is presented in *Table 3.3*.

2.1.2 Preparation of the animal feeds

Study diets were prepared by the mixing together of 100ml of warm water at a temperature of 40°C-50°C per 100g of blended test diets until a liquid consistency was achieved. The mixture was transferred to a Petri dish of 5-10mm in depth and allowed to set overnight at 4°C. The final food had a spongy, oily texture of approximately 55% water. Dietary food intake was measured daily and the animals received freshly prepared food each day.

2.1.3 Blood sample collection and handling protocol

The mice were sacrificed at day 28 of the dietary intervention period. Food was removed from the cages at 6:00pm and the animals were sacrificed the following morning between 8:00am - 10:00am, in the fasted state. The animals were enthusised using Carbon Dioxide (CO_2) and cardiac puncture was performed to draw blood samples. Blood was transferred to a cooled sodium citrate blood vaccutainer tube (BD Vaccutainer, Dublin, Ireland), allowed to clot and centrifuged at 1500rpm for 15 mins at 4°C, where the plasma was harvested and aliquioted before storage at -70°C until further analysis.

2.1.4 Tissue collection protocol

Tissue samples for gene expression analysis were harvested and immediately immersed in 0.5ml RNA*Later* (Ambion, AMS Techonology) and subsequently stored at -70°C. Tissue samples for protein analysis were snap frozen in Liquid Nitrogen (Coopers Cryoservice) and stored at -70°C until further analysis.

2.2 Glucose and Lipid Analyses

2.2.1 Determination of glucose analysis

Plasma glucose concentrations were analysed using an endpoint enzymatic glucose oxidase, peroxidase, choromogen sequence, colorimetric assay (Biomérieux, France). The assay is based on the glucose oxidase methods were glucose reacts with oxygen to produce gluconic acid and hydrogen peroxide. The colour intensity was measured at 505nm and is proportional to the quantity of glucose in the sample. Glucose assay was calibrated on RA-XT chemistry analyser using Biomérieux calimat (Biomérieux Lyotrol N) and bayer control serum 2 were used as quality control samples for the glucose assay.

2.2.2 Determination of cholesterol analysis

Plasma cholesterol levels were measured using an endpoint enzymatic-based assay from Randox Laboratories (Co. Antrim, UK) and adapted to microtitre plates (Nunc, Roskilde, Denmark). The assay is based on the principal that cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidise. 3.5µl of plasma sample was added to 350µl of the assay reagent. An equal amount of the appropriate standard was added to the assay reagent. Solutions were mixed and allowed to sit for 10 mins at room temperature (RT). The absorbance of the sample was then measured against the reagent blank at 490nm on a microplate reader (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK). The cholesterol level was calculated by dividing the absorbance of the sample by the absorbance of the standard and multiplying this by the concentration of the standard, which is known to be 5.17mmol/l (200mg/dl). The level 5.17mmol/l is perceived to be the most desirable concentration of plasma cholesterol. Values between 5.17mmol/l and 6.18mmol/l (200-239mg/dl) are interpreted as borderline concentrations. Whereas, concentrations greater than 6.20mmol/l (240mg/dl) are deemed to be high blood cholesterol volumes.

2.2.3 Determination of TAG analysis

Plasma TAG was determined after enzymatic hydrolysis with lipases. (Randox Laboratories, Co. Antrim, UK). The indicator, quinoneimine was formed from hydrogen peroxoide, 4-aminophenazone and 4-chlorophenol under the catalytic influences of peroxidise. A plasma sample of 3.5µl was added to 350µl of the assay reagent. 3.5µl of the assay standard was also added to the assay reagent. Solutions were mixed and allowed to sit for 10 mins at RT. The absorbance of the sample was then measured against the reagent blank at 490nm on a microplate reader (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK). TAG values were calculated by dividing the absorbance of the standard and multiplying by 2.29mmol/l.

2.2.4 Determination of non-esterified fatty acids (NEFA)

Plasma NEFA analysis was carried out using Randox non-esterified fatty acids (NEFA) kit (Cat # FA 115). This enzymatic assay involves the conversion of NEFA to acyl CoA by the addition of ATP and CoA, through the action of Acyl CoA Synthetase. Acyl CoA oxidase oxidises Acyl CoA to 2,3-trans-Enoyl-CoA and H_2O_2. 4-aminoantipyrine and N-ethyl-N-(2hydroxy-3-sulphopropyl) m-toluidine is coupled with hydrogen peroxide. This is then acted on by a peroxidase enzyme resulting in the production of purple adduct which can be read spectrophotometrically at 505nm. NEFA assay was calibrated on a Technicon RA-XT clinical chemistry analyser using NEFA 1 mmol/l standard, supplied with the Randox NEFA kit. The NEFA quality control plasma was supplied by Wako chemicals (Cat # 416-00201). The normal concentrations of fasting NEFA is between 0.1-0.9 mmol/l.

2.3 Determination of inflammatory mediators

Mouse IL-6 and total adiponectin were analysed by an Ultra-Sensitive sandwich enzyme-linked immunosorbent assay (ELISA) kit (BioSources International, CA, USA and R&D Systems, respectively). A monoclonal antibody specific for each parameter was pre-coated onto the wells of a microtiter plate. Diluted mouse serum samples as well as standards and internal controls were pipetted into the appropriate wells of the 96 well plate. A novel technique called a multiplex ELISA was employed for the analysis of insulin, tumour necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), resistin and plasminogen activated inhibitor-1 (PAI-1) in unison (Linco Research Inc, MO, USA).

2.3.1 Principal of the IL-6 ELISA

During the first incubation, the interleukin (IL)-6 antigen binds to the immobilized capture antibody and after the first wash, a biotinylated monoclonal antibody specific for IL-6 was added. During the second incubation, the antigen binds to the immobilized IL-6 captured during the first incubation. After the removal of the excess second antibody, Streptavaidin-Peroxidase enzyme was added and this binds to the biotinylated antibody to complete the four member sandwich. After a third incubation and wash to remove all unbound enzyme, a substrate solution was added, to produce colour. The intensity of the colour product was directly proportional to the concentration of IL-6 present in the original specimen. The OD absorbance was read at 450nm.

2.3.2 Principal of the adiponectin ELISA

A monoclonal antibody specific for mouse adiponectin was pre-coated on to a microplate. Standards, controls and samples were reconstituted and pipetted into the appropriate wells of the microtitre plate. During the first incubation period of 3 hrs at RT, adiponectin present was bound to the immobilized antibody. After the first incubating period, the plate was washed to remove any unbound substances using a wash buffer supplied with the kit. Subsequently, an enzyme-linked polyclonal antibody specific for mouse adiponectin was added to the wells and incubated for 1 hr at RT. This was followed by another wash to remove any unbound antibody-enzyme reagent and then substrate solution was added to the wells and the plate was protected from light, while incubating at RT for 30 mins. This reaction yielded a blue product which turned to yellow after the addition of the stop solution. The intensity of the colour measured was in direct proportion to the amount of mouse adiponectin bound in the initial step. The OD of the plate was read at 450nm and the wavelength correction was set at 540nm.

2.3.3 Determination of Insulin, TNFα, MCP-1, Resistin and PAI-1 by multiplex ELISA

A multiplex assay kit manufactured by Linco Research, Missouri, USA was employed to simultaneously quantify insulin, TNFα, MCP-1, Resistin and PAI-1 concentrations from mouse serum samples. *Table 2.2* and *Table 2.3* present the multiplex preparation and procedure, respectively. *Table 2.4* illustrates the expected ranges for the ELISA multiplex quality controls and the actual ranges achieved.

Table 2.2: Preparation of all reagents for the multiplex ELISA for the determination of insulin, TNFα, MCP-1, Resistin and PAI-1 concentrations

PREPARATION OF REAGENTS FOR IMMUNOASSAY			
Step 1	**Prepare the mouse adipokine standard cocktail** Reconstitute with 250μl dH_20 to give a 50,000 pg/mL concentration (top standard) Allow vial to set for 5mins, invert several times to mix gently Vortex and mix well		
Step 2	**Preparation of Standard Curve**		
	Standard Concentration (pg/mL)	Volume of Assay Buffer to Add	Volume of Standard to Add
	50,000		
Std 1	12,500	150μl	50μl of 50,000
Std 2	3,125	150μl	50μl of 12,500
Std 3	781.3	150μl	50μl of 3,125
Std 4	195.3	150μl	50μl of 781.3
Std 5	48.8	150μl	50μl of 195.3
Std 6	12.2	150μl	50μl of 48.8
Std 7			
	The assay buffer acts as the 0 standard (background)		
Step 3	**Preparation of Controls** Reconstitute mouse adipokine control I and control II with 250μl dH20 Allow the vials to set for 5mins, insert the vial several times to mix and vortex.		
Step 4	**Preparation of the Wash Buffer** Bring the 10X wash buffer to RT and mix to bring all salts into solution. Dilute 30mL of 10X wash buffer with 270mL dH_20.		
Step 5	**Preparation of Antibody Immobilized Beads** Sonicate each antibody bead bottle for 30secs, vortex for 1min . Add 0.15mL for each antibody bead bottle to the mixing bottle and bring final volume to 3mL with bead diluent.		
Step 6	**Preparation of Serum Matrix** Add 1.0mL of dH_20 to the vial containing the lyophilized serum matrix, gently swirl the bottle and then allow to set for 5-10mins		

Table 2.3: Multiplex ELISA Immunoassay Procedure for the determination of insulin, TNFα, MCP-1, Resistin and PAI-1 concentrations

IMMUNOASSAY PROCEDURE	
Step 1	Block the filter paper by pipetting 200μl of assay buffer into each well of the microtitre plate. Seal and mix on a plate shaker for 10mins at RT
Step 2	Remove assay buffer by vacuum and dry the bottom of the plate using a paper towel
Step 3	Add 10μl of assay buffer to the 0 standard (background)
Step 4	Add 10μl of assay buffer to the sample wells
Step 5	Add 10μl of each standard or control into the appropriate wells
Step 6	Add 10μl of serum matrix to the background, standards and control wells
Step 7	All 10μl of sample into the appropriate wells
Step 8	Vortex bead bottle and add 2.5μl of mixed beads to each well
Step 9	Seal, cover with aluminium foil and incubate with agitation on a plate shaker overnight at 4°C. After overnight, allow plate and reagents to reach RT before continuing
Step 10	Gently remove fluid by vacuum
Step 11	Wash plate 3 times with 200μl/well of wash buffer, remove eash buffer by vacuum filtration between each wash. Dry the bottom of the plate using a paper towel
Step 12	Pipette 50μl of detection antibody into each well
Step 13	Seal, cover with aluminium foil and incubate with agitation on a plate shaker for 30mins.
Step 14	Add 50μl streptavidin-phycperythrin (SAPE) to each well containing the 50μl of detection antibody.
Step 15	Seal, cover with aluminium foil and incubate with agitation on a plate shaker for 30mins at RT
Step 16	Gently remove all contents by vacuum
Step 17	Wash plate 3 times with 200μl/well wash buffer removing wash buffer by vacuum filtration between each wash. Dry the bottom of the plate using a paper towel
Step 18	Add 100μl of sheath fluid to all wells. Seal, cover with aluminium foil and resuspend the beads on a plate shaker for 5mins
Step 19	Read plate on Luminex Instrument (ACS®, STarStation™)

Table 2.4: The expected ranges for quality control for the multiplex ELISA for the determination of insulin, TNFα, MCP-1, Resistin and PAI-1 concentrations

Parameter	QC Level	Units	Expected Range	Range Achieved
Insulin	Control I	pg/mL	1311 - 2724	2390
	Control II		6824 - 14173	12900
MCP-1	Control I	pg/mL	1186 - 2463	2320
	Control II		6330 - 13147	12800
Resistin	Control I	pg/mL	404 - 838	687
	Control II		2122 - 4408	5650
TNFα	Control I	pg/mL	277 – 575	368
	Control II		1326 - 2753	2530
PAI-1	Control I	pg/mL	726 - 1508	1080
	Control II		3569 - 7414	7030

QC (Quality Control); **MCP-1** (Monocyte Chemoattractant Protein-1); **TNFα** (Tumour Necrosis Factor-α); **PAI-1** (Plasminogen Activated Inhibitor-1)

2.4 Immunohistochemical analysis of tissue samples

2.4.1 Tissue preparation

Tissue samples were collected and fixed in formal saline (0.9% NaCl, 10% formaldehyde) for 24 hrs. The tissues were then processed on a Leica TP1020 tissue processor (Leica, Germany) using the following schedule:

Formalin	2 hrs
70% methylated spirit	1 hr
95% methylated spirit	1 hr
Spirit/ Abs. Alc	1 hr
Spirit/ Abs. Alc	1 hr
Spirit/ Abs. Alc	1 hr
Spirit/ Abs. Alc	1 hr
Xylene	1 hr
Xylene	1 hr
Paraffin wax	2 hrs

Paraffin wax	2 hrs

The tissue was embedded in paraffin wax on a Leica EG1140H tissue embedder (Leica, Germany). Tissue sections, 5μM thick, were cut on a Leica Microtome onto poly-1 lysine coated slides which were incubated overnight at 37°C.

2.4.2 Deparaffinisation and re-hyration of tissue samples

The slides were deparaffinised and hydrated as follows:

Xylene	5 min
Xylene	5 mins
Xylene	5 mins
100% Methanol	15 secs
100% Methanol	3 mins
90% Methanol	3 mins
70% Methanol	3 mins
50% Methanol	3 mins
30% Methanol	3 mins
PBS	5 mins

2.4.3 Immunohistochemical staining

The endogenous peroxidise activity was quenched by incubating the slides for 1hr in 0.3% H2O2 in methanol. The slides were washed 3 times for 5 mins in phosphate buffered saline (PBS). The tissue was blocked for 1hr in normal serum from a species in which the secondary antibody was raised (normal serum concentrate, provided with the Vectastain Elite kit, Vector Labs, was diluted in 1X PBS). After blocking, sections were incubated in primary antibody for 1-2 hrs at RT or overnight at 4°C. Specific optimisation was required for each antibody. Sections were washed again 3 times in PBS before being incubated in the appropriate biotinylated secondary antibody provided with the Vectastain kit for 1hr at RT. The slides were washed again as above and incubated in the ABC complex provided with the Vectastain kit for 30 mins at RT. Following this, the slides were washed as before and exposed to diaminobenzibine chromagen (DAB) (Sigma Aldrich, Ireland) for 3-15 mins. Sections were counterstained with haematoxylin (Sigma-Aldrich, UK), washed briefly in tap water and cleared with 3 separate washes of xylene. The slides were mounted in DPX mounting media

(DakoCytomatin, California, USA). Expression of F4/80 (Abcam, Cambridge, UK) by macrophages was analysed.

Table 2.5: Antibodies and the species in which they were raised used in immunohistochemical studies.

Antibody	Source	Company
GLUT4	Goat	Santa Cruz, Biotechnology, CA, USA
F4/80	Rat	Abcam
CD3	Rabbit	Abcam
CD11β	Rat	Abcam

2.5 RNA extraction and analysis

2.5.1 RNA extraction

Total ribonucleic acid (RNA) was isolated from subcutaneous white adipose tissue (WAT) samples using TRI Reagent® (Molecular Research Centre Inc. OH, USA). Tri Reagent combines phenol and guanidine thocyanate in a mono-phase solution which facilitates immediate inhibition of RNase activity, while maintaining high quality RNA integrity. 50mg sections of adipose tissue were homogenised in 1ml TRI Reagent for approximately 30secs at RT using a T18 basic Ultra Turrax® homogeniser (Ultraturrax IKA® T18 Basic Works, Inc, Wilmington, USA). Homogenate was transferred to 1.5ml ribonuclease (RNase)-free microfuge tubes and incubated at RT for 5 mins to allow dissociation of nucleoprotein complexes. The homogenate samples were supplemented with 0.2ml chloroform, shaken vigorously for 15 secs, stored at RT for 15 mins and subsequently centrifuged (Micromax RF 320, Thermo Electron Corporation) at 12,000*g* for 15 mins at 4°C, to allow phase separation into aqueous and organic phases. The colourless aqueous phase contains the RNA and this was pipetted in to a fresh RNase free microfuge tubes. RNA was precipitated by the addition of 0.5ml 100% isopropanol and incubation at RT for 10 mins. The RNA was pelleted by centrifugation at 12,000g for 15 mins at 4°C and stored at -20°C for 2 hrs, to allow for pellet formation. Subsequently, samples were thawed at RT for 10 mins, the supernatant layer was removed and the pellet was washed in 1ml 75% ethanol. Centrifugation was carried out for 5 mins at 12,000*g* at 4°C, after which the ethanol wash was removed and the RNA was allowed to air dry for 3-5 mins. The RNA samples were then resuspended in 20 - 30μl of RNase free

water (Sigma-Aldrich, Poole, Dorset, UK) and removed for storage at -80°C until required for further analysis.

2.5.2 Quantification of RNA

The quantity and purity of the eluted RNA was determined spectrophotometrically by measuring absorbance at 230nm, 260nm and 280nm (Eppendorff biophotometer, Hamburg, Germany) in an UVette® (Eppendorff AG, Hamburg, Germany). RNase free water was used as the diluent control. An $OD_{260/280}$ less than 1.6 implies partially dissolved RNA samples; 1.7-2.0 indicates good purity while an $OD_{260/280}$ of greater than 2 implies purity. An $OD_{260/230}$ greater than 2 indicates no residual guanidinium contamination.

2.5.3 RNA agarose gel electrophoresis

The integrity of the RNA was determined using agarose gel electrophoresis. The gel was prepared by dissolving 1g agarose in 100ml 1X BSSE buffer (0.5M boric acid, 1M Na Tetraborate, 0.05M Na sulphate, 0.01M EDTA) on a hotplate. 5µl ethidium bromide (Sigma-Aldrich) was added after the agarose solution had cooled to approximately 55°C. Following mixing, the gel was poured and allowed to set in the fume hood for 1hr prior to electrophoresis. Approximately 1µg total RNA is required, therefore each sample was calculated in order to provide this amount. The volume of each sample was added to 10µl 2X RNA loading dye. Subsequently, all samples were loaded on the gel and it was run until the bromophenol blue tracing dye had run three quarters of the length of the gel. RNA was visualised using a UV transilluminator (Edvotekm MD, USA). Intact RNA is represented by two clear bands representing 28S (upper band) and 18S (lower band) RNA indicate its integrity.

2.6 Real-time reverse transcription TaqMan® PCR

Real-time reverse transcription Polymerase Chain Reaction (PCR) allows for sensitive detection and quantification of PCR amplification while the reaction is occurring. Data is collected throughout the PCR process and reactions are characterised by the time when amplification of a target is first detected, rather than the amount of target accumulated at the final phase end of the PCR reaction.

TaqMan® chemistry uses a fluorogenic probe to enable the detection of a specific PCR product. When the probe is intact, the proximity of the reporter dye (attached to the 5' end of the probe) and quencher dye (attached to the 3' end of the probe) results in suppression of reporter fluorescence. During the PCR reaction, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. Taq DNA Polymerase cleaves the probe if it is hybridised to the target, displacing the probe and separating the reporter and quencher dyes, thus increasing the flouresence of the reporter dye (Livak *et al*, 1995). This process continues during every amplification cycle. An increase in fluorescence signal emitted is detected only if the target sequence is complimentary to the probe and amplified during PCR, hence non-specific amplification is not detected. Accumulation of PCR product is detected directly by examining the increase in fluorescence emitted by the reporter dye. Thus, samples that contain fewer starting copies of the target gene require a greater number of amplification cycles to generate a detectable signal.

In order to account for small differences in the amount of template added to each reaction, target gene levels were normalised to an endogenous control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference/housekeeping gene in both tissue and cell experiments. Relative quantification was used to analyse changes in gene expression in a given sample. Standard curves for both the target and endogenous reference were generated from pooled RNA from the experimental set of samples. The Ct [the fractional cycle number at which fluorescence signal exceeds a fixed threshold level in the exponential phase of amplification (Higuchi *et al,* 1991)] value was plotted against log standard complementary deoxyribonucleic acid (cDNA) concentration (ng). For each sample the amount of target and endogenous control gene expression were extrapolated from the appropriate standard curve using the Ct readings for each sample. The target amount was then divided by the endogenous reference (GAPDH) amount to obtain the normalised target value.

2.6.1 Real-time TaqMan® PCR

RNA expression was quantified by real time PCR (RT-PCR) using an ABI 7700 Sequence Detection System (Applied Biosystems, Warrington, UK). Each 25μl reaction contained 20ng cDNA, forward and reverse primers, probe and a mastermix solution containing deoxynucleotides, reaction buffer and Taq polymerase. For the gene expression analysis outlined in *Chapter 3*, 2X TaqMan® Universal PCR Mastermix (Applied Biosystems, Warrington, UK) were used. Target genes were investigated using pre-developed primer and

probe kits (Taqman® Gene Expression Assays, Applied Biosystems, Warrington, UK). Reactions in which cDNA was omitted served as a no template control, and cDNA in which the M-MLV RT was omitted served as a no amplication control.

2.6.2 Isolation of total protein extract from white adipose tissue

Total protein from *ob/ob* mice was isolated using the lysis buffer (50mM Tris-HCL pH8.0), 300mM NaCl, 5mM EDTA, 5mM EGTA, 2mM dithiothreitol, 50mM ß-glycerol phosphate, 50mM NaF, 1mM $NaV0_4$, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride (PMSF), 10µg/ml leupeptin, and 14µg/ml aprotinin) as previously described (Takemori *et al*, 2002). 0.3-0.5g adipose tissue was homogenised in 2ml lysis buffer using a T18 basic Ultra Turrax® homogeniser (IKA® Works, Inc, Wilmington, USA). The homogenate was allowed to sit on ice for 30 mins at 4°C, with regular vortexing, followed by centrifugation at 12,000g for 30 mins at 4°C to remove insoluble material. The supernatant was collected as the total protein fraction, vortexed and stored at -20°C for future protein estimation.

2.6.3 Principle of immunoblotting.

Western Blotting or immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. Protein concentration was determined using a Bradford assay using bovine serum albumin (BSA) as a standard (BioRad, Hercules, CA, USA). The proteins are first separated by electrophoresis through SDS-polyacrylamide gels, and then transferred electophoretically from the gel to a membrane (usually nitrocellulose or polyvinylidene fluoride, PVDF). The specificity of the subsequent antibody-antigen interaction enables a single protein to be identified in the midst of a complex mixture of proteins. A suitable secondary antibody, which was conjugated to an enzyme (e.g. horseradish peroxidase), binds to the primary antibody was employed. An appropriate substrate is then added to the enzyme, and together they produce a detectable product, the intensity of which correlates with the abundance of the antigen on the blotting membrane.

2.6.4 Western blotting

The semi-dry method for transfer of electrophoresed proteins to immobilizing membranes as described by Towbin *et al* (1979), was preformed using a Sigma-Aldrich semi-dry blot system (Sigma-Aldrich, Poole, Dorset, UK). During the SDS-PAGE stage, the blot sandwich was prepared as follows: cathode, 2 sheets of Whatman 3mm filter paper (Whatman International

Ltd., Maidstone, UK) pre-soaked in transfer buffer, pre-soaked nitrocellulose membrane, gel, another 2 sheets of pre-soaked filter paper and finally the anode. Care was taken to ensure the absence of air bubbles and to note the orientation of the gel. Electrophoresis transfer was performed at 189mA for 45 mins.

2.6.5 Detection of antigens

After transfer, the blot sandwich was carefully opened, and the membrane removed with a forceps. The choice of block buffer (used to suppress non-specific absorption of antibodies), wash buffer and secondary antibody depends on the antigen under investigation and the choice of antibody. *Table 2.3* outline the conditions used to detect the antigens investigated in experiments outlined in *Chapter 3.* All incubations and wash steps were performed on a roller to ensure thorough mixing.

Table 2.6: Detection of IRS-1 and GLUT4 by Western Blot

STEP	**IRS-1** (IRS-1 antibody, Cell Signalling Technology, Beverly, MA, USA)[1]	**GLUT4** (Chemicon International, Inc, Temecula, CA, USA)
1 hr block at RT with agitation	5% w/v BSA-TBS/Tween 20 (0.1%)	5% w/v BSA-TBS/Tween 20 (0.1%)
6 x 5 mins wash steps with TBS/Tween 20 (0.1%)		
Primary antibody[2,3]	1:1000 dilution in 3% w/v BSA-TBS/Tween 20 (0.1%)[2]	1:1000 dilution in 3% BSA-TBS/Tween 20 (0.1%) (0.1%)[3]
6 x 5 mins wash steps with TBS/Tween 20 (0.1%)		
Secondary Antibody[4]	1:1500 dilution in 3% w/v BSA-TBS/Tween 20 (0.1%)	1:1500 dilution in 3% w/v BSA-TBS/Tween 20 (0.1%)
10 x 5 mins wash steps with TBS/Tween 20 (0.1%)		

[1]Band between 78 and 120kDa represents the insulin receptor as described by Rui *et al*, 2001

[2]Membrane with IRS-1 antibody incubated for 1hr at RT, prior to incubating overnight at 4°C, followed by a further 2 hrs incubation at RT the following day

[3]Membrane with GLUT4 antibody incubated overnight at 4°C, followed by a further 30 mins incubation at RT the following day

[4]Membrane incubated for 1 hr at RT with peroxidase-conjugated secondary antibody (polyclonal goat anti-rabbit immunoglobulin (Ig) G-peroxidase-conjugated immunoglobulins; Sigma-Aldrich, Poole, Dorset, UK)

Antigens were detected by incubating the membrane in a 1:1 solution of luminol/enhancer solution: stable peroxide solution (Supersignal® West Pico Chemiluminescent Substrate, Pierce, Illinois, USA) for 5 mins at RT. Chemiluminescence involves oxidation of luminol in the presence of horseradish peroxidase and hydrogen peroxidase, to form an excited state product (3-aminophthalate), resulting in light emission at 425nm as it decays to the ground state. The enhancer increases the intensity of the light emission and reduces background light emission due to oxidation of luminol by peroxide or other oxidants. This system allows multiple exposures to be performed to obtain the best image. Following the 5 mins incubation, excess reagent was drained from the membrane, which was then placed between two sheets of transparent acetate in an autoradiography cassette (Sigma, Poole, Dorset, UK) before being exposed to Fugi RX film (Amersham Bioscience, Buckinghamshire, England) from 2 mins to overnight exposure, depending on the exposure required. The films were developed using an AGFA CURIX 60, Type 9462/100/140 (AGFA-Gevaert, AG Munich, Germany).

2.6.6 Reuse of western blots

Before probing for a different protein, the blot was initially washed in TBS/Tween 20 (0.1%) or PBS/Tween 20 (0.05%) for 3 x 10 mins, followed by incubation at 50°C for 30 mins in stripping buffer (Restore™, Pierce). After re-washing the blot for a further 6 x 5 mins in TBS/Tween 20 (0.1%), it was ready to use again commencing with the blocking stage in western blot protocol.

2.6.7 Quantification of protein bands

Quantification of protein bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System, Syngene). Molecular weight estimation was confirmed by the inclusion of a suitable standard (Precision Plus Protein™ Standards Dual Colour, Bio-Rad Laboratories Inc.). Results were expressed as arbitrary units or normalised to controls and expressed as fold change relative to control.

2.7 LIPGENE – WorkPackage 1.2 Human Dietary Intervention Study

2.7.1 Recruitment of the Cohort

The Ethics Committee of the St James's Hospital and Federated Dublin Voluntary Hospitals granted approval for the study to be conducted for the TCD cohort. The study was approved by the local ethics committees at each of the 8 clinical intervention centres, all of which conformed to the Helsinki Declaration of 1975 as revised in 1983.

Written informed consent was obtained from all volunteers prior to commencing the study. 15,000 individuals were screened of which 483 subjects with the MetS participated in the 8 research centres throughout the trans EU, LIPGENE 6th Framework project. Within the TCD cohort, the volunteers were recruited primarily from personnel in TCD, out-patients of the Endocrinology Departments of Adelaide and Meath Hospital, Dublin incorporating the National Children's Hospital (AMNCH), Tallaght, Loughlinstown Hospital and employees from a local taxi firm and the general public.

2.7.2 Health and lifestyle questionnaire

A health and lifestyle questionnaire was given to the volunteers on the screening day to assess socio demographic and lifestyle, smoking and alcohol drinking behaviour, daily physical activity and female health, if appropriate. *Table 2.8* outlines the inclusion and exclusion criteria implemented by LIPGENE, *Table 2.9* outlines the endocrine and inflammatory conditions excluded, while *Table 2.10* outlines the hyperlipidmeic medication excluded from the LIPGENE human dietary intervention study.

Table 2.7: LIPGENE – WorkPackage 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 Best Foods, The Netherlands **	UF

* Co-ordinating Centre
§ Core LIPGENE analysis was completed in these centres
** Sole purpose was to create the LIPGENE Dietary Intervention Foods

Table 2.8: 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	High consumers of oily fish (> 2 serving of oily fish per/week 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

supplements (e.g. garlic, anti-oxidants, etc)	
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 /Xenical and Sibutramine /Reductil)
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/week
	Volunteers planning to begin a special diet to loose 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 (weeks) 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.

** see *Table 2.10* below for further information

Table 2.9: Endocrine and inflammatory conditions excluded from the LIPGENE Human Dietary Intervention Study

Endocine Disorders	**Chronic Inflammatory Conditions**
Diabetes (Type 1 and 2)	Inflammatory Bowel Diseases
Hypothyriodism *	Crohns Disease
Hyperthyroidism	Ulcerative Colitis
Adrenal disorders	Rheumatoid Arthritis
Addisons Disease	Lupus
Cushing Disease	Atopic dermatitis

Pheochromocytomas	Psoriasis, Coeliac disease
Multiple Endocrine Neoplasia (MENS)	Fibromyalgia
Other rare conditions encountered	Pelvic inflammatory Disease, Endometriosis
	Severe Asthma
	Other rare conditions encountered

* Subjects diagnosed >1year and on regular thyroxine therapy were included

Table 2.10: Hyperlipidmeic medication excluded from the LIPGENE Human Dietary Intervention Study

Hyperlipidemic Medication	
Chloestyramine	Fenofibrate
Ciprofibrate	Atorvastatin
Isaghula	Pravastatin
Fluvastatin	Gemfibrozil
Rosuvastatin (Crestor)	Nicotinic acid
Acipimox	Cholestin
Zetia	Benzafibrate
Red Rice Yeast (monoscus purpureus)	Gemfibrizol
Supplements	Cerivastatin
Colestipol	Simvastatin
	Ezetimible
Anti-inflammatory Medication*	**NSAIDS†**
Steroids	Ibuprofen (Brufen, Fenbid, Codfen)
Corticosteriods	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 paracetemol before and during the study, however, each subject was instructed to consume for pain relief only.

** A standard dose of 75mg was acceptable

2.8 Collection of anthropometric measurements

Anthropometric and blood pressure measurements were recorded prior to donating of a fasting blood sample. Body weight (nearest 0.1kg), height (nearest 1cm), waist and hip circumference were verified. All body measurements were performed in duplicate with the exception of height, body composition and body weight which were taken in triplicate and the means recorded. Volunteers were instructed to wear light indoor clothing. During measurements volunteers were asked to remove shoes and belts and to empty pockets of

contents. In addition, participants were asked to clear their bladder before measurements were performed.

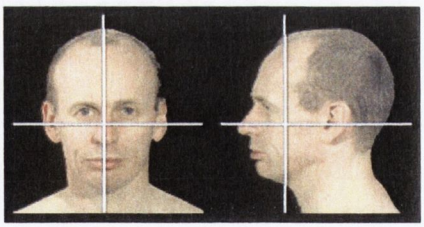
2.8.1 Body weight

The volunteers body weight was measured on a Seca Alpha 770 digital scale (CMS) calibrated in 100g intervals. After a normal expiration, subject's were instructed to briefly hold their breathe and body weight measurements were taken to the nearest 0.1kg, in triplicate measurements and the mean was recorded.

2.8.2 Body height

Height was measured to the nearest cm or mm using a Leicester height measure (CMS). The stadiometer was place on a hard level floor. Measurements were taken in bare or stocking feet. Subjects were asked to remove hats and hair accessories and stand upright with the heels, buttocks, and occiput (back of skull) touching the backboard of the stadiometer. Measurements were taken with the subject's head in the Frankfurt Plane as illustrated in *Figure 2.1* below. The respondent was asked to relax his/her shoulders, inhale and exhale and stand as tall as possible before the height was recorded.

Figure 2.1: Measurements of body height



The measuring arm of the stadiometer was lowered to the top of the subject's head, pushing the hair down. Two measurements were taken to the nearest 0.1 cm, the mean of which was recorded.

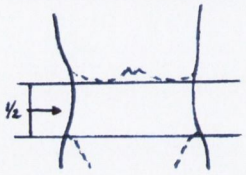
2.8.3 Body mass index (BMI)

Was calculated using the equation: BMI = weight (kg) / height^2 (m^2)

2.8.4 Waist circumference

With the subjects permission measurements were made directly on the skin. Duplicate measurements were taken with the subject in a standing position with the abdomen relaxed, the arms at the sides and the feet together and breathing normally. All values were recorded to the nearest 0.1cm. Subjects were asked to breath deeply in order to locate the bottom of the rib cage. A non-stretch tape measure was used and size were taken at the mid point between the supra iliac crest and lower ribs margin as illustrated in *Figure 2.2* below.

Figure 2.2: Measurement of waist circumference

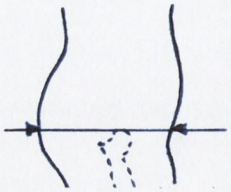


The measurements were taken at the end of a normal expiration, ensuring the tape was taut around the subject's waist, without constricting the skin. All measurements were read by the investigator standing at the subject's left hand side.

2.8.5 Hip circumference

Subjects were requested to stand erect with arms at the sides and feet together and measurements were documented at the level of the greater trochanter without compressing the skin as illustrated in *Figure 2.3*. Duplicate measurements were recorded to the nearest mm.

Figure 2.3: Measurement of hip circumference



2.8.6 Bioelectric impedance analysis

Bioelectric impedance analysis (BIA) was measured in the erect position using a body fat analyser TBF-300 (Tanita Corporation, Illinois, USA). Bioelectric impedance measures the

opposition of body cells and tissues to the flow of electric current (Ellis *et al*, 1999). Impedance is measured as the voltage drop between electrodes, which occurs in fat tissue and bone. In contrast, lean tissues and fluids containing electrolytes conduct the current. An additional opposition or reactance occurs due to the capacitance of cell membranes, tissue interfaces, and non-ionic substances, which is accounted for in the prediction equations (Zarowitz & Pilla, 1989; Ellis *et al*, 1999). Height, weight, age, and gender are included in a number of multiple regression relationships to predict body composition compartments such as fat-free mass and lean body mass. A measure of body fat is indirectly obtained as the difference between body weight and the derived estimate for fat free mass (Ellis *et al*, 1999). Factors known to affect the measurement of BIA including hydration status, consumption of food and beverages, ambient air and skin temperature, recent physical activity, and conductance of the examining table (Ellis *et al*, 1999) where minimised where possible. Duplicate measures were taken, and the mean value recorded. Percentage body fat was calculated using the manufacturers pre-programmed equations.

2.9 Determination of blood pressure

Blood pressure (BP) measurements were taken after the subject has been weighed and prior to the anthropometric measurements. Subjects were invited to sit for 5-7 minutes before measurements were recorded. Measurements were made on the same arm (non-dominant) supported to heart level. Volunteers were instructed to remain quiet, no talking or laughing while measurements were being conducted.

Duplicate BP measurements were taken and if either the systolic or diastolic readings differed by more than 10mmHg, additional readings were obtained until values from two consecutive measurements were within 10mm Hg. BP were measured at the screening visit, and pre-and post-intervention using a calibrated Intelli^{TM} sense mercury sphygmomanometer model no HEM-757 (N.A. Blakeman & Son Surgical Engineers, Omron Healthcare, Inc. Ill, USA).

2.10 Collection and storage of human blood samples

2.10.1 Collection of screening blood samples

All volunteers were requested to adhere to the following criteria prior to their screening visit to the metabolic suite.

1. To observe a 12 hrs overnight fast (no food or drink, except water)
2. Avoid smoking tobacco the morning of each visit until the blood sample was donated

3. Abstain from alcohol 12 hrs proceeding to the blood sample donation
4. Was the volunteer satisfied to donate a blood sample

If the volunteers answered *'yes'* to the four questions, a fasting blood sample of 14mls was drawn when they were in a comfortable lying position. Blood samples were obtained by a trained Phlebotomist who drew blood by venupuncture from the antecubital vein following minimal compression with a latex-free tourniquet. All sterile equipment was provided to the phlebotomist to ensure proper medical conduct at all times, 23G Butterfly Needle, with protective shield (Becton & Dickinson, Plymouth, UK), alcowipe strerets, saturated with 70% v/v isopropyl alcohol (Seton Prebbles Ltd, Oldham, UK), cotton balls (Vernon-Carus Ltd, Lancashire, UK), hypo-allergic adhesive surgical dressing (Mepore, MöInIcke Health Care, Göteborg, Sweden) and Micropore Silk Tape (Promed, UK).

Blood was collected in 1 x 4ml fluoride oxalate coated tube (BD Vacutainer®, Becton & Dickinson, Plymouth, UK) for plasma glucose analysis and 1 x 10ml EDTA-coated evacuated tube (BD Vacutainer, Becton & Dickinson, Plymouth, UK) for TAG, Total cholesterol (TC) and HDL-cholesterol (HDL-c) and LDL-cholesterol (LDL-c) concentrations. The blood tubes were inverted 8 to 10 times to ensure that the coagulant was mixed thoroughly. Subsequently, the blood samples were placed on ice for 30 mins to allow for clotting. Following this, bloods were centrifuged at 3000rpm for 10 mins at 4°C. Plasma was harvested immediately and analysis of plasma TC, TAG and HDL-c was completed within 2 hrs on fresh plasma.

Table 2.11 Baseline screening criteria adapted from the NCEP-ATP III (Assmann *et al*, 2007) and used by LIPGENE Human Dietary Intervention Study to diagnose the MetS

Criteria	Biochemical/Anthropometric marker	Concentrations
1	Fasting glucose concentration	5.5 - 7.0 mmol/L[¥]
2	Serum TAG concentration	≥ 1.5 mmol/l
3	Serum HDL-c concentration	< 1.0 mmol/l (male), < 1.3mmol/l (female)
4	Blood Pressure	130/85 mmHg
5	Waist girth	>102cm (male), > 88cm (female)

HDL-c, High Density Lipoproteins; **TAG**: Tricylglycerol; **mmol/L**: millimoles/Litre; **mmHG**: millimetre of mercury; **cm**: centimetres

[¥] Whilst 7mmol/L was the ideal maximum fasting glucose concentration, individuals with concentrations of up to 8mmol/L were accepted onto the trial, provided that the person was not on any oral hypoglycaemic medication or diet therapy for elevated plasma glucose concentrations.

2.10.2 Collection of intervention blood samples

Following diagnosis of the MetS, volunteers were invited to take part in the 12 week dietary intervention study. 417 volunteers accepted the invitation to take part in and completed the human dietary intervention study. Their first requirement was to denote a 12h fasting blood sample. Volunteers were asked to refrain from vigorous exercise and alcohol the day prior to the sample donation. This clinical assessment was conducted in the Diabetes Day Centre (DDC), AMNCH, Tallaght Hospital, Dublin. On this morning of clinical investigation, anthropometric measures and dietary history recalls were repeated. The volunteers were asked to lie in the supine manner prior to the insertion of two cannulae (BD Adsyte Pro^{TM}, Becton & Dickinson, Madrid, Spain) into the antecubital vein of both forearms. A fasting blood sample was drawn from cannula one and allowed to clot after which, they were centrifuged at 3000rpm for 10 mins at 4°C. Plasma was harvested and all samples were snap frozen in liquid nitrogen (Coopers Cryoservice, Dublin) before been stored in -70°C freezer until analysis.

Following the donation of the >80mls fasting blood sample, volunteers underwent a 180 mins IVGTT as previously described by Boston *et al* (2003).

2.11 LIPGENE Human Dietary Intervention Treatments

Volunteers were randomly stratified to one of four dietary treatments for an intervention period of 12 weeks, these diets are described in *Table 2.12*. Randomisation was completed by the coordinating centre (TCD), according to age, gender and fasting plasma glucose concentration. An equal number of subjects were allocated to each dietary group at each centre using the MINIM (Minimisation Programme for Allocating patients to Clinical Trials, Dept of Clinical Epidemiology, The London Hospital Medical College, UK) randomisation programme. Their habitual diets were manipulated in order to reach the required fat content of the particular diet as described by Shaw *et al* (2007). Spreads, oils, mayonnaise/dressing, baking products and commercial fat were supplied by Unilever Best Foods (Unilever Health Institute, Unilever Bestfoods, Vlaardingen, The Netherlands). Cold storage (4°C) was required for the spreads and baking fats. Oils, mayonnaise and salad dressings were kept at low room temperature (in storage cupboards, out of direct sunlight). Commercial fat products of differing compositions were also provided and used in the manufacture of biscuit products (Fusco Foods, Ltd., Dublin, Ireland). The fatty acid composition and tocopherol content of the LC n-3 PUFA and placebo supplements is described in *Table 2.13*.

Table 2.12: LIPGENE - Human Dietary Intervention Study – Dietary Treatments

	LIPGENE DIETARY TREATMENTS *			
	Diet A	Diet B	Diet C	Diet D
Type of diet	**Control – high fat SFA-rich diet**	**High-fat, MUFA-rich diet**	**Isocaloric, low fat, high complex carbohydrate diet**	**Isocaloric, low fat, high complex carbohydrate diet & 1.24g LC PUFA**
Abbreviation	***HSFA***	***HMUFA***	***LFHCC***	***LFn-3PUFA***
Total Energy %	38	38	28	28
SFA %	16	8	8	8
MUFA %	12	20	11	11
PUFA %	6	6	6	6
Placebo **	NA	NA	HOSO	NA
1g/d LC n-3 PUFA %	NA	NA	NA	1.24g/d LC n-3 PUFA

**HOSO (High oleic, sunflower oil) Placebo was provided by Lipid Nutrition, Looders Crooklan (Wormerveer, The Netherlands)

Table 2.13: Fatty Acid Composition and tocopherol content of the LC n-3 PUFA and placebo HOSO supplement

	LC n-3 PUFA	**Placebo HOSO**
Description:	Marinol™ C-38 is a concentrated natural fish oil with a high content of EPA and DHA	High Oleic Acid Sunflower Seed Oil supplement
Fatty Acid Composition	w/w %	w/w %
C14:0	5	0
C16:0	10.5	3.5
C16:1	4.5	0
C17:0	0.2	0
C18:0	2.5	3.4
C18:1	8	79.2
C18:2	1	11.8
C18:3	0.5	0.2
C20:0	0.2	0.3
C20:1	1.1	0.3
C22:0	0.2	0.9
C24:0	0.2	0.3
C20:5	24	-
C22:6	17.5	-
Total EPA and DHA	38*	-
Mixed Natural Tocopherol	3.0mg	3.0mg

*38% is the standard minimum value of EPA and DHA that is assured in all batches of the product.

2.12 Determination of human inflammatory mediators

Human high sensitivity C-reactive protein (hsCRP), IL-6, TNFα, total adiponectin, resistin soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1) and leptin concentrations were measured by a solid ELISA. For the CRP, IL-6, TNFα, sICAM-1 and sVCAM-1 ELISA a pre-coated microtitre plates with a unique monoclonal antibody specific was provided on purchase, whereas for adiponectin, resistin and leptin, each 96 well ELISA plate required coating with the capture antibody 24 hrs prior to the addition of the human sample. For all ELISAs, diluted samples, specific reconstituted standards and internal controls were individually dispensed into the appropriated wells.

2.12.1 Determination of human CRP

CRP kits (BioCheck Inc, CA, USA) were used which employs a goat anti-CRP antibody (horseradish peroxidase) and was added to the wells after the addition of the diluted sample. This allows the sample to react simultaneously with the two antibodies resulting in the CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45 mins incubation at RT, the wells were washed with water to remove unbound labelled anti-bodies. Tetramethylbenzidine (TBM) reagent was added and incubated for 20 mins, resulting in the development of a blue colour. This colour reaction was stopped with the addition of 1N HCl changing from blue to yellow in colour. The colour produced was directly proportional to the concentration of CRP in the sample, which was measured spectrophotometrically at 450nm (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK).

2.12.2 Determination of human 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).

2.12.3 Determination of human TNFα

TNFα kits were used (R&D Systems, Abingdon, UK) that bind the TNFα 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 (Johannsson *et al*, 1986). In this amplification system, alkaline phosphatase dephosphorylates the reduced form of nicotinamide adenine dinueleotide 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 daiphorase (amplifier). Catalyzed by diaphorase, NADH reduces a tetrazolium saly (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 tetraolium salt and thus the amount of coloured product formed are directly proportional to the amount of TNFα bound in the initial step. A high and low internal control was run with each plate (R&D Systems, MN, USA). 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). The coefficience of variance (CVs) for each biomarker is presented in *Table 2.14*.

2.12.4 Determination of human adiponectin, resistin and leptin

Human serum was used to analyse total adiponectin, resistin and leptin using a DuoSet® ELISA Development System (R&D Systems, MN, USA). Capture antibody made up of mouse anti-human adiponectin, resistin and leptin and detection antibody comprised of biotinylated mouse anti-human adiponectin, resistin and leptin were diluted to a working concentration in PBS without carrier protein and 1% BSA in PBS, respectively. The standard and streptavidin used were provided, the standard was recombinant human adiponectin, resistin and leptin reconstituted with 0.5ml of reagent diluent, whereas the streptavidin-HRP was made up of horseradish-peroxidase. Substrate solution was a mixture of 1:1 colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine) (R&D Systems, MN, USA). The stop solution was 2 N H_2SO_4 (R&D Systems, MN, USA). The wash buffer used was made up of 0.05% Tween in 20 (Sigma Aldrich, Dublin) of Phosphate Buffered Saline (PBS) (Sigma, MO, USA) with a pH of 7.2-7.4. A high and low internal control was run with each plate for adiponectin, resistin and leptin (R&D Systems, MN, USA). Optical density was read immediately on a microplate reader (SPECTRAFluor Plus and XFLOUR Version 3.21,

TECAN, Reading, UK) at 450nm and wavelength was corrected to 595nm. The coefficience of variance (CVs) for each biomarker is presented in *Table 2.14*.

2.12.5 Determination of human sICAM-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 immoblised 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 (stabilized 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.

2.13 Determination of human markers of coagulation – PAI-1 and tPA

Plasma PAI-1 and tPA were determined using Asserachrom® ELISA kits (Diagnostica Stago, France). These kits provide a plastic support, coated with mouse monoclonal anti-human PAI-1 or a tPA antibody which captures the PAI-1 and tPA, respectively. Next, a second mouse monoclonal anti-human antibody coupled with peroxidase binds to another antigenic determinant distant from the first one forming the 'sandwich'. The reaction was stopped using stop solution containing acid and the intensity of the colour produced was a direct relationship to the PAI-1 or tPA concentrations initially present in the human plasma sample. Optical density was read immediately on a microplate reader (SPECTRAFluor Plus and XFLOUR Version 3.21, TECAN, Reading, UK) at 492nm and a standard curve was constructed by plotting the absorbance values, versus standard concentrations. All samples were run in duplicate and readings averaged. The inter-assay CV's for PAI-1 was 6% and tPA was 3%.

Table 2.14: Description of the ELISA kits used in the inflammatory analysis, CV% achieved and dilution factor used.

Sandwich ELISAs					
Parameter	**Sensitivity**	**Manufacturer**	**CV%**		**Dilution**
			High	**Low**	
CRP	hs	BioCheck Inc, CA, USA	9.2	8.46	1*100
IL-6	hs	Biosource Inc, CA, USA	5.74	9.55	1*7
TNFα	hs	R&D Systems, Abingdon, UK	3.77	5.08	1*5
sICAM-1†	hs	R&D Systems, Abington, UK	8.81		1*20
sVCAM-1†	hs	R&D Systems, Abington, UK	5.04		1*5
Adiponectin	Duoset	R&D Systems, MN, USA	2.65	2.81	1*1600
Resistin	Duoset	R&D Systems, MN, USA	2.82	7.06	1*5
Leptin	Duoset	R&D Systems, MN, USA	7.74	5.19	1*20

† only 1 control provided; [hs] high sensitivity; **CV%**, Coefficient of Variance %; **CRP** (C-reactive protein); **IL-6** (Interleukine-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble inter-cellular adhesion molecule-1); **sVCAM-1** (soluble vascular cell adhesion molecule-1).

2.13.1 Determination of human fibrinogen

Quantitative determination of fibrinogen was performed using an automated clotting assay (Fibriquik, Organon Teknika Corporation, North Carolina, USA) using a Coag-A-Mate MTX (Organon Teknika Corporation, North Carolina, USA). The assay is based on the principle that when thrombin is added to a sample of plasma, fibrinogen is converted enzymatically to fibrin, which in turn undergoes polymerisation to form a fibrin network. Factor XIII activated by thrombin catalyses the formation of stabilising cross links to produce a visible clot. The elapsed time from addition of thrombin to the formation of a clot is inversely proportional to fibrinogen level.

Verify Reference Plasma (Biomérieux, North Carolina, USA) was used to calibrate the assay prior to sample analysis. Two sets of coagulation reference plasma; normal and abnormal control sera (Universal Coagulation Reference Plasma and Abnormal Coagulation Reference Plasma, respectively, Pacific Haemostasis, Fischer Diagnostics, Middleton, VA, USA) were used to test assay performance. Duplicate measurements were recorded and readings averaged. The interassay CV for fibrinogen was low control 4.5% and high control 4.8%.

2.14 Determination of human markers of oxidative stress - urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$

As a marker of oxidative stress urinary samples were analysed from free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$ F2-isoprostane) without any extraction by a radioimmunoassay, as described by Basu *et al* (1998*a*). An antibody was raised in rabbits by immunization with 8-iso-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by 1,1'-carbonyldiimmidazole method. Subsequently, the urinary levels of 8-iso-$PGF_{2\alpha}$ were adjusted for creatinine values. Urinary creatinine was measured by a commercial kit (IL™ Test, Monarch, Amherst, NH, USA). Lipid peroxidaiton enzyme 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$ F2α-prostaglandin) was analysed without any extraction by a radioimmunoassay as described by Basu *et al* (1998*b*). An antibody was raised in rabbits by immunization with 15-keto-dihydro-$PGF_{2\alpha}$ coupled to bovine serum albumin at the carboxylic acid by 1,1'-carbonyldiimmidazole method. Subsequently, the urinary levels of 15-keto-dihydro-$PGF_{2\alpha}$ were adjusted for creatinine values. Urinary creatinine was measured by a commercial kit (IL™ Test, Monarch, Amherst, NH, USA). Both urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihyro-$PGF_{2\alpha}$ are expressed as mmol/mmol creatinine.

2.15 Determination of human markers of insulin sensitivity

2.15.1 Determination of human insulin and c-peptide concentrations

Serum insulin and c-peptide concentrations were measured using solid phase, two-site fluoroimmunometric assays (AutoDELFIA Insulin kit and AutoDELFIA C-Peptide kit respectively, Wallac Oy, Turku, Finland) using a 1235 AutoDELFIA automatic immunoassay system (Wallac Oy, Turku, Finland). The assays are based on a direct sandwich technique in which two monoclonal antibodies (derived from mice) are directed against separate antigenic determinants of the protein under investigation (i.e. either the insulin or c-peptide molecule). Samples were simultaneously reacted with immobilised monoclonal antibodies directed against the protein of interest and with europium-labelled monoclonal antibodies directed against different specific antigenic sites on the insulin or c-peptide molecule. Enhancement solution dissociates europium ions from the europium-labelled monoclonal antibody into solution, where they form highly fluorescent chelates with components of the enhancement solution. The fluorescence in each well is measured;, the concentration being directly

proportional to the concentration of insulin/c-peptide in the sample. Insulin results underwent modelling using MINMOD Millennium® software as described by Pacini & Bergman (1986).

2.15.2 Determination of human glucose concentrations

Plasma glucose was determined by endpoint bichromatic analysis, with coupled hexokinase methodology, using a colorimetric assay (IL Test™ Glucose kit), supplied by Instrumentation Laboratory (Warrington, United Kingdom), measured on an ILAB 600 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, United Kingdom). This enzymatic assay involves the conversion of glucose to glucose-6-phosphate by the action of hexokinase, in the presence of ATP. Glucose-6-phosphate dehydrogenase converts glucose-6-phosphate to 6-phosphogluconate, converting NAD^+ to NADH. Although hexokinase catalyses the phosphorylation of hexoses other than glucose, the glucose-6-phosphate dehydrogenase is specific for glucose-6-phosphate, and therefore glucose. The increased absorbance due to the conversion of NAD^+ to NADH is directly proportional to the glucose present in the sample. Absorbance is measured at a primary wavelength of 340nm and a blanking wavelength of 375nm.

2.16 Plasma fatty acid composition analysis

2.16.1 Extraction of total lipids

Total plasma lipids were isolated as described by Noone *et al* (2002). Blood collected in EDTA tubes were immediately centrifuged at 1500rpm for 10 mins. Plasma was harvested and frozen at -70°C until further analysis. 400μl platelet rich plasma was harvested using a plastic pasteur pipette after an overnight thaw at 4°C and transferred into a 10ml conical polypropylene centrifuge tube, 400 μl ultra-pure water was added to this and centrifuged at 670g for 15 mins yielding platelet poor plasma (PPP) and a platelet pellet. For each batch a blank was run containing 800ul ultra-pure water. 400µl of PPP was transferred to a 16x100mm boroscillate glass tube. Lipid extraction was achieved by the addition of 2.5ml cholorform: methanol (v/v 2:1) with 0.01% w/v butylated hydroxy toluene (BHT; Sigma-Aldrich). Samples were vortexed for 1 min and then centrifuged for 10 mins at 2500rpm to separate the aqueous and organic phases. The organic phase (infranatant) was removed using a glass Pasteur pipette and transferred to a 12x75mm boroscillate glass tube. A further lipid extraction of the sample was performed by the addition of 2ml cholorform with 0.01% w/v BHT to the remaining plasma, vortexing for approximately 30secs and centrifuging for 5 mins

at 2500rpm. Infranatants were removed and pooled with the first extract and evaporated to dryness in a vortex evaporator (AGB Scientific Ltd., Dublin, Ireland). The dried samples were flushed with nitrogen (N_2), sealed to prevent lipid oxidation and stored at –20°C.

2.16.2 Transmethylation of lipid extracts

Methyl esters were prepared by adding 0.5ml 0.01M NaOH in dry methanol according to the method of Jiang *et al* (1999). Samples were vortexed for 30secs, and placed in a water bath at 100°C for 40 mins. Tubes were checked for possible leakage and were vortexed to ensure the lipid droplet became completely dissolved. Following this tubes were allowed to cool before addition of 2ml hexane and 2ml ultrapure water. These were vortexed for 10secs, and centrifuged for 5 mins at 2000rpm. 0.5ml of 14% boron trifluoride methanol (Sigma-Aldrich, Poole, Dorset, UK) was then added to the mixture and samples were vortexed for 30secs. Methyl esters were extracted by the addition of 0.5ml hexane to samples and vortexing for 1 min. The supernatant was removed using a glass Pasteur pipette and transferred to a 12x75ml boroscillate glass tube. This extraction procedure was repeated twice to ensure maximum extraction. Pooled extracts were dried in the vortex evaporator, flushed with N_2 and stored at -20°C. Samples were stored at -20°C for no longer than 4 weeks before analysis by gas chromatography (GC).

2.16.3 Gas chromatographic analysis of total plasma lipids

The fatty acid methyl ester (FAME) composition of total plasma lipids were identified using a Shimadzu GC-14A gas lipid chromatograph (Mason Technologies, Dublin, Ireland), which was fitted with a Shimadzu C16-A integrator and a CP Sil 88 fused column (50mm x 0.22mm, 0.2 file thickness; Chrompack Ltd., Middleburg, The Netherlands). The CP-Sil 88 column contained a highly substituted cyanopropyl phase that has been stabilized. The extremely high polarity of this column offers maximum resolution in separations where the boiling point and polarity of the analytes are nearly equal (for example, the separation of positional and geometric isomers). Analytical conditions were as follows:

Carrier gas:	
Oxygen free nitrogen	0.4 kg/cm^3
Column gas:	
Oxygen free nitrogen	0.8 kg/cm^3
Hydrogen	0.6 kg/cm^3
Dry air	0.5 kg/cm^3

Temperature Programme:
Column initial temperature: 180°C
Increase by 5°C/min for 3 mins to 195°C
Hold at 195°C for 40 minutes
Increase by 2°C/min for 12½ mins to 220°C
Hold at 220°C for 20 mins

The dried transmethyated samples were dissolved in 150ul hexane and 8µl was injected onto the column. The injector and detector temperatures were 250°C; the oven temperature was 215°C. 1μl of sample was injected into the GC; and analysis took ~45 mins per sample.

The FAMEs were identified with a flame ionisation detector. Peak identification was performed by comparison of retention times against a FAME standard. Polyunsaturated fatty acids 2 (PUFA2) (from Menhaden oil) and PUFA 3 (from an animal source), both from Supelco, were run every week to determine the relative retention times of the FAs. The retention time of C17 was obtained from the blank sample from each batch. Fatty acid compositions were calculated as a percentage of the total fatty acids. The fatty acids identified were as detailed in *Table 2.15*. Additionally, SFA, MUFA, PUFA, Omega-3 PUFA (n-3PUFA), and omega-6 PUFA (n-6PUFA) were calculated. The FA masses were calculated based on the blank C17 value for each batch. The efficiency of the experiment was also calculated from the C17 value. Samples with an extraction efficiency of less than 30% were rejected. To ensure consistency between batches, a standard serum sample, to serve as an internal control, was extracted as part of each batch. Batches were considered to be acceptable if the standard serum had a CV <10%.

2.17 Determination of human lipoprotein concentrations

2.17.1 Determination of human HDL cholesterol

The IL Test^{TM} HDL-c Kit (Instrumentation Laboratories, Warrington, UK) was used for direct quantification of HDL-c. A detergent specifically solublises HDL-c and a polyanion assists in the selectivity by complexing LDL-c, very low density lipoproteins (VLDL) and chylomicrons (CM) lipoproteins. The released HDL-c interacts with cholesterol esterase and cholesterol oxidase in the presence of a chromogen to produce a colour in proportion to the amount of

HDL-c. Absorbance was measured at 600 nm on the clinical chemistry auto-analyser, ILAB 600.

2.17.2 Determination of human LDL cholesterol

LDL-c was isolated from serum using LDL cholesterol reagent (Randox CH1350, Laboratories Ltd., Co. Antrim) which uses heparin to precipitate LDL-c at its isoelectric point (pH 5.04). A 100μl aliquot of fresh serum was added to 1000μl LDL reagent. This mixture was vortexed for 30secs, left to stand at room temperature for 10 mins and then centrifuged at 2500 g for 15 mins. The supernatant (containing HDL-c and VLDL) was removed and stored at -20°C for future cholesterol analysis on the ILAB. A dilution factor of 11 was used to calculate original plasma concentration of this fraction. Subsequently, the LDL-c concentration was derived by the subtraction of the cholesterol concentration of the isolated fraction from the measured concentration of TC.

2.17.3 Determination of human total cholesterol concentrations

Quantitation of total cholesterol, LDL-c and TRL-c from defrosted plasma and supernatant samples was performed using the IL Test^{TM} Cholesterol Kit (Instrumentation Laboratories, Warrington, UK) on the ILAB. This analysis is based on the formulation of quinoneimine (red colour), which is produced in proportion to cholesterol concentration and measured by absorbance. Control 01 and control 02 (Instrumental Laboratories, Warrington, UK) were used as quality controls.

2.17.4 Determination of human TAG concentrations

Quantitation of plasma and TRL TAG was performed using the IL Test^{TM} TAG Kit (Instrumentation Laboratories, Warrington, UK). This analysis is based on the end point of quinoneimine production (red colour), which is generated in proportion to TAG concentration and measured at absorbance 510 nm on the ILAB.

2.17.5 Determination of human NEFA concentrations

The WAKO NEFA C enzymatic colour kit (Alpha Laboratories, Hampshire, UK) was used to quantitate plasma NEFA concentration. The intensity of coloured product (quinoneimine) is proportional to the concentration of FFA and measured by absorbance using the ILAB. Control 01 and control 02 (Instrumental Laboratories, Warrington, UK) and a NEFA calibrator were used with each analysis (Alpha Laboratories, Hampshire, UK).

Table 2.15: Nomenclature and dietary sources of common fatty acids (Calder, 1998)

Class	Common Name	Chain Length	# double bonds	Main sources
SFA	Caprylic Acid (C8:0)	8	0	Cow and goats milk, palm oil, coconut oil
	Capric Acid (C10:0)	10	0	Coconut oil
	Lauric Acid (C12:0)	12	0	*De novo* synthesis, butter/milk fat, coconut oil
	Myristic Acid (C14:0)	14	0	*De novo* synthesis, butter/milk fat, coconut oil
	Palmitic Acid (C16:0)	16	0	*De novo* synthesis, animal fat, coconut oil, butter, eggs , milk
	Stearic Acid (C18:0)	18	0	*De novo* synthesis, animal fat, coconut oil, butter, eggs , milk
MUFA	Palmitoleic Acid (C16:1)	16	1	Macadamia oil
	Oleic Acid (C18:1)	18	1	Desaturation of stearic acid, olive oil, butter
PUFA	Linoleic Acid (C18:2 n-6)	18	2	Corn, sunflower and safflower oil
	CLA	18	2	Dairy products, ruminant meats
	α-Linoleic Acid (C18:3 n-3)	18	3	Flaxseed oil, soybean oil, linseed oil, green leaves
	γ-linoleic Acid (C18:3 n-6)	18	3	Synthesised from linoleic acid, evening primrose oil
	Eicosapentaeoic Acid (C20:5 n-3)	20	5	Fish oils
	Docosahexaenoic Acid (C22:6:3)	22	6	Fish oils, red meat, offal

SFA: Saturated Fatty Acid, **MUFA**: Monounsaturated Fatty Acid, **PUFA**: Polyunsaturated Fatty Acid (adapted from Calder *et al*, 1998).

2.17.6 Determination of human Apolipoprotein concentrations

Plasma apolipoproteins (Apo) AI, B and E concentrations were measured using an immunonephelometric methods on a BN Prospec® System with commercial kits (Dade Behring, Deerfield, USA). Plasma apo CII and CIII concentrations were measured by

immuno-turbdimetry on a Hitatchi 911 system with commercial kits (Diasys, Bouffmont, France). Assessment of ApoB48 was by a competitive ELISA with a specific ApoB48 rabbit antibody according to Lorec *et al* (2000).

2.18 Power calculations, data preparation and statistical analysis

The statistical power of the LIPGENE dietary intervention study presented in *Chapter 4* and *Chapter 5* was based on insulin sensitivity as the primary biomarker of the MetS. The power of the study was calculated with the ability to detect at least 1 unit difference at a standard deviation (SD) of 2 between diets. The power calculation was based on the assumption that 15% difference in insulin sensitivity was physiologically important and that this difference can be detected at a significance level of 0.05 and a power of 0.8. Thus, a minimum of 98 subjects per dietary group would be required to detect significant differences. The group size was increased to 120 per group to account for an anticipated 20% drop-out rate.

All statistical analysis was conducted using SPSS® for Windows™, version 14.0 (SPSS Inc, Chicago, IL, USA). Results are presented as means ± standard error of the mean (SEM) unless stated otherwise.

2.18.1 Statistical analysis completed on the effects of CLA on insulin sensitivity (Chapter 3), the effects of plasma fatty acids on inflammation and insulin sensitivity (Chapter 4) and the human dietary intervention study (Chapter 5)

(a) One-way analysis of variance (ANOVA) analysis

One-way ANOVA was used to determine a significant effect of the dietary intervention on the change in the biomarkers analysed in the animal and human dietary intervention study. In *Chapter 5*, the change in the intervention data included completers and was calculated by subtracting the post-intervention concentration from the pre-intervention concentration of each parameter. Results are presented in mean ± SEM. In the cross-sectional analysis and the human dietary intervention study as presented in *Chapter 4* and *Chapter 5*, respectively, normality was assessed by Skewness and Kurtosis with a ± 1 cut-off applied. The Inverse Square Root (1/SQRT) and SIN transformation were accepted if normality was not achieved. LOG and SQRT transformation were not applied as the SPSS software states that all values must be greater than 0, otherwise they are identified as a missing value.

(b) Correlation Analysis

Univariate correlation analyses were performed on the results from the *ob/ob* analysis in *Chapter 3*, cross-sectional analysis in *Chapter 4* and the post-dietary intervention results in *Chapter 5* to determine the strength and direction of the linear relationship between variables. Each set of correlation analysis was tested with Pearson's product moment correlations, with Spearman's Rank Order Correlations used as the non parametric alternative.

2.18.2 Statistical analysis completed in both Chapter 4 and Chapter 5

(a) LIPGENE human dietary intervention cohort details

483 MetS individuals were included in the baseline cohort as presented in *Chapter 4*, while 417 MetS individuals completed the dietary intervention study as presented in *Chapater 5*.

For both chapters, age and change of body weight were continuous variables, therefore, they were categorised for post-hoc analysis. Age categories were defined into three groups: 35-50years, 50-65years and 65-70 years. Change in body weight was categorised into 5 categories: Lost 4-8Kg, Lost 2-4Kg, Lost 0-2Kg, Gained 0-2Kg and Gained 2-5Kg.

The distribution of a continuous variable was investigated using Skewness and Kurtosis values by examining the symmetry and elevation of the distribution and a ± 1 cut-off was applied. Histograms and Q-Q plots were also used to assess normality. For variables with skewed distribution, data was subjected to logarithmic (Log), square root transformation (SQRT), Inverse Square Root (1/SQRT) and SIN transformation before statistical analysis.

(b) Calculations and definitions of insulin sensitivity markers

Homeostasis model assessment-insulin resistance ($HOMA_{IR}$) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). In addition, insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) according to the equation $[1/(\log \text{insulin}_0 + \log \text{glucose}_0 + \log \text{NEFA}_0)]$ (Roche, 2004). MINMOD Millennium® was used to calculated the following parameters; insulin sensitivity (S_I), glucose effectiveness (S_G), acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (G0) (Boston *et al*, 2003).

(c) Linear regression analysis to determine the interaction between dietary fatty acids, inflammation and insulin sensitivity (Chapter 4) and the human dietary intervention study (Chapter 5)

Linear regression analysis was performed to investigate the best fitting model to describe the relationship between the dependent variables insulin sensitivity calculated by the MINMOD® Millennium (S_I) and homeostatis model assessment-insulin resistance ($HOMA_{IR}$) and a set of explanatory variables as presented in *Chapter 4* and for dependent variables post-dietary intervention IL-6, adiponectin and resistin. For the human dietary intervention predictors of each variable were assessed regardless of dietary treatment and across each of the LIPGENE dietary groups. Three separate analysis steps were performed to identify significance among the dependent and independent variables. Each dependent variable was measured against the following separate sets of data, 1: LIPGENE partners, 2: demographics and lifestyle, 3: inflammatory data, 4: intravenous glucose tolerance test (IVGTT) data, 5: dietary FAs & 6: plasma FAs. It was necessary that a dummy variable was created for the LIPGENE partners for both *Chapters 4* and *5*, as there were more than 3 LIPGENE partners in each. In addition, it was necessary to create a dummy variable for diet within the human dietary intervention (*Chapter 5*) as again; there were more than 3 diets. Therefore, TCD became the control LIPGENE partner and Diet A (control, high-saturated fatty acid) became the control diet, all other variables were compared to these controls.

Firstly, each variable within the seven datasets were entered individually in a univariate regression analysis manner Secondly, all variables within each datasets were entered simultaneously in a multivariate regression analysis manner, Firstly, each variable from the individual datasets was entered individually in a univariate regression analysis manner against the dependent variables S_I and $HOMA_{IR}$ in the cross-sectional analysis in *Chapter 4* and against the dependent variables post-intervention IL-6, adiponectin and resistin for the human dietary intervention study in *Chapter 5*. Secondly, all variables within each datasets were entered simultaneously in a multivariate regression analysis manner, controlling for age, gender and body weight. The univariate and multivariate results were compared to identify the variables from each dataset that would proceed further to the multiple linear regression modelling analysis. Two approaches were used to decide which variables proceeded; variables that were significant ($p<0.1$) in both models were included and variables that were significant ($p<0.1$) in only one of the models (either the univariate or multivariate model) however strong literature support for there inclusion into the model was required. At the initial stage the

significant cut-off used to denote statistical significance was $p<0.1$, as the regular significance of $p<0.05$ may have failed to identify potential influential predictors within the final model (Hosmer & Lemeshow, 2000).

The explanatory variables were grouped together into the following models; 1: LIPGENE partners; 2: demographics and lifestyle; 3: inflammatory data; 4: dietary FAs & 5: plasma FAs. Multiple linear regression analysis controlling for age, gender and BMI was preformed on each on the models separately, again significance was accepted at $p<0.1$. Multicollinearity was assessed at the stage by means of tolerance values ($p<0.10$) and variance inflation factor (VIF) values (>0.1). Only variables that reached statistically significance and did not indicate multicollinearity were brought forward to the final multiple linear regression model. Age, gender and body weight were controlled for within the final model and from that multicollinearity was also examined. The strength of each explanatory variable was demonstrated using the beta co-efficient score. In the final multiple linear regression model statistical significance was taken at $p<0.05$.

(d) LIPGENE human dietary intervention group comparisons

For the cross-sectional analysis as presented in *Chapter 4*, insulin sensitivity (S_I) as determined by the MINMOD Millennium software, $HOMA_{IR}$, resistin, adiponectin, CRP and SFA/MUFA ratio and SFA/PUFA ratio were divided into three subgroups or tertiles. A one-way ANOVA or non-parametric Kruskal-Wallis test was used to assess significance across all tertiles.

For the human dietary intervention study (*Chapter 5*) the data was divided into three subgroups or tertiles, according to their pre-dietary intervention plasma CRP concentrations. In the low CRP and high CRP tertiles there were 138 subjects and in the medium CRP tertile there were 139 subjects. RM-ANOVA was used to determine the differences between pre- and post-intervention concentrations within the CRP tertiles. A one-way ANOVA or non-parametric Kruskal-Wallis test was used to assess significance across the CRP tertiles. Both statistical tests were carried out irrespective of diet and in relation to the dietary treatments that the subjects were randomly assigned to.

(e) Binary logistic regression analysis on the cross-sectional analysis as presented in Chapter 4

In an attempt to determine patterns between categories of S_I (low, medium and high), binary logistic regression analysis was used to find the best fitting model to describe the relationship between the dependent dichotomous variable 'low S_I versus medium S_I versus high S_I' and a set of explanatory variables. LIPGENE partners, demographics and lifestyle factors, markers of S_I, inflammation, coagulation and oxidative stress as well as dietary fatty acids were analysed to determine the potential predictive power of $HOMA_{IR}$. For the three dependent variables the model development was approached using two separate regression analysis steps. Firstly, the independent influences of each of the food groups on the binary variables were assessed. Secondly, multivariate logistic regression analyses was performed for each of the individual variables, controlling as before for age, gender and body weight. Variables that reached significant ($p<0.1$) in both the univariate and multivariate (controlling for age, gender, BMI) were included in the final model. Multiple binary logistic regression analysis, controlling for age, gender and body weight was performed on the significant variables. In the final model, significance was taken at $p<0.05$. The model fit for the data was assessed by measuring the -2log likelihood (-2LL) ratio and the Hosmer and Lemeshow *'goodness of fit test'*. Poor fit was indicate by a significance value less than $p<0.05$.

The output of the multiple binary logistic regression analysis generate odds ratios (OR) and 95% confidence intervals (CI). The OR represents the change in odds of being in one of the categories of outcome when the value of a predictor increases by one unit (Tabachnick & Fidell, 2007). The CI represents the ranges of values where the analysis can be 95% confident encompasses the true value of the OR (Aiken & West, 1991).

(f) Repeated-Measure Analysis of Variance (RM ANOVA) analysis on the LIPGENE human dietary intervention study as presented in Chapter 5

Analysis was performed using a repeated-measure analysis of variance (RM–ANOVA) to identify significant ($p<0.05$) effects of the dietary intervention. Gender, centre and diet were included as between subject factors. Age and change of body weight were included in the model as the covariates. Statistical differences between all interactions (time*diet group, time*centre, time*change in body weight, time*age, time*gender, gender*centre, time*gender*centre*diet group) were assessed by *post-hoc* analysis: paired and independent t-tests (non parametric alternatives: Wilcoxan and Mann Whitney U, respectively) and one-way

ANOVA (non parametric alternative: Kruskal Wallis). The Bonferroni and Tamhane *post-hoc* tests were used to identify significant differences between the groups as appropriate (Coakes & Steed, 1999). A Bonferroni correction applied if multiple testing occurred in order to account for the increase in type 1 error.

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Chapter 3

The effects of conjugated linoleic acid on inflammatory status in an insulin resistant state

3.1 ABSTRACT

Background: The increasing incidence of the Metabolic Syndrome (MetS) requires alternative strategies to attenuate its risk, given that its inherent characteristics obesity and insulin resistance predispose to Type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD). Adjusting the fatty acid composition of foods, or functional food development may represent an alternative approach to pharmacological therapies.

Aim: This study determined whether enriching beef with the fatty acid isomer *c*9, *t*11-conjugated linoleic acid (CLA) could improve metabolic and molecular markers associated with the MetS within an obese phenotype. **Design:** 30 male, 4 week old *ob/ob* mice were randomly assigned to one of four dietary treatments for 28 days. **Diet A**: control diet, linoleic acid (LA), **Diet B**: high natural beef derived *c*9, *t*11-CLA diet, **Diet C**: low natural beef derived CLA & synthetically prepared *c*9, *t*11-CLA, **Diet D**: low natural beef derived CLA & synthetically prepared TVA.

Results: Feeding a diet enriched with natural beef derived *c*9, *t*11-CLA diet significantly reduced fasting plasma glucose, TAG, NEFA concentrations and $HOMA_{IR}$ ($p<0.0005$), compared to the control diet. These improvements in insulin sensitivity were equivalent to that achieved by a synthetic *c*9, *t*11-CLA diet. Interestingly the high natural beef (Diet B) was also associated with reduced body weight ($p<0.05$). Interestingly plasma adiponectin concentrations were increased and plasma IL-6 levels were decreased following the natural *c*9, *t*11-CLA diet and synthetic *c*9, *t*11-CLA diet ($p<0.001$). The improved metabolic profile was associated with significantly greater white adipose tissue (WAT) GLUT4 ($p<0.0005$). Additionally, insulin receptor substrate (IRS)-1 and IRS-1 serine 307 phosphorylation mRNA expression was significantly increased in the natural and synthetic *c*9, *t*11-CLA diets. Conversely WAT TNFα mRNA expression was significantly reduced following the high-CLA diets ($p<0.05$).

Conclusion: Feeding a diet of *c*9, *t*11-CLA enriched beef improved several components of the MetS, particularly insulin sensitivity, inflammation and lipid metabolism, within the context of obesity. These findings are highly relevant in that they demonstrate that a functional food may alleviate the diabetic phenotype associated with obesity characteristic of the MetS.

3.2 INTRODUCTION

The MetS describes a constellation of metabolic abnormalities that occur simultaneously, including obesity, IR, low high-density lipoprotein cholesterol (HDL-c) concentrations, elevated TAG levels, microalbuminuria and hypertension (Alberti & Zimmett, 1998). The presence of at least three of the above disorders defines the MetS, which in turn predicts the development of T2DM and CVD (Phinney, 2005; Magliano *et al*, 2006). IR is the primary hallmark of the MetS, it occurs as a result of excess adipose tissue (AT) and is associated with disturbances in carbohydrate and lipid metabolism (Shaw *et al*, 2005; Bray & Champagne, 2004). The cost of the medical treatment required by sufferers of the MetS places an enormous burden on the health care system. The total cost of obesity for the EU is estimated to be approximately €32,800 x 10^6 per year (Fry & Finlay, 2005). Therefore, preventative strategies are required to slow rates of progression and to reduce dependence on costly medical management (Shaw *et al*, 2005).

Foods can be regarded as functional if they have been proven to beneficially affect one or more target functions in the body, beyond the traditional nutritional effects. Functional foods act in such a manner that is relevant to improved state of health and well-being and reduction of risk of diseases (Riccardi *et al*, 2005). During biohydrogenation of dietary unsaturated lipids such as polyunsaturated fatty acids (PUFA) in the ruminal gut, a unique fatty acid (FA) termed CLA is produced. CLA refers to all the positional and geometric isomers of LA (Banni, 2002). LA contains two double bonds in positions 9 and 12 and CLA is formed when reactions shift the location of one or both of the double bonds of LA in such a manner that the two double bonds are no longer separated by two single bonds. Bacterial synthesis of CLA occurs through the microbial isomerisation of dietary LA in the digestive tracts of ruminant animals and for this reason CLA is found naturally in meat and dairy products (Pariza, 2002). At least eighteen different isomers have been identified and these differ in the relative position of the two double bonds (Eulitz *et al*, 1999). The *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) isomer is the most abundant, accounting for >90%, whereas its counterpart, *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA) makes up <10% in natural food products (Choi & Song, 2005). The structures of the *c*9, *t*11-CLA and the *t*10, *c*12-CLA isomers are illustrated in *Figure 1.12*. TVA is the main precursor of CLA and is formed through incomplete biohydrogenation of dietary FA. TVA has been shown to bioconvert to CLA within the AT of mice (Santora *et al*, 2000). Due to the natural original of CLA in meat and dairy products, CLA can be consumed as part of a daily diet and has received much attention

as a potential functional food. In recent years CLA has been reported to have anti-carcinogenic (Beppu *et al*, 2006), anti-artherogenic (Toomey, 2005; Arbonés-Mainar *et al.* 2006), anti-obesity (Bhattacharya *et al*, 2005), anti-adipogenic (Brandebroug & Hu, 2005) and anti-inflammatory effects (Loscher *et al*, 2005). All of these potentially positive target functions highlight the importance of CLA at a time when the increasing prevalence of obesity and the MetS affects not only adults but also a worrisome proportion of adolescents and children globally (Klein-Platat *et al*, 2005).

CLA has attracted considerable attention because of its reported beneficial effects on body composition and obesity, by decreasing body fat and increasing lean body mass both *in vivo* and *in vitro*. (Laso *et al*, 2007; Simon *et al*, 2006; Gaullier *et al*, 2005; Hargrave *et al*, 2005). To date, clarity still remains illusive on the mechanistic effects of CLA on body composition, although several reports have endeavoured to describe this phenomenon (House *et al*, 2005; Corino *et al*, 2005; Kovacs & Mela, 2006). The first report to suggest that CLA may have potential as an anti-obesity nutrient was published in 1997, when mice supplemented with 0.5% CLA had a 60% decrease in body fat after 4-5 weeks (weeks) (Park *et al*, 1997). Accumulating evidence suggests that the *t*10, *c*12-CLA isomer appears to be responsible for the reduction of fat pad weight due to its ability to suppress body fat accumulation (Wargent *et al*, 2005; Evans *et al*, 2002). Therefore, this isomer may have potential in the treatment and management of obesity.

IR is the primary hallmark of the MetS, it occurs as result of excess AT and is associated with disturbances in carbohydrate and lipid metabolism (Evans *et al*, 2002; Mensink, 2005). Inconsistencies exist in the studies which examined the isomer specific effects of CLA supplementation on insulin sensitivity. For example, a study on healthy humans observed no effects of CLA when blending *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers (3.0grams per day (g/d) CLA) together for 8 weeks on fasting glucose or insulin concentrations (Moloney *et al*, 2004). Another study examining the effects of the *c*9, *t*11-CLA isomer alone showed decreased insulin sensitivity and increased lipid perioxidation in obese middle aged men at high risk for CVD (Risérus *et al*, 2004*c*). Whereas, a study on young sedentary men showed a significant improvement in insulin sensitivity after supplementation for 8 weeks with mixed isomers of CLA (35.9% *c*9, *t*11-CLA and 36.8% *t*10, *c*12-CLA) (Eyjolfson *et al*, 2004).

The MetS is often associated with a sub-acute pro-inflammatory state and therefore the effects of CLA on markers of the inflammatory response are important to investigate (Ghanim

et al, 2004; Dandona *et al*, 2004; Roche *et al*, 2005). The pro-inflammatory, insulin de-sensitising effects of tumour necrosis factor α (TNFα) within the context of the MetS are well characterised. TNFα impedes insulin signalling and has been implicated in the pathogenesis of T2DM (Dandona *et al*, 2004). Human obesity is positively related to TNFα expression in AT and muscle and plasma levels of TNFα are positively associated with body mass index (BMI), fasting glucose and TAG concentrations, and inversely associated with HDL-c (Ohya *et al*, 2005). The immune modulating effects of CLA were also evaluated in a population with high risk of coronary heart disease (CHD). Subjecs were supplemented with 3g/d of either *c*9, *t*11-CLA or *t*10, *c*12-CLA for 13 weeks and results showed no significant effect on plasma C-reactive protein (CRP), TNFα, interleukin (IL)-6 or IL-8 concentrations (Ramakers *et al*, 2005).

Numerous studies have been carried out to investigate the effects of CLA on blood lipid profiles and have found inconsistent results (Naumann *et al*, 2006; Abronés-Mainer *et al*, 2006; Tricon *et al*, 2004; Warren *et al*, 2003; Clément *et al*, 2002). However, these studies have found undesirable effects for the *t*10, *c*12-CLA isomer compared to the *c*9, *t*11-CLA isomer, whereby it promoted arthrosclerosis (Arbonés-Mainer *et al*, 2006), increased plasma TAG and ratios of low density lipoprotein (LDL): high density lipoprotein (HDL) and total cholesterol (TC):HDL-c (Tricon *et al*, 2004) and liver weights were significantly increased (Warren *et al*, 2003; Clément *et al*, 2002). A separate study using the ApoE knockout$^{-/-}$ mouse model examined the effect of dietary supplementation with an 80:20% *c*9, *t*11 and *t*10, *c*12-CLA blend, a preparation which reflects the natural sources of CLA (Chin *et al*, 1994). Administration of this isomeric blend with a 1% cholesterol diet to ApoE$^{-/-}$ animals with pre-established atherosclerosis induced almost complete regression of early atherosclerotic lesions (Toomey *et al*, 2005). This effect was mediated without decreasing plasma cholesterol or TAG levels. Interestingly, the *c*9, *t*11-CLA isomer also decreased pro-inflammatory cytokine levels and in particular the expression of plasma TNFα mRNA *in vivo*. Moreover, it was shown that the *c*9, *t*11-CLA isomer exclusively reduced atherogenesis in hamsters coincident with decreased inflammatory-related gene expression via peroxisome proliferator activator α/γ (PPARα/γ) activation (Arbonés-Mainer *et al*, 2004).

The aim of the present study was to investigate the effects of natural beef *c*9, *t*11 CLA, a synthetic source of beef enriched *c*9, *t*11 CLA and a synthetic source of TVA on metabolic and molecular markers in the IR prone *ob/ob* mouse model. The *ob/ob* mouse model is a well

characteristised model for obesity and IR. It has been reported that at 28 days of age, the *ob/ob* mouse contains 3 times as much fat compared to a lean mouse of same age (Thurlby & Trayhurn, 1978). In the present study, the dietary feeds varied in compositions of natural beef derived *c*9, *t*11-CLA or natural beef derived *c*9, *t*11-CLA with additional synthetic *c*9, *t*11-CLA sources or TVA. To the knowledge of the author, this study is the first to determine the specific effects of dietary supplementation of *c*9, *t*11-CLA isomer in its biologically active form, synthetic form or TVA, on body fat composition, markers of insulin sensitivity, inflammatory status and lipid metabolism at a cellular and molecular level from the WAT in the *ob/ob* mouse.

3.3 RESEARCH DESIGN AND METHODS

3.3.1 Animal experimentation, intervention details and tissue collection protocol

The animal experimentation was conducted as per *Section 2.1 to 2.1.2.* Plasma and white adipose tissue was collected as per *Section 2.1.3* to *Section 2.1.4*

Table 3.1: Description of the diets fed to the *ob/ob* mice

Group	*Diet Description*	*n*
Diet A	***Control Diet***	
	Low natural beef derived *c*9, *t*11-CLA & Linoleic Acid (LA)	7
Diet B	***High CLA Beef Diet***	
	High natural beef derived *c*9, *t*11-CLA	8
Diet C	***Synthetic Diet***	
	Low natural beef derived *c*9, *t*11-CLA & synthetically derived *c*9,*t*11-CLA	7
Diet D	***Synthetic TVA Diet***	
	Low natural beef derived *c*9, *t*11-CLA & synthetically derived *trans* Vaccenic Acid (TVA)	8

LA: (Linoleic Acid); **CLA:** (Conjugated Linoleic Acid); *c*9, *t*11-CLA: (*cis*-9, *trans*-11-CLA); **TVA:** (*trans*Vaccenic Acid); **n:** (number).

Table 3.2 Percentage energy from macronutrients in the diets fed to the ob/ob mice

		Control Diet	*High CLA Beef Diet*	*Synthetic CLA Diet*	*Synthetic TVA Diet*
Total Energy	kcal/g	4.7	4.7	4.7	4.7
% Energy from Fat	%	50	52	51	52
% Energy from Protein	%	23	23	24	23
% Energy from Carbohydrate	%	26	26	25	25

3.3.2 Determination of glucose, cholesterol, TAG and NEFA analysis

Serum glucose concentrations, cholesterol, TAG and NEFA levels were analysed as per *Section 2.2.1* to *Section 2.2.4.*

3.3.3 Determination of inflammatory mediators

Mouse IL-6 and total adiponectin were analysed as per *Section 2.3.1* and *Section 2.3.2*, respectively). Analysis of serum insulin, TNFα, monocyte chemoattractant protein-1 (MCP-1), resistin and plasminogen activator inhibitor-1 (PAI-1) concentrations as per *Section 2.3.3.* The ranges achieved for the inflammatory quality controls are presented in *Table 2.4.*

3.3.4 Immunohistochemistry, tissue preparation and staining

The preparation of tissue for immunohistochemistry analysis, deparaffinized and hydratation technique and staining for specific antibodies is detailed in *Section 2.4.1* to *Section 2.4.3.*

Table 3.3: The fatty acid composition (w/w %) of the control (linoleic acid), *cis*-9 *trans*-11 high CLA (*c*9, *t*11-CLA) enriched diet, low *cis*-9 *trans*-11 CLA diets and synthetic CLA or TVA

	Diet A **Control Linoleic Acid**	***Diet B*** **High natural *c*9, *t*11-CLA**	***Diet C*** **Synthetic *c*9, *t*11-CLA**	***Diet D*** ***trans*** **Vaccenic Acid (TVA)**
Fatty acids				
C14:0 (Mysteric Acid)	2.43	2.43	2.55	2.38
C16:0 (Palmitic Acid)	22.55	21.09	22.78	22.34
C16:1 (Palmitoleic Acid)	4.49	3.94	4.46	4.6
C18:0 (Stearic Acid)	9.04	9.7	9.7	9.43
C18:1 (*trans*-11) (Vaccenic Acid)	2.18	7.26	2.24	5.39
C18:1 (*cis*9) (Oleic Acid)	4.33	4	4.46	4.61
C18:2 (*trans* 10, *cis*12-CLA)	0	0	0	0
C18:2 (*trans*-10-CLA)	0	0.045	0.069	0
C18:2 (*cis* 9, *trans* 11-CLA)	0.48	2.14	1.93	0.48

3.3.5 mRNA expression analysis and Real-time TaqMan® PCR

The extraction of RNA from the WAT, the complete RT-PCR procedure is described in *Section 2.5*

3.3.6 Western blotting and quantification of protein bands

Proteins were separated by SDS-PAGE and Western blotting was performed as per *Section 2.6.3.* Quantification of protein bands was performed using GeneSnap Acquisition and GeneTools Analysis Software (GeneGenesis Gel Documentation and Analysis System, Syngene). Molecular weight estimation was confirmed by the inclusion of a suitable standard (Precision Plus Protein™ Standards Dual Colour, Bio-Rad Laboratories Inc.).

3.3.7 Data preparation and statistical analysis

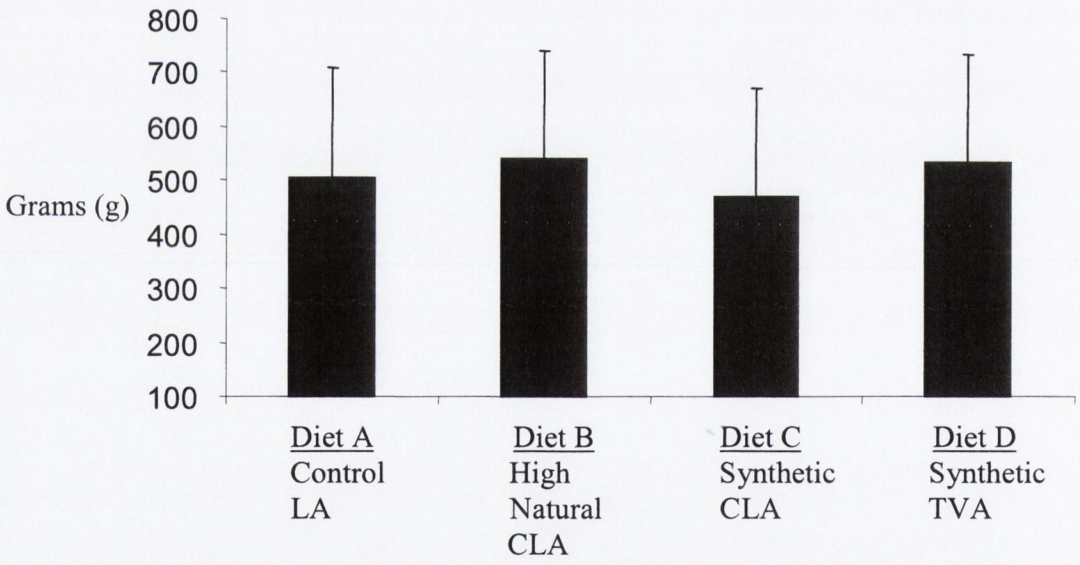
All statistical analysis was conducted using SPSS® for Windows™, version 14.0 (SPSS Inc, Chicago, IL, USA). Results are presented as means ± standard error of the mean (SEM) unless stated otherwise. All statistical analysis for *Chapter 3* is described in *Section 2.18* and *Section 2.18.1.*

3.4 RESULTS

3.4.1 Measurement of total food consumed

Four-week old male *ob/ob* mice were fed a controlled diet of chow for a 7-day period, after which, the animals were randomly assigned to one of the four dietary treatments as described in *Table 3.1*, percentage energy from macronutrients is presented in *Table 3.2* and the fatty acid composition (w/w%) of each diet is detailed in *Table 3.3*. Food intake was measured at the same time on a daily basis for the 28-day intervention period. Whilst there was a significant difference observed at day 7, where the Synthetic Diet (Diet C) group consumed less food, no overall significant difference in food consumption was observed across the four dietary treatments during the 28 day study (*Table 3.4; Figure 3.1a).*

Figure 3.1a: Average amount of food consumed per mouse per group over 28 days
Values are presented in means ± SEM



3.4.2 Measurement of total weight change

Animals were weighed on arrival into the BioResources facilities (TCD) to ensure all animals were thriving normally prior to the start of the study. Mice were weighed again at the end of the acclimatizing period, day zero (0) of the intervention period. Body weight measurements were recorded every second day during the study and the final measurements were taken on the morning of day 28 and their change in body weight was recorded. At day 0, no significant difference in body weight was noted between the four dietary treatment groups, therefore, any

differences in body weight observed during the study was not as consequence of altered starting body weight. On day 7, no significant differences were observed across the diet groups, however on day 14, it was noted that those mice receiving the Synthetic CLA (Diet C) were significantly heavier compared to the control, LA (Diet A; $p<0.05$). At day 21, no significant difference was noted and at day 28, again no significant differences were observed across the four dietary treatments. Nevertheless, when body weight change was calculated (day 28 body weight minus day 0 body weight), the increase in body weight was 16.77g, 12.58g, 15.13g and 13.32g for Diet A, Diet B, Diet C and Diet D, respectively (*Table 3.5*). After statistical analysis, it was noted that the high natural CLA (Diet B) mice gained significantly less weight compared to control, LA (Diet A) group ($p<0.05$) despite no significant increases in food consumption. Additionally, there was a trend for the low CLA and synthetic TVA (Diet D) to be lower compared to the control, LA (Diet A) group, although it was not statistically significant (p=0.07). Therefore, supplementation with natural *c*9, *t*11-CLA had an effect on weight gain in the study, with body weight change among the high natural CLA (Diet B) group being significantly less ($p<0.05$) compared to the control, LA (Diet A) group at 4.19g as illustrated in *Figure 3.1b.*

Figure 3.1b: Body weight change over intervention period

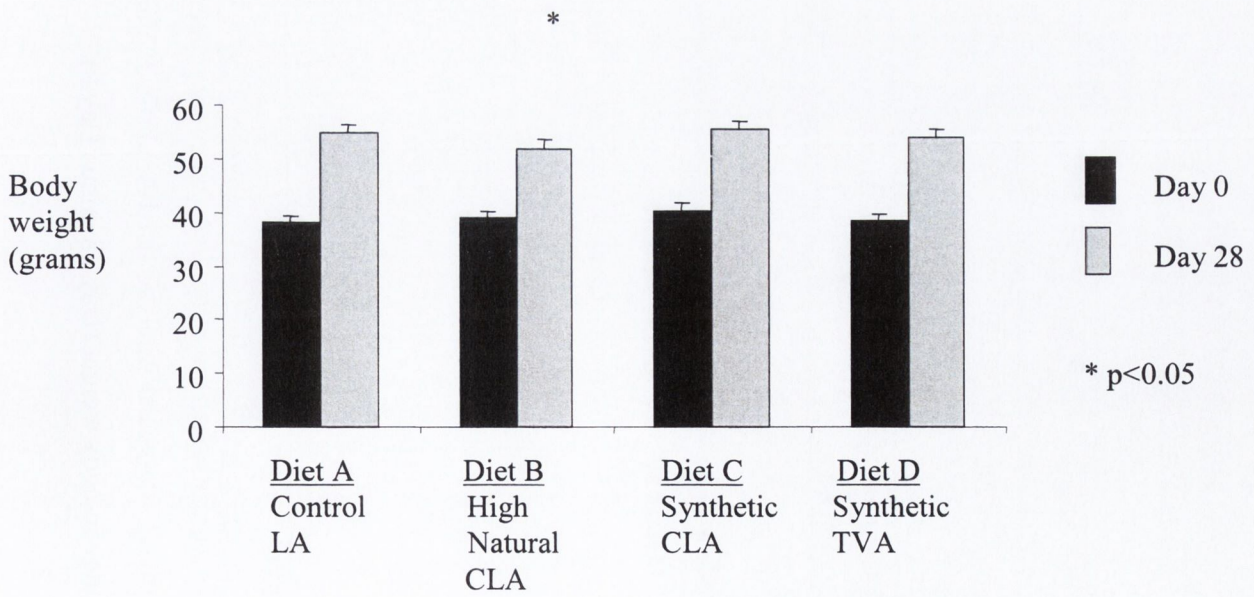


Table 3.4: Mean and SEM of the food consumption for all the mice across the four dietary treatments over 28 days (g)

	Control - Linoleic Acid Beef Diet A			High natural *c*9, *t*11-CLA beef Diet B			Synthetic *c*9, *t*11-CLA beef Diet C			Synthetic TVA beef Diet D		
	n	Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)
Day 7	8	306.36 [a]	(14.03)	7	292.01 [ab]	(4.58)	8	243.47 [b]	(33.44)	7	287.09 [ab]	(5.04)
Day 14	8	226.07 [ns]	(6.73)	7	202.86 [ns]	(5.77)	8	207.76 [ns]	(8.25)	7	210.9 [ns]	(9.01)
Day 21	8	281.38 [ns]	(2.84)	7	251.94 [ns]	(18.05)	8	279.27 [ns]	(3.57)	7	242.4 [ns]	(29.56)
Day 28	8	197.94 [ns]	(2.59)	7	195.43 [ns]	(25.62)	8	207.33 [ns]	(6.71)	7	191.7 [ns]	(14.85)

[abc...] denotes significance across the diets (when two superscripts contain the same letter there is no significant difference between two of the four diets), Statistical Analysis used Analysis of Variance (ANOVA); **CLA** (Conjugated Linoleic Acid); **TVA** (*trans* Vaccenic Acid);

Table 3.5: Mean and SEM of the gain in body weight across the four dietary treatments over 28 days

	Control - Linoleic Acid beef Diet A			High natural *c*9, *t*11-CLA beef Diet B			Synthetic *c*9, *t*11-CLA beef Diet C			Synthetic TVA beef Diet D		
	n	Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)	n	Mean	(SEM)
Day 0	8	37.93 [ns]	(1.18)	7	38.84 [ns]	(1.21)	8	40.28 [ns]	(0.71)	8	38.24 [ns]	(1.4)
Day 7	8	46.82 [ns]	(1.64)	7	48.07 [ns]	(0.9)	8	48.82 [ns]	(1.41)	8	45.26 [ns]	(1.51)
Day 14	8	48.32 [a]	(1.68)	7	48.94 [ab]	(0.82)	8	51.93 [b]	(0.86)	7	49.56 [ab]	(0.76)
Day 21	8	53.45 [ns]	(1.32)	7	51.58 [ns]	(0.99)	8	54.32 [ns]	(0.85)	7	52.49 [ns]	(0.97)
Day 28	8	54.7 [ns]	(1.13)	7	51.93 [ns]	(0.98)	8	55.41 [ns]	(1.28)	7	53.8 [ns]	(1.69)
Body Weight Change	8	16.77 [a]	(1.02)	7	12.58 [b]	(1.2)	8	15.14 [ab]	(1.37)	7	13.24 [ab]	(1.52)

[abc...] denotes significance across the diets (when two superscripts contain the same letter there is no significant difference between two of the four diets), Statistical Analysis used Analysis of Variance (ANOVA); **CLA** (Conjugated Linoleic Acid); **TVA** (*trans* Vaccenic Acid); Change in body weight (Day 28 minus Day 0).

3.4.3 Effects of the CLA enriched beef on markers of insulin sensitivity

Insulin concentrations were determined as part of the Mutliplex technology (Linco Research, USA) as described in *Section 2.3.3*. Results of this analysis showed no significant difference between the dietary groups after a one-way ANOVA with Bonferroni *post-hoc* testing. However, both the high natural CLA and synthetic CLA groups (Diet B and Diet C, respectively) showed significantly reduced serum glucose concentrations when compared to the control LA diet (Diet A; $p<0.0005$).

The insulin data was combined with the glucose concentrations in order to calculate a number of surrogate markers of IR such as $HOMA_{IR}$ [(glucose*insulin)/22.5] (Dandona *et al*, 2004). Although it should be acknowledged that to date, the $HOMA_{IR}$ model has not been validated for use in animal models. Additionally, insulin sensitivity was calculated with use of the revised QUICKI according to the equation [1/(log $insulin_0$ + log $glucose_0$ + log $NEFA_0$)] (Roche, 2004). Results show that $HOMA_{IR}$ concentrations were significantly reduced in the high natural CLA (Diet B) and the synthetic CLA (Diet C), compared to the control, LA (Diet A) ($p<0.0005$) and synthetic TVA (Diet D) ($p<0.0005$). Moreover, $HOMA_{IR}$ was also found to be significantly decreased in the synthetic TVA (Diet D) compared to the control, LA (Diet A) ($p<0.05$). Furthermore, the revised QUICKI index of insulin sensitivity demonstrated that the high natural CLA (Diet B) and the synthetic CLA (Diet C) were significantly increased compared to the control, LA (Diet A) and the synthetic TVA (Diet D) ($p<0.0005$). Moreover, the high natural CLA (Diet B) was significantly increased as compared to synthetic CLA (Diet C) ($p<0.001$) and no significant effect was noted for the synthetic TVA (Diet D) compared to the control, LA (Diet A) (*Table 3.6*).

3.4.4 Effects of the CLA enriched beef on lipoprotein metabolism

Table 3.7 presents the effect of the CLA enriched diets on lipoprotein metabolism. After statistical analysis, results showed that the high natural CLA (Diet B) and synthetic CLA (Diet C) had significantly reduced serum NEFA concentrations ($p<0.0005$). Analysis of TC, showed a significant increase for both the high natural CLA (Diet B) and synthetic CLA (Diet C) after CLA feeding ($p<0.05$). Serum TAG concentrations were found to be significantly decreased in the high natural CLA (Diet B) and synthetic CLA (Diet C) compared to the control, LA (Diet A) ($p<0.05$).

Table 3.6: The effects of varying composition of CLA beef diets on markers of insulin sensitivity in the *ob/ob* mouse after 28 days

Mediator	Units	Control Linoleic Acid beef *Diet A*			High natural *c*9, *t*11-CLA beef *Diet B*			Synthetic *c*9, *t*11-CLA beef *Diet C*			Synthetic TVA beef *Diet D*		
		n	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**
Glucose	*mmol/L*	6	19.63 ^{a}	(0.31)	6	14.07 ^{b}	(0.43)	7	15.28 ^{b}	(0.56)	7	18.08 ^{a}	(0.29)
Insulin †	*ng/mL*	8	3.0 ^{ns}	(0.48)	7	4.02 ^{ns}	(0.61)	8	3.28 ^{ns}	(0.49)	7	2.28 ^{ns}	(0.54)
$HOMA_{IR}$	*na*	6	2.47 ^{a}	(0.08)	5	1.22 ^{b}	(0.06)	7	1.51 ^{b}	(0.11)	7	2.09 ^{c}	(0.07)
Revised QUICKI†	*na*	6	0.63 ^{a}	(0.00)	6	1.00 ^{b}	(0.03)	7	0.85 ^{c}	(0.03)	7	0.67 ^{ad}	(0.01)

Values are presented in means ± SEM (Standard Error of the Mean); **n** (number of samples); $^{abc...}$ denotes significance across the diets (when two superscripts contain the same letter there is no significant difference between two of the four diets), Statistical Analysis used Analysis of Variance **(ANOVA);** † (Non Parametric Alternative: Kruskal-Wallis). **CLA** (Conjugated Linoleic Acid); **TVA** (*trans* Vaccenic Acid); **$HOMA_{IR}$** (Homeostatis Model Assessment - Insulin Resistance) [(glucose*insulin)/22.5]; Revised Quantitative insulin sensitivity check index **(QUICKI)** [$1/(\log \text{insulin}_0 + \log \text{glucose}_0 + \log \text{NEFA}_0)$] ; **na** (non applicable).

Table 3.7: The effects of varying composition of CLA beef diets on lipoprotein concentrations in the *ob/ob* mouse after 28 days

Mediator	Units	Control - Linoleic Acid beef *Diet A*			High natural c9,t11 CLA beef *Diet B*			Synthetic c9,t11-CLA beef *Diet C*			Synthetic TVA beef *Diet D*		
		n	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**	**n**	**Mean**	**(SEM)**
NEFA	*mmol/L*	6	0.70 ^{a}	(0.03)	6	0.36 ^{b}	(0.03)	7	0.47 ^{b}	(0.03)	7	0.66 ^{a}	(0.02)
Cholesterol	*mmol/L*	6	5.14 ^{a}	(0.17)	6	5.83 ^{b}	(0.07)	7	5.87 ^{b}	(0.18)	7	5.66 ^{ab}	(0.19)
TAG	*mmol/L*	6	1.50 ^{a}	(0.03)	6	1.39 ^{b}	(0.02)	7	1.37 ^{b}	(0.02)	7	1.51 ^{a}	(0.03)

Values are presented in means ± SEM (Standard Error of the Mean); **n** (number of samples); **NEFA** (Non Esterified Fatty Acids); **TAG** (Triglycerides); **CLA** (Conjugated Linoleic Acid); **TVA** (*trans* Vaccenic Acid); $^{abc...}$ denotes significance across the diets (when two superscripts contain the same letter there is no significant difference between two of the four diets); Statistical Analysis used Analysis of Variance (**ANOVA**);

3.4.5 Effects of CLA enriched beef on markers of inflammation

The effects of the four dietary treatments on markers of inflammation that are associated to insulin resistance are presented in *Table 3.8*. Both CLA diets, the high natural CLA (Diet B) and synthetic CLA (Diet C), showed positive improvements in inflammatory status, whereby, a significant increase was noted for total serum adiponectin concentrations ($p<0.0005$ and $p<0.001$ respectively). Also, a significant decrease in serum IL-6 concentrations was observed within the high natural CLA (Diet B) and synthetic CLA (Diet C) groups ($p<0.001$ and $p<0.0005$ respectively). Serum resistin, TNFα, PAI-1 and MCP-1 concentrations (as determined by Multiplex ELISA, *Section 2.3.3)* showed no significance differences between any of the dietary treatments.

3.4.6 Relationship between markers of insulin sensitivity, inflammation and blood lipids irrespective and according to each dietary treatment

Correlation analysis showed very strong relationship between insulin sensitivity, biomarkers of inflammatory status and lipoprotein concentrations, irrespective of dietary treatment as exemplified in *Table 3.9.* Glucose and insulin concentrations were found to be strongly and positively correlated with $HOMA_{IR}$, serum IL-6 concentrations, NEFA and TAG levels. Whereas serum glucose and insulin levels display very strong inverse relationships with adiponectin concentrations, cholesterol levels and QUICKI index of insulin sensitivity. When assessed according to dietary treatment, glucose and insulin were correlated strongly with each other and with $HOMA_{IR}$, reaching significance ($p<0.0005$) for each of the four diets. No other significance was noted according to dietary treatments (*Data not shown*).

3.4.7 Gene expression analysis from the white adipose tissue

3.4.7a Molecular markers of insulin sensitivity in the white adipose tissue

AT plays a central role in systemic insulin sensitivity, therefore the effects of differing high natural CLA (Diet B) and synthetic CLA diets on the expression of key genes involved in regulating insulin sensitivity and glucose homeostasis were investigated (*Figure 3.2a* and *Figure 3.2b*). As no significant findings were observed for synthetic TVA (Diet D) in terms of body composition and circulating markers of insulin sensitivity, inflammation and blood lipids therefore molecule analysis was not performed for synthetic TVA (Diet D) on WAT.

However, analysis for the other CLA treatments demonstrated that IRS-1 expression was increased (1.6 fold compared to the control, LA Diet A) following the high natural CLA (Diet

B) and reached statistical significance ($p<0.05$). Similarly, IRS-1 was increased 1.5 fold following treatment with the synthetic CLA (Diet C) when compared to the control, LA (Diet A) which also reached statistical significance ($p<0.01$). There was no significant difference between the high natural CLA (Diet B) and the synthetic CLA (Diet C). GLUT4 expression was also increased in response to the high natural CLA (Diet B) and the synthetic CLA (Diet C) when compared to the control diet. High natural CLA (Diet B) was increased 1.5 fold, and reached significance (p=0.002), whilst the synthetic CLA (Diet C) was increased 1.6 fold, again reaching significance (p=0.003). As with IRS-1, there was no significant difference between the high natural CLA beef (Diet B) and the synthetic CLA (Diet C).

3.4.7b Molecular markers of inflammation status in the white adipose tissue

As inflammation is one of the most interesting areas in the MetS, the effects of differing compositions of high natural CLA (Diet B) and synthetic CLA (Diet C) on the gene expression of two key regulators of the immune response was investigated by Taqman® PCR analysis (*Figure 3.3a and Figure 3.3b*). Results for the expression of IL-6 from the WAT showed a decrease (0.6 fold) for the high natural CLA (Diet B), although this did not reach statistical significance (p=0.13). Genetic expression of IL-6 was also decreased following the synthetic CLA (Diet C) by 0.6 fold, but again this did not reach statistical significance (p=0.06). TNFα gene expression was also analysed as a key marker of inflammatory status, and showed a similar expression profile to that of IL-6. Both the high natural CLA (Diet B) and synthetic CLA (Diet C) displayed a significant 0.6 fold decrease in expression ($p<0.05$ for both diets).

Table 3.8: The effects of varying composition of CLA beef diets on markers of inflammation in the *ob/ob* mouse after 28 days

Mediator	*Units*	Control - Linoleic Acid beef *Diet A*				High natural c9, t11-CLA beef *Diet B*				Synthetic *c*9, *t*11-CLA beef *Diet C*				Synthetic TVA beef *Diet D*			
		n	**Mean**		**(SEM)**	**n**	**Mean**		**(SEM)**	**n**	**Mean**		**(SEM)**	**n**	**Mean**		**(SEM)**
Adiponectin *	*mg/mL*	5	7198	^{a}	(284)	5	9961	^{b}	(101)	6	9212	^{b}	(288)	7	8056	^{a}	(322)
IL-6 *	*pg/mL*	7	1626	^{a}	(69.18)	5	1168	^{b}	(75.29)	6	1151	^{b}	(44.7)	7	1690	^{a}	(54.3)
Resistin ¥ †	*pg/mL*	8	5155.6	^{ns}	(3911)	7	2214.3	^{ns}	(231)	8	1912.7	^{ns}	(176)	7	1845.7	^{ns}	(192)
TNFα ¥ †	*pg/mL*	8	5.00	^{ns}	(0.50)	7	5.49	^{ns}	(0.47)	8	5.00	^{ns}	(0.54)	7	5.86	^{ns}	(0.74)
PAI-1 ¥	*pg/mL*	8	1892.0	^{ns}	(844.3)	7	4421.1	^{ns}	(1547.2)	8	4323.5	^{ns}	(852.6)	7	3539.4	^{ns}	(789.5)
MCP-1 ¥	*pg/mL*	8	153.9	^{ns}	(17.6)	7	185.9	^{ns}	(17.3)	8	193.0	^{ns}	(30.6)	7	184.1	^{ns}	(28.8)

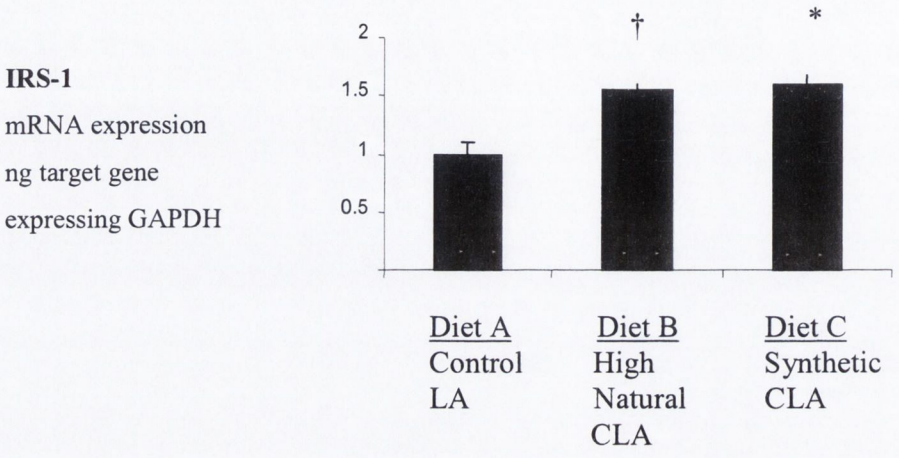
$^{abc...}$ denotes significance across the diets (when two superscripts contain the same letter there is no significant difference between two of the four diets); ^{ns} (non significant); **n** (number of samples); * Quanitikine ELISA analysed separately and statistically measured by 1-way ANOVA analysis, ¥ Multiplex ELISA, all measured simultaneously and statistically measured by t-tests. Values are presented in means ± **SEM** (Standard Error of the Mean); **CLA** (Conjugated Linoleic Acid); **TVA** (*trans* Vaccenic Acid); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **PAI-1** (Plasminogen Activated Inhibitor-1), **MCP-1** (Monocyte Chemoattractant Protein-1); Statistical Analysis used Analysis of Variance (**ANOVA**); † (Non Parametric Alternative: Kruskal-Walli).

Table 3.9: Correlation matrix displaying the significant relationships between the markers of insulin sensitivity, inflammation and lipoprotein metabolism, irrespective of dietary treatment

		Glucose	Insulin		$HOMA_{IR}$		QUICKI		Adiponectin		IL-6		NEFA		Cholesterol		TAG	
Glucose	r	Na	1.00	***	1.00	***	-0.90	***	-0.76	***	0.76	***	0.77	***	-0.39	*	0.65	***
	sig		0.00		0.00		0.00		0.00		0.00		0.00		0.05		0.00	
Insulin	r		na		1.00	***	-0.90	***	-0.76	***	0.76	***	0.77	***	-0.39	*	0.65	***
	sig				0.00		0.00		0.00		0.00		0.00		0.05		0.00	
$HOMA_{IR}$	r				na		-0.89	***	-0.76	***	0.76	***	0.77	***	-0.39	*	0.65	***
	sig						0.00		0.00		0.00		0.00		0.05		0.00	
QUICKI	r						na		0.75	***	-0.73	***	-0.93	***	0.40	*	-0.58	***
	sig								0.00		0.00		0.00		0.04		0.00	
Adiponectin	r								Na		-0.81	***	-0.72	***	0.17	ns	-0.56	**
	sig										0.00		0.00		0.43		0.01	
IL-6	r										na		0.69	***	-0.13	ns	0.70	***
	sig												0.00		0.56		0.00	
NEFA	r												na		-0.47	*	0.58	***
	sig														0.02		0.00	
Cholesterol	r														na		-0.18	ns
	sig																0.39	
TAG	r																na	
	sig																	

* (p<0.05), ** (p<0.01), *** (p<0.0005); [ns] (non signifiant); [na] (non applicable

IRS-1 expression from the white adipose tissue in the *ob/ob* mice following CLA feeding



GLUT4 expression from the white adipose tissue in the *ob/ob* mice following CLA feeding

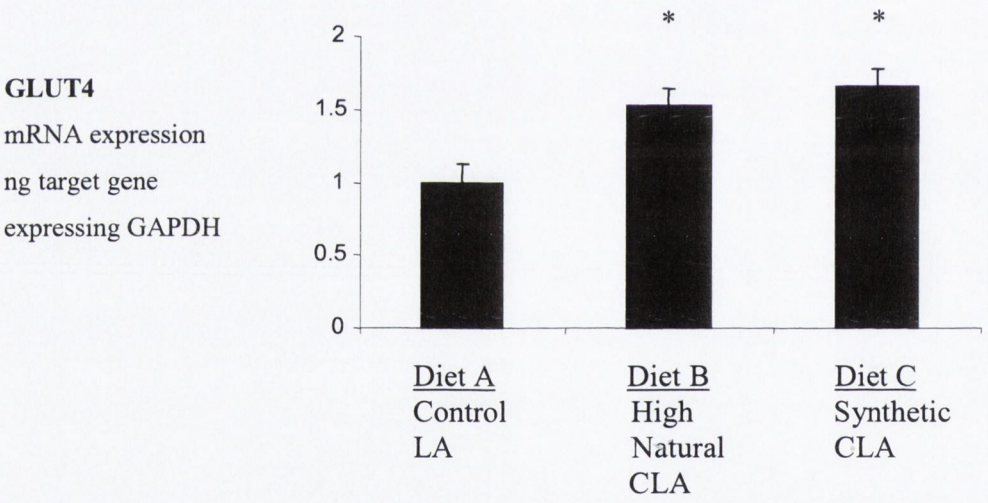
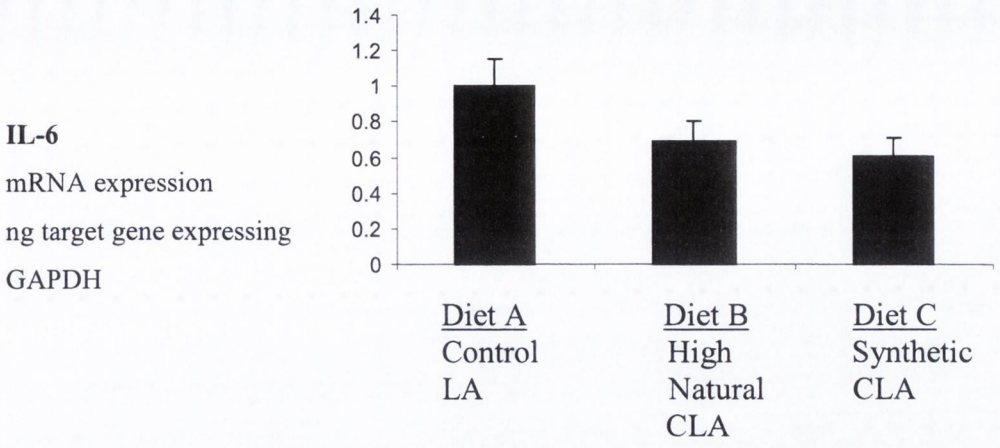


Figure 3.2a and 3.2b: mRNA expression of IRS-1 and GLUT4, key proteins involved in insulin signalling cascade on WAT following a control (LA), high natural *c*9, *t*11-CLA beef derived CLA diet or low natural beef derived CLA & synthetic *c*9, *t*11-CLA Diet.

Target mRNA levels were normalised to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed relative to the control, LA (Diet A). Values present group means ± SEM with n=8 in the control, LA (Diet A), n=7 in the high natural CLA (Diet B) and n=8 in the synthetic CLA (Diet C); † ($p<0.05$); * ($p<0.01$).

IL-6 expression from the white adipose tissue in the *ob/ob* mice following CLA feeding



TNFα expression from the white adipose tissue in the *ob/ob* mice following CLA feeding

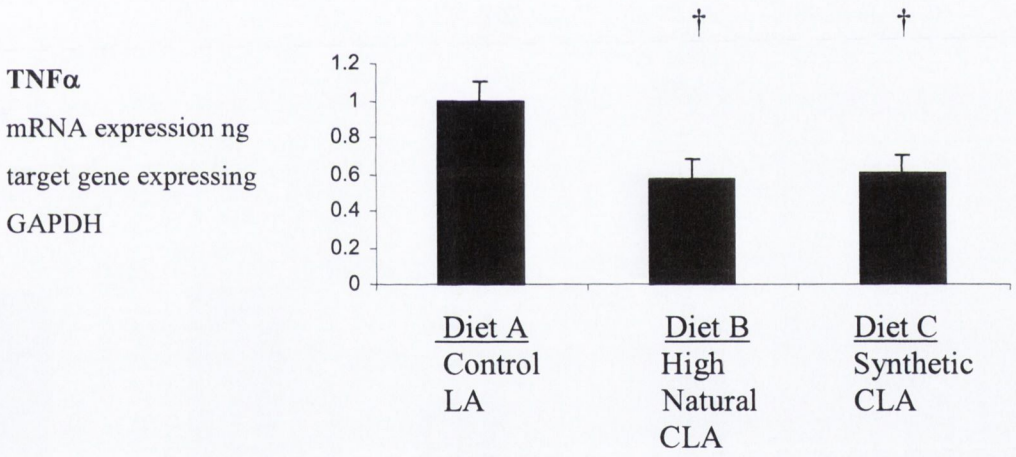


Figure 3.3a and Figure 3.3b: mRNA expression of IL-6 and TNFα, key markers involved in inflammation in WAT following a control (LA), high natural *c*9, *t*11-CLA derived beef diet or low natural beef derived CLA & synthetic *c*9, *t*11-CLA Diet.

Target mRNA levels were normalised to GAPDH and expressed relative to the control, LA (Diet A). Values present group means ± SEM with n=8 in the control, LA (Diet A), n=7 in the high natural CLA enriched beef (Diet B) and n=8 in the synthetic CLA (Diet C); † ($p<0.05$).

3.4.8 Expression of proteins involved in the insulin signalling cascade from the white adipose tissue – GLUT4 and IRS-1

Key targets involved in glucose uptake into the AT, GLUT4 and IRS-1 were investigated to determine whether feeding a high natural CLA (Diet B) or synthetic CLA (Diet C) alters the expression of these proteins. This study demonstrated that GLUT4 expression was increased after the high natural CLA (Diet B) and the synthetic CLA (Diet C) compared to the control, LA (Diet A) as illustrated in *Figure 3.4*.

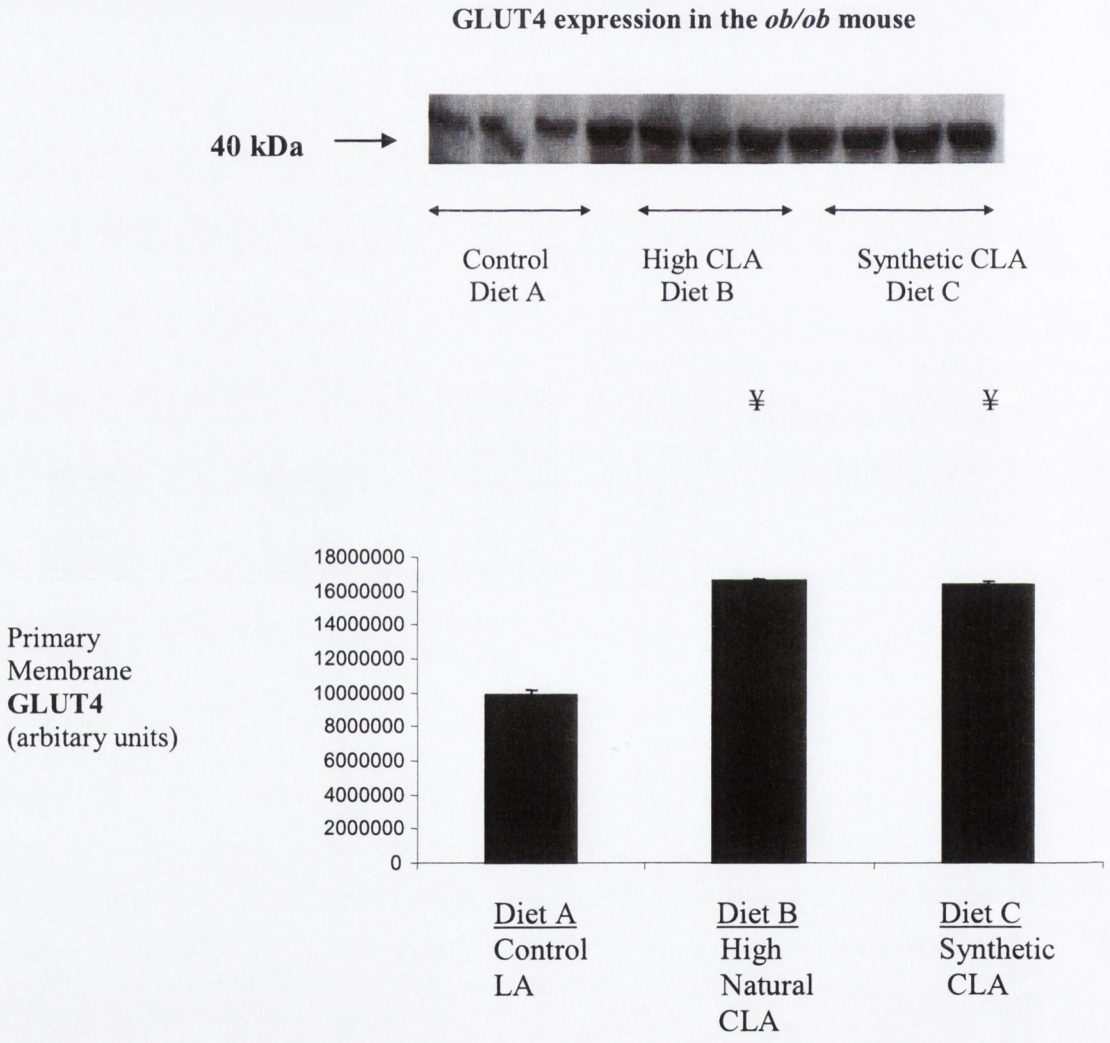


Figure 3.4: GLUT4 expression in WAT from control (LA), natural *c*9, *t*11-CLA derived beef and synthetic *c*9, *t*11-CLA diets normalised for the β-Actin loading control. The representative western blot and histogram from densitometric analysis expressed as arbitrary units, means ± SD are illustrated above; ¥ ($p<0.0005$).

3.4.9 Expression of IRS-1 from the white adipose tissue

IRS-1 expression was also investigated after 28 days of feeding a CLA beef enriched diet to *ob/ob* mice. Results demonstrated that IRS-I expression was significantly increased in the high natural CLA beef (Diet B) when compared to the control, LA (Diet A) ($p<0.05$). Additionally, IRS-1 expression was significantly increased in the synthetic *c*9, *t*11-CLA (Diet C) when compared to the control, LA (Diet A) ($p<0.01$; *Figure 3.5*).

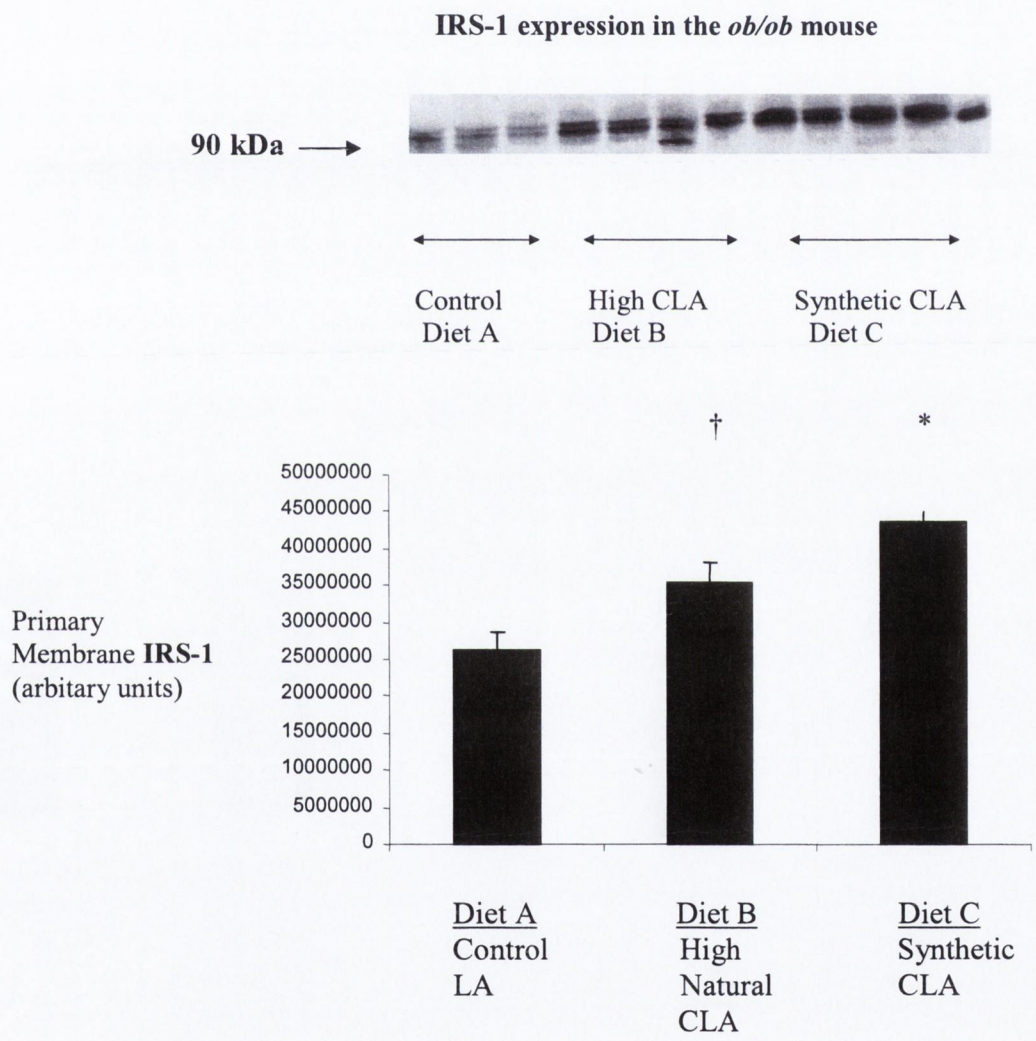


Figure 3.5: IRS-1 expression in WAT from control (LA), high natural *c*9, *t*11-CLA derived beef and synthetic *c*9, *t*11-CLA diets normalised for the β-Actin loading control. The representative western blot and histogram from densitometric analysis expressed as arbitrary units, means ± SD are illustrated above. † ($p<0.05$); * ($p<0.01$).

3.4.10 Expression of IRS-1 Serine 307 from the white adipose tissue

IRS-1 Serine (Ser)307, the phosphorylated form of IRS-1 was also investigated in this study after 28 days of CLA beef enriched feeding to *ob/ob* mice. Results demonstrated that both CLA enriched diets, the high natural CLA beef (Diet B) and synthetic CLA (Diet C) increased expression of IRS-1 serine 307 from the WAT ($p<0.05$) (*Figure 3.6*).

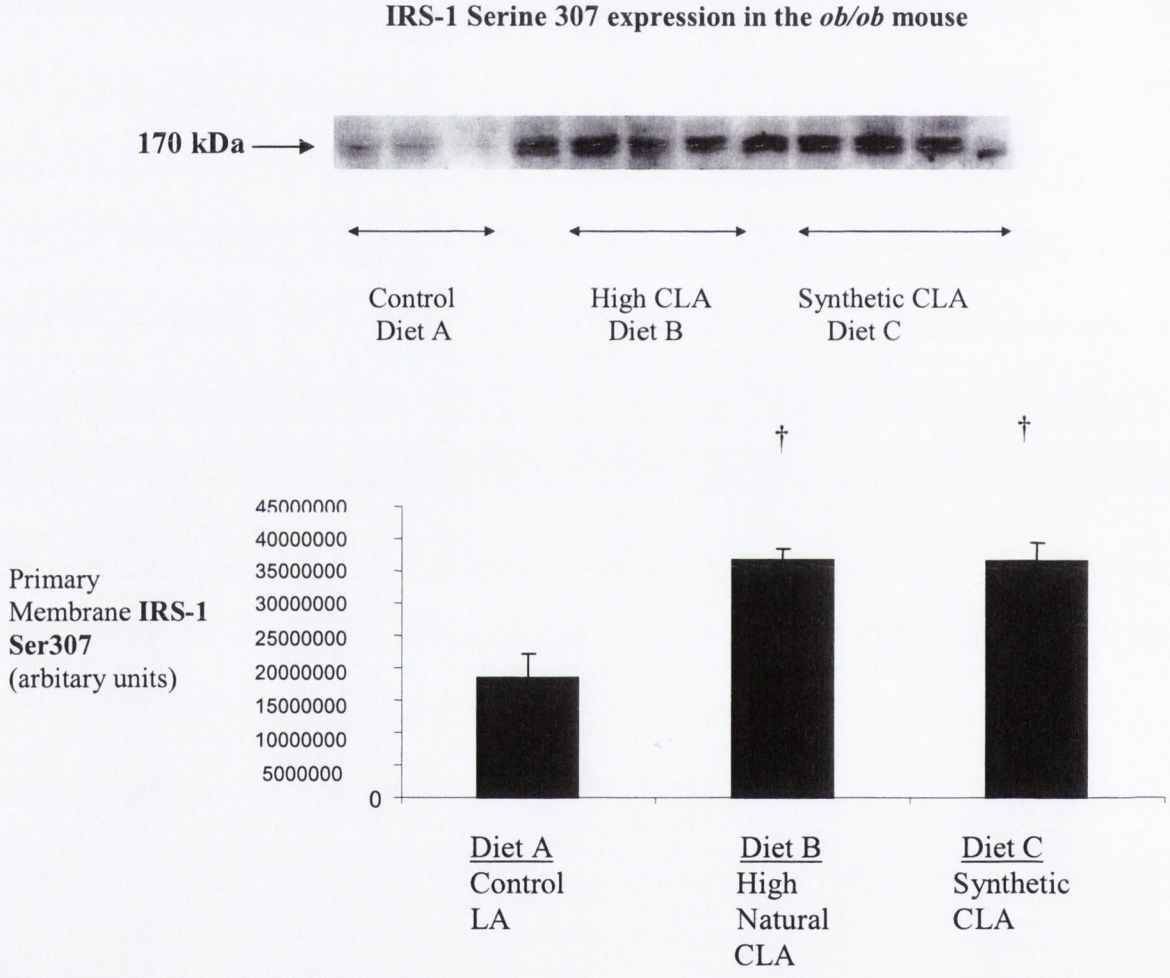


Figure 3.6: IRS-1 Ser307 expression in WAT from control (LA), high natural *c*9, *t*11-CLA derived beef and synthetic *c*9, *t*11-CLA diets. The representative western blot and histogram from densitometric analysis expressed as arbitrary units, means ± SD are illustrated above. † ($p<0.05$).

3.4.11 Expression of β-actin from the white adipose tissue

β-actin or nuclear histone, is one of six different actin isoforms which has been identified. Actins are highly conserved proteins that are involved in cell motility, structure and integrity. β-Actin is a relatively stable cytoskeletal protein generally thought to be present at a constant level in cells, regardless in most cases of experimental treatment or technical procedure. For this reason, measurement of β-Actin was used as an internal control for the CLA feeding study to identify technical error.

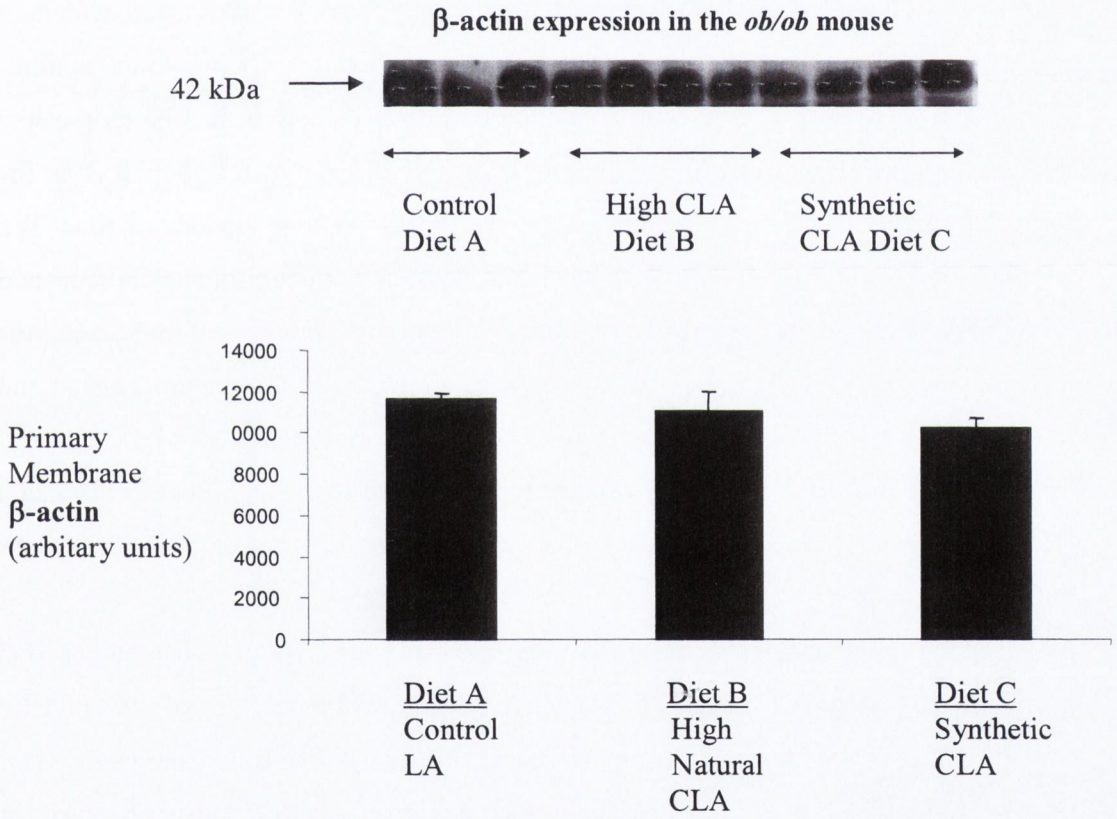


Figure 3.7: mRNA expression of β-Actin, following a control (LA), high natural *c*9, *t*11-CLA derived beef diet or low natural beef derived CLA & synthetic *c*9, *t*11-CLA Diet. Values present group means ± SD with n=8 in the control, LA (Diet A), n=7 in the high CLA enriched beef (Diet B) and n=8 in the synthetic CLA (Diet C). Results demonstrated no statistical significance differences among the three dietary treatments examined which suggests equal loading of sample.

3.5 DISCUSSION

Metabolic diseases such as abdominal obesity, IR, dyslipidmeia and hypertension are increasing globally at an alarming rate. These metabolic disorders make up what is now commonly referred to as the MetS, which increases the risk of developing CVD and T2DM (Villegas *et al*, 2004). The aetiology of MetS is a combination of genetic, perinatal and environmental factors. The latter may contribute more in our modern society than in the past as it is characterised by an ongoing positive energy balance (increased energy intake and decreased physical activity), increased stress, smoking and alcohol intake (Katan *et al*, 1995, Pfeuffer & Schrezenmeir, 2000). CLA has received much attention due to its potential anti-diabetic (Eyjolfson *et al*, 2004), anti-inflammatory (Yu *et al*, 2002) and chemoprotective properties (Belury, 1995) demonstrated both *in vivo* and *in vitro*. CLA is found naturally in the diet in the lipid portion of ruminant meat and dairy products and is now under the spotlight to be considered as a functional food (Toomey *et al*, 2006). A functional food is an improving agent that does not have side effects and can be taken for long periods of time. Within the context of the CVD disease risk, CLA has been shown to have anti-diabetic effects and induce the regression of atherosclerosis in a number of rodent models. For example, a mixture of *c*9, *t*11-CLA and *t*10, *c*12-CLA respectively has been shown to inhibit the progression and induce the regression of established atherosclerosis in $ApoE^{-/-}$ mouse models (Toomey *et al*, 2006; Arbonés-Mainar *et al*, 2006), whilst a diet supplemented with ~ 4.5 % *c*9, *t*11-CLA in *ob/ob* mice was shown to improve lipid metabolism after 4-weeks (Roche *et al*, 2002; Moloney *et al*, 2007).

This present study was designed using the C57BL/6J *ob/ob* mouse model to elucidate the relationship between natural *c*9, *t*11-CLA beef, a synthetic form of *c*9, *t*11-CLA and a synthetic form of TVA on body composition, insulin sensitivity and inflammatory status. The *c*9, *t*11-CLA was solely investigated due to the positive effects previously observed on insulin sensitivity, inflammatory status and blood lipid profiles. The *ob/ob* homozygous mice are leptin deficient, hyper-insulinemic and become extremely obese and are therefore an excellent rodent model of the MetS and T2DM. In this study mice were randomly assigned to one of four dietary treatments varying in compositions of CLA for a 28 day period. Results of this study show potential beneficial effects of natural *c*9, *t*11-CLA in relation to body weight, whereby the high natural CLA beef group gained significantly less weight, when compared to the control, LA group despite no significant increases in food intake. Previously studies have noted similar results and have postulated that this may be due to an increase in energy

expenditure and thermogenesis (Watras *et al*, 2007; West *et al*, 2000). In addition, this has been attributed to decreased lipolysis and FA oxidation and reduced deposition of FA in AT. Previous studies investigation fat-lowering effects of CLA in mice have shown that CLA reduces body fat while increasing lean body mass (Ohya *et al*, 2005; Smedman *et al*, 2005; Risérus *et al*, 2004; Gonzalez *et al*, 2006). Additionally, CLA has been found to reduce body fat in different rodent models (DeLany & West, 2000; Pariza, 2002). Research demonstrated that administration of 0.5% dietary CLA resulted in decreased body fat of 57% and 60% in male and female mice, respectively (Park *et al*, 1997). Contrary to this, a study in rats fed a diet supplemented with 0.5% CLA from 3 to 8 weeks of age, demonstrated significantly higher body weights compared to the controls, despite food intake not being affected (Chin *et al*, 1994). When mice were fed CLA preparations enriched with either the *c*9, *t*11-CLA or *t*10, *c*12-CLA isomer, favourable changes in body composition, as determined by reduced body fat, enhanced whole body water, and enhanced whole body protein, were only associated with the feeding of the *t*10, *c*12-CLA (Park *et al*, 1999). Several studies investigating the anti-obesity effects of CLA isomers have also been performed in humans. Recently, a double-blind, placebo-controlled study set out to examine the modifications in body composition of 48 healthy obese humans, randomized to receive either placebo (8 g safflower oil/d), 3.2 g/d CLA or 6.4 g/d CLA of mixed isomers for 12 weeks. Results showed that lean body mass increased by 0.64 kg in the 6.4 g/d CLA group ($p<0.05$). Furthermore, significant decreases in serum HDL-c and significant increases in serum alkaline phosphatase, CRP and IL-6 also occurred in the 6.4 g/d CLA group, although all values remained within normal limits. The study concluded that although CLA may increase lean body mass in obese humans, it may also increase markers of inflammation in the short term (Steck *et al*, 2007). Another, recent double blind, placebo-controlled study was carried out on 118 healthy, overweight and obese adults. Subjects were randomised into two groups supplemented with either (3 x 4 g/d) CLA or placebo for 6 months. Results showed that CLA significantly decreased body fat mass at month 3 and at month 6, compared to the placebo treatment. Interestingly, the reduction in fat mass was located mostly in the legs. Additionally, the waist/hip ratio decreased significantly compared with placebo. Lean body mass increased significantly within the CLA group and all changes were independent of diet and physical exercise (Gaullier *et al*, 2007).

Several studies have reported that the *t*10, *c*12-CLA isomer appears to be accountable for the reduction of body fat accumulation (Wargent *et al*, 2005; Evans *et al*, 2002). Risérus *et*

al (2002) reported that the *t*10, *c*12-CLA isomer (approximately 2g/d) for 12-weeks was responsible for a marked trend towards a decrease in body fat, sagittal abdominal diameter, waist circumference, BMI and weight in 60 obese men with signs of the MetS, whereas only sagittal abdominal diameter and body fat decreased after a CLA mixture (50:50 blend of *c*9, *t*11-CLA and *t*10, *c*12-CLA). Within the same group, 3 months supplementation of (3g/d) pure *c*9, *t*11-CLA was not found to affect body composition in 25, abdominally-obese men (Risérus *et al*, 2004). A recent study examining the effects of CLA in patients with T2DM, supplementation (8g/d) of mixed CLA isomers (37% *c*9, *t*11-CLA and 39% *t*10, *c*12-CLA) or a supplement containing (8g/d) safflower oil for 8 weeks, found that the *t*10, *c*12-CLA isomer were inversely correlated with changes in body weight and serum leptin ($p<0.05$) (Belury *et al*, 2003). Several studies have been completed on the effects of CLA supplementation on healthy, normal-weight individuals and demonstrated no significant effects of body composition (Zambell *et al*, 2001; Kreider *et al*, 2002; Petridou *et al*, 2003; Tricon *et al*, 2004), while three seperate studies have reported modest reductions in fat mass after CLA supplementation (Mougios *et al,* 2001; Smedman & Vessby, 2001; Thom *et al*, 2001). The majority of studies have found that the *t*10, *c*12-CLA is responsible for the fat-lower effects of CLA, however, the current study suggests that *c*9, *t*11-CLA isomer may also have the potential of reducing body fat and increase lean body mass. Further longer-term research is required to determine the isomer specific effects may have potential in the treatment and management of excessive body fat in the overweight or obese state.

According to the World Health Organisation (WHO), impaired glucose tolerance (IGT) or IR are the central criteria for diagnosis of the MetS, combined with two of the following conditions: obesity, dyslipidemia, hypertension and microalbuminuria (Alberi *et al*, 2006). IR is also the major underlying pathophysiological defect leading to the development of T2DM. Yet approximately two-thirds of people with IR do not develop overt T2DM. Nevertheless, they are at the same increase risk of CVD events as diabetic individuals (Abuissa *et al*, 2005). Consumption of high-caloric diet and lack of exercise have increased the worldwide prevalence of T2DM and obesity (Kasuga, 2006). Given this escalating prevalence of obesity, it would be advantageous to identify potential therapeutic nutrients/ functional foods to improve insulin sensitivity within the context of obesity. Studies to date have indicated that the effects of CLA on IR and glucose homeostatis are variable and dependent on species and the type of isomer present. Two very recent studies have

endeavoured to investigate the impact of CLA on markers of insulin sensitivity and have published inconsistent results (Thrush *et al*, 2007; Lambert *et al*, 2007). Therefore, to determine whether the biological and synthetic form of *c*9, *t*11-CLA affects glucose metabolism and IR, serum glucose and insulin were measured and $HOMA_{IR}$ and revised QUICKI was calculated within the present study. Interestingly, results demonstrated that after intervention with the natural *c*9, *t*11-CLA enriched diet and the synthetic *c*9, *t*11-CLA diet, significant reductions in plasma glucose concentrations were found, as well as a decreased $HOMA_{IR}$ index of IR and an improved revised QUICKI indicator of insulin sensitivity in the well characterised *ob/ob* mouse model. The improved metabolic profile may be partly ascribed to the upregulated GLUT4, IRS-1 and IRS-1 serine phosphorylated mRNA expression in the WAT following the *c*9, *t*11-CLA enriched diet and the synthetic CLA diet. Glucose uptake in peripheral tissues is largely mediated by intrinsic membrane proteins known to facilitate glucose transport (GLUTs). GLUT4 is selectively expressed in insulin-sensitive tissues such as muscle and AT (Herman & Kahn, 2006). Therefore, an impairment in GLUT4 translocation and/or insulin signalling through free fatty acids (FFA) in muscle and adipocytes may affect basal and insulin stimulated glucose uptake, which may result in IR and hyperglycemia, the hallmark of T2DM (Park *et al*, 2005). In addition, FFA may impair steps in insulin-signal transduction, leading to reduced insulin receptor substrate associated phosphatidlylinositol-3-kinase (P13K-AKT) activity. This is an important step in triggering the translocation of GLUT4 molecules to the cell surface and with decreased activity of P13K-AKT this leads to IR (Medina-Gomez *et al*, 2007).

Anti-diabetic results were found in a recent study whereby the isomeric effects of *c*9, *t*11-CLA was demonstrated on markers of insulin sensitivity in *ob/ob* mice. The study concluded that these positive effects may be mediated through the anti-inflammatory effects of *c*9, *t*11-CLA seen in the WAT (Moloney *et al*, 2007). However, a diet-controlled study on T2DM human subjects observed no effects of CLA when blending *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers (3.0g/d CLA) together for 8 weeks on fasting plasma glucose or insulin concentrations (Moloney *et al*, 2004). A very similar study investigating the effect of a blend (50:50 or 80:20%) of *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers (3.0g/d CLA) for 8 weeks in 51 healthy subjects (18 males, 33 females) demonstrated no significant effects on fasting plasma glucose or insulin concentrations, however, the 50:50 blend reduced plasma TAG concentrations, whereas the 80:20 blend reduced plasma VLDL concentrations (Noone *et al*,

2002). Results from this study suggest that blends of CLA isomers may be biologically inactive in healthy humans or perhaps the dose given was insufficient to achieve alterations in insulin sensitivity. In support of this hypothesis, Medinae *et al* (2000) conducted their study in a metabolic suite and gave 17 healthy women 3.0g CLA per day for 9 weeks. Neither plasma glucose or insulin concentrations were significantly altered following supplementation. All other human intervention studies investigating the effect of CLA on insulin sensitivity have been conduced on a free-living basis. In a very recent study 38 healthy young men were given a diet rich in butter as a source of milk fat with 115 g/d of CLA-rich fat (5.5 g/d CLA oil, a mixture of 39.4% *c*9, *t*1-CLA and 38.5% *t*10, *c*12-CLA) or control fat with a low content of CLA for 5-weeks. Results showed that the CLA diet resulted in increased lipid peroxidation measured as an 83% higher 8-iso-prostaglandin F2α (8-iso-$PGF_{2\alpha}$). No other significant differences were observed for markers of insulin sensitivity, inflammation or lipoprotein metabolism (Raff *et al*, 2008). Kamphius *et al* (2003) recruited 54 overweight subjects (26 men, 28 women) age 20-50 years and submitted them to a very low calorie diet for 3 weeks followed by supplementation with either CLA or placebo to test the effects of CLA following weight loss. At the end of the 13 week intervention, neither plasma glucose nor insulin concentrations were significantly altered. Smedman & Vessby (2001) gave 25 abdominally obese (age 39-64 years), 4.2g of 50:50 CLA blend. Again, no significant modifications were noted for plasma glucose and insulin concentrations. Risérus *et al* (2001) gave 25 abdominally obese males (39-64 years) supplements containing the same dose and blend as Smedman & Vessby (2001). Results showed an increase in plasma glucose concentrations after the CLA and placebo supplements, but no difference was observed between the groups and plasma insulin concentrations remained unchanged. In contrast to this theory about CLA isomeric blends, young adults with a sedentary lifestyle, supplemented with 4g/d CLA (35.5% *c*9, *t*11; 36.8% *t*10, *c*12) for 8-weeks showed reduced fasting insulin concentrations and improved insulin sensitivity compared to placebo (Eyjolfson *et al*, 2004).

The current study on *ob/ob* mice used a single blend of *c*9, *t*10-CLA and noted that this is indeed biologically active as proven with the significant modifications in markers of insulin sensitivity. Another determined the isomer specific effect of CLA in $ApoE^{-/-}$ mice and showed that the *c*9, *t*10-CLA isomer reduced glucose and insulin concentrations and improved the indexes of insulin sensitivity (revised QUICKI) and IR ($HOMA_{IR}$) (de Roos *et al*, 2005). A recent crossover study determining the health benefits of single blends of CLA enriched dairy products (1.421 g/d *c*9, *t*11-CLA) versus control dairy products (0.151d/g *c*9,*t*11-CLA) for 6-

weeks showed that consumption of *c*9,*t*11-CLA fortified foods had no significant impact on insulin, glucose or lipid metabolism healthy middle aged men (Tricon *et al*, 2006). In contrast, another study set out to examine the effects of the *c*9, *t*11-CLA isomer alone in 25 Caucasian men with abdominal obesity, age 35-64 years at high risk for CVD. Surprisingly, the *c*9, *t*11-CLA isomer decreased insulin sensitivity by 15% as measured using the euglycaemic-hyerinsulinaemic clamp. Mean insulin-mediated glucose uptake was also significantly lower after *c*9, *t*11-CLA supplementation compared to the control. In addition, the *c*9, *t*11-CLA had no significant effect on either fasting plasma glucose or insulin concentrations however, increased lipid perioxidation in obese middle aged men (Risérus *et al*, 2004*c*). This was the first study to demonstrate a negative effect of the predominant isomer on insulin sensitivity. In comparison to the studies examining single *c*9, *t*11-CLA isomer, another study concluded that the *c*9, *t*11-CLA was the more effective isomer in the treatment of IR, and this was associated with upregulated acyl-CoA oxidase (ACO) and uncoupling protein-2 (UCP-2) mRNA expression in liver and skeletal muscle (Choi & Song, 2005). It should be noted that a number of aforementioned studies (Smedman & Vessby, 2001; Risérus *et al*, 2001; Risérus *et al*, 2002; Risérus *et al*, 2004*c*; Gaullier *et al,* 2004; Malpuech-Brugere *et al*, 2004) may be criticised due to their choice of placebo supplement.

The data generated from *in vivo* and *in vitro* studies have been important in defining the potentially diverse effects of CLA isomers on insulin sensitivity. Similar to isomer specific effects on body fat composition, the effects on insulin sensitivity appear to be isomer specific. However, in contrast to the fat-lowering effects of the *t*10, *c*12-CLA isomer, it appears to be the *c*9, *t*11-CLA isomer may have potential insulin sensitizing abilities. Moreover the human studies carried out would suggest that the effect of CLA supplementation may also be dependent upon the age and background insulin sensitivity of the individual.

The effects of *c*9, *t*11-CLA in its natural and synthetic form were investigated on markers of the inflammatory response in this present study. Results show anti-inflammatory effects were observed for the natural *c*9, *t*11-CLA diet and synthetic *c*9, *t*11-CLA diet, whereby serum concentrations of IL-6 were significantly decreased compared to the control diet. Inflammation is a process in response to tissue or organ damage from exogenous and endogenous factors, aiming at the restoration of impaired homeostasis (Coppack, 2001). The MetS is now associated with an ongoing sub-acute pro-inflammatory state (Ghanim *et al*, 2004; Dandona *et al*, 2004; Roche *et al*, 2005). Obesity is associated with chronic low grade inflammation of WAT. WAT is now seen as a highly dynamic organ, not only is it the

physiological site for energy storage as lipids, but it is a secretory site for several hormones collectively termed 'adipocytokines' (Trayhurn & Wood, 2004). To date, over fifty adipocytokines have been identified, including TNFα and IL-6, these molecules may have local effects on WAT physiology but also systemic effects on other organs and directly antagonise insulin signalling (Bastard *et al*, 2006). IL-6 expression can be induced by many transcription factors such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and cAMP response element (CRE)-binding protein (CREB) depending on cell type and stimulus (Chung *et al*, 2005, Vanden *et al*, 2003). Activation of NF-κB is critical for FA-induced IL-6 secretion and IR in myotubes *in vitro* (Sinha *et al*, 2004; Weigert *et al*, 2004) and muscle *in vivo* (Itani *et al*, 2002). NF-κB is a ubiquitous transcription factor regulating the expression of genes promoting inflammation and cell survival. The activation of NF-κB is critical for the induction of cytokine and cell adhesion molecule (CAM) expression. Inhibition of the NF-κB mediated inflammatory response may therefore attenuate pro-atherogenic processes such as CAM expression and chemokine release in the endothelium. NF-κB-dependent transcription normally begins with the phosphorylation of the inhibitory κB proteins (IκBs) and their subsequent degradation. IκB proteins are key regulators of NF-κB, sequestering the NF-κB dimer in the cytoplasm. Phosphorylation of IκBα by IκBα kinase (IKK) triggers its polyubiquitination and proteosomal degradation, thereby releasing NF-κB, especially p50/p65, to the nucleus (Chung *et al*, 2005; Shoelson *et al*, 2003). PPARγ is a nuclear receptor that is critically required for insulin sensitivity (Rosen *et al*, 1999). CLA is a known agonist of PPARγ (Cimini *et al*, 2005) and the anti-inflammatory effects of CLA may be mediated at least in part by PPARγ dependent inhibition of NF-κB activation (Zhao *et al*, 2005). Neither NF-κB nor IκB expression from the WAT were determined as part of this CLA study. It has also been demonstrated that TNFα expression is regulated by PPARγ agonists (Choi *et al*, 2005). No significance differences were noted for circulating TNFα concentrations in the present study, yet significant reductions were noted for TNFα mRNA expression levels from the WAT following the high natural CLA and synthetic CLA diets.

Additional anti-inflammatory results of the present study show that feeding an enriched diet of *c*9, *t*11-CLA beef and synthetic *c*9, *t*11-CLA beef significantly increases circulating levels of adiponectin compared to the control diet. Adiponectin is an adipocyte-derived protein, has emerged as a key molecule underlying the development of the MetS (Nedvidková *et al*, 2005). It is a recently discovered protein and is synthesized and secreted

from both WAT and BAT in copious amounts, accounting for 0.01% of total plasma proteins (Matsuzama *et al*, 2003, Garaulet *et al*, 2007). Unlike many other adipokines, circulating levels have been noted to be low in obesity and diabetic status of humans but levels have been reported to improve significantly after weight-loss (Mantzoros *et al*, 2005). Adiponectin is one of the most abundant adipose tissue specific factors and appears to improve insulin sensitivity and inhibit vascular inflammation through the activation of AMP-activated protein kinase (Trayhurn & Wood, 2004).

In recent years a number of studies have examined the effect of CLA on animal models of atherosclerosis. Atherosclerosis is a progressive disease of medium and large arteries characterised by the accumulation of lipids and fibrous elements leading to the formation of an atherosclerotic plaque resulting from disturbances in TAG metabolism (Alipour *et al*, 2007). It is the primary cause of heart disease and stroke and in the Western world it is responsible for 50% of all deaths it is thought to be one of the primary causes of death in the world (Ross, 1999). Since the 1990's it has been characterised as an inflammatory disease as some of the hallmarks of the disease include infiltration of inflammatory cells such as monocytes and macrophages and proliferation of smooth muscle cells (SMC) (Ross, 1993) Early studies demonstrated that rabbits fed an atherogenic diet (containing14% fat and 0.1% cholesterol) supplemented with (0.5g/d) CLA for 22-weeks significantly lowered plasma TAG and plasma LDL cholesterol concentrations and developed less atherosclerosis compared to controls (Kritchevsky *et al*, 2000). To measure the relationship between *c*9, *t*11-CLA biological form and lipoprotein metabolism, serum glucose, TC and TAG were measured in this current study. Results found that both plasma NEFA and TAG concentrations were significantly decreased following both the natural and synthetic *c*9, *t*11-CLA diets compared to the control. On the other hand, TC was significantly increased in both these diets, compared to the control diet, which may be due to the high percentage of PUFA contained in the diets. In this study GLUT4 mRNA expression from the WAT were increased in the high CLA beef. GLUT4 promotes intracellular flux of glucose and facilitates reduced plasma levels of glucose. Our group reported similar results, whereby 16 *ob/ob* mice were randomly assigned to receive a *c*9, *t*11-CLA diet or placebo for 6 weeks. Results showed that feeding a *c*9, *t*11-CLA enriched diet reduced fasting glucose, insulin and TAG concentrations and increased AT plasma membrane GLUT4 and insulin receptor expression compared with the control LA enriched diet. Interestingly, after the *c*9, *t*11-CLA diet, AT macrophage infiltration was less,

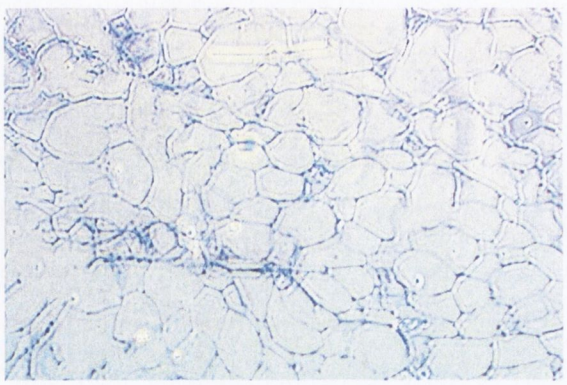
with marked downregulation of several inflammatory markers in AT, including reduced TNFα and NF-κB p65 expression (Moloney *et al*, 2007). Another very recent study was conducted on 50 Wister rats and investigated the effects the effect of CLA on insulin resistance and its molecular mechanisms in the WAT. Animals were randomly designed to the control, high-fat and high-fat with CLA (0.75, 1.50, and 3.00g in per 100g diet) groups. The results showed that supplementation with CLA significantly reduced body weight gain and white fat pad weight in the rats, the levels of plasma FFA, TAG, leptin, insulin and blood glucose concentration in the obese rats of CLA group were also decreased compared to the rats in the high-fat group. Dietary CLA increased the mRNA expression of PPARγ, fatty acid binding proteins (aP2), fatty acid transporter protein (FATP), acyl-CoA synthetase (ACS) and adiponectin in the AT of obese rats. The study concluded that CLA may ameliorate IR by activating PPARγ, and increasing the expression of PPARγ target genes such as ap2, FATP, and adiponectin in the WAT (Zhou *et al*, 2008).

Preliminary work has commenced examining the presence of macrophages in sections of WAT from this *ob/ob* feeding study. The details of the procedure are outlined in *Section 2.4.* It will be intriguing to investigate whether the attenuation of inflammatory mediator expression and the associated improvement in insulin sensitivity and inflammatory status could be due to reduced infiltration of macrophage into adipose tissue in mice fed natural or synthetic *c*9, *t*11-CLA or TVA. In this study, several attempts were made to stain the sections of WAT using different antibodies and using different concentrations of each antibody. Firstly, primary antibodies against rat monoclonal CD11b was investigated as it has been implicated in various adhesive interactions of monocytes, marcophages and granulocytes as well as in mediating the uptake of complement coated particles. In addition, rabbit polyclonal CD3 was also stained for as CD3 subunits are structurally related to members of the immunoglobulins (Ig) superfamily encoded by closely linked genes on human chromosome 11. The CD3 antigen is present on 68-82% of normal peripheral blood lymphocytes, 65-85% of thymocytes and Purkinje cells in the cerebellum. Decreased percentages of T lymphocytes may be observed in some autoimmune diseases. Expression of GLUT4 antibody and rat monoclonal F4/80 were also stained for as GLUT4 is an insulin-regulated glucose transporter found in adipose is responsible for insulin-regulated glucose disposal, while F4/80 is heterogeneous and is reported to vary during macrophage maturation and activation. The F4/80 antigen is expressed

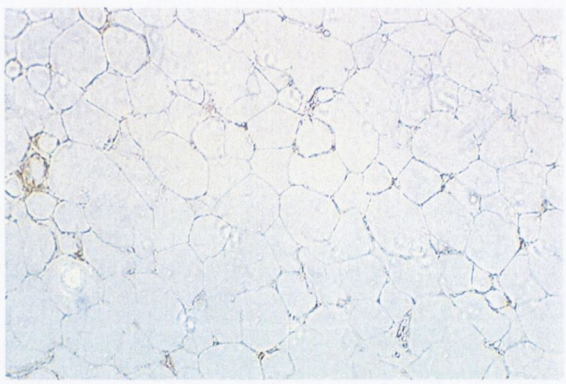
on a wide range of mature tissue macrophages including kupffer cells, langerhans, microglia, macrophages located in the gut lamina propria, peritoneal cavity, lung, thymus, bone marrow stroma and macrophages in the red pulp of the spleen. The ligands and biological functions of the F4/80 antigen have not yet been determined but recent studies suggest a role for F4/80 in the generation of efferent CD8+ve regulatory T cells.

However, results demonstrated very weak macrophages accumulation for each antibody, it was suggested that this was due to technical error as oppose to the lack of each antibody present in the adipose tissue. Due to the time constrains of this post-graduate doctorate this work will be carried out in the near future. However, *Figure 3.7* illustrates the preliminary work carried out after staining for F4/80 for control, LA (Diet A) and high natural *c*9, *t*11-CLA (Diet B).

Figure 3.7: Preliminary work investigating the presence of macrophages in white adipose tissue



Control, Linoleic Acid (Diet A)
No primary antibody



High natural *c*9, *t*11-CLA (Diet B)
Stained for F4/80

Throughout the study, very few significant findings were noted for the synthetic TVA diet, in terms of body composition, and circulating markers of insulin sensitivity, inflammation and blood lipids. Therefore, the synthetic TVA diet was not investigated as a cellular level in the WAT, due to the non-significant results demonstrated at the preliminary stage. A recent *in vitro* study published interesting results in relation to the factors influencing the biohydrogenation of TVA to CLA. Results showed that the presence of glucose and cellobiose

to cultured media inhibited the rate of biohydration of LA to TVA and CLA. Optimal conditions of pH, were also an influencing factor for biohydrogenation (Nam & Garnsworthy, 2007). Similar results regarding the high glucose concentrations and pH sensitivity were also noted by Martin and Jenkin (2002). As *ob/ob* mice are genetically prone to IR and study food was provided *ad libitum*, concentrations of circulating glucose were higher compared to lean mice, therefore, it is possible that the TVA did not bioconvert to CLA, or simply because the amount to TVA provided was inadequate, hence the null effects on insulin sensitivity, inflammation and lipoprotein metabolism.

In conclusion, this is the first study to compare the effects of feeding either natural beef derived *c*9, *t*11-CLA, synthetically prepared *c*9, *t*11-CLA or synthetically prepared TVA on metabolic markers of insulin sensitivity, inflammatory status and lipid metabolism in rodents. The results of the study are very promising in terms of the CLA supplementation in the MetS, as body weight gain was less, insulin sensitivity, inflammatory status and blood lipids were significantly improved on a diet containing *c*9, *t*11-CLA in both the natural and synthetic form. These results further support the use of CLA as a functional food as the result show that CLA is indeed metabolically active. However, several limitations occur whereby, this was a study was conducted in rodents and was of short-term duration. It remains to be seen whether supplementation with natural and/or synthetic forms of *c*9, *t*11-CLA would be as metabolically active and have similar positive outcomes in man. In addition, this study failed to analyse the expression of NF-κB and IκB expression from the WAT, perhaps this analysis could be performed in the near future. It is without doubt that further longer term research interventions are required to elucidate CLA as a functional food in man and evidence regarding effectiveness of CLA in humans is not conclusive.

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Chapter 4

The interaction between dietary fatty acids, inflammation and insulin sensitivity in the MetS

4.1 ABSTRACT

Background: The metabolic syndrome (MetS) is an association of metabolic alterations that represents a significant risk factor for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). Diets high in saturated fatty acids (SFA) intake are typical of the 'western diet' and are associated with increasing obesity levels which may eventually lead to development of the MetS, T2DM and ultimately CVD.

Objective: To investigate the interaction between plasma and dietary fatty acid composition, inflammation and insulin resistance (IR) in individuals with the MetS. **Design:** Cross-sectional study involving 483 MetS individuals as defined by a modified version of the NCEP-ATP III criteria were recruited as part of the LIPGENE human dietary intervention study.

Results: No interaction was demonstrated between plasma dietary fatty acids (FAs) and inflammation in an IR state. However, in relation to the dietary FAs and insulin sensitivity, results showed that caprylic acid (C8:0) and *trans* fatty acids (TFA) were found to have higher levels in the low insulin sensitivity as calculated by MINMOD® Millennium (S_I) tertile compared to the high S_I tertile ($p<0.05$). Additionally, oleic acid (C18:1) was found to be lower in the high S_I tertile compared to the medium S_I tertile. Furthermore, analysis noted that when individuals were categorized into tertiles of S_I, those with lower insulin sensitivity had a significantly higher body weight and body mass index (BMI) measured by weight (kg) / height^2 (kg/m^2), higher concentrations of inflammatory markers, as well as lower circulating adiponectin concentrations. Females had higher CRP concentrations compared to males and those with BMI >30 have increased CRP concentrations compared to leaner MetS volunteers. Tumour necrosis factor α (TNFα) and adiponectin concentrations were positively associated with insulin sensitivity, whereas BMI, tissue type plasminogen activator (tPA) and alcohol intake were negatively associated with insulin sensitivity.

Conclusions: In conclusion, despite no significant interaction between plasma FA composition and inflammation on insulin sensitivity, overweight and obesity are associated with decreased insulin sensitivity. Moreover, reduced insulin sensitivity is also associated with higher consumption of saturated fatty acids and higher inflammatory status, both which increase the risks for T2DM and CVD.

4.2 INTRODUCTION

It has become conventional knowledge that the prevalence of overweight and obesity is increasing at an epidemic rate, particularly in highly industrialized societies of Europe and North America (Goralski & Sinal, 2007). In Europe, approximately, 10-20% of men and 10-25% of women and 65% of the adult population in the USA are currently considered overweight or obese (Urek *et al,* 2007). Obesity is defined as an increase in body weight beyond the limitations of skeletal and physical requirements as the result of an excessive accumulation of fat or adipose tissue (AT) in the body (Hauner, 2007). It is a complex metabolic disorder that is associated with higher rates of abnormal glucose tolerance leading to hyperglycaemia, hypertension and hyperlipidemia. Clustered together, these metabolic aberrations are commonly referred to as the MetS. The concept of risk factor clustering was first introduced by Reaven (1988), who highlighted the existence of this syndrome due to the escalating incidence of the above metabolic disorders in unison. Thus, this saw the birth of an unknown phenomenon of heart threatening abnormalities generally occurring in IR individuals. IR is a subnormal biological state where the tissues of the body have a reduced sensitivity to the actions of the insulin hormone and therefore greater amounts of insulin are required to elicit a quantitatively normal response. As a consequence hyperinsulinaemia develops which ultimately leads to the progressive deterioration in pancreatic β-cell function predicting the development of T2DM (Mauvais-Jarvis *et al*, 2002). Furthermore, it has been hypothesis that an increase in a number of non-traditional risk factors including C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1) and fibrinogen concentrations as well as other markers of inflammation and fibrolysis are components of the MetS cluster (Hanley *et al*, 2004; Festa *et al*, 2000).

Dietary FAs are hydrocarbon chains that can be SFA such as mysteric acid (C14:0) and stearic acid (C18:0). A diet high in SFA intake is common in industrialised societies and associated with increasing obesity levels (Astrup *et al*, 2000). Monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are unsaturated fatty acids. MUFA includes palmitoleic acid (C16:1) and oleic acid (C18:1), whereas PUFA includes linoleic acid (LA, C18:2:6) and α-linolenic acid (ALA, C18:3:3). LA is an n-6 PUFA, whereas ALA is an n-3 PUFA, however both are essential FA as they cannot be synthesized *de novo* and therefore must be obtained from the diet. The metabolism of the both FA is competitive, since both pathways employ the same set of enzymes. The major end product of the n-6 pathway is

arachidonic acid (AA, C20:4:6), whereas, the major end products of the n-3 pathway are eicosapentaeoic acid (EPA, C20:5:3) and docosahexaeoic acid (DHA, C22:6:3) (Calder & Yaqoob, 2007) (*Figure 1.11*). The so-called western diet is typified by high intakes of LA and low consumption of ALA food products (Goyens *et al*, 2006). In previous studies, ALA has been reported to decrease CVD risk factors, possibly by favourably modifying vascular inflammation and endothelial dysfunction (Zhao *et al*, 2004). However, Pischon *et al* (2003) concluded that a combination of both n-6 and n-3 FAs are associated with lower inflammatory concentrations. A recent cross-over study in healthy men investigating the effects of a high LA diet versus a low LA diet (10.5% and 3.8%, respectively) while maintaining an intake of 1% energy from ALA for 4 weeks, resulted in higher plasma EPA levels (Liou *et al*, 2007).

There are indications from cross-sectional and dietary intervention studies in humans that a high intake of SFA may contribute to the development of obesity (Astrup *et al.* 2000). However, results from cross-sectional studies have been similarly mixed. A positive correlation between insulin sensitivity, derived from postload insulin and glucose measurements and total dietary fat, which was reported by Lovejoy *et al* (1992), was apparent only before adjusting for BMI. Two other studies also found no association with fasting insulin after accounting for BMI (Parker *et al*, 1993; Mayer-Davies *et al*, 1997), whereas the CARDIA study found that total fat was unrelated to fasting insulin in univariate and multivariate analyses (Manolio *et al*, 1991). In contrast, several studies, including the Hoorn study (Mooy *et al*, 1998) found a positive association between total dietary fat and fasting or postprandial insulin, independent of BMI (Vitelli *et al,* 1996).

Warensjö *et al* (2006*b*) found cross-sectional FA composition to be significantly different between subjects with or without MetS, whereby the proportions of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:1), γ-LA (C18:3:6), dihomo-γ-LA (C20:3:6) and Δ6 desaturase activity was notably higher, on the other hand LA (C18:2:6) and Δ5 desaturase activity was particularly lower in subjects with MetS at the ages of 50 and 70years. Furthermore the study showed that the generated FAs factors, which represent dietary fat quality and endogenous FA metabolism, may be important in the development of MetS, wherein a high factor (low LA) and a low factor (n-3 PUFA) significantly predicted the development of MetS over 20 years, independent of lifestyle factors. The study suggests that changes in FA composition and desaturase activities may appear long before the onset of MetS. Another recent cross-sectional study set out to determine how dietary FA composition could be used to identify Asian Indian men and women who are at greater risk of obesity and

dyslipidemia. These results showed that for men the lean controls had lower waist circumference, WHR, total cholesterol (TC), triglycerides (TAG), LDL-c, fasting plasma glucose and intakes of TFA compared with obese and dyslipidemic cases. These results suggest that clinicians should consider obesity measures, metabolic profiles and dietary FA simultaneously to better comprehend the existent of the risk factors associated with the MetS (Ghosh, 2007*a*). Similar results were noted for the female cohort of this study and concluded that dietary FA composition is related to indicators of obesity and the recent shift in dietary habits may have caused an increase in the prevalence of obesity and dyslipidemia (Ghosh, 2007*b*). Moreover, a cross-sectional, population-based Spanish study found a favourable relationship between intakes of MUFA and β-cell insulin secretion (Rojo-Martínez *et al*, 2006). Yet, another cross-sectional Spanish study found that oleic acid (C18:1) levels decreased with age, whereas LA proportion increased noticeably with age in the skeletal muscle and AA proportion increased with age in the AT. It was suggested that these findings may be explained on the basis of the changing dietary habits of children, who are progressively abandoning the 'Mediterranean diet' and favouring the high SFA 'western diet'. Although the study was carried out on children, there was an age-independent positive correlation between insulinemia/$HOMA_{IR}$ index and AA content in AT TAG ($r = 0.47$, $P < 0.001$) (Aldámiz-Echevarría *et al*, 2007).

The Nurses' Health Study was among the largest prospective investigations into the risk factors for major chronic diseases in 121,000 female nurses residing in the USA. Every 2 years, a follow-up questionnaire was sent to update information on potential risk factors and to identify newly diagnosed cases of chronic diseases. The results of the study documented 2507 incidents of T2DM after 14 years. However, no significant associations were noted between total fat intake, SFA and MUFA and the risk of T2DM. Nevertheless, a positive relationship was observed between TFA and risk of T2DM. The study estimated that replacing 2% of energy from TFA with PUFA would lead to a 40% lower risk of developing T2DM (Salmerón *et al*, 2001). These results concurred with another survey that investigated the relation between dietary FAs and T2DM in a prospective cohort of 35,988 older non- diabetic women. The main findings of the study showed that 1890 incident cases of T2DM occurred after 11 years and T2DM incidence was negatively associated with dietary PUFA, vegetable fat, and TFA intake (Meyer *et al*, 2001). A sub-cohort of 730 from the Nurses' Health Study I were selected as control subjects for an earlier nested case-control study of diabetes. These women had no incidence of CVD, cancers, or T2DM at the time of blood draw. Results showed that

CRP levels were 73% higher among those in the highest quintile of TFA, compared with the lowest quintile. IL-6 levels were 17% higher, sICAM-1 10%, and sVCAM-1 levels 10% higher. The study concluded that higher intake of TFA could adversely affect endothelial function, which might partially explain why the positive relation between TFA and CVD risk occurred (Lopez-Garcia *et al*, 2005).

Adiposity contributes to a pro-inflammatory milieu and a reduction of fat mass correlates with a decrease in serum levels of many adipokines, namely TNFα and IL-6 (Von Sahacky, 2007). The pro-inflammatory cytokine, TNFα is widely known to be over-expressed in WAT of obese and IR humans. As well as having the ability to cause IR effects on insulin-mediated cellular signalling pathways, TNFα can cause the stimulation of other pro-inflammatory cytokines such as IL-6 concentrations (Lo *et al*, 2007; Grimble, 1996). Whereas, IL-6 is a well known inducer of CRP production from the liver (Anty *et al,* 2006; Yudkin *et al*, 2000). LC n-3 PUFA have a variety of anti-inflammatory and immune-modulating effects, therefore can inhibit the synthesis and release of pro-inflammatory cytokines such as TNFα and IL-6 (Barré, 2007). TNFα has neurotoxic actions, where as LC n-3 PUFA are potent neuroprotectors. Based on this, it is suggested that the principle mechanism LC n-3 PUFA can be due to the suppression of TNFα and IL-6 synthesis and release (Das, 2000).

Blood coagulation and fibrinolysis play an important role in the development and complications of coronary heart disease (CHD). In the human body, blood clots are constantly formed and dissolved and this phenomenon is tightly regulated by the coagulation and fibrinolytic systems. When this tight balance is disturbed, a tendency for bleeding or thrombosis occurs. Fibrinogen concentration, tPA and plasminogen activator inhibitor-1 (PAI-1) antigen concentrations are positively associated with CHD incidence, IR, MetS and T2DM (Aso *et al*, 2005). Byberg *et al* (2001*a*) carried out a cross-sectional study to investigate the associations between PAI-1 activity and dietary nutrient intake, focusing on fat quality. Interestingly, results showed that the intake of both MUFA and PUFA acids was positively associated with PAI-1 activity, whereas the injestion of SFA was not. Additionally, in serum cholesterol esters (CE), higher proportions of palmitoleic acid (C16:1) and dihomo-gamma-linoleic acid (dihomo-γ-LA, C18:3:6) and a lower proportion of LA were connected with higher PAI-1 levels. An intervention trial was performed on 150 hyperlipidmeic subjects, to investigate the effect of supplementing their diet with EPA, DHA or biologically equivalent levels of the precursor, ALA on blood coagulation levels. Results after 4 weeks showed no

effect and concluded that dietary fat quality was not a strong regulator for many of the markers of blood coagulation and fibrinolysis (Finnegan *et al,* 2003).

Lipid peroxidation is a free radical generating process whereby lipids are oxidized without the release of energy. PUFA are more prone to lipid peroxidation than SFA or MUFA. Increased markers of lipid peroxidation have been observed in smokers and are known to be involved in a number of human pathologies including IR and diabetes-related complications (Davi *et al*, 1999; Nourooz-Zadeh *et al*, 1997; Risérus *et al,* 2002). Oxidative stress is often seen as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to tissue damage. One of the most valuable biomarker of lipid peroxidation in the human body is the isoprostanes, which are specific products arising from the peroxidation of unsaturated FAs residues in lipids (Basu, 2004). A study on healthy subjects supplemented with n-3 PUFA for 3 months demonstrated a significant reduction in 8-iso-$PGF_{2\alpha}$ isoprostane levels, yet 15-keto-dihydro-$PGF_{2\alpha}$ prostaglandin levels remained unchanged (Nälsén *et al*, 2006). There results are in line with reduced levels of 8-iso-$PGF_{2\alpha}$ isoprostane seen in T2DM supplemented with EPA and DHA or intake of fish oils (Mori *et al*, 2000; Mori *et al*, 2003).

Dietary surveys often fail to adequately measure the intake of food and nutrients due to reporting bias, particularly under-reporting (Livingstone & Black, 2003). Therefore, biomarkers such as AT, red blood cells (RBC), CE's and plasma FAs are extensively used to assess dietary intake in epidemiological studies as biomarkers of individual FA play an important role in the development of chronic disease (Baylin *et al,* 2005; Kaaka *et al*, 1997). Plasma phospholipids FA are believed to reflect short-term (weeks to months) dietary fatty intake (Raatz *et al,* 2001; Kwon *et al*, 1991; Riboli *et al*, 1987). Many studies have demonstrated that the levels of specific FAs in plasma are reflective of FAs consumed in the diet (Judd *et al*, 1989; Lopez *et al*, 1991; Ma *et al*, 1995; Manku *et al*, 1983). Plasma FA composition was the primary biomarker to reflect FA intake in this cross-sectional study.

LIPGENE is a 5 year (2004-2009), trans-EU project entitled *Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis*. The objective was to apply an integrated approach to examine how variations in the composition of dietary fats interact with common genetic variations to influence the development of the MetS (Buttriss and Nugent, 2005). This chapter focuses on the human dietary intervention study, involving 8 EU partners as described in *Table 2.7*. It investigates the LIPGENE population in a cross-sectional/observational nature carried out at one time-point to determine the interaction

between plasma and dietary FA composition and markers of inflammation, endothelial dysfunction, coagulation and oxidative stress on IR in the MetS volunteers recruited across the 8 participating EU centres. After collection of the baseline samples for cross-sectional analysis, the LIPGENE MetS volunteers underwent a 12 week human dietary intervention as presented in *Chapter 5* of this thesis.

4.3 RESEARCH DESIGN AND METHODS

The Ethics Committee of the St James's Hospital under the Federated Dublin Voluntary Hospitals Amalgamation Act, 1961 granted approval for the study to be conducted on the TCD cohort. Written informed consent was obtained from all volunteers prior to commencing the investigation. 15,593 individuals were screened of which 483 MetS subjects participated in the 8 EU research centres; Trinity College Dublin (TCD), University of Reading (UoR), University of Oslo (UO), INSERM, France, University of Maastricht (UM), Córdoba (HURS-UCO), Poland (JUMC) and Uppsala University (UU) outlined in *Table 2.7*. Within in TCD cohort, the volunteers were recruited primarily from personnel in TCD, out-patients of the Endocrinology Departments of Adelaide and Meath Hospital, Dublin incorporating the National Children's Hospital (AMNCH), Tallaght, Dublin 24 and Loughlinstown Hospital, Co. Dublin and employees from a local taxi-firm and the general public.

Table 4.1 Baseline screening criteria adapted from the NCEP-ATP III (Assmann *et al*, 2007) and used by LIPGENE Human Dietary Intervention Study to diagnose the MetS

Criteria	Biochemical/Anthropometric marker	Concentrations
1	Fasting glucose concentration	5.5 - 7.0 mmol/L
2	Serum TAG concentration	≥ 1.5 mmol/l
3	Serum HDL-c concentration	< 1.0 mmol/l (male), < 1.3mmol/l (female)
4	Blood Pressure	130/85 mmHg
5	Waist girth	>102cm (male), > 88cm (female)

HDL-c, High Density Lipoproteins; **TAG**: Tricylglycerol; **mmol/L**: millimoles/Litre; **mmHG**: millimetre of mercury; **cm**: centimetres.

4.3.1 Collection of anthropometric and blood pressure measurements

All anthropometric measurements and blood pressure measurements were taken as described in *Section 2.8* and *Section 2.9,* respectively.

4.3.2 Dietary assessment

A 3-day weighed food diary was completed prior to commencing the LIPGENE cross-sectional study. The volunteers were instructed to include 2 week days (Monday to Thursday) and 1 weekend day (Saturday or Sunday). Friday was not included as there was much ambiguity as to whether it is considered a week day or weekend day. The food was weighed using calibrated Soehnle Vita Food Scales (Soehnle-Waagen GmbH & Co, Germany). Volunteers were asked to include food packaging and home made recipes when possible. Various dietary analysis programs were used across the 8 EU centres to ensure culturally specific foods were included (**TCD**: WISP version 3.0; **UoR**: Food Base version 3.1; **UO**: Kostberegningssystems (nutrient calculation software developed in the Department of Nutrition, UO; **INSERM**: Nutrilog; **UM**: Komeet & Orion software; **HURS-UCO**: DietSource version 2.0; **JUMC**: Dietetian software; **UU**: MATs connected to the Swedish Board of Food Administration database for foods).

4.3.3 Clinical investigations and collection of bloods samples

Following diagnosis of the MetS, 483 volunteers accepted invitation to participate in the study. Within TCD, volunteers attending the Diabetes Day Centre, AMNCH, Tallaght Hospital after observation of a 12 hrs overnight fast (no food or drink, except water), avoiding smoking tobacco on the morning of each visit, abstaining from alcohol 12 hrs proceeding to the blood sample donation and avoiding vigorous exercise on the day prior to the clinical investigation. Volunteers were asked to donate a fasting blood sample prior to undergoing intravenous glucose tolerance test (IVGTT) lasting 180 mins as described by Bergman *et al* (1985). In brief, a bolus of 50% glucose solution (Phoenix Pharma Ltd, Gloucester, UK) (0.3g/kg BW) was infused into cannula 1 over a 1 min period and this was followed by 20mls of saline. 20 mins later, a dose of insulin was infused into the same cannula (0.03U/kg BW). Meanwhile, blood samples from cannula 2 occurred at the following intervals over the 3 hrs period: -5, 0, 2, 4, 8, 19, 22, 30, 40, 70, 90 and 180 mins. At each time point each subject's blood glucose was monitored using a gluco-trend machine to ensure the level did not drop below 2.0mmol/L. If blood glucose dropped below this level, the IVGTT was discontinued and an isotonic high glucose drink was administered immediately. The blood tubes were inverted 8 to 10 times to ensure that the coagulant was mixed thoroughly. Subsequently, blood samples from ethylenediaminetetra-acetic acid (EDTA), sodium citrate and fluoride oxalate tubes were placed on ice for 30 mins to allow for clotting, whilst serum and serum separator

vaccunainer tubes were left at room temperature (RT) to allow for clotting. Following this, blood tubes were centrifuged as described in *Section 2.10* and plasma or serum was harvested immediately and stored at -70°C until further analysis.

4.3.4 Determination of human inflammatory and coagulation markers

Human CRP, IL-6, TNFα, soluble intercellular adhesion molecule-1 (sICAM-1), vascular adhesion molecule-1 (sVCAM-1), resistin, total adiponectin and leptin, concentrations were measured by on a basis on solid phase sandwich enzyme linked immunosorbent assay (ELISA) as described in *Section 2.12.* Plasma tPA and PAI-1 concentrations were determined using the commercially available kits (ASSERACHROM® tPA, ASSERACHROM® PAI-1, respectively) by enzyme immunoassay (EIA) procedure by ELISA (Diagnostica Stago, France) as described in *Section 2.13.* Fibrinogen clotting activity was measured by using an automated clotting assay as described in *Section 2.13.1.*

4.3.5 Determination of human urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$

As a marker of oxidative stress urinary samples were analysed from free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$ F2-isoprostane) without any extraction by a radioimmunoassay. Lipid peroxidaiton enzyme 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$ F2α-prostaglandin) was analysed without any extraction by a radioimmunoassay, both techniques are described in *Section 2.14.*

4.3.6 Determination of human insulin and c-peptide and glucose concentrations

Serum insulin and c-peptide concentrations were measured using a solid phase, two site fluoroimmunometric assays (AutoDELFIA Insulin kit, Wallac Oy, Finland and AutoDELFIA C-peptide kit, Wallac Oy, Finland) (*Section 2.15*).

4.3.7 Determination of human plasma fatty acids composition

Plasma fatty acid composition was determined according to the Bligh and Dyer method as described in *Section 2.16* (Bligh & Dyer, 1959). A more detailed description of these FAs is presented in *Table 2.15.*

4.3.8 Determination of human HDL, LDL, total cholesterol, TAG, NEFA and apolipoprotein concentrations

The IL Test™ HDL-c Kit (Instrumentation Laboratories, Warrington, UK) was used for direct quantification of high density lipoprotein-cholesterol (HDL-c). Low density lipoprotein-cholesterol (LDL-c) was isolated from serum using LDL-c reagent (Randox, Laboratories Ltd., Co. Antrim) (*Section 2.17.1* and *Section 2.17.2*, respectively). Quantitation of total cholesterol, LDL-c and TRL from defrosted plasma and supernatant samples was performed using the IL Test™ Cholesterol Kit (Instrumentation Laboratories, Warrington, UK) on the ILAB (*Section 2.17.3*). Quantitation of plasma TAG and non-esterified fatty acids (NEFA) was performed using the IL Test™ TAG Kit (Instrumentation Laboratories, Warrington, UK) (*Section 2.17.4* and *Section 2.17.5*). Plasma apolipoproteins (Apo) AI, B and E concentrations were measured using an immunonephelometric methods on a BN Prospec® System with commercial kits (Dade Behring, Deerfield, USA) (*Section 2.17.6*).

4.3.9 Data preparation and statistical analysis

All data preparation and statistical analysis such as one-way ANOVA, correlation analysis, linear regression analysis and binary logistic regression analysis was conducted using SPSS® for Windows™, version 14.0 (SPSS Inc, Chicago, IL, USA) and is described in detail in *Section 2.18.1* and *Section 2.18.2*. Results are presented as means ± standard error of the mean (SEM) unless stated otherwise.

IVGTT definitions: Homeostasis model assessment-insulin resistance ($HOMA_{IR}$) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) according to the equation $[1/(\log \text{insulin}_0 + \log \text{glucose}_0 + \log \text{NEFA}_0)]$ (Roche, 2004). Insulin and glucose results from the IVGTT were entered into MINMOD Millennium, a programme based on Bergman's minimal model which enables the estimation of 2 key indices of glucose/insulin dynamics: insulin sensitivity (S_I) and glucose effectiveness (S_G). Additional analysis from the MINMOD modelling include, acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (Zero) (G0) were all calculated by the Bergman MINMOD Millennium® (Boston *et al*, 2003).

4.4 RESULTS

4.4.1 Subject characteristics and anthropometric measurement details

Baseline characteristics of the group, irrespective of gender are presented in *Table 4.2* and according to gender in *Table 4.3*. After a one-way ANOVA: significant differences were noted between the gender groups, whereby females were older than the males. Despite the males being taller and heavier in weight, the females had a significantly higher BMI ($p<0.05$), this may be simply due to the fact that there were more females in the baseline cohort compared to males.

Table 4.2: Baseline characteristics of the LIPGENE human dietary intervention cohort. Values represent mean (±SEM)

	n	*Mean*	*SEM*
Age (y)	483	54.55	0.41
Height (m)	483	1.68	0.00
Weight (kg)	483	91.62	0.65
BMI (kg/m^2)	483	32.35	0.19

Table 4.3: Characteristics of the LIPGENE cohort according to gender
Values represent mean (±SEM)

	Male			**Female**			
	n	*Mean*	*SEM*	*n*	*Mean*	*SEM*	*ANOVA*
Age (y)	218	53.4	0.67	265	55.55	0.49	$p<0.01$
Height (m)	218	1.76	0	265	1.62	0	$p<0.000$
Weight (kg)	218	99	0.93	265	85.67	0.73	$p<0.000$
BMI (kg/m^2)	218	31.95	0.26	265	32.72	0.27	$p<0.05$

4.4.2 Cross-sectional study cohort stratified according to markers of insulin sensitivity (S_I)

Tertiles of insulin sensitivity (S_I) were created in order to determine the effects of inflammation, plasma and dietary FA composition on low, medium and high concentrations of S_I. *Table 4.4* displays the minimum and maximum value for the tertiles groups for each parameter investigated. *Table 4.5* presents the results for the effects of S_I tertiles on mediators of inflammation and demonstrates that plasma sICAM-1, PAI-1 and tPA concentrations were significantly higher in low S_I tertile compared to medium and high S_I tertiles ($p<0.001$, $p<0.01$ and $p<0.005$, respectively). These results suggest that lower S_I may result in higher circulating levels of inflammatory biomarkers. Interestingly, adiponectin concentrations were

found to be significantly lower in the low S_I tertile compared to the high S_I tertile (p=0.004). No significant differences were noted for plasma CRP, IL-6, TNFα, sVCAM-1, fibrinogen, leptin and urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ when stratified according to S_I.

Plasma and dietary FA composition were investigated across the ranges of S_I as presented in *Table 4.6*. Contrary to the hypothesis, no major differences were noted for plasma FA composition when stratified according to S_I, however in relation to the dietary FA, caprylic acid (C8:0) was found to have higher levels in the low S_I tertile compared to the high S_I tertile ($p<0.05$). Additionally, oleic acid (C18:1) was found to be lower in the high S_I tertile compared to the medium S_I tertile and TFA were found to be significantly higher in the low S_I tertile compared to the high S_I tertile (p=0.006). Anthropometric measurements were also studied according to the S_I ranges and results show that body weight and BMI were significantly higher in the low S_I tertile compared to the medium and high S_I groups ($p<0.0005$). BMR was also noted to be higher in the low S_I group compared to the high S_I group only ($p<0.0005$) (*Table 4.6*).

Furthermore, metabolic markers of S_I as determined by Bergman's MINMOD Millennium® software package (Boston *et al*, 2003) was investigated across the tertiles of S_I. These include S_I, glucose effectiveness (S_G), acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (G0). The results presented in *Table 4.7* demonstrate that according to S_I are unsurprising, whereby S_I, S_G, P2, P3 were considerably higher within the high S_I tertile compared to the low S_I tertile. Additionally, plasma c-peptide, fasting insulin, fasting glucose, insulin/c-peptide ratio, $HOMA_{IR}$ and AIRg were much higher in the low S_I tertile compared to the high S_I tertile. QUICKI was calculated as $1/[\log(I_0) + \log(G_0)]$, where I = insulin and G = glucose (Katz *et al*, 2000), however, this showed no significant differences between groups of S_I.

Table 4.4: The ranges of the tertiles created for markers of insulin sensitivity and inflammation

Parameter	Group	n	Units	Minimum Value	Maximum Value
S_I	Tertile 1	144	(mU/L)-1 min-1	0.19	1.96
	Tertile 2	145		1.97	3.14
	Tertile 3	144		3.15	15.29
$HOMA_{IR}$	Tertile 1	157		0.33	1.39
	Tertile 2	158		1.39	1.63
	Tertile 3	158		1.63	11.57
Resistin	Tertile 1	160	pg/mL	1463.0	7782.0
	Tertile 2	161		7785.0	11550.0
	Tertile 3	161		11596.0	345330.0
Adiponectin	Tertile 1	160	mg/L	0.15	2.55
	Tertile 2	161		2.55	4.12
	Tertile 3	161		4.13	22.86
CRP	Tertile 1	160	mg/L	.09	2.63
	Tertile 2	161		2.65	5.96
	Tertile 3	161		5.97	28.39

S_I: Insulin Sensitivity; **$HOMA_{IR}$**: Homeostatis Model Assessment – Insulin Resistance; **CRP**: C reactive protein; **n**: number of volunteers; **mg/L**: milligrams per Litre

4.4.3 Cross-sectional study cohort stratified according to $HOMA_{IR}$

Bonora *et al* (2001) proposed that $HOMA_{IR}$ seemed to be a reliable tool in the assessment of IR, and it can be used as a valid alternative to the glucose clamp or other sophisticated techniques in epidemiological settings. $HOMA_{IR}$ was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Low $HOMA_{IR}$ values indicate high S_I, whereas high $HOMA_{IR}$ values indicate low S_I (IR).

In this study tertiles of $HOMA_{IR}$ were created consisting of low, medium and high values as presented in *Table 4.4.* Markers of inflammation, plasma and dietary FA composition, anthropometric measurements and S_I parameters were investigated across each of the tertiles

Table 4.5: Mean(±SEM) of the markers of inflammation, endothelial dysfuction and oxidative stress according to tertiles of S_I

	Tertiles of S_I										
	Tertile 1				Tertile 2				Tertile 3			
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
CRP (mg/L)	143	5.17	ns	0.36	144	4.96	ns	0.32	144	5.12	ns	0.36	*ns*
IL-6 (pg/mL)	143	4.76	ns	0.31	144	4.97	ns	0.37	144	5.37	ns	0.37	*ns*
TNFα (pg/mL)	143	4.45	ns	0.24	144	5.26	ns	0.48	144	5.62	ns	0.47	*ns*
sICAM-1 (ng/mL)	143	301.11	a	6.40	144	275.56	b	6.29	144	269.32	b	5.75	*p=0.001*
sVCAM-1 (ng/mL)	143	609.83	ns	14.75	144	580.49	ns	14.06	144	595.02	ns	12.12	*ns*
Resistin (pg/mL)	143	9905.40	ns	423.65	144	9801.01	ns	426.59	144	10578.01	ns	345.59	*ns*
Adiponectin (μg/mL)	143	3.24	a	0.15	144	3.73	a	0.22	144	4.23	b	0.23	*p=0.004*
PAI-1 (ng/mL)	143	63.09	a	3.26	144	57.41	ab	3.19	144	47.58	b	2.92	*p=0.003*
tPA (ng/mL)	143	9.72	a	0.31	144	8.72	b	0.38	144	7.59	b	0.29	*p=0.000*
Fibrinogen (mg/dL)	142	320.94	ns	7.33	143	325.07	ns	6.74	144	332.17	ns	6.81	*ns*
Leptin (ng/mL)	143	22.97	ns	1.83	144	20.57	ns	1.53	142	19.97	ns	1.50	*ns*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	143	0.46	ns	0.01	142	0.47	ns	0.01	142	0.48	ns	0.01	*ns*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	143	0.16	ns	0.01	142	0.17	ns	0.01	142	0.17	ns	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [ns] (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

CRP (C-reactive protein) ; **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1);

sVCAM-1 (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_{2}\alpha$ (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

Tertile Ranges: Low S_I tertile (0.19 - 1.96 (mU/L)-1min-1); **Medium S_I tertile** (1.97 - 3.14(mU/L)-1min-1); **High S_I tertile** (≥3.15(mU/L)-1min-1)

Table 4.6: Mean(±SEM) of the plasma fatty acids, dietary fatty acids and anthropometric measurements according to tertiles of S_I

	Tertiles of S_I									
	Tertile 1			Tertile 2			Tertile 3			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
%										
Plasma Fatty Acids										
C14:0 (Mysteric acid)	143	2.09 ^{ns}	0.09	140	1.78 ^{ns}	0.06	142	1.86 ^{ns}	0.08	*ns*
C16:0 (Palmitic acid)	143	27.22 ^{ns}	0.41	140	26.28 ^{ns}	0.42	142	26.38 ^{ns}	0.42	*ns*
C16:1 (Palmitoleic acid)	143	1.36 ^{ns}	0.07	140	1.30 ^{ns}	0.07	142	1.53 ^{ns}	0.11	*ns*
C18:0 (Stearic acid)	143	4.01 ^{ns}	0.16	140	4.39 ^{ns}	0.18	142	4.28 ^{ns}	0.19	*ns*
C18:1 (Oleic acid)	143	26.64 ^{ns}	0.35	140	26.74 ^{ns}	0.39	142	25.96 ^{ns}	0.36	*ns*
C18:2 (n-6) (Linoleic acid)	143	26.47 ^{ns}	0.46	140	27.55 ^{ns}	0.44	142	27.79 ^{ns}	0.44	*ns*
C18:3 (n-6) (γ-Linoleic acid)	143	0.06 ^{ns}	0.01	140	0.06 ^{ns}	0.01	142	0.06 ^{ns}	0.00	*ns*
C18:4 (n-6) (Stearidonic acid)	143	0.04 ^{ns}	0.00	140	0.03 ^{ns}	0.00	142	0.04 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	143	0.24 ^{ns}	0.02	140	0.20 ^{ns}	0.01	142	0.24 ^{ns}	0.03	*ns*
C20:3 (n-6) (Arachidonic acid)	143	0.87 ^{ns}	0.04	140	0.89 ^{ns}	0.04	142	0.83 ^{ns}	0.04	*ns*
C20:4 (n-6) (Arachidonic acid)	143	6.97 ^{ns}	0.20	140	6.70 ^{ns}	0.20	142	6.79 ^{ns}	0.20	*ns*
C20:4 (n-3) (Arachidonic acid)	143	0.06 ^{ns}	0.01	140	0.05 ^{ns}	0.01	142	0.05 ^{ns}	0.01	*ns*
C20:5 (n-3) (EPA)	143	0.87 ^{ns}	0.07	140	1.00 ^{ns}	0.07	142	0.97 ^{ns}	0.08	*ns*
C22:4 (n-6) (Docosatetraenoic acid)	143	0.18 ^{ns}	0.01	140	0.17 ^{ns}	0.01	142	0.22 ^{ns}	0.02	*ns*
C22:5 (n-3) (Docosapentaenoic acid)	143	0.48 ^{ns}	0.03	140	0.43 ^{ns}	0.03	142	0.51 ^{ns}	0.04	*ns*
C22:6 (n-3) (DHA)	143	2.19 ^{ns}	0.09	140	2.13 ^{ns}	0.10	142	2.19 ^{ns}	0.12	*ns*
SFA	143	33.32 ^{ns}	0.52	140	32.44 ^{ns}	0.56	142	32.52 ^{ns}	0.56	*ns*
MUFA	143	28.24 ^{ns}	0.37	140	28.23 ^{ns}	0.42	142	27.72 ^{ns}	0.42	*ns*
PUFA	143	38.44 ^{ns}	0.60	140	39.32 ^{ns}	0.56	142	39.76 ^{ns}	0.58	*ns*
LC (n-6) PUFA	143	34.55 ^{ns}	0.55	140	35.37 ^{ns}	0.52	142	35.69 ^{ns}	0.52	*ns*
LC (n-3) PUFA	143	3.89 ^{ns}	0.15	140	3.95 ^{ns}	0.16	142	4.07 ^{ns}	0.19	*ns*
Dietary Fatty Acids										
TotalFat	133	87.60 ^{ns}	2.70	137	87.06 ^{ns}	2.77	134	79.69 ^{ns}	2.36	*ns*
TotalFat%	133	36.14 ^{ns}	0.63	137	36.54 ^{ns}	0.63	134	35.21 ^{ns}	0.58	*ns*
SAFA	133	30.02 ^{ns}	1.06	137	30.73 ^{ns}	1.18	134	27.44 ^{ns}	0.97	*ns*
SAFA%	132	12.19 ^{ns}	0.25	137	12.78 ^{ns}	0.30	134	12.09 ^{ns}	0.28	*ns*
MUFA	133	32.21 ^{ns}	1.24	137	33.02 ^{ns}	1.23	134	28.81 ^{ns}	0.99	*ns*
MUFA%	133	13.44 ^{ns}	0.44	137	13.92 ^{ns}	0.41	134	12.91 ^{ns}	0.38	*ns*
PUFA	133	13.82 ^{ns}	0.65	137	13.46 ^{ns}	0.57	134	13.32 ^{ns}	0.63	*ns*
PUFA%	133	5.67 ^{ns}	0.19	137	5.65 ^{ns}	0.18	134	5.81 ^{ns}	0.21	*ns*
LC (n-3) PUFA	86	1.49 ^{ns}	0.15	88	1.57 ^{ns}	0.12	103	1.49 ^{ns}	0.11	*ns*
LC (n-6) PUFA	86	9.65 ^{ns}	0.66	88	9.16 ^{ns}	0.59	103	8.89 ^{ns}	0.63	*ns*
C8:0 (Caprylic acid)	43	0.27 ^{a}	0.03	46	0.21 ^{ab}	0.03	69	0.19 ^{b}	0.02	*p=0.032*
C10:0 (Capric acid)	59	0.58 ^{ns}	0.05	65	0.56 ^{ns}	0.05	86	0.47 ^{ns}	0.04	*ns*
C12:0 (Lauric acid)	69	1.31 ^{ns}	0.10	85	1.40 ^{ns}	0.12	98	1.06 ^{ns}	0.09	*ns*
C14:0 (Mysteric acid)	96	2.53 ^{ns}	0.17	108	2.71 ^{ns}	0.19	115	2.37 ^{ns}	0.14	*ns*
C16:0 (Palmitic acid)	96	14.43 ^{ns}	0.64	108	14.68 ^{ns}	0.64	115	12.83 ^{ns}	0.52	*ns*
C16:1 (Palmitoleic acid)	96	1.28 ^{ns}	0.06	108	1.44 ^{ns}	0.08	115	1.23 ^{ns}	0.06	*ns*
C18:0 (Stearic acid)	96	6.68 ^{ns}	0.36	108	7.00 ^{ns}	0.45	115	5.98 ^{ns}	0.31	*ns*
C18:1 (Oleic acid)	96	27.42 ^{ab}	1.51	108	28.33 ^{a}	1.39	115	23.13 ^{b}	1.08	*p=0.010*
C18:2:6 (LA)	120	10.67 ^{ns}	0.59	128	10.01 ^{ns}	0.51	128	9.49 ^{ns}	0.57	*ns*
C18:3:3 (ALA)	120	1.23 ^{ns}	0.09	128	1.24 ^{ns}	0.07	128	1.12 ^{ns}	0.07	*ns*
C20:4:6 (AA)	59	0.13 ^{ns}	0.02	65	0.14 ^{ns}	0.01	86	0.11 ^{ns}	0.01	*ns*
C20:5:3 (EPA)	110	0.12 ^{ns}	0.02	108	0.14 ^{ns}	0.02	116	0.15 ^{ns}	0.02	*ns*
C22:6:3 (DHA)	110	0.18 ^{ns}	0.03	108	0.23 ^{ns}	0.03	116	0.24 ^{ns}	0.03	*ns*
trans Fatty Acids	66	2.80 ^{a}	0.24	55	2.23 ^{ab}	0.32	66	1.82 ^{b}	0.20	*p=0.006*
Anthropometry										
Body Weight (Kg)	133	95.60 ^{a}	1.25	138	91.31 ^{b}	1.14	134	87.44 ^{b}	1.33	*p=0.000*
Height (m)	133	114.55 ^{ns}	6.83	138	119.26 ^{ns}	6.57	134	104.38 ^{ns}	7.01	*ns*
BMI (kg/m^2)	144	33.56 ^{a}	0.36	143	31.97 ^{b}	0.31	144	31.52 ^{b}	0.34	*p=0.000*
BMR (MJ/d)	133	7.50 ^{a}	0.10	137	7.23 ^{ab}	0.10	132	6.94 ^{b}	0.09	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{ns}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

caprylic acid **(C8:0)**; capric acid **(C10:0)**, lauric acid **(C12:0)**; mysteric acid **(C14:0)**; palmitic acid **(C16:0)**; palmitoleic acid **(C16:1)**; Stearic Acid **(18:0)**; Oleic Acid **(18:1)**; Linoleic Acid (LA, **18:2:6**); a-Linoleic acid (ALA, **18:3:3**); Arachidonic Acid (AA, **20:4:6**); EPA **(20:5:3)**; DHA **(22:6:3)**;

BMI (Body Mass Index); **BMR** (Basal Metabolic Rate: *Men* (30-59 years) 11.5 x body weight (kg) + 873 standard error of estimation = 167

BMR (Megajoules per day)

Tertile Ranges: Low S_I tertile (0.19 - 1.96 (mU/L)-1min-1); **Medium S_I tertile** (1.97 - 3.14(mU/L)-1min-1); **High S_I tertile** (>3.15(mU/L)-1min-1)

Table 4.7: Mean(±SEM) of the markers of Insulin sensitivity as calculated by MINMOD Millenium® according to tertiles of S_I

	Tertiles of S_I										
	Tertile 1			Tertile 2			Tertile 3				
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
C-peptide (ng/ml)	143	3.01	a	0.07	142	2.67	b	0.07	142	2.29	c	0.06	*p=0.000*
S_I (mU/L)-1min-1	144	1.32	a	0.04	145	2.54	b	0.03	145	4.84	c	0.17	*p=0.000*
S_G (min-1)	144	0.01	a	0.00	145	0.02	b	0.00	145	0.02	c	0.00	*p=0.000*
AIRg (mUL^{-1} min^{-1})	144	470.74	a	38.84	145	352.21	b	21.73	145	252.37	c	15.10	*p=0.000*
DI	144	608.24	a	47.92	145	887.96	b	54.84	145	1141.03	c	65.77	*p=0.000*
G0 (mg/dl^{-1})	144	299.09	ns	2.74	145	300.22	ns	3.38	145	295.62	ns	3.80	*ns*
P2 (min^{-1})	144	0.04	a	0.00	145	0.05	b	0.00	145	0.05	b	0.00	*p=0.000*
P3 (mU/L) min^{-2}	144	0.00	a	0.00	145	0.00	b	0.00	145	0.00	c	0.00	*p=0.000*
Fasting Insulin (uIU/ml)	144	13.15	a	0.54	145	9.58	b	0.40	145	7.22	c	0.29	*p=0.000*
Fasting Glucose (mmol/l)	144	5.99	a	0.07	145	6.10	a	0.10	145	5.83	b	0.07	*p=0.027*
$HOMA_{IR}$	144	1.62	a	0.04	145	1.74	a	0.09	145	1.54	b	0.04	*p=0.028*
QUICKI	144	0.23	ns	0.00	145	0.23	ns	0.00	145	0.24	ns	0.00	*ns*
Insulin/c-peptide ratio	143	4.37	a	0.13	142	3.55	b	0.12	142	3.16	c	0.08	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Tertile Ranges: Low S_I tertile (0.19 - 1.96 (mU/L)-1min-1); **Medium S_I tertile** (1.97 - 3.14(mU/L)-1min-1); **High S_I tertile** (>3.15(mU/L)-1min-1)

as presented in *Table 4.8 - Table 4.10.* Results for the inflammatory markers demonstrated that concentrations of plasma PAI-1 and tPA were significantly higher in high $HOMA_{IR}$ tertile compared to the low tertiles ($p<0.01$ and $p<0.0005$, respectively) and plasma leptin concentrations were substantially higher in high $HOMA_{IR}$ tertile compared to the medium tertile ($p<0.05$). Similar to the results of the S_I stratification, no significant findings were noted in relation to plasma FA (*Table 4.9*). In regards to the dietary FA, total energy from MUFA was found to be appreciably higher in the high $HOMA_{IR}$ group compared to the low $HOMA_{IR}$ group (p=0.002). Moreover, oleic acid (C18:1) and DHA (C22:6:3) was noted to be significantly higher in the high $HOMA_{IR}$ group compared to the low $HOMA_{IR}$ group (p=0.002 and $p<0.05$, respectively). In relation to the anthropometry, those in the high $HOMA_{IR}$ tertile were found to be taller compared to those in the low $HOMA_{IR}$ tertile (p=0.004).

Metabolic markers of S_I were investigated across the $HOMA_{IR}$ tertiles and as expected results for c-peptide, G0, fasting insulin, fasting glucose, $HOMA_{IR}$ and QUICKI were significantly higher in the high $HOMA_{IR}$ tertile compared to the low tertile ($p<0.0005$). However, S_I, AIRg, DI, P2 and P3 values were significantly higher in the low $HOMA_{IR}$ tertile compared to the high tertile ($p<0.0005$) (*Table 4.10*). It is possible that more associations were noted with the $HOMA_{IR}$ tertiles compared to the tertiles of S_I as $HOMA_{IR}$ is an analysis of the fasting insulin, whereas S_I accounts for both fasting and the postprandial analysis of insulin.

4.4.4 Cross-sectional study cohort stratified according to resistin

Resistin is a peptide hormone that belongs to a family to tissue-specific resistin-like molecules originally named for its resistance to insulin. Therefore, tertiles of resistin were created to examine any associations between inflammation, plasma and dietary FAs and IR. Although the first report proposed that serum resistin levels to be increased in the obese state, several publications later have demonstrated resistin more of an innocent bystander than a real player in metabolic diseases (Steppan *et al*, 2001; Ukkola, 2002).

This cross-sectional study cohort was divided according to their plasma resistin concentrations, in a similar manner to S_I and $HOMA_{IR}$ categories. The ranges for plasma resistin tertiles are presented in *Table 4.4*. When the metabolic markers of S_I were stratified according to the tertiles of plasma resistin concentrations, a number of significant results emerged as presented in *Table 4.11*. Results show that plasma c-peptide concentrations were considerably higher in high resistin tertile compared to low tertile ($p<0.05$). Similarly, fasting glucose and $HOMA_{IR}$ levels were also noted to be higher in the high resistin range compared

Table 4.8: Mean(±SEM) of the markers of inflammation, endothelial dysfuction and oxidative stress according to tertiles of $HOMA_{IR}$

	Tertiles of $HOMA_{IR}$										
	Tertile 1				Tertile 2				Tertile 3			
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
CRP (mg/L)	156	5.08	ns	0.31	158	4.49	ns	0.30	158	5.60	ns	0.38	*ns*
IL-6 (pg/mL)	156	5.21	ns	0.34	158	5.00	ns	0.32	158	4.81	ns	0.33	*ns*
TNFα (pg/mL)	156	5.27	ns	0.54	158	4.90	ns	0.31	158	5.07	ns	0.25	*ns*
sICAM-1 (ng/mL)	156	278.71	ns	5.88	158	280.77	ns	6.04	158	290.36	ns	5.88	*ns*
sVCAM-1 (ng/mL)	156	603.44	ns	12.81	158	593.05	ns	13.72	158	599.01	ns	13.15	*ns*
Resistin (pg/mL)	156	9397.15	ns	355.98	158	10288.40	ns	425.21	158	10577.04	ns	377.37	*ns*
Adiponectin (µg/mL)	156	3.93	ns	0.21	158	3.44	ns	0.17	158	3.79	ns	0.20	*ns*
PAI-1 (ng/mL)	156	46.54	a	2.78	158	56.42	ab	3.13	158	60.90	b	2.97	*p=0.002*
tPA (ng/mL)	156	7.73	a	0.26	158	8.65	b	0.32	158	9.66	b	0.39	*p=0.000*
Fibrinogen (mg/dL)	155	335.36	ns	7.05	158	320.72	ns	6.54	157	323.54	ns	6.72	*ns*
Leptin (ng/mL)	154	19.19	ab	1.29	157	20.52	a	1.65	158	24.72	b	1.78	*p=0.040*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	155	0.47	ns	0.01	156	0.47	ns	0.01	155	0.47	ns	0.01	*ns*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	155	0.17	ns	0.01	156	0.16	ns	0.00	155	0.17	ns	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [ns] (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1);

sVCAM-1 (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activated Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_{2}\alpha$ (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

Tertile Ranges: Low $HOMA_{IR}$ tertile (0.33 - 1.39); **Medium $HOMA_{IR}$ tertile** (1.39 - 1.63); **High $HOMA_{IR}$ tertile** (>1.63)

Table 4.9: Mean(±SEM) of the plasma fatty acids, dietary fatty acids and anthropometric measurements according to tertiles of $HOMA_{IR}$

	Tertiles of $HOMA_{IR}$									
	Tertile 1			Tertile 2			Tertile 3			
%	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
Plasma Fatty Acids										
C14:0 (Mysteric acid)	155	1.96 ^{ns}	0.09	155	1.92 ^{ns}	0.08	155	1.82 ^{ns}	0.06	*ns*
C16:0 (Palmitic acid)	155	25.97 ^{ns}	0.37	155	26.60 ^{ns}	0.40	155	26.91 ^{ns}	0.40	*ns*
C16:1 (Palmitoleic acid)	155	1.58 ^{ns}	0.11	155	1.32 ^{ns}	0.07	155	1.24 ^{ns}	0.06	*ns*
C18:0 (Stearic acid)	155	3.88 ^{ns}	0.17	155	4.30 ^{ns}	0.16	155	4.31 ^{ns}	0.17	*ns*
C18:1 (Oleic acid)	155	26.53 ^{ns}	0.34	155	25.98 ^{ns}	0.36	155	26.46 ^{ns}	0.34	*ns*
C18:2 (n-6) (Linoleic acid)	155	27.96 ^{ns}	0.43	155	27.50 ^{ns}	0.45	155	27.07 ^{ns}	0.43	*ns*
C18:3 (n-6) (γ-Linoleic acid)	155	0.06 ^{ns}	0.01	155	0.06 ^{ns}	0.00	155	0.06 ^{ns}	0.00	*ns*
C18:4 (n-6) (Stearidonic acid)	155	0.04 ^{ns}	0.00	155	0.04 ^{ns}	0.00	155	0.04 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	155	0.25 ^{ns}	0.02	155	0.21 ^{ns}	0.02	155	0.20 ^{ns}	0.01	*ns*
C20:3 (n-6) (Arachidonic acid)	155	0.83 ^{ns}	0.04	155	0.86 ^{ns}	0.03	155	0.87 ^{ns}	0.04	*ns*
C20:4 (n-6) (Arachidonic acid)	155	6.91 ^{ns}	0.19	155	6.99 ^{ns}	0.20	155	6.75 ^{ns}	0.18	*ns*
C20:4 (n-3) (Arachidonic acid)	155	0.06 ^{ns}	0.01	155	0.05 ^{ns}	0.00	155	0.05 ^{ns}	0.01	*ns*
C20:5 (n-3) (EPA)	155	0.91 ^{ns}	0.07	155	1.05 ^{ns}	0.08	155	0.96 ^{ns}	0.07	*ns*
C22:4 (n-6) (Docosatetraenoic acid)	155	0.21 ^{ns}	0.02	155	0.17 ^{ns}	0.01	155	0.17 ^{ns}	0.01	*ns*
C22:5 (n-3) (Docosapentaenoic acid)	155	0.52 ^{ns}	0.04	155	0.45 ^{ns}	0.02	155	0.44 ^{ns}	0.02	*ns*
C22:6 (n-3) (DHA)	155	2.05 ^{ns}	0.09	155	2.22 ^{ns}	0.10	155	2.35 ^{ns}	0.10	*ns*
SFA	155	31.81 ^{ns}	0.48	155	32.81 ^{ns}	0.53	155	33.04 ^{ns}	0.52	*ns*
MUFA	155	28.36 ^{ns}	0.40	155	27.51 ^{ns}	0.39	155	27.91 ^{ns}	0.35	*ns*
PUFA	155	39.83 ^{ns}	0.55	155	39.68 ^{ns}	0.59	155	39.05 ^{ns}	0.52	*ns*
LC (n-6) PUFA	155	35.97 ^{ns}	0.50	155	35.58 ^{ns}	0.54	155	34.92 ^{ns}	0.49	*ns*
LC (n-3) PUFA	155	3.85 ^{ns}	0.15	155	4.10 ^{ns}	0.17	155	4.13 ^{ns}	0.16	*ns*
Dietary Fatty Acids										
TotalFat	147	85.53 ^{ns}	2.68	149	82.42 ^{ns}	2.32	148	88.07 ^{ns}	2.60	*ns*
TotalFat%	147	35.75 ^{ns}	0.61	149	35.72 ^{ns}	0.58	148	37.23 ^{ns}	0.59	*ns*
SAFA	147	30.04 ^{ns}	1.10	149	28.62 ^{ns}	0.89	148	29.60 ^{ns}	1.08	*ns*
SAFA%	146	12.43 ^{ns}	0.29	149	12.43 ^{ns}	0.25	148	12.31 ^{ns}	0.24	*ns*
MUFA	147	30.55 ^{ns}	1.16	149	30.53 ^{ns}	1.03	148	34.48 ^{ns}	1.21	*ns*
MUFA%	147	12.83 ^{a}	0.37	149	13.32 ^{ab}	0.37	148	14.76 ^{b}	0.44	*p=0.002*
PUFA	147	13.57 ^{ns}	0.62	149	13.82 ^{ns}	0.63	148	13.36 ^{ns}	0.49	*ns*
PUFA%	147	5.65 ^{ns}	0.19	149	5.88 ^{ns}	0.20	148	5.67 ^{ns}	0.16	*ns*
LC (n-3) PUFA	106	1.59 ^{ns}	0.14	93	1.44 ^{ns}	0.12	108	1.50 ^{ns}	0.10	*ns*
LC (n-6) PUFA	106	9.13 ^{ns}	0.59	93	9.06 ^{ns}	0.71	108	9.50 ^{ns}	0.50	*ns*
C8:0 (Caprylic acid)	76	0.21 ^{ns}	0.02	51	0.20 ^{ns}	0.02	42	0.24 ^{ns}	0.03	*ns*
C10:0 (Capric acid)	93	0.49 ^{ns}	0.04	72	0.50 ^{ns}	0.03	65	0.60 ^{ns}	0.06	*ns*
C12:0 (Lauric acid)	107	1.07 ^{ns}	0.09	89	1.27 ^{ns}	0.10	80	1.33 ^{ns}	0.10	*ns*
C14:0 (Mysteric acid)	120	2.53 ^{ns}	0.15	110	2.32 ^{ns}	0.12	123	2.63 ^{ns}	0.18	*ns*
C16:0 (Palmitic acid)	120	13.96 ^{ns}	0.59	110	13.03 ^{ns}	0.52	123	14.63 ^{ns}	0.58	*ns*
C16:1 (Palmitoleic acid)	120	1.27 ^{ns}	0.07	110	1.24 ^{ns}	0.06	123	1.45 ^{ns}	0.07	*ns*
C18:0 (Stearic acid)	120	6.67 ^{ns}	0.35	110	5.88 ^{ns}	0.28	123	6.87 ^{ns}	0.39	*ns*
C18:1 (Oleic acid)	120	24.45 ^{a}	1.27	110	24.70 ^{b}	1.26	123	30.19 ^{b}	1.31	*p=0.002*
C18:2:6 (LA)	140	9.98 ^{ns}	0.54	133	10.08 ^{ns}	0.58	139	9.93 ^{ns}	0.44	*ns*
C18:3:3 (ALA)	140	1.20 ^{ns}	0.08	133	1.19 ^{ns}	0.07	139	1.18 ^{ns}	0.07	*ns*
C20:4:6 (AA)	93	0.13 ^{ns}	0.01	72	0.10 ^{ns}	0.01	65	0.14 ^{ns}	0.01	*ns*
C20:5:3 (EPA)	126	0.13 ^{ns}	0.02	116	0.13 ^{ns}	0.02	124	0.15 ^{ns}	0.02	*ns*
C22:6:3 (DHA)	126	0.21 ^{a}	0.03	116	0.20 ^{ab}	0.03	124	0.24 ^{b}	0.03	*p=0.035*
trans Fatty Acids	82	2.49 ^{ns}	0.25	63	2.16 ^{ns}	0.23	53	2.04 ^{ns}	0.22	*ns*
Anthropometry										
Body Weight (Kg)	148	90.76 ^{ns}	1.40	149	91.70 ^{ns}	1.18	148	91.81 ^{ns}	1.03	*ns*
Height (m)	148	98.59 ^{a}	6.91	149	115.45 ^{ab}	6.38	148	128.88 ^{b}	5.77	*p=0.004*
BMR (MJ/d)	146	7.21 ^{ns}	0.10	148	7.21 ^{ns}	0.09	148	7.19 ^{ns}	0.09	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

caprylic acid **(C8:0)**; capric acid **(C10:0)**, lauric acid **(C12:0)**; mysteric acid **(C14:0)**; palmitic acid **(C16:0)**; palmitoleic acid **(C16:1)**;

Stearic Acid **(18:0)**; Oleic Acid **(18:1)**; Linoleic Acid (LA, **18:2:6**); a-Linoleic acid (ALA, **18:3:3**); Arachidonic Acid (AA, **20:4:6**); EPA **(20:5:3)**; DHA **(22:6:3)**;

BMI (Body Mass Index); **BMR** (Basal Metabolic Rate); **BMR** (Megajoules per day)

Tertile Ranges: Low $HOMA_{IR}$ tertile (0.33 - 1.39); **Medium $HOMA_{IR}$ tertile** (1.39 - 1.63); **High $HOMA_{IR}$ tertile** (>1.63)

Table 4.10:Mean(±SEM) of the markers of Insulin sensitivity as calculated by the MINMOD Millenium® and analysed according to tertiles of $HOMA_{IR}$

	Tertiles of $HOMA_{IR}$										
	Tertile 1				Tertile 2				Tertile 3			
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
C-peptide (ng/ml)	154	2.40	a	0.06	158	2.61	ab	0.07	156	2.89	b	0.07	*p=0.000*
S_I (mU/L)-1min-1	144	3.07	a	0.15	146	2.97	ab	0.17	142	2.66	b	0.16	*p=0.023*
$S_{G\,(}$min-1)	144	0.02	ns	0.00	146	0.02	ns	0.00	142	0.02	ns	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	144	472.03	a	26.60	146	378.59	b	33.54	142	219.96	c	17.32	*p=0.000*
DI	144	1207.25	a	60.05	146	891.28	b	55.99	142	529.83	c	48.45	*p=0.000*
G0 (mg/$dl^{-1)}$	144	285.11	a	3.59	146	293.68	b	3.03	142	316.74	c	2.82	*p=0.000*
P2 ($min^{-1)}$	144	0.05	a	0.00	146	0.04	a	0.00	142	0.04	b	0.00	*p=0.000*
P3 (mU/L) min^{-2}	144	0.00	a	0.00	146	0.00	a	0.00	142	0.00	b	0.00	*p=0.000*
Fasting Insulin (uIU/ml)	157	8.49	a	0.35	158	9.98	b	0.49	158	11.10	c	0.47	*p=0.000*
Fasting Glucose (mmol/l)	157	5.20	a	0.03	158	5.82	b	0.01	158	6.94	c	0.08	*p=0.000*
$HOMA_{IR}$	157	1.20	a	0.01	158	1.50	b	0.01	158	2.22	c	0.08	*p=0.000*
QUICKI	157	0.24	a	0.00	158	0.23	b	0.00	158	0.23	c	0.00	*p=0.000*
Insulin/c-peptide ratio	153	3.47	ns	0.10	158	3.77	ns	0.13	157	3.84	ns	0.13	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Tertile Ranges: Low $HOMA_{-IR}$ tertile (0.33 - 1.39); **Medium $HOMA_{IR}$ tertile** (1.39 - 1.63); **High $HOMA_{IR}$ tertile** (>1.63)

Table 4.11: Mean(±SEM) of the markers of Insulin sensitivity as calculated by MINMOD Millenium® according to tertiles of Resistin

	Tertiles of Resistin									
	Tertile 1			Tertile 2			Tertile 3			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
C-peptide (ng/ml)	154	2.52 [a]	0.07	159	2.62 [ab]	0.07	155	2.77 [b]	0.07	*p=0.048*
S_I (mU/L)-1min-1	139	2.53 [a]	0.12	150	3.20 [b]	0.18	142	2.96 [ab]	0.17	*p=0.023*
$S_{G\,(}$min-1)	139	0.02 [ns]	0.00	150	0.02 [ns]	0.00	142	0.02 [ns]	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	139	354.56 [ns]	23.85	150	360.43 [ns]	23.44	142	358.24 [ns]	35.92	*ns*
DI	139	793.13 [ns]	53.56	150	971.52 [ns]	58.17	142	866.02 [ns]	65.95	*ns*
G0 (mg/dl $^{-1)}$	139	295.23 [ns]	3.33	150	296.82 [ns]	3.53	142	303.41 [ns]	3.16	*ns*
P2 ($min^{-1)}$	139	0.04 [ns]	0.00	150	0.05 [ns]	0.00	142	0.04 [ns]	0.00	*ns*
P3 (mU/L) min^{-2}	139	0.00 [ns]	0.00	150	0.00 [ns]	0.00	142	0.00 [ns]	0.00	*ns*
Fasting Insulin (uIU/ml)	156	9.31 [ns]	0.41	159	9.61 [ns]	0.43	157	10.61 [ns]	0.49	*ns*
Fasting Glucose (mmol/l)	156	5.85 [a]	0.07	159	5.95 [ab]	0.07	157	6.18 [b]	0.09	*p=0.040*
$HOMA_{IR}$	156	1.54 [a]	0.04	159	1.60 [ab]	0.04	157	1.78 [b]	0.09	*p=0.038*
QUICKI	156	0.23 [ns]	0.00	159	0.23 [ns]	0.00	157	0.23 [ns]	0.00	*ns*
Insulin/c-peptide ratio	155	3.70 [ns]	0.12	157	3.59 [ns]	0.12	156	3.79 [ns]	0.13	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [n]s (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Tertile Ranges: Low Resistin tertile (1463 - 7782mg/L) ; **Medium Resistin tertile** (7785 - 11550mg/L); **High Resistin tertile** (>11596mg/L)

to the low resistin range ($p<0.05$). No noteworthy differences were noted for S_G, AIRg, DI, G0, P2, P3, fasting insulin, QUICKI and insulin/c-peptide ratio. When plasma and dietary FA composition was investigated across the resistin tertiles, no significant differences emerged for plasma FA and in relation to the dietary FA, only caprylic acid (C8:0) and TFA were substantially higher in the low resistin tertile compared to the high tertile of resistin ($p<0.01$). In relation to anthropometric measurements, only BMI was found to be noticably higher in the high resistin tertile compared to the low resistin tertile ($p<0.05$) (*Data not shown*).

Tertiles of resistin were investigated in relation to the inflammatory mediators as presented in *Table 4.12.* A number of prominent results emerged, wherein plasma IL-6, TNFα, sVCAM-1, resistin, PAI-1, tPA and urinary 8-iso-$PGF_{2\alpha}$ concentrations were all especially higher in high resistin tertile compared to the low resistin tertile. However, no particular differences were noted for plasma CRP, fibrinogen, leptin concentrations or urinary 15-keto-dihydro-$PGF_{2\alpha}$.

4.4.5 Cross-sectional study cohort stratified according to adiponectin

Adiponectin is an adipocyte-derived protein abundantly present in plasma, it has the ability to increase S_I by stimulating fatty acid oxidation, decreasing plasma TAG and improves glucose metabolism (Nedvídková *et al,* 2005). Therefore, tertiles of adiponectin were created in a similar manner to those above and ranges of the low, medium and high tertiles of adiponectin are presented in *Table 4.13* and *Table 4.14*. The majority of studies have found adiponectin to possess anti-inflammatory properties, therefore, it was no surprise to find that levels of S_I were significantly higher in the high adiponectin tertile compared to the low adiponectin tertile (p=0.01). Additionally, levels of G0, P2 and P3 were considerably elevated in the high adiponectin tertile compared to the low adiponectin tertile ($p\leq 0.02$). Contrary to this, c-peptide and fasting insulin levels were found to be significantly higher in the low adiponectin tertile compared to the high adiponectin tertile ($p\leq 0.02$). Whereas, no particular differences were noted for levels of S_G, AIRg, DI, fasting glucose, $HOMA_{IR}$, QUICKI insulin/c-peptide ratio (*Table 4.13*).

When plasma FAs were examined across the tertiles of adiponectin, a multitude of results emerged as presented in *Table 4.14*. In particular, myristic acid (C14:0), eicosenoic acid (C20:1), eicosatetraenoic acid (C20:4:3), EPA (C20:5:3) and Long Chain (LC) n-3 PUFA were considerably increased in the low adiponectin tertile compared to the high adiponectin tertile. Contrary to this, oleic acid (C18:0) and ALA (C18:3:6) were significantly higher in

Table 4.12: Mean(±SEM) of the markers of inflammation, endothelial dysfuction and oxidative stress according to tertiles of Resistin

	Tertiles of Resistin										
	Tertile 1				Tertile 2				Tertile 3			
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
hsCRP (mg/L)	160	4.59	ns	0.28	161	4.92	ns	0.31	161	5.77	ns	0.37	*ns*
IL-6 (pg/mL)	160	4.13	a	0.28	161	5.24	ab	0.35	161	5.62	b	0.33	*p=0.003*
TNFα (pg/mL)	160	4.19	a	0.28	161	5.53	b	0.53	161	5.40	b	0.25	*p=0.000*
sICAM-1 (ng/mL)	160	284.94	ns	6.09	161	275.30	ns	5.58	161	289.44	ns	5.87	*ns*
sVCAM-1 (ng/mL)	160	567.29	a	13.74	161	594.31	ab	11.72	161	637.80	b	13.50	*p=0.000*
Resistin (pg/mL)	160	5179.02	a	132.30	161	9666.45	b	87.97	161	15321.05	c	305.72	*p=0.000*
Adiponectin (μg/mL)	160	3.31	ns	0.14	161	4.01	ns	0.25	161	3.79	ns	0.17	*ns*
PAI-1 (ng/mL)	160	50.33	a	3.06	161	53.12	ab	2.96	161	60.13	b	2.82	*p=0.019*
tPA (ng/mL)	160	8.33	a	0.26	161	8.28	ab	0.34	161	9.43	b	0.37	*p=0.013*
Fibrinogen (mg/dL)	160	328.00	ns	7.41	160	322.80	ns	6.18	160	332.78	ns	6.53	*ns*
Leptin (ng/mL)	159	18.79	ns	1.33	161	22.79	ns	1.57	159	22.84	ns	1.77	*ns*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	158	0.46	ab	0.01	158	0.46	a	0.01	157	0.49	b	0.01	*p=0.035*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	158	0.17	ns	0.01	158	0.17	ns	0.01	157	0.17	ns	0.00	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [ns] (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1);

sVCAM-1 (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_{2}\alpha$ (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

Tertile Ranges: Low Resistin tertile (1463 - 7782mg/L) ; **Medium Resistin tertile** (7785 - 11550mg/L); **High Resistin tertile** (>11596mg/L)

Table 4.13:Mean(±SEM) of the markers of Insulin sensitivity as calculated by MINMOD Millenium® according to tertiles of Adiponectin

	Tertiles of Adiponectin										
	Tertile 1			Tertile 2			Tertile 3				
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
C-peptide (ng/ml)	156	2.83	a	0.07	154	2.68	a	0.07	158	2.40	b	0.07	*p=0.000*
S_I (mU/L)-1min-1	145	2.79	a	0.17	140	2.65	a	0.13	146	3.26	b	0.17	*p=0.011*
S_G (min-1)	145	0.02	ns	0.00	140	0.02	ns	0.00	146	0.02	ns	0.00	*ns*
AIRg (mUL^{-1} min^{-1})	145	366.82	ns	22.99	140	367.55	ns	28.40	146	339.54	ns	32.39	*ns*
DI	145	897.21	ns	61.16	140	827.57	ns	63.61	146	910.91	ns	54.41	*ns*
G0 (mg/dl $^{-1}$)	145	292.94	a	3.53	140	300.10	b	3.16	146	302.42	b	3.32	*p=0.026*
P2 (min^{-1})	145	0.04	a	0.00	140	0.04	a	0.00	146	0.05	b	0.00	*p=0.024*
P3 (mU/L) min^{-2}	145	0.00	a	0.00	140	0.00	ab	0.00	146	0.00	b	0.00	*p=0.000*
Fasting Insulin (uIU/ml)	157	10.51	a	0.46	158	9.89	ab	0.42	157	9.13	b	0.45	*p=0.028*
Fasting Glucose (mmol/l)	157	6.06	ns	0.09	158	6.03	ns	0.07	157	5.88	ns	0.07	*ns*
$HOMA_{IR}$	157	1.71	ns	0.08	158	1.65	ns	0.04	157	1.56	ns	0.04	*ns*
QUICKI	157	0.23	ns	0.00	158	0.23	ns	0.00	157	0.23	ns	0.00	*ns*
Insulin/c-peptide ratio	156	3.68	ns	0.12	157	3.65	ns	0.11	155	3.75	ns	0.13	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Tertile Ranges: Low Adiponectin tertile (0.15-2.55μg/mL) ; **Medium Adiponectin tertile** (2.55-4.12μg/mL); **High Adiponectin tertile** (>4.13μg/mL)

high adiponectin tertile compared to the low adiponectin tertile ($p<0.01$). Additionally, DHA (C22:6:3) was significantly higher in the medium adiponectin tertile compared to the high adiponectin tertile ($p<0.05$).

In regards to dietary FAs across the adiponectin tertiles, again results identified that the majority of dietary fats are higher in the low adiponectin tertile compared to the high adiponectin tertile as presented in *Table 4.14*. In particular, total fat, total SFA, total MUFA and total PUFA were all highly increased in the low adiponectin tertile compared to the high tertile ($p<0.0005$). Additionally, LC n-3 PUFA and LC n-6 PUFA were significantly higher in the low adiponectin tertile compared to the high tertile ($p<0.0005$). Furthermore, many SFA were found to be significantly higher in the low adiponectin tertile compared to the high tertile, such as capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Many PUFA were also demonstrated to be higher in the low adiponectin tertile compared to the high adiponectin tertile, such as LA (C18:2:6), ALA (C18:3:3) and DHA (C22:6:3).

When inflammatory markers were investigated over adiponectin tertiles, significance only emerged for fibrinogen and leptin concentrations, whereby concentrations of both mediators were significantly higher in the high adiponectin tertile compared to the low adiponectin tertile ($p<0.02$) (*Data not shown*).

4.4.6 Cross-sectional study cohort stratified according to CRP

CRP is a sensitive physiological marker of subclinical systemic inflammation and is associated with hyperglycaemia, IR and overt T2DM. It is the principal downstream mediator of the acute phase response and is primarily derived via IL-6 dependent hepatic biosynthesis (Pradhan *et al*, 2001). Therefore, metabolic markers of S_I were stratified according to CRP tertiles. Results demonstrated significance for QUICKI and insulin/c-peptide ratio only, whereby QUICKI levels were higher in the low CRP tertile compared to the high CRP tertile ($p<0.03$). Contrary to this, insulin/c-peptide ratio was higher in the high CRP tertile compared to the low CRP tertile ($p<0.02$) (*Data not shown*). When plasma FA were analysed according to CRP tertiles, several interesting differences were noted, wherein myristic acid (C14:0), palmitic acid (C16:0) and total SFA were significantly higher in the low CRP tertile compared to the high CRP tertile ($p\leq 0.0001$). Furthermore, oleic acid (C18:0) and total MUFA were found to be higher in the medium tertile compared to the low adiponectin tertile ($p\leq 0.03$)

Table 4.14: Mean(±SEM) of the plasma fatty acids, dietary fatty acids and anthropometric measurements according to tertiles of Adiponectin

	Tertiles of Adiponectin									
	Tertile 1			Tertile 2			Tertile 3			
%	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
Plasma Fatty Acids										
C14:0 (Mysteric acid)	155	1.99 ^{a}	0.07	159	1.93 ^{ab}	0.08	160	1.79 ^{b}	0.08	*p=0.027*
C16:0 (Palmitic acid)	155	27.30 ^{ns}	0.36	159	26.02 ^{ns}	0.36	160	26.18 ^{ns}	0.43	*ns*
C16:1 (Palmitoleic acid)	155	1.29 ^{ns}	0.07	159	1.44 ^{ns}	0.07	160	1.43 ^{ns}	0.09	*ns*
C18:0 (Stearic acid)	155	3.77 ^{a}	0.15	159	4.24 ^{ab}	0.16	160	4.48 ^{b}	0.18	*p=0.008*
C18:1 (Oleic acid)	155	25.75 ^{ns}	0.38	159	26.74 ^{ns}	0.31	160	26.73 ^{ns}	0.34	*ns*
C18:2 (n-6) (Linoleic acid)	155	27.57 ^{ns}	0.49	159	27.17 ^{ns}	0.39	160	27.59 ^{ns}	0.41	*ns*
C18:3 (n-6) (γ-Linoleic acid)	155	0.05 ^{a}	0.00	159	0.06 ^{ab}	0.00	160	0.08 ^{b}	0.01	*p=0.000*
C18:4 (n-6) (Stearidonic acid)	155	0.04 ^{ns}	0.00	159	0.04 ^{ns}	0.00	160	0.04 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	155	0.25 ^{a}	0.02	159	0.19 ^{b}	0.02	160	0.22 ^{a}	0.01	*p=0.005*
C20:3 (n-6) (Arachidonic acid)	155	0.76 ^{a}	0.04	159	0.93 ^{b}	0.03	160	0.88 ^{ab}	0.03	*p=0.003*
C20:4 (n-6) (Arachidonic acid)	155	6.87 ^{ns}	0.18	159	6.95 ^{ns}	0.18	160	6.78 ^{ns}	0.20	*ns*
C20:4 (n-3) (Arachidonic acid)	155	0.08 ^{a}	0.01	159	0.05 ^{b}	0.00	160	0.04 ^{b}	0.00	*p=0.002*
C20:5 (n-3) (EPA)	155	1.06 ^{a}	0.07	159	1.03 ^{ab}	0.07	160	0.82 ^{b}	0.07	*p=0.013*
C22:4 (n-6) (Docosatetraenoic acid)	155	0.18 ^{ns}	0.01	159	0.18 ^{ns}	0.01	160	0.19 ^{ns}	0.01	*ns*
C22:5 (n-3) (Docosapentaenoic acid)	155	0.53 ^{a}	0.03	159	0.46 ^{b}	0.03	160	0.42 ^{c}	0.03	*p=0.000*
C22:6 (n-3) (DHA)	155	2.26 ^{ab}	0.10	159	2.29 ^{a}	0.09	160	2.03 ^{b}	0.10	*p=0.024*
SFA	155	33.06 ^{ns}	0.46	159	32.19 ^{ns}	0.47	160	32.45 ^{ns}	0.56	*ns*
MUFA	155	27.29 ^{ns}	0.42	159	28.36 ^{ns}	0.34	160	28.38 ^{ns}	0.37	*ns*
PUFA	155	39.65 ^{ns}	0.59	159	39.45 ^{ns}	0.49	160	39.17 ^{ns}	0.56	*ns*
LC (n-6) PUFA	155	35.44 ^{ns}	0.55	159	35.29 ^{ns}	0.44	160	35.52 ^{ns}	0.50	*ns*
LC (n-3) PUFA	155	4.21 ^{a}	0.16	159	4.15 ^{ab}	0.16	160	3.66 ^{b}	0.16	*p=0.008*
Dietary Fatty Acids										
TotalFat	148	93.34 ^{a}	2.81	148	80.53 ^{b}	2.30	148	82.50 ^{b}	2.37	*p=0.000*
TotalFat%	148	36.88 ^{ns}	0.57	148	35.16 ^{ns}	0.59	148	36.68 ^{ns}	0.62	*ns*
SAFA	148	32.57 ^{a}	1.11	148	28.89 ^{ab}	0.93	148	27.01 ^{b}	0.98	*p=0.000*
SAFA%	148	12.77 ^{a}	0.24	148	12.60 ^{ab}	0.26	147	11.84 ^{b}	0.27	*p=0.011*
MUFA	148	33.59 ^{ns}	1.16	148	29.79 ^{ns}	1.08	148	32.27 ^{ns}	1.16	*ns*
MUFA%	148	13.30 ^{ns}	0.33	148	13.08 ^{ns}	0.39	148	14.53 ^{ns}	0.46	*ns*
PUFA	148	16.32 ^{a}	0.67	148	12.37 ^{b}	0.54	148	12.01 ^{b}	0.45	*p=0.000*
PUFA%	148	6.41 ^{a}	0.19	148	5.40 ^{b}	0.19	148	5.35 ^{b}	0.16	*p=0.000*
LC (n-3) PUFA	84	1.98 ^{a}	0.17	96	1.59 ^{a}	0.12	127	1.15 ^{b}	0.08	*p=0.000*
LC (n-6) PUFA	84	11.42 ^{a}	0.75	96	9.72 ^{a}	0.60	127	7.40 ^{b}	0.42	*p=0.000*
C8:0 (Caprylic acid)	36	0.25 ^{ns}	0.03	51	0.23 ^{ns}	0.02	82	0.20 ^{ns}	0.02	*ns*
C10:0 (Capric acid)	68	0.63 ^{a}	0.05	73	0.55 ^{ab}	0.04	89	0.43 ^{b}	0.04	*p=0.002*
C12:0 (Lauric acid)	87	1.40 ^{a}	0.11	95	1.19 ^{ab}	0.08	94	1.06 ^{b}	0.10	*p=0.019*
C14:0 (Mysteric acid)	103	2.78 ^{a}	0.14	118	2.70 ^{a}	0.14	132	2.14 ^{b}	0.16	*p=0.000*
C16:0 (Palmitic acid)	103	15.75 ^{a}	0.62	118	14.36 ^{a}	0.54	132	12.15 ^{b}	0.52	*p=0.000*
C16:1 (Palmitoleic acid)	103	1.44 ^{a}	0.07	118	1.35 ^{a}	0.06	132	1.21 ^{b}	0.07	*p=0.002*
C18:0 (Stearic acid)	103	7.25 ^{a}	0.36	118	6.57 ^{a}	0.28	132	5.87 ^{b}	0.38	*p=0.000*
C18:1 (Oleic acid)	103	28.84 ^{ns}	1.35	118	25.86 ^{ns}	1.20	132	25.36 ^{ns}	1.32	*ns*
C18:2:6 (LA)	143	12.23 ^{a}	0.57	132	9.75 ^{b}	0.52	137	7.87 ^{c}	0.38	*p=0.000*
C18:3:3 (ALA)	143	1.48 ^{a}	0.09	132	1.16 ^{a}	0.06	137	0.93 ^{b}	0.05	*p=0.000*
C20:4:6 (AA)	68	0.12 ^{ab}	0.01	73	0.15 ^{a}	0.02	89	0.10 ^{b}	0.01	*p=0.017*
C20:5 (EPA)	124	0.16 ^{ns}	0.02	110	0.15 ^{ns}	0.02	132	0.11 ^{ns}	0.01	*ns*
C22:6 (DHA)	124	0.24 ^{a}	0.03	110	0.24 ^{ab}	0.04	132	0.16 ^{b}	0.02	*p=0.036*
trans Fatty Acids	70	2.64 ^{a}	0.24	55	2.86 ^{a}	0.37	73	1.65 ^{b}	0.15	*p=0.001*
Anthropometry										
Body Weight (Kg)	148	94.91 ^{a}	1.13	148	90.07 ^{b}	1.12	148	89.08 ^{b}	1.34	*p=0.001*
Height (m)	148	136.20 ^{a}	5.76	148	98.16 ^{b}	6.77	148	109.32 ^{b}	6.38	*p=0.000*
BMI (kg/m^2)	157	32.11 ^{ab}	0.32	157	31.84 ^{a}	0.33	157	33.06 ^{b}	0.35	*p=0.000*
BMR	148	7.53 ^{a}	0.09	148	7.07 ^{b}	0.09	145	6.99 ^{b}	0.09	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

caprylic acid **(C8:0)**; capric acid **(C10:0)**, lauric acid **(C12:0)**; mysteric acid **(C14:0)**; palmitic acid **(C16:0)**; palmitoleic acid **(C16:1)**; Stearic Acid **(18:0)**; Oleic Acid **(18:1)**; Linoleic Acid (LA, **18:2:6**); a-Linoleic acid (ALA, **18:3:3**); Arachidonic Acid (AA, **20:4:6**); EPA **(20:5:3)**; DHA **(22:6:3)**; **BMI** (Body Mass Index); **BMR** (Basal Metabolic Rate)

Tertile Ranges: Low Adiponectin tertile (0.15-2.55μg/mL) ; **Medium Adiponectin tertile** (2.55-4.12μg/mL); **High Adiponectin tertile** (>4.13μg/mL)

(*Table 4.15*). Dietary FAs were also examined in this cross-sectional investigation according to CRP tertiles. Results of this analysis show that total energy intake from MUFA was significantly higher in high CRP tertile compared to the low CRP tertile (p=0.002). Contrary to these findings, LA (C18:2:6) and AA (C20:4) were noted to be higher in the low CRP tertile compared to the high CRP tertile ($p<0.05$) (*Table 4.15*). No noteworthy difference was noted for anthropometric measurements across the CRP tertiles.

Inflammatory markers were measured according to the CRP tertiles and results show that CRP and fibrinogen concentrations were noticeably different across all the CRP tertiles, with the high CRP demonstrating the highest concentrations for both pararmeters ($p<0.0005$). IL-6 concentrations were noted to be higher in the low CRP tertile compared to the medium CRP tertile (p=0.02), whereas PAI-1 concentrations were noted to be higher in the medium CRP tertile compared to the low CRP tertile ($p<0.0005$). Finally, leptin concentrations were noted to be higher in the high and medium tertiles compared to the low tertile ($p<0.0005$) (*Data not shown).*

4.4.7 Cross-sectional study cohort stratified according to SFA/MUFA ratio

As a cross-sectional measure of habitual dietary fat intake, the ratio of SFA/MUFA was calculated and subsequently, tertiles of low, medium and high levels were created. Measures of S_I, inflammatory biomarkers, plasma and dietary FAs were then stratified according to SFA/MUFA ratio as presented in *Table 4.16* and *Table 4.17*. Results of the inflammatory analysis show that c-peptide concentrations and DI was considerably higher in the medium and high tertile compared to the low SFA/MUFA tertile ($p \leq 0.02$). Additionally, AIRg levels were higher in the high SFA/MUFA tertile compared to the low tertile ($p<0.05$) and fasting glucose concentrations were found to be higher in the low SFA/MUFA tertile compared to the medium and high tertiles ($p<0.03$) (*Table 4.16*).

The inflammatory mediators were investigated according to the tertiles of SFA/MUFA and results show that the CRP and leptin concentrations were significantly higher in the low SFA/MUFA ratio tertile compared to the medium and high tertile ($p<0.0005$). Contrary to this, IL-6 concentrations were higher in the medium and high SFA/MUFA ratio tertiles compared to the low tertile ($p<0.02$) and finally, adiponectin concentrations were higher in the low SFA/MUFA concentrations compared to the high tertile ($p<0.03$) (*Table 4.17*).

Plasma FAs were stratified according to SFA/MUFA ratio tertiles and numerous noteworthy results were established. In particular, myristic acid (C14:0), palmitic acid (C16:0),

Table 4.15: Mean(±SEM) of the plasma fatty acids, dietary fatty acids and anthropometric measurements according to tertiles of CRP

	Tertiles of CRP									
	Tertile 1			Tertile 2			Tertile 3			
%	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
Plasma Fatty Acids										
C14:0 (Mysteric acid)	158	2.07 ^{a}	0.07	157	1.91 ^{ab}	0.08	159	1.73 ^{b}	0.08	*p=0.000*
C16:0 (Palmitic acid)	158	27.64 ^{a}	0.41	157	25.89 ^{b}	0.38	159	25.94 ^{b}	0.35	*p=0.001*
C16:1 (Palmitoleic acid)	158	1.26 ^{ns}	0.06	157	1.45 ^{ns}	0.08	159	1.46 ^{ns}	0.09	*ns*
C18:0 (Stearic acid)	158	4.35 ^{ns}	0.17	157	4.09 ^{ns}	0.16	159	4.05 ^{ns}	0.16	*ns*
C18:1 (Oleic acid)	158	25.67 ^{a}	0.35	157	27.01 ^{b}	0.34	159	26.56 ^{ab}	0.34	*p=0.029*
C18:2 (n-6) (Linoleic acid)	158	27.43 ^{ns}	0.46	157	27.27 ^{ns}	0.42	159	27.62 ^{ns}	0.41	*ns*
C18:3 (n-6) (γ-Linoleic acid)	158	0.06 ^{ns}	0.01	157	0.07 ^{ns}	0.01	159	0.06 ^{ns}	0.00	*ns*
C18:4 (n-6) (Stearidonic acid)	158	0.03 ^{ns}	0.00	157	0.04 ^{ns}	0.00	159	0.04 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	158	0.19 ^{a}	0.01	157	0.23 ^{a}	0.03	159	0.23 ^{b}	0.01	*p=0.019*
C20:3 (n-6) (Arachidonic acid)	158	0.80 ^{ns}	0.03	157	0.87 ^{ns}	0.03	159	0.91 ^{ns}	0.04	*ns*
C20:4 (n-6) (Arachidonic acid)	158	6.45 ^{ns}	0.20	157	6.93 ^{ns}	0.18	159	7.23 ^{ns}	0.18	*ns*
C20:4 (n-3) (Arachidonic acid)	158	0.05 ^{ns}	0.00	157	0.05 ^{ns}	0.00	159	0.06 ^{ns}	0.01	*ns*
C20:5 (n-3) (EPA)	158	0.99 ^{ns}	0.07	157	0.98 ^{ns}	0.08	159	0.94 ^{ns}	0.06	*ns*
C22:4 (n-6) (Docosatetraenoic acid)	158	0.18 ^{ab}	0.01	157	0.17 ^{a}	0.02	159	0.20 ^{b}	0.01	*p=0.016*
C22:5 (n-3) (Docosapentaenoic acid)	158	0.42 ^{ns}	0.02	157	0.50 ^{ns}	0.03	159	0.49 ^{ns}	0.03	*ns*
C22:6 (n-3) (DHA)	158	2.13 ^{ns}	0.10	157	2.24 ^{ns}	0.10	159	2.21 ^{ns}	0.09	*ns*
SFA	158	34.06 ^{a}	0.55	157	31.89 ^{b}	0.48	159	31.73 ^{b}	0.45	*p=0.001*
MUFA	158	27.11 ^{a}	0.37	157	28.70 ^{b}	0.37	159	28.25 ^{ab}	0.38	*p=0.009*
PUFA	158	38.83 ^{ns}	0.61	157	39.41 ^{ns}	0.51	159	40.03 ^{ns}	0.51	*ns*
LC (n-6) PUFA	158	34.91 ^{ns}	0.55	157	35.32 ^{ns}	0.48	159	36.01 ^{ns}	0.47	*ns*
LC (n-3) PUFA	158	3.91 ^{ns}	0.17	157	4.09 ^{ns}	0.17	159	4.01 ^{ns}	0.14	*ns*
Dietary Fatty Acids										
TotalFat	149	86.33 ^{ns}	2.78	150	84.11 ^{ns}	2.24	150	85.10 ^{ns}	2.53	*ns*
TotalFat%	149	35.28 ^{ns}	0.62	150	36.55 ^{ns}	0.57	150	36.83 ^{ns}	0.57	*ns*
SAFA	149	31.33 ^{ns}	1.14	150	29.19 ^{ns}	0.97	150	27.70 ^{ns}	0.91	*ns*
SAFA%	148	12.69 ^{ns}	0.27	150	12.56 ^{ns}	0.26	150	11.98 ^{ns}	0.24	*ns*
MUFA	149	30.31 ^{ns}	1.11	150	31.87 ^{ns}	1.07	150	32.93 ^{ns}	1.22	*ns*
MUFA%	149	12.49 ^{a}	0.36	150	13.97 ^{b}	0.41	150	14.30 ^{b}	0.40	*p=0.002*
PUFA	149	14.98 ^{ns}	0.68	150	12.58 ^{ns}	0.48	150	12.98 ^{ns}	0.56	*ns*
PUFA%	149	6.07 ^{ns}	0.21	150	5.47 ^{ns}	0.18	150	5.60 ^{ns}	0.15	*ns*
LC (n-3) PUFA	90	1.66 ^{ns}	0.13	101	1.51 ^{ns}	0.11	121	1.39 ^{ns}	0.11	*ns*
LC (n-6) PUFA	90	10.15 ^{ns}	0.68	101	8.65 ^{ns}	0.50	121	8.90 ^{ns}	0.58	*ns*
C8:0 (Caprylic acid)	50	0.22 ^{ns}	0.02	54	0.24 ^{ns}	0.02	70	0.21 ^{ns}	0.02	*ns*
C10:0 (Capric acid)	78	0.58 ^{ns}	0.05	71	0.55 ^{ns}	0.05	86	0.45 ^{ns}	0.03	*ns*
C12:0 (Lauric acid)	97	1.34 ^{ns}	0.10	89	1.18 ^{ns}	0.09	95	1.08 ^{ns}	0.09	*ns*
C14:0 (Mysteric acid)	109	2.77 ^{ns}	0.15	119	2.49 ^{ns}	0.15	130	2.30 ^{ns}	0.15	*ns*
C16:0 (Palmitic acid)	109	14.57 ^{ns}	0.60	119	14.00 ^{ns}	0.57	130	13.12 ^{ns}	0.53	*ns*
C16:1 (Palmitoleic acid)	109	1.27 ^{ns}	0.06	119	1.33 ^{ns}	0.06	130	1.34 ^{ns}	0.07	*ns*
C18:0 (Stearic acid)	109	6.84 ^{ns}	0.34	119	6.31 ^{ns}	0.30	130	6.28 ^{ns}	0.39	*ns*
C18:1 (Oleic acid)	109	24.78 ^{ns}	1.18	119	26.93 ^{ns}	1.25	130	26.99 ^{ns}	1.40	*ns*
C18:2:6 (LA)	138	11.40 ^{a}	0.58	135	9.10 ^{b}	0.44	144	9.26 ^{b}	0.51	*p=0.010*
C18:3:3 (ALA)	138	1.34 ^{ns}	0.07	135	1.14 ^{ns}	0.06	144	1.08 ^{ns}	0.07	*ns*
C20:4:6 (AA)	78	0.13 ^{a}	0.01	71	0.11 ^{ab}	0.01	86	0.11 ^{b}	0.01	*p=0.053*
C20:5:3 (EPA)	119	0.13 ^{ns}	0.02	117	0.17 ^{ns}	0.03	135	0.12 ^{ns}	0.02	*ns*
C22:6:3 (DHA)	119	0.20 ^{ns}	0.03	117	0.25 ^{ns}	0.03	135	0.20 ^{ns}	0.03	*ns*
trans Fatty Acids	73	2.36 ^{ns}	0.22	61	2.44 ^{ns}	0.29	69	2.13 ^{ns}	0.26	*ns*
Anthropometry										
Body Weight (Kg)	159	90.18 ^{ns}	1.16	150	91.89 ^{ns}	1.25	150	92.09 ^{ns}	1.21	*ns*
Height (m)	159	119.35 ^{ns}	6.49	150	108.17 ^{ns}	6.49	150	112.43 ^{ns}	6.35	*ns*
BMR	159	7.31 ^{ns}	0.09	150	7.18 ^{ns}	0.10	147	7.09 ^{ns}	0.08	*ns*
BMI	159	30.68 ^{a}	0.29	161	32.72 ^{b}	0.33	161	33.57 ^{b}	0.32	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{n}s (non significant)

$^{abc...}$ denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

caprylic acid **(C8:0)**; capric acid **(C10:0)**, lauric acid **(C12:0)**; mysteric acid **(C14:0)**; palmitic acid **(C16:0)**; palmitoleic acid **(C16:1)**; Stearic Acid **(18:0)**; Oleic Acid **(18:1)**; Linoleic Acid (LA, **18:2:6**); a-Linoleic acid (ALA, **18:3:3**); Arachidonic Acid (AA, **20:4:6**); EPA **(20:5:3)**; DHA **(22:6:3)**; **BMI** (Body Mass Index); **BMR** (Basal Metabolic Rate)

Tertile Ranges: Low CRP tertile (0.09-2.63mg/L); **Medium CRP tertile** (2.65-5.96mg/L); **High CRP tertile** (>5.97mg/L)

Table 4.16:Mean(±SEM) of the markers of Insulin sensitivity as calculated by MINMOD Millenium® according to tertiles of SAFA/MUFA ratio

	Tertiles of SFA/MUFA									
	Tertile 1			Tertile 2			Tertile 3			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
C-peptide (ng/ml)	149	2.45 [a]	0.07	148	2.70 [b]	0.07	143	2.77 [b]	0.08	*p=0.006*
S_I (mU/L)-1min-1	127	2.81 [ns]	0.14	141	3.11 [ns]	0.19	136	2.79 [ns]	0.15	*ns*
$S_{G\,(}$min-1)	127	0.02 [ns]	0.00	141	0.02 [ns]	0.00	136	0.02 [ns]	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	127	299.10 [a]	20.81	141	370.29 [ab]	26.74	136	412.27 [b]	37.37	*p=0.05*
DI	127	730.21 [a]	50.09	141	974.11 [b]	68.97	136	939.49 [b]	61.12	*p=0.033*
G0 (mg/$dl^{-1)}$	127	298.76 [ns]	3.60	141	296.63 [ns]	3.19	136	300.82 [ns]	3.73	*ns*
P2 ($min^{-1)}$	127	0.04 [ns]	0.00	141	0.04 [ns]	0.00	136	0.04 [ns]	0.00	*ns*
P3 (mU/L) min^{-2}	127	0.00 [ns]	0.00	141	0.00 [ns]	0.00	136	0.00 [ns]	0.00	*ns*
Fasting Insulin (uIU/ml)	150	9.47 [ns]	0.42	149	9.57 [ns]	0.42	146	10.48 [ns]	0.53	*ns*
Fasting Glucose (mmol/l)	150	6.18 [a]	0.09	149	5.93 [b]	0.07	146	5.90 [b]	0.07	*p=0.027*
$HOMA_{IR}$	150	1.77 [ns]	0.08	149	1.60 [ns]	0.05	146	1.58 [ns]	0.04	*ns*
QUICKI	150	0.23 [ns]	0.00	149	0.23 [ns]	0.00	146	0.23 [ns]	0.00	*ns*
Insulin/c-peptide ratio	149	3.87 [ns]	0.14	148	3.53 [ns]	0.12	144	3.67 [ns]	0.12	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [ns] (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Table 4.17:Mean(±SEM) of the markers of inflammation, endothelial dysfuction and oxidative stress according to tertiles of SAFA/MUFA ratio

	Tertiles of SFA/MUFA										
	Tertile 1				Tertile 2				Tertile 3		
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
CRP (mg/L)	150	6.17	a	0.38	149	4.81	b	0.33	150	4.47	b	0.30	*p=0.000*
IL-6 (pg/mL)	150	3.98	a	0.25	149	5.59	b	0.38	150	5.51	b	0.35	*p=0.013*
TNFα (pg/mL)	150	4.85	ns	0.42	149	5.12	ns	0.28	150	5.18	ns	0.45	*ns*
sICAM-1 (ng/mL)	150	292.40	ns	6.00	149	279.24	ns	5.87	150	278.85	ns	6.23	*ns*
sVCAM-1 (ng/mL)	150	603.08	ns	13.17	149	581.16	ns	14.44	150	612.73	ns	13.37	*ns*
Resistin (pg/mL)	150	10009.28	ns	393.29	149	9994.83	ns	404.16	150	10247.40	ns	409.54	*ns*
Adiponectin (μg/mL)	150	4.03	a	0.21	149	3.66	ab	0.20	150	3.37	b	0.19	*p=0.029*
PAI-1 (ng/mL)	150	53.13	ns	3.01	149	56.41	ns	3.10	150	53.56	ns	3.13	*ns*
tPA (ng/mL)	150	8.64	ns	0.34	149	8.89	ns	0.38	150	8.47	ns	0.30	*ns*
Fibrinogen (mg/dL)	149	324.88	ns	7.97	149	335.52	ns	6.69	149	326.32	ns	6.25	*ns*
Leptin (ng/mL)	149	29.03	a	2.09	149	17.54	b	1.25	150	18.95	b	1.35	*p=0.000*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	147	0.48	ns	0.01	149	0.48	ns	0.01	149	0.46	ns	0.01	*ns*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	147	0.17	ns	0.01	149	0.17	ns	0.01	149	0.17	ns	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); [ns] (non significant)

[abc...] denotes significance across the tertiles (when two superscripts contain the same letter there is no significant difference between the tertiles)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1);

sVCAM-1 (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_2\alpha$ (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

palmitoleic acid (C16:1) and DHA (C22:6:3) levels were significantly higher in the high SFA/MUFA tertile compared to the low tertile ($p \leq 0.003$). Additionally, AA (C20:4:3) and EPA (C20:5:3) were found to be higher in the medium and high SFA/MUFA tertile compared to the low tertile. Contrary to this, oleic acid (18:1), AA (C20:4:6) and DHA (C22:6:3) were found to be significantly higher in the low SFA/MUFA tertile compared to the medium and high tertiles ($p \leq 0.001$) (*Data not shown*).

When the dietary FA were stratified according to the SFA/MUFA tertiles, numerous interestingly differences were found. Some of these significant differences include levels of energy intake from total fat, total MUFA, and energy intake from total MUFA were found to be significantly higher in the low SFA/MUFA tertile compared to the medium and high tertile ($p < 0.0005$). Contrary to this, total SFA, caprylic acid (C8:0), capric acid (C10:0) and lauric acid (C14:0) was demonstrated to be considerably elevated higher in high SFA/MUFA tertile compared to the medium and low tertiles ($p < 0.0005$). Additionally, EPA (C20:5:3) and DHA (C22:6:3) were found to be significantly higher in the low SFA/MUFA tertile compared to the high tertile ($p \leq 0.01$) (*Data not shown*).

4.4.8 Cross-sectional study cohort stratified according to SFA/PUFA ratio

Metabolic markers of insulin sensitivity, inflammation and plasma and dietary FA were stratified according to SFA/PUFA ratio. This ratio was devised in a similar method to the SFA/MUFA manner and low, medium and high tertiles were created of the SFA/PUFA ratio. When markers of S_I were investigated across the SFA/PUFA tertile, no substantial difference was noted (*Table 4.18*). However, when inflammatory markers were stratified across the SFA/PUFA tertiles, significant difference was noted for CRP and leptin, whereby the medium tertile contained significantly higher concentrations compared to the low tertile ($p \leq 0.05$). A significant divergence was also noted for resistin concentrations, wherein the concentrations for the low and medium tertile were high compared to the high tertile ($p < 0.0005$) (*Data not shown*). Several important differences were noted for the plasma fatty acid data, in particular, palmitoleic acid (C16:1) was higher in the low SFA/PUFA tertile compared to the high tertile, contrary to this, oleic acid (C18:1) and AA (C20:3:6) was found to be significantly higher in the high tertile compared to the low tertile ($p \leq 0.02$). When dietary FAs were stratified according to SFA/PUFA, several significant differences were also noted. Total SFA, total PUFA, caprylic acid (C8:0), capric acid (C10:0) and myristic acid (C14:0) were significantly

Table 4.18:Mean(±SEM) of the markers of Insulin sensitivity as calculated by MINMOD Millenium® according to tertiles of SAFA/PUFA ratio

	Tertiles of SFA/PUFA										
	Tertile 1			Tertile 2			Tertile 3				
	n	Mean		SEM	n	Mean		SEM	n	Mean		SEM	*ANOVA*
C-peptide (ng/ml)	147	2.56	ns	0.07	150	2.75	ns	0.07	143	2.62	ns	0.08	*ns*
S_I (mU/L)-1min-1	135	3.13	ns	0.20	135	2.72	ns	0.13	134	2.87	ns	0.15	*ns*
$S_{G\,(}$min-1)	135	0.02	ns	0.00	135	0.02	ns	0.00	134	0.02	ns	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	135	331.86	ns	25.42	135	381.45	ns	36.38	134	372.91	ns	25.66	*ns*
DI	135	872.72	ns	63.63	135	869.32	ns	61.02	134	915.53	ns	61.06	*ns*
G0 (mg/$dl^{-1)}$	135	300.58	ns	3.87	135	297.24	ns	3.37	134	298.31	ns	3.25	*ns*
P2 ($min^{-1)}$	135	0.04	ns	0.00	135	0.05	ns	0.00	134	0.04	ns	0.00	*ns*
P3 (mU/L) min^{-2}	135	0.00	ns	0.00	135	0.00	ns	0.00	134	0.00	ns	0.00	*ns*
Fasting Insulin (uIU/ml)	149	9.01	ns	0.40	151	10.31	ns	0.51	145	10.18	ns	0.46	*ns*
Fasting Glucose (mmol/l)	149	6.04	ns	0.08	151	6.04	ns	0.08	145	5.92	ns	0.08	*ns*
$HOMA_{IR}$	149	1.68	ns	0.08	151	1.67	ns	0.06	145	1.59	ns	0.04	*ns*
QUICKI	149	0.23	ns	0.00	151	0.23	ns	0.00	145	0.23	ns	0.00	*ns*
Insulin/c-peptide ratio	147	3.47	ns	0.10	151	3.71	ns	0.14	143	3.89	ns	0.13	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ^{ns} (non significant)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

different across all the tertiles, with the high tertile containing the highest levels and the low tertile demonstrating the lowest levels of each fatty acid. Contrary to this, LC n-3 PUFA, LC n-6 PUFA and DHA (C22:6:3) were found to be significantly higher in the low tertile compared to the high SFA/PUFA tertile. In regards to anthropometry assessments, BMI was found to be considerably higher in the low tertile compared to the high SFA/PUFA tertile ($p<0.05$) (*Data not shown*).

4.4.9 Univariate correlations in cross-sectional study cohort

Correlation analysis was carried out to determine the strength and direction of the linear relationship between two variables. Inflammatory mediators were correlated against each other as exhibited in *Table 4.19.* In general, the relationships are relatively weak, however several moderate to strong relationships were noted. When c-peptide was analysed against PAI-1, a strong relationship was noted (r=0.39; P=0.0005), additionally when CRP and fibrinogen were correlated a positively strong relationship was identified (r=0.39; P=0.0005). Markers of endothelial dysfunction sICAM-1 and sVCAM-1 demonstrated a moderate relationship (r=0.26; P=0.0005), whereas, urinary markers of oxidative stress 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ presented a very strong and positive relationship when correlated with each other (r=0.43; P=0.0005).

Correlations were also performed between markers of inflammation and S_I as presented in *Table 4.20.* Once again, relatively weak associations were recorded. However, several strong inverse relationships were noted for c-peptide when correlated with metabolic markers of S_I, namely S_I itself (r= -0.38; p=0.0005), P2 (r= -0.24; p=0.0005), P3 (r= -0.42; p=0.0005) and a very strong positive connection with fasting insulin (r= 0.70; p=0.0005). Additionally, when PAI-1 was correlated fasting insulin, a positive relationship was observed (r= 0.41; p=0.0005) (*Data not shown*).

Plasma FAs were correlated against the inflammatory mediators and results show that once again the relationships are weak throughout the data as presented in *Table 4.21.* Only one plasma fatty acid, namely SFA showed a strong relationship with coagulation marker PAI-1 (r= 0.27; p=0.0005).

Finally, inflammatory biomarkers were connected with dietary FAs and anthropometric measurements as presented in *Table 4.22.* In general, the correlations demonstrated weak associations, with the exception of two, whereby a strong positive relationship was observed

Table 4.19: Correlation matrix displaying the inflamatory markers, coagulation markers and oxidative stress markers correlated with each other in the cross-sectional LIPGENE study

		C-peptide		CRP		IL-6		TNFα		sICAM-1		sVCAM-1		Resistin		Adiponectin		PAI-1		Fibrinogen		tPA		Leptin		8-iso-$PGF_{2\alpha}$		15-keto-dihydro-$PGF_{2\alpha}$	
		r		r		r		r		r		r		r		r		r		r		r		r		r		r	
C-peptide	*r*	na		0.03	ns	0.03	ns	-0.01	ns	0.17	***	0.07	ns	0.13	***	-0.20	***	0.39	***	0.00	ns	0.35	***	0.21	***	0.01	ns	-0.09	*
CRP	*r*			na		-0.11	*	-0.02	ns	0.07	ns	0.02	ns	0.08	ns	0.13	***	0.09	*	0.39	***	0.05	ns	0.33	***	0.02	ns	0.02	ns
IL-6	*r*					na		0.10	*	0.00	ns	0.12	**	0.14	***	-0.08	ns	-0.09	*	-0.02	ns	0.04	ns	-0.13	**	0.00	ns	0.03	ns
TNFα	*r*							na		0.68	ns	0.03	ns	0.09	*	-0.07	ns	-0.06	ns	-0.02	ns	0.02	ns	-0.03	ns	-0.11	**	0.02	ns
sICAM-1	*r*									na		0.26	***	0.03	ns	-0.02	ns	0.08	ns	0.07	ns	0.15	***	0.16	***	0.19	***	0.04	ns
sVCAM-1	*r*											na		0.21	***	0.00	ns	-0.11	*	0.00	ns	0.05	ns	0.05	ns	0.03	ns	0.08	ns
Resistin	*r*													na		0.06	ns	0.12	**	0.02	ns	0.06	ns	0.02	ns	0.05	ns	0.01	ns
Adiponectin	*r*															na		-0.04	ns	0.13	**	-0.11	**	0.08	ns	-0.03	ns	-0.02	ns
PAI-1	*r*																	na		0.01	ns	0.23	***	0.16	***	0.12	**	-0.03	ns
Fibrinogen	*r*																			na		0.06	ns	0.12	**	0.00	ns	0.02	ns
tPA	*r*																					na		0.07	ns	0.00	ns	-0.10	*
Leptin	*r*																							na		0.07	ns	0.01	ns
8-iso-$PGF_{2\alpha}$	*r*																									na		0.43	***
15-keto-dihydro-$PGF_{2\alpha}$	*r*																											na	

* (p<0.05), ** (p<0.01), *** (p<0.0005) ; ns (non significant)

CRP (C-reactive protein); IL-6 (Interleukin-6); TNFα (Tumour Necrosis Factor-α); sICAM-1 (soluble Adhesion Molecule-1); sVCAM (soluble Vascular Adhesion Molecule-1); PAI-1 (Plasminogen Activator Inhibitor-1); tPA (tissue Plasminogen activator)

8-Iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F_2alpha); 15-keto-$PGF_{2\alpha}$ (15-keto-prostaglandin-F_2alpha);

Table 4.20: Correlation matrix displaying the inflammatory markers, coagulation markers and oxidative stress markers correlated with markers of insulin sensitivity in the LIPGENE cross-sectional study

		S_I	S_G	AIRg	DI	G0	P2	P3	Fasting Insulin	Fasting Glucose	$HOMA_{IR}$	QUICKI	Insulin/ c-Peptide
		r	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*
C-peptide	*r*	-0.38 ***	0.00 ns	0.13 **	-0.12 *	0.12 **	-0.24 ***	-0.42 ***	0.70 ***	0.22 ***	0.22 ***	-0.09 ns	0.13 **
CRP	*r*	0.01 ns	-0.02 ns	0.01 ns	0.01 ns	0.00 ns	-0.03 ns	-0.04 ns	0.10 *	0.00 ns	0.00 ns	-0.13 **	0.12 **
IL-6	*r*	0.00 ns	0.05 ns	0.10 *	0.08 ns	0.06 ns	0.06 ns	0.07 ns	0.01 ns	-0.04 ns	-0.04 ns	0.01 ns	-0.03 ns
TNFα	*r*	0.08 ns	0.08 ns	-0.04 ns	0.04 ns	0.10 *	0.10 *	0.11 *	-0.01 ns	0.08 ns	0.08 ns	-0.02 ns	-0.11 *
sICAM-1	*r*	-0.18 ***	0.05 ns	-0.01 ns	-0.12 *	0.11 *	-0.05 ns	-0.15 ***	0.21 ***	0.06 ns	0.06 ns	-0.08 ns	0.17 ***
sVCAM-1	*r*	-0.04 ns	-0.03 ns	-0.06 ns	-0.06 ns	-0.03 ns	-0.01 ns	-0.03 ns	0.06 ns	0.00 ns	0.00 ns	-0.08 ns	0.03 ns
Resistin	*r*	0.05 ns	0.03 ns	-0.02 ns	0.01 ns	0.07 ns	0.02 ns	0.04 ns	0.11 *	0.11 *	0.11 *	-0.08 ns	0.03 ns
Adiponectin	*r*	0.17 ***	0.07 ns	-0.06 ns	0.08 ns	0.13 **	0.10 *	0.18 ***	-0.14 ***	-0.05 ns	-0.05 ns	0.03 ns	0.00 ns
PAI-1	*r*	-0.19 ***	0.01 ns	0.08 ns	-0.07 ns	0.12 **	-0.07 ns	-0.20 ***	0.41 ***	0.19 ***	0.19 ***	0.01 ns	0.24 ***
tPA	*r*	-0.24 ***	0.01 ns	0.08 ns	-0.06 ns	0.15 ***	-0.13 **	-0.25 ***	0.32 ***	0.21 ***	0.20 ***	-0.06 ns	0.10 *
Fibrinogen	*r*	0.07 ns	0.00 ns	0.05 ns	0.09 *	-0.01 ns	-0.01 ns	0.01 ns	0.05 ns	-0.07 ns	-0.07 ns	0.09 *	0.08 ns
Leptin	*r*	-0.04 ns	-0.02 ns	-0.02 ns	-0.07 ns	0.14 ***	0.00 ns	-0.04 ns	0.23 ***	0.08 ns	0.08 ns	-0.19 ***	0.21 ***
8-Iso-$PGF_{2\alpha}$	*r*	-0.01 ns	0.02 ns	-0.04 ns	-0.05 ns	0.01 ns	-0.02 ns	-0.05 ns	0.03 ns	-0.02 ns	-0.01 ns	-0.06 ns	0.05 ns
15-keto-dihydro-$PGF_{2\alpha}$	*r*	0.09 ns	0.01 ns	-0.06 ns	0.02 ns	-0.05 ns	-0.01 ns	0.07 ns	-0.09 *	-0.07 ns	-0.07 ns	0.03 ns	-0.01 ns

* ($p<0.05$), ** ($p<0.01$), *** ($p<0.0005$) ; ns (non significant)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1); **sVCAM** (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator); **8-Iso-$PGF_{2\alpha}$** (8-iso-prostaglandin F_2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F_2alpha); **S_I** (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));G0 (Distribution of Glucose at Time 0); P2 (Removal rate of insulin from the **interstitual space); P3** (Movement of circulating insulin to interstitial space); **$HOMA_{IR}$** (Homeostatis Model Assessment); **QUICKI** (Quantitative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Table 4.21: Correlation matrix displaying the plasma fatty acids with the markers of inflammation, coagulation and oxidative stress in the LIPGENE cross-sectional study

		C14:0		C16:0		C16:1		C18:0		C18:1		C18:2n6		C18:4n3		C20:1		C20:3n6		C20:4n3		C20:4n6		C20:5n3		C22:4n6		C22:5n3		C22:6n3		SFA		MUFA		PUFA		LC n-6 PUFA		LC n-3 PUFA	
		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r	
CRP	r	-0.18	***	-0.13	***	0.05	**	-0.04	ns	0.11	**	-0.02	ns	0.07	ns	0.09	*	0.10	*	0.01	ns	0.14	***	-0.02	ns	0.04	ns	0.07	ns	0.06	ns	-0.14	***	0.13	**	0.04	ns	0.04	ns	0.05	ns
IL-6	r	0.04	ns	0.14	***	-0.01	ns	0.04	ns	-0.08	ns	-0.02	ns	-0.05	ns	0.04	ns	-0.11	*	-0.07	ns	-0.12	**	-0.01	ns	0.03	ns	-0.05	ns	-0.10	*	0.14	***	-0.07	ns	-0.07	ns	-0.07	ns	-0.06	ns
TNFα	r	-0.07	ns	-0.06	ns	0.05	ns	-0.07	ns	0.02	ns	0.03	ns	-0.04	ns	-0.05	ns	-0.02	ns	-0.05	ns	0.04	ns	0.15	***	-0.06	ns	-0.03	ns	0.10	*	-0.07	ns	0.03	ns	0.05	ns	0.03	ns	0.11	*
sICAM-1	r	0.09	*	0.03	ns	0.02	ns	-0.11	**	0.04	ns	0.00	ns	-0.01	ns	-0.09	ns	-0.08	ns	-0.12	*	0.04	ns	-0.17	***	0.00	ns	-0.03	ns	0.01	ns	0.01	ns	0.03	ns	-0.02	ns	0.01	ns	-0.08	ns
sVCAM-1	r	-0.05	ns	-0.02	ns	0.02	ns	-0.27	***	0.03	ns	0.11	**	-0.11	*	0.05	ns	-0.29	***	-0.07	ns	0.11	*	-0.04	ns	0.03	ns	0.06	ns	-0.03	ns	-0.10	*	0.04	ns	0.08	ns	0.11	*	-0.03	ns
Resistin	r	0.02	ns	0.22	***	0.07	ns	-0.03	ns	-0.15	***	-0.01	ns	-0.05	ns	0.07	ns	-0.21	***	-0.05	ns	-0.09	*	-0.03	ns	0.03	ns	-0.01	ns	-0.01	ns	0.17	***	-0.12	**	-0.06	ns	-0.06	ns	-0.01	ns
Adiponectin	r	-0.10	*	0.00	ns	0.02	ns	0.18	***	-0.02	ns	0.01	ns	0.01	ns	-0.07	ns	0.09	ns	-0.09	ns	-0.09	*	-0.09	*	0.03	ns	-0.23	***	-0.09	*	0.02	ns	-0.01	ns	-0.03	ns	-0.01	ns	-0.12	**
PAI1	r	0.20	***	0.22	***	0.01	ns	0.20	***	-0.09	*	-0.19	***	0.10	*	0.00	ns	0.05	ns	0.13	**	-0.11	**	-0.10	*	0.14	***	0.04	ns	-0.02	ns	0.27	***	-0.09	*	-0.17	***	-0.19	***	-0.02	ns
tPA	r	0.11	*	0.12	**	0.06	ns	0.02	ns	0.10	*	-0.17	***	0.02	ns	-0.01	ns	-0.03	ns	-0.03	ns	-0.06	ns	-0.07	ns	0.02	ns	-0.02	ns	-0.02	ns	0.13	**	0.10	*	-0.17	***	-0.18	***	-0.04	ns
Fibrinogen	r	-0.11	*	-0.05	ns	0.06	ns	0.05	ns	0.05	ns	0.01	ns	0.06	ns	0.02	ns	0.07	ns	0.01	ns	-0.02	ns	0.01	ns	0.04	ns	-0.01	ns	-0.05	ns	-0.05	ns	0.05	ns	0.00	ns	0.01	ns	-0.02	ns
Leptin	r	-0.08	ns	-0.14	***	0.07	ns	-0.09	ns	0.11	**	0.02	ns	-0.07	ns	-0.03	ns	0.07	ns	-0.06	ns	0.13	***	-0.07	ns	-0.15	***	-0.04	ns	0.07	ns	-0.13	***	0.12	**	0.04	ns	0.06	ns	0.00	ns
8-iso-PGF$_{2\alpha}$	r	0.13	**	0.05	ns	0.13	***	-0.05	ns	-0.08	ns	0.07	ns	-0.04	ns	0.01	ns	-0.16	***	0.05	ns	-0.02	ns	-0.09	*	0.03	ns	-0.05	ns	-0.08	ns	0.04	ns	-0.05	ns	0.00	ns	0.04	ns	-0.09	*
15-keto-dihydso-PGF$_{2\alpha}$	r	0.06	ns	0.07	ns	0.04	ns	-0.03	ns	-0.14	***	0.10	*	-0.03	ns	0.09	ns	-0.18	***	0.04	ns	-0.02	ns	-0.10	*	0.06	ns	-0.03	ns	-0.13	***	0.05	ns	-0.11	**	0.04	ns	0.07	ns	-0.12	**

* (p<0.05), ** (p<0.01), *** (p<0.0005) ; ns (non significant)

CRP (C-reactive protein); IL-6 (Interleukin-6); TNFα (Tumour Necrosis Factor-α); sICAM-1 (soluble Adhesion Molecule-1); sVCAM-1 (soluble Vascular Adhesion Molecule-1); PAI-1 (Plasminogen Activator Inhibitor-1); tPA (tissue Plasminogen activator)

8-iso-PGF$_{2\alpha}$ (8-iso-prostaglandin F$_{2\alpha}$/abfrac); 15-keto-PGF$_{2\alpha}$ (15-keto-prostaglandin-F$_{2\alpha}$/abfrac)

myristic acid (C14:0); palmitic acid (C16:0); palmitoleic acid (C16:1); Stearic acid (18:0); Oleic acid (18:1); Linoleic acid (18:2:6); Arachidonic acid (20:4:6); EPA (20:5:3); DHA (22:6:3)

Table 4.22: Correlation matrix displaying the dietary fatty acids and anthropometric measurements with the markers of inflammation, coagulation and oxidative stress in the LIPGENE cross-sectional study

		Total Fat	Total Fat %	SAFA	SAFA %	MUFA	MUFA %	PUFA	PUFA %	n-3	n-6	C8:0	C10:0	C12:0	C18:3	C18:1	C18:2	C20:4	C20:5	C22:6	trans Fatty Acids	Body Weight	Height	BMR
		r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r	r
CRP	r	-0.01 ns	0.09 *	-0.11 *	-0.09 ns	0.08 ns	0.17 ***	-0.12 **	-0.10 *	-0.10 ns	-0.11 ns	0.00 ns	-0.10 ns	-0.09 ns	-0.15 ***	0.07 ns	-0.17 ***	-0.15 *	0.02 ns	0.01 ns	0.00 ns	0.07 ns	-0.03 ns	-0.06 ns
IL-6	r	-0.05 ns	-0.06 ns	0.00 ns	0.00 ns	-0.09 ns	-0.11 *	0.01 ns	0.00 ns	0.17 ***	0.10 ns	-0.05 ns	0.08 ns	-0.03 ns	0.10 *	-0.01 ns	0.09 ns	0.22 ***	0.10 ns	0.10 ns	0.03 ns	-0.02 ns	-0.11 *	-0.02 ns
TNFα	r	-0.12 **	-0.09 *	-0.10 *	-0.07 ns	-0.10 *	-0.06 ns	-0.07 ns	-0.03 ns	0.09 ns	-0.02 ns	0.00 ns	0.05 ns	0.11 ns	-0.01 ns	-0.05 ns	-0.08 ns	0.12 ns	0.06 ns	0.15 **	-0.08 ns	0.00 ns	0.04 ns	-0.02 ns
sICAM-1	r	0.00 ns	0.05 ns	-0.04 ns	-0.01 ns	0.04 ns	0.06 ns	-0.06 ns	-0.05 ns	-0.10 ns	-0.06 ns	-0.01 ns	0.01 ns	-0.04 ns	-0.21 ***	0.04 ns	-0.11 *	0.01 ns	-0.05 ns	0.02 ns	-0.20 **	0.06 ns	-0.04 ns	-0.04 ns
sVCAM-1	r	-0.12 **	-0.02 ns	-0.13 ***	-0.08 ns	-0.14 ***	-0.08 ns	-0.08 ns	0.00 ns	-0.10 ns	-0.03 ns	0.02 ns	0.02 ns	-0.12 *	-0.15 ***	-0.10 *	-0.10 *	-0.06 ns	0.04 ns	0.05 ns	-0.05 ns	-0.06 ns	-0.05 ns	-0.06 ns
Resistin	r	-0.09 *	-0.06 ns	-0.10 *	-0.06 ns	-0.09 *	-0.07 ns	0.04 ns	0.08 ns	0.05 ns	0.08 ns	-0.21 ***	-0.06 ns	0.08 ns	0.01 ns	-0.05 ns	0.04 ns	0.06 ns	-0.01 ns	0.02 ns	-0.23 ***	0.01 ns	0.15 ***	-0.03 ns
Adiponectin	r	-0.09 ns	-0.01 ns	-0.13 ***	-0.09 *	-0.04 ns	0.04 ns	-0.09 *	-0.04 ns	-0.23 ***	-0.19 ***	-0.20 **	-0.18 **	-0.06 ns	-0.19 ***	-0.15 **	-0.20 ***	-0.17 *	-0.11 ns	-0.15 ns	-0.30 ***	-0.14 ***	-0.02 ns	-0.12 **
PAI-1	r	0.07 ns	0.02 ns	0.12 *	0.11 *	0.07 ns	0.03 ns	0.12 **	0.10 *	0.08 ns	-0.01 ns	0.02 ns	0.04 ns	0.08 ns	0.10 *	0.07 ns	0.07 ns	-0.01 ns	-0.11 ns	-0.12 *	0.07 ns	0.26 ***	0.07 ns	0.16 ***
tPA	r	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.00 ns	-0.02 ns	-0.02 ns	0.09 ns	0.08 ns	0.00 ns	0.00 ns	-0.04 ns	0.01 ns	0.08 ns	0.01 ns	0.15 *	-0.03 ns	0.03 ns	-0.03 ns	0.20 ***	-0.10 *	0.17 ***
Fibrinogen	r	-0.11 *	-0.02 ns	-0.08 ns	0.01 ns	-0.11 *	-0.04 ns	-0.12 **	-0.06 ns	-0.15 **	-0.10 ns	-0.09 ns	-0.19 ***	-0.13 *	-0.10 *	-0.13 **	-0.15 ***	-0.13 ns	-0.15 **	-0.18 **	-0.13 ns	0.01 ns	-0.22 ***	-0.10 *
Leptin	r	-0.08 ns	0.21 ***	-0.18 ***	0.03 ns	0.04 ns	0.27 ***	-0.20 ***	-0.08 ns	-0.07 ns	-0.07 ns	0.02 ns	0.01 ns	-0.08 ns	-0.19 ***	0.14 **	-0.21 ***	-0.15 *	0.08 ns	0.13 *	0.04 ns	0.09 ns	0.01 ns	-0.25 ***
8-iso $PGF_{2\alpha}$	r	0.00 ns	0.00 ns	0.03 ns	0.03 ns	0.03 ns	0.04 ns	0.02 ns	0.03 ns	-0.07 ns	-0.02 ns	-0.08 ns	-0.10 ns	-0.05 ns	-0.07 ns	-0.01 ns	0.01 ns	0.03 ns	0.00 ns	-0.03 ns	0.04 ns	-0.04 ns	0.06 ns	-0.06 ns
15-keto-dihydro-$PGF_{2\alpha}$	r	-0.04 ns	-0.02 ns	0.00 ns	0.04 ns	-0.05 ns	-0.03 ns	-0.02 ns	0.01 ns	-0.13 *	-0.08 ns	-0.14 ns	-0.10 ns	-0.04 ns	-0.05 ns	-0.09 ns	-0.03 ns	0.02 ns	-0.03 ns	-0.11 *	0.07 ns	-0.12 **	0.11 *	-0.14 ***

* (p<0.05), ** (p<0.01), *** (p<0.0005), ns (non significant)

CRP (C reactive protein), IL-6 (Interleukin-6), TNF-α (Tumour Necrosis Factor-α), sICAM-1 (soluble Adhesion Molecule-1), sVCAM-1 (soluble Vascular Adhesion Molecule-1), PAI-1 (Plasminogen Activator Inhibitor-1), tPA (tissue Plasminogen activator)

8-iso PGF-2α (8-iso-prostaglandin F-2alpha), 15-keto PGF-1,15-keto-prostaglandin F-alpha.

myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), Stearic acid (18:0), Oleic acid (18:1), Linoleic acid (18:2 n-6), Arachidonic acid (20:4 n-6), EPA (20:5 n-3), DHA (22:6 n-3)

for c-peptide and body weight (r= 0.30; p=0.0005) and a moderate relationship was recorded for leptin and total energy intake from MUFA (r= 0.27; p=0.0005).

4.4.10 Linear regression analysis to determine predictors of S_I in the cross-sectional study cohort

Table 4.23 presents the independent relationship of each variable to S_I measured, using univariate and multivariate regression analysis. Although many variables were significant predictors of pre-intervention S_I, some failed to be key in subsequent multiple linear regression analysis, controlling for age, gender and body weight, in particular, insulin/c-peptide ratio, sICAM-1, PAI-1 and dietary FA; LA (C18:2), ALA (C18:3) and AA (C20:4) as well as plasma FA; oleic acid (C18:1). *Table 4.24* presents the multivariate analysis of each of the main variables brought forward from the initial univariate and multivariate analysis. Many variables reached significance with model 2 and these significant variables were inputted into the final model as presented in *Table 4.25*. The overall significance shows that gender, TNFα and adiponectin were all positively associated with predicting S_I, whereas BMI, tPA and alcohol intake were negatively associated with predicting S_I. The r^2 of the model was 0.696 and adjusted r^2 was 0.79, signifying an excellent fit for the population.

4.4.11 Linear regression analysis to determine predictors of $HOMA_{IR}$ in the cross-sectional study cohort

Linear regression analysis was also carried out to determine the predictors of $HOMA_{IR}$ from the inflammatory, S_I, dietary and plasma FA data obtained from the LIPGENE cross-sectional study cohort. Analysis was performed in a similar manner to above, in brief, univariate analysis and multivariate analysis (controlling for age, gender and body weight) was carried out for each variable. Those variables that reached significant ($p \leq 0.1$) in both analyses and/or reached significance in one univariate or multivariate and with evidence in recent literature to support to hypothesis that the variable may influence $HOMA_{IR}$ were carried forward to the next multivariate analyses. Significant variables ($p \leq 0.1$) from the second step were then carried over to the final multivariate analysis (*model 1 and model 2 of the analysis not presented*). Results of the final model show that gender, BMI, fasting glucose and QUICKI were all positively associated with predicting $HOMA_{IR}$, whereas LIPGENE partner HURS-UCO, Córdoba and tPA were negatively associated with predicting $HOMA_{IR}$ (*Table 4.26*).

Table 4.23: Model 1 of the Linear Regresion analysis showing the relationship of each of the individual variables to S_I using univaratiate and multivariate controlling for age, gender and body weight in the LIPGENE cross-sectional data

	Predictors of S_I							
	Univariate				Multivariate			
					controlling for age, gender & body weight			
	β	*sig*	95% CI		β	*sig*	95% CI	
			Lower	Upper			Lower	Upper
LIPGENE Centre †								
TCD v UoR	+	ns	-0.46	0.64	-	0.09	-1.32	0.09
TCD v UO	+	ns	-0.42	0.64	-	0.06	-1.35	0.02
TCD v INSERM	-	ns	-0.72	0.57	-	0.03	-1.64	-0.09
TCD v UM	-	0.04	-1.12	-0.02	-	0.00	-1.75	-0.32
TCD v HURS-UCO	-	ns	-0.87	0.11	-	0.03	-1.40	-0.05
TCD v JUMC	+	ns	-0.47	0.54	-	ns	-1.19	0.14
TCD v UU	+	ns	-0.30	0.87	-	0.03	-1.61	-0.09
Demographics & Lifestyle								
Age	+	ns	-0.01	0.03	-	ns	-0.03	0.02
Gender	+	0.00	0.38	1.10	+	0.00	0.50	1.36
Height	-	ns	0.00	0.00	-	0.00	-0.02	0.00
BMI	-	0.00	-0.14	-0.06	-	0.00	-0.15	-0.04
BMR	-	0.00	-0.57	-0.25	+	ns	0.02	1.55
Playsport (Yes v No)	-	ns	-0.60	0.51	+	ns	-0.47	0.62
Drink Alcohol (Yes v No)	-	0.02	-0.90	-0.09	-	0.04	-1.02	-0.04
Insulin Sensitivity								
S_G	+	ns	-16.10	40.54	-	0.05	-50.50	-0.46
Fasting Insulin	-	0.00	-0.15	-0.09	+	ns	-0.01	0.05
Fasting Glucose	-	ns	-0.26	0.11	+	ns	-0.28	0.49
$HOMA_{IR}$	-	ns	-0.30	0.19	-	ns	-0.51	0.36
QUICKI	+	ns	-4.90	26.11	-	ns	-16.33	6.98
Insulin/c-peptide ratio	-	0.00	-0.45	-0.22	-	ns	-0.12	0.08
C-peptide	-	0.00	-0.79	-0.40	-	0.00	-0.57	-0.11
Inflammatory Markers								
CRP	+	ns	-0.03	0.05	+	ns	-0.03	0.06
IL-6	+	ns	-0.03	0.06	+	ns	-0.05	0.04
TNFα	+	0.02	0.01	0.08	+	0.03	0.00	0.08
sICAM-1	-	0.02	-0.01	0.00	-	ns	0.00	0.00
sVCAM-1	-	ns	0.00	0.00	-	ns	0.00	0.00
Resistin	+	ns	0.00	0.00	+	ns	0.00	0.00
Adiponecin	+	0.00	0.04	0.19	+	0.05	0.00	0.15
Leptin	-	ns	-0.01	0.01	-	ns	-0.01	0.01
Coagulation Markers								
PAI-1	-	0.00	-0.01	0.00	-	ns	-0.01	0.00
tPA	-	0.00	-0.13	-0.05	-	0.03	-0.10	-0.01
Fibrinogen	+	ns	0.00	0.00	+	ns	0.00	0.00
Oxidative Stress								
8-Iso-$PGF_{2\alpha}$	+	ns	-0.99	1.45	-	ns	-1.62	0.93
15-Keto-hydro-$PGF_{2\alpha}$	+	0.08	-0.33	5.26	+	ns	-1.03	4.85
Dietary Fatty Acids								
TotalFat	-	ns	-0.01	0.00	-	ns	-0.14	0.05
TotalFat%	-	ns	-0.03	0.02	-	ns	-0.26	0.25
SAFA	-	ns	-0.02	0.01	+	*	0.03	0.52
SAFA%	+	ns	-0.06	0.06	-	ns	-0.83	0.06
MUFA	-	0.09	-0.03	0.00	+	0.00	0.03	0.14
MUFA%	-	ns	-0.05	0.02	+	ns	-0.15	0.21
PUFA	+	ns	-0.03	0.03	-	ns	-0.32	0.15
PUFA%	+	ns	-0.03	0.14	+	ns	-0.14	0.96
LC n-6 PUFA	+	ns	-0.15	0.23	+	ns	-0.24	0.42
LC n-3 PUFA	-	ns	-0.04	0.03	-	ns	-0.44	-0.05
C8:0	-	0.01	-4.22	-0.62	-	0.00	-12.28	-4.53
C10:0	-	0.02	-1.51	-0.16	-	0.00	-7.17	-2.86
C12:0	-	ns	-0.41	0.11	-	ns	-0.81	0.62
C14:0	-	ns	-0.20	0.06	+	0.00	0.26	1.02
C16:0	-	0.08	-0.06	0.00	+	ns	-0.11	0.27
C16:1	-	ns	-0.45	0.15	+	0.00	1.38	3.88
C18:0	-	ns	-0.10	0.01	+	ns	-0.06	0.27
C18:1	-	0.03	-0.03	0.00	-	0.02	-0.11	-0.01
C18:2	-	ns	-0.04	0.02	-	0.03	-0.16	-0.01
C18:3	+	ns	-0.21	0.27	+	0.00	0.87	2.63
C20:4	-	ns	-2.70	2.10	-	0.00	-24.34	-10.72
C20:5	+	ns	-0.65	1.16	-	ns	-6.42	0.70
C22:6	+	ns	-0.43	0.83	+	ns	0.09	5.00
trans Fatty Acids	-	0.03	-0.28	-0.02	-	0.00	-0.93	-0.33
Plasma Fatty Acids								
C14:0	-	ns	-0.32	0.06	-	0.10	-0.49	0.04
C16:0	-	ns	-0.06	0.02				
C16:1	+	ns	-0.04	0.32	+	0.02	0.05	0.55
C18:0	+	ns	-0.05	0.12	+	0.03	0.03	0.45
C18:1	-	ns	-0.08	0.01	+	ns	-0.04	0.11
C18:2:6	+	0.09	0.00	0.06	+	ns	-0.01	0.11
C18:3:6	+	ns	-2.70	2.93	-	ns	-3.94	2.59
C18:4:6	+	ns	-2.14	5.79	+	ns	-1.23	8.40
C20:1	-	ns	-0.94	0.62	-	ns	-1.30	0.43
C20:3:6	-	ns	-0.52	0.30	-	0.03	-1.35	-0.09
C20:4:6	-	ns	-0.10	0.06	+	ns	-0.10	0.12
C20:4:3	-	ns	-3.07	2.09	-	ns	-3.88	3.07
C20:5:3	+	sig	-0.11	0.30	+	ns	-1.05	1.26
C22:4:6	+	sig	-0.36	1.71	+	ns	-0.36	2.33
C22:5:3	+	sig	-0.29	0.73	+	ns	-1.05	1.47
C22:6:3	+	ns	-0.13	0.18	+	ns	-0.88	1.38
SFA	-	ns	-0.04	0.02		¥		
MUFA	-	ns	-0.06	0.02		¥		
PUFA	+	ns	-0.01	0.05		¥		
LC n-6 PUFA	+	ns	-0.01	0.05		¥		
LC n-3 PUFA	+	ns	-0.05	0.13	-	ns	-1.19	0.97

sIgnificance ($p \leq 0.10$); **ns** (non significance); ¥ (fatty acid result not given); **ns** (non significant)

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Oslo); **INSERM,** Marseille, France; **UM** (University of Maastricht); **HURS-UCO** (Cordoba, Spain); **JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1); **sVCAM-1** (soluble Vascular Adhesion Molecule-1); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F_2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F_2alpha);

S_I (Insulin Sensitivity); S_G (Glucose Effectiveness);HOMAIR (Homeostatis Model Assessment);

QUICKI (Quantitative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids);

caprylic acid **(C8:0)**; capric acid **(C10:0)**, lauric acid **(C12:0)**; mysteric acid **(C14:0)**; palmitic acid **(C16:0)**; palmitoleic acid **(C16:1)**; Stearic Acid **(18:0)**; Oleic Acid **(18:1)**; Linoleic Acid **(18:2:6)**; Arachidonic Acid **(20:4:6)**; EPA **(20:5:3)**; DHA **(22:6:3)**

Table 4.24: Model 2 of the Linear Regresion analysis showing the relationship of each of the individual varaibles of S_I using multivatate analysis controlling for age, gender body weight in the LIPGENE cross-sectional data

	Multivariate			
	Controlling for age, gender, body weight			
	β	*sig*	95% CI	
			Lower	Upper
Model 1: LIPGENE Partners				
r^2 = .339; Adjusted r^2 = .093				
Body weight	-	0.00	-0.16	-0.07
Age	+	ns	-0.02	0.02
Gender	+	0.00	0.43	1.15
TCD v UoR	-	0.09	-1.32	0.09
TCD v UO	-	0.06	-1.35	0.02
TCD v INSERM	-	0.03	-1.64	-0.09
TCD v UM	-	0.00	-1.75	-0.32
TCD v HURS-UCO	-	0.03	-1.40	-0.05
TCD v JUMC	-	ns	-1.19	0.14
TCD v UU	-	0.03	-1.61	-0.09
Model 2 : Inflammatory markers				
r^2 = .350; Adjusted r^2 = .112				
Body weight	-	0.00	-0.15	-0.07
Age	-	ns	-0.03	0.01
Gender	+	0.00	0.38	1.08
TNFα	+	0.02	0.01	0.08
Adiponectin	+	0.00	0.04	0.18
Model 3: Insulin Sensitivity				
r^2 = .132; Adjusted r^2 = .124				
Body weight	-	0.00	-0.12	-0.04
Age	-	ns	-0.02	0.02
Gender	+	0.00	0.38	1.07
C-peptide	-	0.00	-0.65	-0.24
Model 4: Coagulation markers				
r^2 = .328; Adjusted r^2 = .108				
Body weight	-	0.00	-0.14	-0.06
Age	+	ns	-0.02	0.02
Gender	+	0.00	0.34	1.06
tPA	-	0.01	-0.10	-0.01
Model 5: Oxidative stress				
r^2 = .311; Adjusted r^2 = .097				
Body weight	-	0.00	-0.15	-0.07
Age	-	ns	-0.03	0.01
Gender	+	0.00	0.46	1.16
15-keto-dihydro-$PGF_{2\alpha}$	+	ns	-1.10	4.29
Model 6: Dietary Fatty Acids				
r^2 = .139; Adjusted r^2 = .095				
Body weight	-	0.00	-0.19	-0.04
Age	-	ns	-0.04	0.03
Gender	+	0.02	0.14	1.40
C8:0	-	ns	-14.82	9.79
C10:0	-	ns	-1.55	0.47
C16:1	+	0.09	-0.09	1.22
C18:1	-	ns	-0.05	0.02
trans Fatty Acids	-	ns	-0.29	0.04
Model 7: Lifestyle				
r^2 = .107; Adjusted r^2 = .092				
Body weight	-	0.00	-0.15	-0.04
Age	-	ns	-0.03	0.02
Gender	+	0.00	0.50	1.36
Alcohol intake (Yes v No)	-	0.04	-1.02	-0.04

significance ($p \leq 0.1$); **ns** (non signficance)

TCD (Trinity College Dublin); **UoR** (University of Reading); **UO** (University of Oslo); **INSERM (**Marseille, France); **UM** (University of Maastricht)**; HURS-UCO** (Cordóba, Spain)**; JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

TNFα (Tumour Necrosis Factor-α); **tPA** (tissue Plasminogen activator); **15-keto-PGF2a** (15-keto-prostaglandin-F2alpha); caprylic acid **(C8:0)**; capric acid **(C10:0)**, palmitoleic acid **(C16:1)**; Oleic Acid (18:1)

Table 4.25: Model 3 of the Linear Regresion analysis showing the relationship of each of the individual variable of S_I using multivaraite analysis controlling for age gender and body weight in the LIPGENE cross-sectional data

	Predictors of S_I			
	Multivariate *controlling for age, gender, body weight*			
r^2 = .696; Adjusted r^2 = .079	β	***sig***	**95% CI**	
			Lower	Upper
Age	+	ns	-0.02	0.03
Gender	+	0.00	0.34	1.28
Body weight	-	0.00	-0.15	-0.04
TCD v UoR	+	ns	-0.72	0.72
TCD v UO	-	ns	-0.95	0.51
TCD v INSERM	-	ns	-1.04	0.56
TCD v UM	-	ns	-0.96	0.62
TCD v JUMC	+	ns	-0.68	0.77
TCD v UU	-	ns	-1.18	0.40
TNFα	+	0.03	0.00	0.09
Adiponectin	+	0.03	0.01	0.18
tPA	-	0.03	-0.11	-0.01
Dietary Fatty Acid - C16:1	+	ns	-0.18	0.56
Alcohol intake (Yes v No)	-	0.03	-1.05	-0.05

significance ($p \leq 0.05$); **ns** (non signficance)

TCD (Trinity College Dublin); **UoR** (University of Reading); **UO** (University of Oslo); **INSERM** (Marseille, France); **UM** (University of Maastricht); **JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

TNFα (Tumour Necrosis Factor-α); **tPA** (tissue Plasminogen activator); **15-keto-PGF2a** (15-keto-prostaglandin-F2alpha); palmitoleic acid (C16:1)

Table 4.26: Model 3 of the Linear Regresion analysis showing the relationship of each of the individual variable of $HOMA_{IR}$ using multivaraite analysis controlling for age gender and body weight in the LIPGENE cross-sectional data

	Predictors of $HOMA_{IR}$			
	Multivaraite			
	Controlling for age, gender, body weight			
	β	*sig*	95% CI	
r^2 = .885; Adjusted r^2 =.883			Lower	Upper
Age	-	ns	0.00	0.00
Gender	+	0.03	0.01	0.11
Body weight	+	ns	-0.01	0.01
TCD V HURS-UCO	-	0.04	-0.14	0.00
sVCAM-1	-	ns	0.00	0.00
Resistin	+	ns	0.00	0.00
tPA	-	0.04	-0.01	0.00
Fasting Glucose	+	0.00	0.72	0.78
QUICKI	+	0.00	1.23	6.35

TCD (Trinity College Dublin); **HURS-UCO** (Cordóba, Spain);
sVCAM-1 (soluble Vascular Adhesion Molecule-1); **tPA** (tissue Plasminogen activator);
QUICKI (Quantitiative Insulin Sensitive Check Index)

4.4.12 Binary logistic regression analysis to determine predictors of S_I in the cross-sectional study cohort

Binary logistic regression was performed to determine the presence or absence of influencing factors relevant when the dependent variable S_I was split into three categories, low, medium and high S_I as described in *Section 4.4.1.* From these tertiles binary logistic regression was performed in order to determine the predictors of S_I, between the low and medium tertile group, low and high tertile group and medium and high tertile group as presented in *Table 4.27 - Table 4.29.*

Analysis was carried out in a similar manner to the linear regression analysis, in a univariate and subsequent, multivariate manner, controlling for age, gender and body weight. In general, there were very few noteworthy predictors between the low and medium tertiles of S_I, from both univariate and multivariate analyses ($p \leq 0.1$) (*model 1 and model 2 of the analysis not presented*). In the final model, only fasting insulin and dietary fatty acid, palmitoleic acid (C16:1) were found to be significant predictors of low versus medium ranges of S_I. The fasting insulin was found to be positively significant, whereas the palmitoleic acid (C16:1) was found to be inversely significant (*Table 4.27*).

Binary logistic analysis was performed to determine the important predictors of low versus high tertiles of S_I in a similar manner to the above method. Interestingly, this analysis highlighted many more significant variables compared to the low versus medium S_I categories (*model 1 and model 2 of the analysis not presented*). The overall predictors of low versus high S_I categories demonstrated that gender was inversely associated with low versus high, on the other hand, LIPGENE partners, UoR, UO, INSERM, HURS-UCO and UU as well as sICAM-1, alcohol intake and tPA were positively associated with predicting low versus high S_I (*Table 4.28*).

Binary logistic regression analysis was performed to determine the influence of each individual variable within the inflammatory, S_I, plasma and dietary fatty acid data to highlight significant predictors of medium versus high S_I (*model 1 and model 2 of the analysis not presented*). The final model shows that only age and basal metabolic rate (BMR) were positively significant predictors of medium to high S_I as presented in *Table 4.29.*

Table 4.27: Model 3 of the Binary Linear Regresion analysis showing the relationship of each of the individual variable for Low versus High S_I using multivaraite analysis controlling for age gender and body weight in the LIPGENE cross-sectional data

	Low v Medium S_I				
(-2 Log Likelihood = 249.	Multivariate				
Hosmer & Lemeshow (.6	*controlling for age, gender, body weight*				
	β	*sig*	OR	95% C.I	
				Lower	Upper
Age	-	0.73	0.99	0.96	1.03
Gender	-	0.14	0.61	0.32	1.17
Body weight	+	0.14	1.06	0.98	1.16
sICAM-1	+	0.19	1.00	1.00	1.01
Fasting Insulin	+	0.01	1.09	1.02	1.16
Dietary FA - C16:1	-	0.02	0.59	0.37	0.93
Plasma FA - C14:0	+	0.79	1.05	0.74	1.48

significance ($p < 0.05$); **OR** (Odds Rato);

sICAM-1 (soluble Adhesion Molecule-1); mysteric acid (C14:0); palmitoleic acid (C16:1); Oleic Acid (18:1)

Table 4.28: Model 3 of the Binary Linear Regresion analysis showing the relationship of each of the individual variable for Low versus High S_I using multivaraite analysis controlling for age gender and body weight in the LIPGENE cross-sectional data

	Predictors of Low v High S_I				
	Multivariate				
	Controlling for age, gender, body weight				
(-2 Log Likelihood = 230.27); Hosmer & Lemeshow (.300)	β	sig	OR	95.0% C.I	
				Lower	Upper
Age	-	0.06	0.96	0.92	1.00
Gender	-	0.04	0.45	0.21	0.97
Body weight	+	0.02	1.11	1.01	1.22
TCD v UoR	+	0.02	3.59	1.25	10.27
TCD V UO	+	0.02	4.01	1.31	12.33
TCD V INSERM	+	0.00	9.56	2.47	37.04
TCD V HURS-UCO	+	0.07	3.45	0.92	12.93
TCD v UU	+	0.00	9.19	2.41	35.05
Alcohol Intake (Yes v No)	+	0.04	2.53	1.05	6.08
sICAM-1	+	0.02	1.01	1.00	1.01
tPA	+	0.01	1.15	1.03	1.27
Dietary Fatty Acis - C18:1	+	0.86	1.00	0.97	1.04

significance ($p \leq 0.05$); **OR** (Odds Rato);

TCD (Trinity College Dublin); **UoR** (University of Reading); **UO** (University of Oslo); **INSERM** (Marseille, France);

HURS-UCO (Cordoba, Spain); **UU** (Uppsala University, Sweden)

sICAM-1 (soluble Adhesion Molecule-1); tPA (tissue Plasminogen activator);

S_I (Insulin Sensitivity); Oleic Acid **(18:1);**

Table 4.29: Model 3 of the Binary Linear Regresion analysis showing the relationship of each of the individual variable for Medium versus High S_I using multivaraite analysis controlling for age gender and body weight in the LIPGENE cross-sectional data

		Medium v High S_I			
		Multivariate			
		controlling for age, gender, body weight			
(-2 Log Likelihood = 256.09	β	*sig*	OR	95.0% C.I.	
Hosmer & Lemeshow (.251)				Lower	Upper
Age	+	0.02	1.05	1.01	1.09
Gender	+	ns	4.20	0.90	19.56
Body weight	-	ns	0.94	0.84	1.05
Playsport (Yes v No)	+	ns	1.99	0.89	4.48
BMR	+	0.02	2.51	1.16	5.40

significance ($p \leq 0.05$); **ns** (non significant); OD (Odds Ratio);

S_I (Insulin Sensitivity); **BMR** (Basal Metabolic Rate);

4.4.13 Cross-sectional variation in the concentrations of inflammatory biomarkers across the LIPGENE partners

When the inflammatory markers were investigated according to participating centre a significant number of differences were noted as presented in *Table 4.30.* Interestingly plasma CRP was demonstrated to be highest in HURS-UCO and lowest in UO ($p<0.0005$). Concentrations of plasma sICAM-1 were also noted to be highest in HURS-UCO, however lowest in UU and leptin levels were highest in HURS-UCO and lowest in UoR ($p<0.0005$). UoR were noted to have the highest circulating levels of plasma IL-6, sVCAM-1, tPA and fibrinogen concentrations. Whereas UO had the lowest levels of IL-6 and fibrinogen concentrations, UU had the lowest levels of sVCAM-1 and INSERM had the lowest levels of tPA concentrations. Additionally, INSERM had the lowest levels of resistin, PAI-1 and 8-iso-$PGF_{2\alpha}$ concentrations, suggesting that the habitual Mediterranean diet may result in lower inflammatory status, endothelial dysfunction and oxidative stress.

4.4.14 Cross-sectional variation in the concentrations of S_I parameters across the LIPGENE partners

Eight EU centres participated in the LIPGENE cross-sectional study. The MINMOD Millennium® modelling software was used to determine levels of Insulin sensitivity (S_I), after each MetS volunteer underwent a clinical IVGTT investigation. The MINMOD Millennium® (Boston *et al,* 2003) also provided values for the following measurements: glucose effectiveness (S_G), acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (Zero) (G0). Homeostasis model assessment-insulin resistance ($HOMA_{IR}$) and Quantitative insulin sensitivity check index (QUICKI) were also assessed for each MetS volunteer.

Results showed that interestingly, UO had the highest levels of c-peptide, S_G and G0 but had the lowest levels of insulin/c-peptide ratio. TCD had the highest levels of S_I and DI, whereas UM had the lowest levels of S_I and HURS-UCO had the lowest levels of DI. Furthermore, HURS-UCO had the lowest levels of fasting glucose concentrations and therefore had the highest $HOMA_{IR}$ score, which is opposite to INSERM, who had the highest fasting glucose and lower $HOMA_{IR}$ index of S_I ($p<0.0005$) (*Table 4.31*).

Table 4.30: The Mean(±SEM) for the inflammatory markers, endothelial markers and oxidative stress concentrations across the 8 centres involved in the LIPGENE cross-sectional study

	TCD n=59		UoR n=59		UO n-64		INSERM n=41		UM n=59		HURS-UCO n=77		JUMC n=72		UU n=51	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
CRP (mg/L)	6.32 ^{ac}	0.66	4.28 ^{a}	0.45	3.89 ^{bd}	0.45	4.99 ^{acd}	0.53	4.13 ^{ad}	0.48	6.87 ^{c}	0.59	5.25 ^{ac}	0.40	4.41 ^{ab}	0.52
IL-6 (pg/mL)	3.60 ^{a}	0.46	8.50 ^{b}	0.70	7.00 ^{bd}	0.56	4.10 ^{ad}	0.43	4.64 ^{cd}	0.29	3.85 ^{ac}	0.30	3.81 ^{ac}	0.34	4.61 ^{ac}	0.76
TNFα (pg/mL)	4.72 ^{ac}	0.24	4.91 ^{ac}	0.23	5.91 ^{d}	0.28	4.29 ^{e}	1.36	3.38 ^{e}	1.01	4.95 ^{abd}	0.29	4.89 ^{abd}	0.41	7.34 ^{abcd}	0.85
sICAM-1 (ng/mL)	305.72 ^{ad}	10.02	288.96 ^{abd}	7.81	293.91 ^{abd}	11.02	263.10 ^{bc}	12.05	255.79 ^{bc}	7.51	306.69 ^{d}	7.30	294.61 ^{abd}	9.33	233.54 ^{ce}	7.51
sVCAM-1 (ng/mL)	593.10 ^{ns}	21.46	647.15 ^{ns}	24.08	599.73 ^{ns}	21.39	605.24 ^{ns}	25.09	594.75 ^{ns}	17.99	606.57 ^{ns}	17.40	587.27 ^{ns}	23.90	562.42 ^{ns}	19.61
Resistin (pg/mL)	11273.93 ^{ac}	551.20	11511.42 ^{a}	465.79	12136.73 ^{a}	887.21	5298.27 ^{b}	366.68	9816.61 ^{ac}	485.46	10081.82 ^{ac}	460.87	9709.90 ^{ac}	542.58	8994.55 ^{c}	664.92
Adiponectin (µg/mL)	6.22 ^{a}	0.44	3.89 ^{b}	0.18	3.20 ^{c}	0.45	3.34 ^{bc}	0.27	2.16 ^{a}	0.17	3.90 ^{b}	0.21	3.83 ^{b}	0.19	2.84 ^{dc}	0.15
PAI-1 (ng/mL)	60.10 ^{acde}	4.98	52.60 ^{ade}	4.78	52.16 ^{d}	5.37	24.15 ^{b}	2.66	76.25 ^{c}	4.79	47.87 ^{ad}	3.71	68.06 ^{ce}	3.74	43.62 ^{abcd}	4.99
tPA (ng/mL)	7.83 ^{a}	0.75	10.71 ^{c}	0.48	8.78 ^{be}	0.44	7.05 ^{a}	0.48	7.93 ^{ad}	0.46	9.35 ^{def}	0.55	9.15 ^{bf}	0.39	7.71 ^{ade}	0.52
Fibrinogen (mg/dL)	344.41 ^{ac}	13.48	355.41 ^{a}	11.74	288.60 ^{b}	7.20	334.71 ^{abc}	11.10	310.97 ^{abc}	9.94	309.88 ^{bc}	10.79	351.67 ^{a}	9.14	333.64 ^{abc}	10.97
Leptin (ng/mL)	19.63 ^{ac}	2.08	15.75 ^{a}	1.92	15.81 ^{ac}	1.51	16.07 ^{a}	2.33	16.67 ^{a}	2.68	39.10 ^{b}	3.27	22.52 ^{bc}	1.73	19.00 ^{ac}	2.01
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	0.52 ^{ns}	0.02	0.44 ^{ns}	0.02	0.44 ^{ns}	0.02	0.42 ^{ns}	0.02	0.50 ^{ns}	0.02	0.47 ^{ns}	0.02	0.49 ^{ns}	0.02	0.45 ^{ns}	0.02
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	0.18 ^{ns}	0.01	0.16 ^{ns}	0.01	0.16 ^{ns}	0.01	0.16 ^{ns}	0.01	0.19 ^{ns}	0.01	0.16 ^{ns}	0.01	0.16 ^{ns}	0.01	0.18 ^{ns}	0.01

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{a,b,c}... denotes significance across centres (when 2 superscripts contain the same letter there is no significant difference between 2 of the 8 categories); ;

hsCRP (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6), **TNFα** (Tumour Necrosis Factor-α), **sICAM-1** (soluble Intracellular Adhesion Molecule-1); **sVCAM-1** (soluble Vascular Adhesion Molecule); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F_2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Oslo); **INSERM** (Marseille, France); **UM** (University of Maastricht); **HURS-UCO** (Cordóba, Spain); **JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

Table 4.31: *The Mean(±SEM)* for the markers of Insulin Sensitivity across the 8 centres involved in the LIPGENE cross-sectional study

	TCD		UoR		UO		INSERM		UM		HURS-UCO		JUMC		UU	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
C-peptide (ng/ml)	2.40 ^{ace}	0.11	2.79 ^{cd}	0.10	3.22 ^{d}	0.12	2.19 ^{ae}	0.13	2.84 ^{abcdf}	0.11	2.51 ^{abcd}	0.11	2.73 ^{abc}	0.09	2.12 ^{ef}	0.10
S_I (mU/L)-1min-1	3.47 ^{a}	0.35	2.94 ^{abe}	0.20	3.00 ^{abd}	0.32	2.83 ^{abd}	0.31	2.42 ^{d}	0.21	2.58 ^{ed}	0.19	2.93 ^{abe}	0.19	3.15 ^{ab}	0.30
S_G (min-1)	0.015 ^{ace}	0.00	0.016 ^{ce}	0.00	0.020 ^{d}	0.00	0.015 ^{abcd}	0.00	0.014 ^{e}	0.00	0.015 ^{abcd}	0.00	0.015 ^{abcd}	0.00	0.016 ^{abcd}	0.00
AIRg (mUL^{-1} min^{-1})	426.68 ^{abc}	81.10	393.19 ^{abc}	40.64	326.97 ^{a}	37.38	372.86 ^{abc}	51.65	464.20 ^{c}	43.85	297.60 ^{ab}	29.80	311.71 ^{ab}	32.46	282.39 ^{ab}	33.89
DI	1096.06 ^{a}	105.70	989.67 ^{ab}	92.98	864.86 ^{abc}	104.43	913.60 ^{ab}	128.09	974.92 ^{ab}	106.95	645.90 ^{c}	64.60	820.15 ^{bc}	80.87	785.64 ^{bc}	101.43
G0 (mg/dl^{-1})	304.13 ^{ac}	6.41	296.54 ^{a}	4.52	320.28 ^{c}	7.66	273.61 ^{d}	6.07	285.19 ^{e}	3.67	300.97 ^{abc}	4.54	293.95 ^{abe}	3.83	302.58 ^{abce}	4.82
P2 (min^{-1})	0.045 ^{ac}	0.00	0.039 ^{abd}	0.00	0.052 ^{c}	0.00	0.036 ^{bd}	0.00	0.037 ^{d}	0.00	0.048 ^{abcd}	0.01	0.044 ^{abc}	0.00	0.046 ^{abcd}	0.01
P3 (mU/L) min^{-2}	0.000 ^{a}	0.00	0.000 ^{ab}	0.00	0.000 ^{ab}	0.00	0.000 ^{ab}	0.00	0.000 ^{b}	0.00	0.000 ^{ab}	0.00	0.000 ^{ab}	0.00	0.000 ^{ab}	0.00
Fasting Insulin (uIU/ml)	9.28 ^{a}	0.85	11.22 ^{bd}	0.77	10.51 ^{ab}	0.67	8.68 ^{ad}	0.68	10.29 ^{ab}	0.78	9.78 ^{ab}	0.59	10.98 ^{b}	0.65	6.97 ^{c}	0.64
Fasting Glucose (mmol/l)	5.85 ^{acde}	0.12	5.90 ^{ac}	0.16	6.12 ^{de}	0.11	5.67 ^{cd}	0.15	5.87 ^{ae}	0.08	6.37 ^{b}	0.12	5.84 ^{ae}	0.08	6.07 ^{ae}	0.16
$HOMA_{IR}$	1.56 ^{acd}	0.07	1.66 ^{ad}	0.17	1.69 ^{c}	0.06	1.46 ^{d}	0.09	1.54 ^{ac}	0.04	1.86 ^{b}	0.08	1.53 ^{ac}	0.04	1.73 ^{ac}	0.14
QUICKI	0.23 ^{abc}	0.00	0.24 ^{b}	0.00	0.23 ^{abc}	0.00	0.24 ^{abc}	0.00	0.24 ^{ac}	0.00	0.23 ^{a}	0.00	0.24 ^{c}	0.00	0.23 ^{abc}	0.00
Insulin/c-peptide ratio	3.73 ^{ad}	0.26	3.88 ^{ad}	0.18	3.16 ^{bc}	0.11	3.88 ^{ad}	0.20	3.53 ^{ab}	0.16	4.06 ^{ad}	0.22	3.98 ^{d}	0.17	3.25 ^{b}	0.21

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{a,b,c}... denctes significance across centres (when 2 superscripts contain the same letter there is no significant difference between 2 of the 8 categories); ;

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

$HOMA_{IR}$ (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Oslo); **INSERM** (Marseille, France); **UM** (University of Maastricht); **HURS-UCO** (Cordóba, Spain); **JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

4.4.15 Cross-sectional variation in the concentrations of plasma fatty acids across the LIPGENE partners

When plasma FAs were investigated across the LIPGENE participating centres, a number of significant results emerged as presented in *Table 4.32.* UoR were noticed to have the highest circulating levels of plasma myristic acid (C14:0), palmitoleic acid (C16:1), eicosenoic acid (C20:1), EPA (C20:5:3) and DHA (C22:4:3). However, UoR were also noted to have the lowest circulating levels of plasma γ-LA (C18:2:6), AA (C20:4:6), DHA (C22:6:3) and LC n-3 PUFA levels. UO were noted to have the highest levels of plasma γ-LA (C18:2:6), EPA (C20:5:6), DHA (C22:6:3) and LC n-3 PUFA. Whereas UO, had the lowest levels of eicosenoic acid (C20:1) compared to the other centres. Several interesting findings were also noted for UM, whereby they had the highest plasma levels of palmitic acid (16:0) and total SFA and the lowest levels of oleic acid (C18:1), AA (C20:3:6) and total MUFA. In relation to the Mediterranean partners, INSERM had the highest levels of plasma LC n-6 PUFA, however they were noted to have the lowest levels of LC n-3 PUFA. Moreover, HURS-UCO had the lowest levels of myristic acid (C14:0), palmitoleic acid (C16:1), stearic acid (C18:0), AA (C20:4:3), EPA (C20:5:3) and total SFA.

4.4.16 Cross-sectional variation in the concentrations of dietary fatty acids across the LIPGENE partners

When dietary FAs were investigated across the participating LIPGENE centres, a number of significant differences were noted as presented in *Table 4.32*. UoR were found to have lower total fat and MUFA, whereas UO had the highest levels of SFA. HURS-UCO reported the highest levels of total fat and MUFA and the lowest levels of energy intake from both SFA and PUFA.

4.4.17 Gender variation in markers of inflammation, insulin sensitivity, dietary and plasma fatty acids in the cross-sectional cohort

A number of significant gender interactions were noted for inflammatory markers as presented in *Table 4.33.* Females were noted to have higher circulating levels of CRP, adiponectin, fibrinogen, leptin ($p<0.0005$). Markers of oxidative stress 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ were also increased in females ($p<0.05$). Whereas, their male counterparts were noted to have higher plasma tPA concentrations only ($p<0.0005$).

Table 4.32: The Mean(±SEM) for theplasma fatty acid, dietary fatty acids and anthropometic measurements across the 8 centres involved study
LIPGENE cross-sectional study

	TCD		UoR		UO		INSERM		UM		HURS-UCO		JUMC		UU	
%	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Plasma Fatty Acids																
C14:0 (Mysteric acid)	2.21 ^{ac}	0.13	2.70 ^{c}	0.16	1.96 ^{b}	0.09	1.63 ^{b}	0.08	2.11 ^{ab}	0.12	1.19 ^{d}	0.07	1.90 ^{ab}	0.13	1.59 ^{ab}	0.06
C16:0 (Palmitic acid)	29.29 ^{ad}	0.73	28.77 ^{acd}	0.84	26.55 ^{bc}	0.38	24.40 ^{be}	0.37	30.50 ^{d}	0.61	24.46 ^{be}	0.50	23.59 ^{be}	0.23	24.66 ^{be}	0.39
C16:1 (Palmitoleic acid)	1.68 ^{ac}	0.13	2.20 ^{c}	0.21	1.23 ^{ad}	0.11	1.50 ^{afg}	0.14	0.97 ^{bdef}	0.10	0.73 ^{b}	0.05	1.25 ^{af}	0.07	1.88 ^{cg}	0.09
C18:0 (Stearic acid)	4.53 ^{ab}	0.45	4.46 ^{ab}	0.39	3.84 ^{ab}	0.18	4.01 ^{ab}	0.09	4.56 ^{ab}	0.38	3.50 ^{a}	0.15	4.59 ^{b}	0.07	3.80 ^{ab}	0.08
C18:1 (Oleic acid)	24.38 ^{ad}	0.42	26.65 ^{c}	0.48	23.59 ^{d}	0.46	27.48 ^{bc}	0.67	23.26 ^{a}	0.51	29.04 ^{b}	0.49	27.42 ^{bc}	0.44	29.38 ^{b}	0.46
C18:2 (n-6) (Linoleic acid)	26.92 ^{abc}	0.70	25.34 ^{a}	0.63	29.53 ^{c}	0.70	28.55 ^{abc}	0.84	27.75 ^{abc}	0.82	28.63 ^{bc}	0.69	26.32 ^{ab}	0.50	26.64 ^{abc}	0.57
C18:3 (n-6) (γ-Linoleic acid)	0.091 ^{a}	0.01	0.090 ^{ac}	0.01	0.028 ^{b}	0.00	0.083 ^{ac}	0.01	0.040 ^{b}	0.01	0.057 ^{abc}	0.01	0.057 ^{abc}	0.01	0.053 ^{bc}	0.01
C18:4 (n-6)	0.034 ^{abc}	0.00	0.054 ^{c}	0.01	0.038 ^{abc}	0.00	0.031 ^{abc}	0.01	0.042 ^{bc}	0.01	0.020 ^{ab}	0.00	0.053 ^{bc}	0.01	0.015 ^{a}	0.00
C20:1 (Eicosenoic acid)	0.26 ^{ac}	0.02	0.35 ^{c}	0.06	0.11 ^{b}	0.01	0.18 ^{ab}	0.02	0.33 ^{cd}	0.04	0.22 ^{abcd}	0.02	0.12 ^{b}	0.01	0.19 ^{ab}	0.01
C20:3 (n-6) (Arachidonic acid)	0.59 ^{ac}	0.05	0.69 ^{cd}	0.05	0.91 ^{bd}	0.07	1.11 ^{b}	0.05	0.57 ^{c}	0.04	0.86 ^{d}	0.05	1.17 ^{be}	0.03	0.97 ^{bde}	0.05
C20:4 (n-6)	6.80 ^{ad}	0.45	5.40 ^{cdg}	0.29	6.49 ^{ad}	0.22	7.94 ^{af}	0.32	6.33 ^{adf}	0.36	7.66 ^{ag}	0.18	8.31 ^{bfg}	0.23	5.78 ^{cdef}	0.17
C20:4 (n-3)	0.048 ^{bc}	0.01	0.051 ^{b}	0.00	0.050 ^{b}	0.01	0.021 ^{bc}	0.00	0.143 ^{a}	0.02	0.016 ^{c}	0.00	0.054 ^{b}	0.01	0.044 ^{bc}	0.01
C20:5 (n-3) (EPA)	0.64 ^{b}	0.08	0.69 ^{b}	0.08	1.72 ^{ad}	0.15	0.65 ^{b}	0.06	0.67 ^{b}	0.09	0.47 ^{b}	0.06	1.30 ^{c}	0.10	1.59 ^{dc}	0.13
C22:4 (n-6)	0.22 ^{ad}	0.03	0.35 ^{c}	0.04	0.13 ^{b}	0.01	0.18 ^{ab}	0.03	0.28 ^{ac}	0.02	0.10 ^{b}	0.01	0.15 ^{bd}	0.01	0.08 ^{b}	0.00
C22:5 (n-3)	0.33 ^{a}	0.03	0.71 ^{c}	0.10	0.46 ^{ab}	0.03	0.44 ^{ab}	0.02	0.62 ^{bc}	0.04	0.36 ^{a}	0.03	0.46 ^{ab}	0.02	0.35 ^{a}	0.02
C22:6 (n-3) (DHA)	1.70 ^{a}	0.16	1.16 ^{a}	0.08	3.11 ^{c}	0.18	1.80 ^{a}	0.10	1.61 ^{a}	0.12	2.59 ^{bc}	0.09	2.82 ^{b}	0.13	2.51 ^{b}	0.13
SFA	36.03 ^{a}	1.11	35.93 ^{a}	1.14	32.35 ^{bc}	0.40	30.04 ^{b}	0.41	37.17 ^{a}	0.96	29.14 ^{b}	0.45	30.08 ^{bc}	0.31	30.06 ^{bc}	0.39
MUFA	26.31 ^{a}	0.50	29.20 ^{bc}	0.62	24.93 ^{a}	0.52	29.15 ^{c}	0.75	24.57 ^{a}	0.56	29.99 ^{bc}	0.49	28.79 ^{bc}	0.45	31.46 ^{bc}	0.49
PUFA	37.66 ^{ab}	1.10	34.87 ^{a}	0.89	42.72 ^{c}	0.77	40.80 ^{bc}	0.98	38.26 ^{ab}	1.12	40.87 ^{bc}	0.67	41.12 ^{bc}	0.56	38.49 ^{ab}	0.66
LC (n-6) PUFA	34.62 ^{abc}	0.97	31.87 ^{a}	0.79	37.09 ^{bc}	0.73	37.86 ^{b}	0.97	34.97 ^{abc}	0.99	37.31 ^{b}	0.70	36.00 ^{bc}	0.53	33.53 ^{c}	0.60
LC (n-3) PUFA	3.04 ^{a}	0.24	3.01 ^{a}	0.17	5.63 ^{b}	0.33	2.94 ^{a}	0.17	3.29 ^{a}	0.20	3.56 ^{a}	0.14	5.12 ^{b}	0.22	4.96 ^{b}	0.26
Dietary Fatty Acids																
TotalFat	90.73 ^{ab}	4.64	81.52 ^{a}	3.94	87.97 ^{ab}	4.47	77.57 ^{a}	3.60	87.46 ^{ab}	4.45	97.53 ^{b}	2.96	77.74 ^{a}	3.58	71.92 ^{a}	3.50
TotalFat%	35.08 ^{ac}	0.94	31.83 ^{c}	0.90	35.74 ^{a}	0.76	37.45 ^{a}	1.05	34.94 ^{ac}	0.90	42.81 ^{de}	0.70	37.47 ^{ae}	0.81	32.71 ^{bcd}	0.72
SAFA	31.94 ^{ab}	1.96	28.63 ^{ab}	1.75	33.93 ^{a}	1.83	27.58 ^{ab}	1.54	32.03 ^{ab}	1.75	25.29 ^{b}	0.97	27.17 ^{ab}	1.41	28.97 ^{ab}	1.59
SAFA%	12.22 ^{abc}	0.40	11.06 ^{ac}	0.44	13.78 ^{b}	0.40	13.21 ^{b}	0.58	12.75 ^{b}	0.37	10.99 ^{ac}	0.25	13.18 ^{b}	0.47	13.02 ^{b}	0.38
MUFA	32.02 ^{ac}	1.64	24.61 ^{c}	1.22	30.52 ^{ac}	1.64	24.67 ^{ac}	1.39	28.11 ^{ac}	1.65	47.90 ^{b}	1.47	31.19 ^{a}	1.57	26.71 ^{ac}	1.38
MUFA%	12.50 ^{a}	0.47	9.61 ^{c}	0.30	12.44 ^{ab}	0.35	12.02 ^{ab}	0.57	11.20 ^{ab}	0.41	21.14 ^{d}	0.45	14.99 ^{a}	0.42	12.06 ^{ab}	0.34
PUFA	15.45 ^{acd}	0.97	12.05 ^{ab}	0.74	16.55 ^{c}	1.10	10.28 ^{b}	0.90	17.76 ^{d}	1.02	10.76 ^{b}	0.39	13.50 ^{abc}	1.09	11.08 ^{ab}	0.67
PUFA%	6.00 ^{ad}	0.30	4.75 ^{cd}	0.25	6.68 ^{acd}	0.27	4.91 ^{cd}	0.34	7.14 ^{ab}	0.31	4.73 ^{bc}	0.13	6.47 ^{ad}	0.39	5.05 ^{bcd}	0.21
LC (n-3) PUFA	0.71	0.06	1.68	0.15	2.67	0.19	0.98	0.10	†	†	1.20	0.07	1.70	0.22	†	†
LC (n-6) PUFA	5.04	0.42	10.36	0.67	13.42	0.92	7.52	0.91	†	†	8.22	0.36	11.33	1.55	†	†
C8:0 (Caprylic acid)	0.21	0.02	0.21	0.02	†		0.32	0.03	†	†	†	†	0.17	0.01	†	†
C10:0 (Capric acid)	0.38	0.05	0.49	0.05	0.75	0.05	0.55	0.05	†	†	†	†	0.41	0.04	†	†
C12:0 (Lauric acid)	1.06	0.12	0.92	0.09	1.61	0.12	0.68	0.07	†	†	†	†	0.78	0.09	1.81	0.15
C14:0 (Mysteric acid)	1.79	0.19	3.07	0.26	3.31	0.19	2.25	0.21	†	†	1.83	0.19	2.66	0.23	2.87	0.18
C16:0 (Palmitic acid)	10.05	0.75	14.73	0.80	16.57	0.89	9.94	0.91	†	†	14.53	0.56	14.61	1.02	15.18	0.83
C16:1 (Palmitoleic acid)	0.73	0.06	1.22	0.07	1.52	0.08	0.72	0.06	†	†	1.86	0.08	1.62	0.13	1.18	0.07
C18:0 (Stearic acid)	5.01	0.41	6.97	0.43	8.50	0.53	5.07	0.75	†	†	6.34	0.51	6.41	0.56	6.21	0.35
C18:1 (Oleic acid)	16.49	1.11	21.58	1.07	27.21	1.51	13.04	1.24	†	†	43.21	1.46	25.93	1.98	24.35	1.27
C18:2:6 (LA)	6.34	0.43	10.00	0.67	13.28	0.91	6.89	1.16	14.15	0.87	8.22	0.36	11.17	1.54	8.73	0.57
C18:3; (ALA)	0.72	0.05	1.09	0.07	1.95	0.15	0.89	0.09	1.28	0.10	0.81	0.06	1.40	0.14	1.48	0.09
C20:4:6 (AA)	0.06	0.01	0.21	0.02	0.14	0.01	0.01	0.00	†	†	†	†	0.16	0.02	†	†
C20:5 (EPA)	0.04	0.01	0.20	0.05	0.24	0.03	0.10	0.04	0.08	0.03	0.16	0.02	0.10	0.04	†	†
C22:6 (DHA)	0.07	0.02	0.25	0.06	0.41	0.05	0.16	0.06	0.11	0.03	0.27	0.03	0.20	0.07	†	†
trans Fatty Acids	1.35	0.14	1.96	0.18	†	†	3.69	0.57	2.97	0.28	†	†	†	†	†	†
Anthropometry																
Body Weight (Kg)	91.41 ^{ab}	2.57	92.39 ^{a}	1.81	94.66 ^{a}	1.87	87.54 ^{ab}	2.40	94.45 ^{a}	1.75	90.62 ^{ab}	1.40	95.34 ^{a}	1.80	83.31 ^{b}	1.84
Height (m)	167.60 ^{a}	1.20	1.71 ^{b}	0.01	173.63 ^{d}	1.19	1.68 ^{b}	0.01	172.97 ^{cd}	1.03	161.08 ^{a}	1.02	1.67 ^{b}	0.01	166.91 ^{a}	1.15
BMR	7.36 ^{a}	0.17	7.35 ^{a}	0.15	7.37 ^{a}	0.15	7.14 ^{a}	0.18	7.58 ^{a}	0.12	7.01 ^{a}	0.11	7.29 ^{a}	0.15	6.34 ^{b}	0.14

Values present the adjusted Mean ± SEM (Standard Error of the Mean); † (concentrations not given)

^{a,b,c} ... denotes significance across centres (when 2 superscripts contain the same letter there is no significant difference between 2 of the 8 categories); ;

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Oslo); **INSERM** (Marseille, France); **UM** (University of Maastricht); **HURS-UCO** (Cordóba, Spain); **JUMC** (Krakow, Poland); **UU** (Uppsala University, Sweden)

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids);

caprylic acid (C8:0); capric acid (C10:0), lauric acid (C12:0); mysteric acid (C14:0); palmitic acid (C16:0); palmitoleic acid (C16:1); Stearic acid (18:0); Oleic acid (18:1); Linoleic acid (LA, 18:2:6); alpha Linoleic Acid (ALA; 18:3:???)

Arachidonic Acid (AA, 20:4:6); EPA (20:5:3); DHA (22:6:3); BMR (Basal Metabolic Rate)

Table 4.33: The Mean(±SEM) for the inflammatory markers, coagulation markers and oxidative stress markers across gender in the LIPGENE cross-sectional study

	Male			Female			
	n	**Mean**	**SEM**	**n**	**Mean**	**SEM**	***t-test***
CRP (mg/L)	218	4.29 [a]	0.27	264	5.75 [b]	0.26	*p=0.000*
IL-6 (pg/mL)	218	5.02 [ns]	0.27	264	4.98 [ns]	0.26	*ns*
TNFα (pg/mL)	218	4.93 [ns]	0.39	264	5.14 [ns]	0.23	*ns*
sICAM-1 (ng/mL)	218	277.07 [ns]	4.89	264	288.30 [ns]	4.66	*ns*
sVCAM-1 (ng/mL)	218	600.05 [ns]	11.98	264	599.71 [ns]	9.79	*ns*
Resistin (pg/mL)	218	9864.56 [ns]	343.97	264	10231.66 [ns]	287.33	*ns*
Adiponectin (μg/mL)	218	3.28 [a]	0.17	264	4.06 [b]	0.14	*p=0.000*
PAI-1 (ng/mL)	218	55.74 [ns]	2.61	264	53.54 [ns]	2.26	*ns*
tPA (ng/mL)	218	9.70 [a]	0.33	264	7.84 [b]	0.20	*p=0.000*
Fibrinogen (mg/dL)	218	311.70 [a]	5.54	262	341.31 [b]	5.28	*p=0.000*
Leptin (ng/mL)	218	12.10 [a]	0.87	261	29.32 [b]	1.31	*p=0.000*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	213	0.45 [a]	0.01	260	0.48 [b]	0.01	*p=0.031*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	213	0.16 [a]	0.00	260	0.17 [b]	0.00	*p=0.042*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

[a,b,c]... denotes significance across gender (when 2 superscripts contain the same letter there is no significant difference between the 2 genders)

SEM (Standard Error of the Mean); **CRP** (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6),

TNFα (Tumour Necrosis Factor-a), **sICAM-1** (soluble Intracellular Adhesion Molecule-1);

sVCAM-1 (soluble Vascular Adhesion Molecule);

PAI-1 (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-Iso-$PGF_{2}\alpha$ (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha);

mmol/mmol crea (millimole per millimole of creatinine)

In relation to the metabolic markers of S_I, females had higher levels of S_I and P3 compared to males ($p<0.0005$), however males were noted to have higher levels of c-peptide and QUICKI index of S_I ($p<0.0005$) as well as having higher AIRg levels compared to females ($p<0.05$) (*Table 4.34*).

Gender interactions were also observed for plasma FAs, whereby females had high α-LA and AA (C20:3:6). On the other hand, males had higher levels of palmitic acid (C16:1), eicosenoic acid (C20:1), DHA (C22:4:6) and SFA concentrations ($p\leq 0.05$) (*Table 4.35*). Interestingly, when dietary FA composition was established according to gender, several significant interactions were noted, with males having higher levels in all dietary FA compared to females. Levels of total fat, SFA, MUFA, PUFA, LC n-3 PUFA, LC n-6 PUFA, caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), LA (C18:2), ALA (C18:3) and AA (C20:4) were higher in males subjects.

When anthropometric measurements were analysed across the centres, it was no surprise to find that males were significantly higher in body weight and BMR compared to females ($p<0.0005$) (*Table 4.35*).

4.4.18 Age variation in markers of inflammation, insulin sensitivity, dietary and plasma fatty acids in the cross-sectional cohort

As age is a continuous variable, the following age categories were created 35-50 years, 50-65 and >65 years. Markers of inflammation, insulin sensitivity, plasma and dietary FA composition and anthropometric measurements were then analysed according to age categories as presented in *Table 4.36 – Table 4.38*.

Results of the inflammatory biomarkers show that sVCAM-1 were higher in the >65 year category compared to the two younger categories ($p\leq 0.001$). Concentrations of plasma tPA were observed to be higher in the 50-65 years and the >65 years compared to the younger subjects in the 35-50 age category. However, plasma PAI-1 concentrations were noted to be higher in the 35-50 and 50-65 age categories compared to the >65 age categories ($p<0.01$) (*Table 4.36*).

When parameters of S_I were analysed according to age categories, the younger age group (35-50 years) were noted to have higher levels of AIRg, DI, fasting insulin and insulin/c-Peptide ratio levels ($p<0.0005$). Additionally, the 35-50 age group were observed to have lower levels

Table 4.34: The Mean(±SEM) for the markers of insulin sensitivity across gender in the LIPGENE cross-sectional study

	Male			Female			
	n	Mean	SEM	n	Mean	SEM	*t-test*
C-peptide (ng/ml)	216	2.73 [a]	0.06	252	2.56 [b]	0.06	*p=0.040*
S_I (mU/L)-1min-1	203	2.48 [a]	0.11	231	3.27 [b]	0.14	*p=0.000*
$S_{G\,(}$min-1)	203	0.015 [ns]	0.00	231	0.016 [ns]	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	203	400.60 [a]	28.14	231	320.90 [b]	17.34	*p=0.016*
DI	203	875.19 [ns]	52.94	231	883.66 [ns]	44.66	*ns*
G0 (mg/$dl^{-1)}$	203	295.66 [ns]	2.47	231	300.64 [ns]	2.90	*ns*
P2 ($min^{-1)}$	203	0.04 [ns]	0.00	231	0.05 [ns]	0.00	*ns*
P3 (mU/L) min^{-2}	203	0.000 [a]	0.00	231	0.000 [b]	0.00	*p=0.000*
Fasting Insulin (uIU/ml)	203	10.18 [ns]	0.38	232	9.80 [ns]	0.38	*ns*
Fasting Glucose (mmol/l)	203	6.01 [ns]	0.06	232	5.95 [ns]	0.07	*ns*
$HOMA_{IR}$	203	1.63 [ns]	0.03	232	1.64 [ns]	0.06	*ns*
QUICKI	203	0.24 [a]	0.00	232	0.23 [b]	0.00	*p=0.000*
Insulin/c-peptide ratio	201	3.63 [ns]	0.10	227	3.75 [ns]	0.09	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

[a,b, c]... denotes significance across gender (when 2 superscripts contain the same letter there is no significant difference between the 2 genders)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI)); **G0** (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the interstitual space; **P3** (Movement of circulating insulin to interstitial space); **$HOMA_{IR}$** (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); Insulin/c-peptide: ratio of insulin to c-peptide

Table 4.35: The Mean(±SEM) for the plasma fatty acids, dietary fatty acids and anthropometic measurements across gender in the LIPGENE cross-sectional study

	Male			Female			
%	n	Mean	SEM	n	Mean	SEM	*t-test*
Plasma Fatty Acids							
C14:0 (Mysteric acid)	214	2.00 ^{ns}	0.07	261	1.82 ^{ns}	0.06	*ns*
C16:0 (Palmitic acid)	214	27.22 ^{a}	0.35	261	25.87 ^{b}	0.28	*p=0.002*
C16:1 (Palmitoleic acid)	214	1.20 ^{a}	0.06	261	1.55 ^{b}	0.07	*p=0.000*
C18:0 (Stearic acid)	214	4.07 ^{ns}	0.15	261	4.24 ^{ns}	0.12	*ns*
C18:1 (Oleic acid)	214	26.22 ^{ns}	0.31	261	26.56 ^{ns}	0.26	*ns*
C18:2 (n-6) (Linoleic acid)	214	27.15 ^{ns}	0.38	261	27.71 ^{ns}	0.33	*ns*
C18:3 (n-6) (γ-Linoleic acid)	214	0.05 ^{a}	0.00	261	0.07 ^{b}	0.00	*p=0.007*
C18:4 (n-6)	214	0.04 ^{ns}	0.00	261	0.03 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	214	0.25 ^{a}	0.02	261	0.20 ^{b}	0.01	*p=0.028*
C20:3 (n-6) (Arachidonic acid)	214	0.78 ^{a}	0.03	261	0.93 ^{b}	0.03	*p=0.000*
C20:4 (n-6)	214	6.98 ^{ns}	0.18	261	6.79 ^{ns}	0.14	*ns*
C20:4 (n-3)	214	0.06 ^{ns}	0.01	261	0.05 ^{ns}	0.00	*ns*
C20:5 (n-3) (EPA)	214	0.90 ^{ns}	0.06	261	1.03 ^{ns}	0.06	*ns*
C22:4 (n-6)	214	0.21 ^{a}	0.01	261	0.16 ^{b}	0.01	*p=0.001*
C22:5 (n-3)	214	0.50 ^{ns}	0.02	261	0.44 ^{ns}	0.02	*ns*
C22:6 (n-3) (DHA)	214	2.12 ^{ns}	0.09	261	2.25 ^{ns}	0.07	*ns*
SFA	214	33.29 ^{a}	0.45	261	31.93 ^{b}	0.37	*p=0.017*
MUFA	214	27.66 ^{ns}	0.33	261	28.31 ^{ns}	0.29	*ns*
PUFA	214	39.05 ^{ns}	0.49	261	39.77 ^{ns}	0.41	*ns*
LC (n-6) PUFA	214	35.17 ^{ns}	0.44	261	35.66 ^{ns}	0.38	*ns*
LC (n-3) PUFA	214	3.88 ^{ns}	0.14	261	4.11 ^{ns}	0.12	*ns*
Dietary Fatty Acids							
TotalFat	208	96.14 ^{a}	2.33	246	75.99 ^{b}	1.57	*p=0.000*
TotalFat%	208	35.79 ^{ns}	0.51	246	36.70 ^{ns}	0.46	*ns*
SAFA	208	33.64 ^{a}	0.96	246	25.82 ^{b}	0.62	*p=0.000*
SAFA%	208	12.37 ^{ns}	0.21	245	12.46 ^{ns}	0.21	*ns*
MUFA	208	35.11 ^{a}	1.01	246	28.86 ^{b}	0.80	*p=0.000*
MUFA%	208	13.17 ^{ns}	0.32	246	13.98 ^{ns}	0.32	*ns*
PUFA	208	15.70 ^{a}	0.54	246	11.69 ^{b}	0.37	*p=0.000*
PUFA%	208	5.86 ^{ns}	0.16	246	5.61 ^{ns}	0.14	*ns*
LC (n-3) PUFA	138	1.70 ^{a}	0.12	179	1.36 ^{b}	0.07	*p=0.01*
LC (n-6) PUFA	138	10.19 ^{a}	0.54	179	8.50 ^{b}	0.42	*p=0.013*
C8:0 (Caprylic acid)	79	0.25 ^{a}	0.02	98	0.20 ^{b}	0.01	*p=0.048*
C10:0 (Capric acid)	108	0.59 ^{a}	0.04	131	0.47 ^{b}	0.03	*p=0.05*
C12:0 (Lauric acid)	118	1.32 ^{ns}	0.09	167	1.12 ^{ns}	0.07	*ns*
C14:0 (Mysteric acid)	148	2.83 ^{a}	0.14	215	2.27 ^{b}	0.11	*p=0.000*
C16:0 (Palmitic acid)	148	15.92 ^{a}	0.55	215	12.44 ^{b}	0.36	*p=0.000*
C16:1 (Palmitoleic acid)	148	1.48 ^{a}	0.06	215	1.20 ^{b}	0.05	*p=0.000*
C18:0 (Stearic acid)	148	7.52 ^{a}	0.31	215	5.74 ^{b}	0.25	*p=0.000*
C18:1 (Oleic acid)	148	29.43 ^{a}	1.24	215	24.19 ^{b}	0.89	*p=0.000*
C18:2:6 (LA)	190	11.61 ^{a}	0.49	232	8.59 ^{b}	0.34	*p=0.000*
C18:3: (ALA)	190	1.32 ^{a}	0.07	232	1.08 ^{b}	0.04	*p=0.000*
C20:4:6 (AA)	108	0.14 ^{a}	0.01	131	0.10 ^{b}	0.01	*p=0.004*
C20:5 (EPA)	180	0.15 ^{ns}	0.02	195	0.13 ^{ns}	0.01	*ns*
C22:6 (DHA)	180	0.22 ^{ns}	0.03	195	0.21 ^{ns}	0.02	*ns*
trans Fatty Acids	114	2.53 ^{ns}	0.21	92	2.10 ^{ns}	0.19	*ns*
Anthropometry							
Body Weight (Kg)	209	98.17 ^{a}	1.04	246	85.74 ^{b}	0.77	*p=0.000*
Height (m)	209	118.30 ^{ns}	5.69	246	107.63 ^{ns}	4.85	*ns*
BMR	208	8.19 ^{a}	0.06	243	6.34 ^{b}	0.03	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{a,b,c}... denotes significance across gender (when 2 superscripts contain the same letter there is no significant difference between the 2 genders)

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids);

caprylic acid (C8:0); capric acid (C10:0), lauric acid (C12:0); mysteric acid (C14:0); palmitic acid (C16:0); palmitoleic acid (C16:1); Streric acid (18:0); Oleic acid (18:1); Linoleic acid (18:2:6); Arachidonic Acid (20:4:6); EPA (20:5:3); DHA (22:6:3);

BMR (Basal Metabolic Rate)

Table 4.36: The Mean(±SEM) for the inflammatory markers, coagulation markers and oxidative stress markers across age categories in the LIPGENE cross-sectional study

	Age 35-49			Age 50-65			Age >65			
	n	**Mean**	**SEM**	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	***ANOVA***
CRP (mg/L)	128	4.84 [ns]	0.36	287	5.16 [ns]	0.25	65	5.22 [ns]	0.51	*ns*
IL-6 (pg/mL)	128	4.93 [ns]	0.38	287	4.96 [ns]	0.24	65	5.27 [ns]	0.52	*ns*
TNFα (pg/mL)	128	4.64 [ns]	0.46	287	4.95 [ns]	0.21	65	6.26 [ns]	0.97	*ns*
sICAM-1 (ng/mL)	128	286.69 [ns]	7.03	287	280.96 [ns]	4.26	65	284.28 [ns]	8.98	*ns*
sVCAM-1 (ng/mL)	128	573.01 [a]	13.75	287	593.18 [a]	9.45	65	675.71 [b]	24.36	*p=0.001*
Resistin (pg/mL)	128	9847.38 [ns]	418.62	287	10188.84 [ns]	279.67	65	9802.63 [ns]	694.05	*ns*
Adiponectin (μg/mL)	128	3.59 [ns]	0.22	287	3.83 [ns]	0.15	65	3.33 [ns]	0.21	*ns*
PAI-1 (ng/mL)	128	58.93 [a]	3.38	287	54.71 [a]	2.20	65	45.10 [b]	4.48	*p=0.004*
tPA (ng/mL)	128	7.77 [a]	0.31	287	8.83 [b]	0.27	65	9.77 [b]	0.44	*p=0.039*
Fibrinogen (mg/dL)	127	316.50 [ns]	8.57	286	329.90 [ns]	4.76	65	340.70 [ns]	9.90	*ns*
Leptin (ng/mL)	126	18.47 [ns]	1.54	286	23.69 [ns]	1.28	65	17.77 [ns]	1.82	*ns*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	125	0.45 [ns]	0.01	282	0.48 [ns]	0.01	64	0.45 [ns]	0.02	*ns*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	125	0.16 [ns]	0.00	282	0.17 [ns]	0.00	64	0.17 [ns]	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

[ns] (non significant); [a,b,c]... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 3 age categories)

hsCRP (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Intercellular Adhesion Molecule-1)

sVCAM-1 (soluble Vascular Adhesion Molecule); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-iso-PGF_{2a} (8-iso-prostaglandin F2alpha); **15-keto-PGF2a** (15-keto-prostaglandin-F2alpha);

mmol/mmol crea (millimole per millimole of creatinine)

of fasting glucose and $HOMA_{IR}$ concentrations compared to the older age categories ($p \leq$ 0.005) (*Table 4.37).*

According to plasma FAs, those in the 35-50 age category were recognised to have higher levels of myristic acid (C14:0) and γ-LA (C18:3:6) ($p<0.05$). However, the >65 age category demonstrated higher levels in EPA (C20:5:6) and DHA (C22:6:3) concentrations ($p \leq 0.05$) (*Table 4.38*).

4.4.19 BMI variation in markers of inflammation, insulin sensitivity, dietary and plasma fatty acids in the cross-sectional cohort

BMI categories were also created as a method of stratifying the cross-sectional cohort, normal weight (BMI 20-25 kg/m^2), overweight (BMI 25-30 kg/m^2), obese (BMI 30-35 kg/m^2) and morbidly obese (BMI >35 kg/m^2). Inflammation, S_I, plasma and dietary FA composition and anthropometric measurements were examined according to BMI categories as presented in *Table 4.39 - Table 4.41.* Several interactions were noted for BMI categories and inflammation mediators, whereby the morbidly obese category was found to have higher levels of plasma CRP, sICAM-1, PAI-1, fibrinogen and leptin concentrations ($p \leq 0.02$) (*Table 4.39*).

Table 4.40 presents the data in regards to S_I parameters according to BMI categories. Results show that concentrations of c-peptide, AIRg, G0 and insulin/c-peptide ratio were higher in the morbidly obese category ($p \leq 0.02$). Unsurprisingly, S_I and P3 concentrations were higher in the normal weight group (BMI 20-25 kg/m^2) ($p<0.0005$).

No noteworthy interactions were noted for plasma FAs and BMI categories and few interactions were noted for dietary FA, whereby total fat and total MUFA were higher in the morbidly obese category ($p \leq 0.001$) (*Table 4.41).*

Table 4.37: The Mean(±SEM) for the markers of Insulin sensitivity acorss the age categories in the LIPGENE cross-sectional study

	Age 35 - 50			Age 50 - 65			Age >65			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
C-peptide (ng/ml)	125	2.61 ^{ns}	0.07	277	2.66 ^{ns}	0.05	64	2.56 ^{ns}	0.12	*ns*
S_I (mU/L)-1min-1	118	2.59 ^{ns}	0.13	259	3.06 ^{ns}	0.13	57	2.84 ^{ns}	0.21	*ns*
$S_{G\,(}$min-1)	118	0.02 ^{ns}	0.00	259	0.02 ^{ns}	0.00	57	0.02 ^{ns}	0.00	*ns*
AIRg (mUL^{-1} $min^{-1)}$	118	513.26 ^{a}	43.33	259	296.31 ^{b}	15.33	57	318.23 ^{b}	33.57	*p=0.000*
DI	118	1130.42 ^{a}	74.22	259	778.11 ^{b}	41.01	57	822.26 ^{b}	83.41	*p=0.000*
G0 ($mg/dl^{-1)}$	118	295.62 ^{ns}	4.27	259	300.82 ^{ns}	2.36	57	292.48 ^{ns}	4.70	*ns*
P2 ($min^{-1)}$	118	0.05 ^{ns}	0.00	259	0.04 ^{ns}	0.00	57	0.04 ^{ns}	0.00	*ns*
P3 (mU/L) min^{-2}	118	0.00 ^{ns}	0.00	259	0.00 ^{ns}	0.00	57	0.00 ^{ns}	0.00	*ns*
Fasting Insulin (uIU/ml)	118	11.31 ^{a}	0.52	260	9.88 ^{a}	0.36	57	7.64 ^{b}	0.53	*p=0.000*
Fasting Glucose (mmol/l)	118	5.69 ^{a}	0.07	260	6.07 ^{b}	0.05	57	6.16 ^{b}	0.18	*p=0.000*
$HOMA_{IR}$	118	1.46 ^{a}	0.04	260	1.68 ^{b}	0.04	57	1.81 ^{b}	0.18	*p=0.005*
QUICKI	118	0.24 ^{ns}	0.00	260	0.23 ^{ns}	0.00	57	0.23 ^{ns}	0.00	*ns*
Insulin/c-peptide ratio	117	4.18 ^{a}	0.15	254	3.63 ^{b}	0.09	57	2.96 ^{c}	0.12	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{ns} (non significant); ^{a,b,c}... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 3 age categories)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

HOMA-IR (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Table 4.38: The Mean(±SEM) for the plasma fatty acids, dietary fatty acids and anthropometric measurements across age categories in the LIPGENE cross-sectional study

	Age 35 - 50			Age 50 - 65			Age >65			
%	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
Plasma Fatty Acids										
C14:0 (Mysteric acid)	126	2.08 ^{a}	0.09	285	1.83 ^{b}	0.06	64	1.90 ^{ab}	0.12	*p=0.032*
C16:0 (Palmitic acid)	126	27.07 ^{ns}	0.47	285	26.28 ^{ns}	0.28	64	26.21 ^{ns}	0.52	*ns*
C16:1 (Palmitoleic acid)	126	1.39 ^{ns}	0.09	285	1.37 ^{ns}	0.06	64	1.49 ^{ns}	0.14	*ns*
C18:0 (Stearic acid)	126	4.23 ^{ns}	0.21	285	4.23 ^{ns}	0.11	64	3.73 ^{ns}	0.26	*ns*
C18:1 (Oleic acid)	126	26.45 ^{ns}	0.37	285	26.19 ^{ns}	0.25	64	27.27 ^{ns}	0.62	*ns*
C18:2 (n-6) (Linoleic acid)	126	26.69 ^{ns}	0.48	285	27.83 ^{ns}	0.32	64	27.33 ^{ns}	0.62	*ns*
C18:3 (n-6) (γ-Linoleic acid)	126	0.065 ^{ab}	0.01	285	0.062 ^{a}	0.00	64	0.048 ^{b}	0.01	*p=0.040*
C18:4 (n-6)	126	0.038 ^{ns}	0.00	285	0.035 ^{ns}	0.00	64	0.039 ^{ns}	0.01	*ns*
C20:1 (Eicosenoic acid)	126	0.23 ^{ns}	0.02	285	0.20 ^{ns}	0.01	64	0.26 ^{ns}	0.04	*ns*
C20:3 (n-6) (Arachidonic acid)	126	0.83 ^{ns}	0.04	285	0.90 ^{ns}	0.03	64	0.75 ^{ns}	0.05	*ns*
C20:4 (n-6)	126	7.07 ^{ns}	0.25	285	6.92 ^{ns}	0.13	64	6.32 ^{ns}	0.26	*ns*
C20:4 (n-3)	126	0.05 ^{ns}	0.00	285	0.05 ^{ns}	0.00	64	0.06 ^{ns}	0.01	*ns*
C20:5 (n-3) (EPA)	126	0.81 ^{ns}	0.08	285	0.98 ^{ns}	0.05	64	1.22 ^{ns}	0.14	*ns*
C22:4 (n-6)	126	0.21 ^{ns}	0.02	285	0.17 ^{ns}	0.01	64	0.19 ^{ns}	0.03	*ns*
C22:5 (n-3)	126	0.47 ^{ns}	0.04	285	0.44 ^{ns}	0.02	64	0.57 ^{ns}	0.06	*ns*
C22:6 (n-3) (DHA)	126	2.03 ^{ns}	0.11	285	2.24 ^{ns}	0.07	64	2.31 ^{ns}	0.14	*ns*
SFA	126	33.38 ^{ns}	0.64	285	32.33 ^{ns}	0.36	64	31.84 ^{ns}	0.71	*ns*
MUFA	126	28.07 ^{ns}	0.41	285	27.77 ^{ns}	0.27	64	29.02 ^{ns}	0.67	*ns*
PUFA	126	38.55 ^{ns}	0.68	285	39.91 ^{ns}	0.40	64	39.15 ^{ns}	0.72	*ns*
LC (n-6) PUFA	126	34.86 ^{ns}	0.60	285	35.87 ^{ns}	0.37	64	34.64 ^{ns}	0.66	*ns*
LC (n-3) PUFA	126	3.68 ^{ns}	0.19	285	4.03 ^{ns}	0.12	64	4.50 ^{ns}	0.25	*ns*
Dietary Fatty Acids										
TotalFat	118	94.18 ^{a}	3.29	271	82.63 ^{b}	1.75	65	79.79 ^{b}	3.15	*p=0.004*
TotalFat%	118	36.16 ^{ns}	0.68	271	36.58 ^{ns}	0.45	65	35.28 ^{ns}	0.79	*ns*
SAFA	118	32.52 ^{a}	1.38	271	28.54 ^{a}	0.71	65	27.31 ^{ab}	1.13	*p=0.024*
SAFA%	118	12.30 ^{ns}	0.30	270	12.54 ^{ns}	0.20	65	12.14 ^{ns}	0.36	*ns*
MUFA	118	34.19 ^{ns}	1.37	271	31.22 ^{ns}	0.84	65	29.37 ^{ns}	1.40	*ns*
MUFA%	118	13.19 ^{ns}	0.41	271	13.92 ^{ns}	0.31	65	13.09 ^{ns}	0.50	*ns*
PUFA	118	15.12 ^{a}	0.76	271	12.83 ^{b}	0.39	65	13.55 ^{ab}	0.86	*p=0.018*
PUFA%	118	5.79 ^{ns}	0.21	271	5.63 ^{ns}	0.13	65	6.00 ^{ns}	0.31	*ns*
LC (n-3) PUFA	95	1.42 ^{ns}	0.13	189	1.54 ^{ns}	0.08	33	1.56 ^{ns}	0.19	*ns*
LC (n-6) PUFA	95	9.40 ^{ns}	0.71	189	9.14 ^{ns}	0.40	33	9.30 ^{ns}	0.96	*ns*
C8:0 (Caprylic acid)	66	0.23 ^{ns}	0.02	94	0.23 ^{ns}	0.02	17	0.15 ^{ns}	0.02	*ns*
C10:0 (Capric acid)	81	0.53 ^{ns}	0.05	135	0.53 ^{ns}	0.03	23	0.46 ^{ns}	0.07	*ns*
C12:0 (Lauric acid)	86	1.20 ^{ns}	0.11	159	1.19 ^{ns}	0.07	40	1.24 ^{ns}	0.12	*ns*
C14:0 (Mysteric acid)	100	2.71 ^{ns}	0.21	213	2.43 ^{ns}	0.10	50	2.39 ^{ns}	0.16	*ns*
C16:0 (Palmitic acid)	100	14.49 ^{ns}	0.76	213	13.65 ^{ns}	0.39	50	13.50 ^{ns}	0.68	*ns*
C16:1 (Palmitoleic acid)	100	1.37 ^{ns}	0.09	213	1.28 ^{ns}	0.04	50	1.34 ^{ns}	0.09	*ns*
C18:0 (Stearic acid)	100	7.46 ^{ns}	0.55	213	6.13 ^{ns}	0.20	50	5.89 ^{ns}	0.33	*ns*
C18:1 (Oleic acid)	100	25.49 ^{ns}	1.49	213	26.84 ^{ns}	0.98	50	25.79 ^{ns}	1.70	*ns*
C18:2:6 (LA)	109	10.16 ^{ns}	0.65	251	9.74 ^{ns}	0.37	62	10.39 ^{ns}	0.79	*ns*
C18:3: (ALA)	109	1.21 ^{ns}	0.09	251	1.17 ^{ns}	0.05	62	1.23 ^{ns}	0.09	*ns*
C20:4:6 (AA)	81	0.115 ^{ns}	0.01	135	0.118 ^{ns}	0.01	23	0.146 ^{ns}	0.02	*ns*
C20:5 (EPA)	104	0.107 ^{a}	0.02	226	0.146 ^{b}	0.02	45	0.171 ^{ab}	0.04	*p=0.038*
C22:6 (DHA)	104	0.166 ^{a}	0.03	226	0.238 ^{b}	0.02	45	0.237 ^{b}	0.04	*p=0.008*
trans Fatty Acids	66	2.20 ^{ns}	0.24	111	2.56 ^{ns}	0.22	29	1.83 ^{ns}	0.25	*ns*
Anthropometry										
Body Weight (Kg)	119	98.24 ^{a}	1.54	271	89.93 ^{b}	0.81	65	85.40 ^{b}	1.59	*p=0.000*
Height (m)	119	104.55 ^{ns}	7.68	271	112.61 ^{ns}	4.75	65	126.81 ^{ns}	9.00	*ns*
BMR	117	7.93 ^{a}	0.11	269	7.00 ^{b}	0.06	65	6.67 ^{b}	0.12	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{ns} (non significant); ^{a,b,c}... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 3 age categories)

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids);

caprylic acid (C8:0); capric acid (C10:0), lauric acid (C12:0); mysteric acid (C14:0); palmitic acid (C16:0); palmitoleic acid (C16:1); Streric acid (18:0); Oleic acid (18:1); Linoleic acid (18:2:6); Arachidonic Acid (20:4:6); EPA (20:5:3); DHA (22:6:3); BMR (Basal Metabolic Rate)

Table 4.39: The Mean(±SEM) for the inflammatory markers, coagulation markers and oxidative stress markers across BMI categories in the LIPGENE cross-sectional study

	BMI 20-25			BMI 25-30			BMI 30-35			BMI >35			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
CRP (mg/L)	13	3.18 [a]	0.73	136	3.80 [a]	0.28	200	5.08 [b]	0.27	131	6.67 [c]	0.44	*p=0.000*
IL-6 (pg/mL)	13	5.77 [ab]	1.82	136	5.96 [a]	0.39	200	4.69 [b]	0.27	131	4.44 [b]	0.31	*p=0.027*
TNFα (pg/mL)	13	5.42 [ns]	2.00	136	5.08 [ns]	0.46	200	5.40 [ns]	0.37	131	4.44 [ns]	0.25	*ns*
sICAM-1 (ng/mL)	13	275.21 [ab]	21.71	136	272.87 [a]	5.52	200	279.94 [a]	5.50	131	300.81 [b]	6.61	*p=0.015*
sVCAM-1 (ng/mL)	13	622.42 [ns]	37.70	136	615.84 [ns]	13.97	200	583.50 [ns]	12.37	131	607.08 [ns]	13.97	*ns*
Resistin (pg/mL)	13	7552.31 [ns]	1031.89	136	9782.94 [ns]	406.99	200	10223.32 [ns]	368.20	131	10347.18 [ns]	391.67	*ns*
Adiponectin (µg/mL)	13	3.57 [ns]	0.41	136	3.48 [ns]	0.19	200	3.73 [ns]	0.18	131	3.88 [ns]	0.23	*ns*
PAI-1 (ng/mL)	13	38.99 [ac]	14.49	136	42.06 [a]	2.92	200	56.40 [c]	2.35	131	66.04 [b]	3.62	*p=0.000*
tPA (ng/mL)	13	5.95 [ns]	0.94	136	8.14 [ns]	0.36	200	9.00 [ns]	0.32	131	9.07 [ns]	0.30	*ns*
Fibrinogen (mg/dL)	13	315.13 [ab]	21.37	136	320.30 [a]	6.48	199	317.93 [a]	6.00	130	353.23 [b]	8.01	*p=0.002*
Leptin (ng/mL)	13	6.56 [a]	0.97	136	13.37 [a]	1.13	198	20.13 [b]	1.21	130	33.75 [c]	2.12	*p=0.000*
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	13	0.438 [ns]	0.03	134	0.469 [ns]	0.01	197	0.468 [ns]	0.01	129	0.475 [ns]	0.01	*ns*
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	13	0.184 [ns]	0.02	134	0.178 [ns]	0.01	197	0.158 [ns]	0.00	129	0.171 [ns]	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

[ns] (non significant); [a,b, c]... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 4 BMI categories)

hsCRP (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α); **sICAM-1** (soluble Intercellular Adhesion Molecule-1)

sVCAM-1 (soluble Vascular Adhesion Molecule); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen activator)

8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F2alpha);

mmol/mmol crea (millimole per millimole of creatinine)

Table 4.40: The Mean(±SEM) for the markers of Insulin sensitivity acorss the BMI categories in the LIPGENE cross-sectional study

	BMI 20 - 25			BMI 25 - 30			BMI 30 - 35			BMI >35			
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	*ANOVA*
C-peptide (ng/ml)	12	1.82 ^{a}	0.22	133	2.34 ^{a}	0.06	196	2.70 ^{b}	0.06	126	2.94 ^{b}	0.08	*p=0.000*
S_I (mU/L)-1min-1	10	4.94 ^{a}	1.18	121	3.18 ^{b}	0.18	186	2.88 ^{bc}	0.13	113	2.47 ^{c}	0.16	*p=0.000*
$S_{G\,(}$min-1)	10	0.02 ^{ns}	0.00	121	0.02 ^{ns}	0.00	186	0.02 ^{ns}	0.00	113	0.02 ^{ns}	0.00	*ns*
AIRg (mUL^{-1} min$^{-1)}$	10	148.21 ^{a}	28.61	121	301.67 ^{c}	22.67	186	384.33 ^{bc}	29.39	113	394.54 ^{b}	28.93	*p=0.021*
DI	10	818.64 ^{ns}	287.63	121	874.62 ^{ns}	66.19	186	917.57 ^{ns}	53.70	113	826.89 ^{ns}	62.38	*ns*
G0 (mg/dl $^{-1)}$	10	281.56 ^{ab}	9.71	121	288.16 ^{a}	3.03	186	301.25 ^{b}	3.36	113	306.01 ^{b}	3.36	*p=0.002*
P2 (min$^{-1)}$	10	0.042 ^{ns}	0.00	121	0.048 ^{ns}	0.00	186	0.043 ^{ns}	0.00	113	0.040 ^{ns}	0.00	*ns*
P3 (mU/L) min^{-2}	10	0.000 ^{a}	0.00	121	0.000 ^{a}	0.00	186	0.000 ^{ab}	0.00	113	0.000 ^{b}	0.00	*p=0.000*
Fasting Insulin (uIU/ml)	12	5.05 ^{a}	0.91	134	7.42 ^{a}	0.27	199	10.28 ^{b}	0.39	126	12.24 ^{c}	0.60	*p=0.000*
Fasting Glucose (mmol/l)	12	5.82 ^{ns}	0.24	134	5.93 ^{ns}	0.08	199	6.04 ^{ns}	0.08	126	5.98 ^{ns}	0.08	*ns*
$HOMA_{IR}$	12	1.54 ^{ns}	0.13	134	1.59 ^{ns}	0.04	199	1.69 ^{ns}	0.07	126	1.62 ^{ns}	0.04	*ns*
QUICKI	12	0.24 ^{ns}	0.00	134	0.23 ^{ns}	0.00	199	0.23 ^{ns}	0.00	126	0.23 ^{ns}	0.00	*ns*
Insulin/c-peptide ratio	12	2.65 ^{ab}	0.19	133	3.21 ^{a}	0.09	195	3.80 ^{bc}	0.12	126	4.14 ^{c}	0.15	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{ns} (non significant); ^{a,b,c}... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 4 BMI categories)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

HOMA-IR (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **Insulin/c-peptide**: ratio of insulin to c-peptide

Table 4.41: The Mean(±SEM) for the plasma fatty acids, dietary fatty acids and anthropometric measurements across BMI categories in the LIPGENE cross-sectional study

	BMI 20 - 25			BMI 25 - 30			BMI 30 - 35			BMI>35			
%	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	**n**	**Mean**	**SEM**	***ANOVA***
Plasma Fatty Acids													
C14:0 (Mysteric acid)	13	1.84 ^{ns}	0.28	132	1.99 ^{ns}	0.08	199	1.90 ^{ns}	0.07	129	1.83 ^{ns}	0.09	*ns*
C16:0 (Palmitic acid)	13	24.99 ^{ns}	0.90	132	26.87 ^{ns}	0.42	199	26.19 ^{ns}	0.33	129	26.59 ^{ns}	0.45	*ns*
C16:1 (Palmitoleic acid)	13	1.88 ^{ns}	0.29	132	1.40 ^{ns}	0.09	199	1.39 ^{ns}	0.07	129	1.33 ^{ns}	0.08	*ns*
C18:0 (Stearic acid)	13	4.00 ^{ns}	0.42	132	4.02 ^{ns}	0.18	199	4.12 ^{ns}	0.15	129	4.32 ^{ns}	0.18	*ns*
C18:1 (Oleic acid)	13	27.14 ^{ns}	0.64	132	25.78 ^{ns}	0.37	199	26.30 ^{ns}	0.33	129	27.18 ^{ns}	0.35	*ns*
C18:2 (n-6) (Linoleic acid)	13	28.29 ^{ns}	1.37	132	27.95 ^{ns}	0.43	199	27.52 ^{ns}	0.41	129	26.83 ^{ns}	0.47	*ns*
C18:3 (n-6) (γ-Linoleic acid)	13	0.08 ^{ns}	0.02	132	0.05 ^{ns}	0.01	199	0.06 ^{ns}	0.00	129	0.07 ^{ns}	0.01	*ns*
C18:4 (n-6)	13	0.03 ^{ns}	0.01	132	0.03 ^{ns}	0.00	199	0.04 ^{ns}	0.00	129	0.03 ^{ns}	0.00	*ns*
C20:1 (Eicosenoic acid)	13	0.14 ^{ns}	0.02	132	0.22 ^{ns}	0.02	199	0.22 ^{ns}	0.02	129	0.22 ^{ns}	0.02	*ns*
C20:3 (n-6) (Arachidonic acid)	13	0.96 ^{ns}	0.12	132	0.80 ^{ns}	0.04	199	0.85 ^{ns}	0.03	129	0.91 ^{ns}	0.04	*ns*
C20:4 (n-6)	13	6.51 ^{ns}	0.43	132	6.55 ^{ns}	0.22	199	7.12 ^{ns}	0.17	129	6.89 ^{ns}	0.21	*ns*
C20:4 (n-3)	13	0.03 ^{ns}	0.01	132	0.06 ^{ns}	0.01	199	0.05 ^{ns}	0.00	129	0.05 ^{ns}	0.01	*ns*
C20:5 (n-3) (EPA)	13	1.16 ^{ns}	0.29	132	1.06 ^{ns}	0.09	199	1.00 ^{ns}	0.06	129	0.81 ^{ns}	0.07	*ns*
C22:4 (n-6)	13	0.15 ^{ns}	0.03	132	0.19 ^{ns}	0.01	199	0.20 ^{ns}	0.01	129	0.16 ^{ns}	0.01	*ns*
C22:5 (n-3)	13	0.40 ^{ns}	0.03	132	0.50 ^{ns}	0.04	199	0.48 ^{ns}	0.02	129	0.43 ^{ns}	0.03	*ns*
C22:6 (n-3) (DHA)	13	2.07 ^{ns}	0.41	132	2.22 ^{ns}	0.11	199	2.26 ^{ns}	0.09	129	2.08 ^{ns}	0.09	*ns*
SFA	13	30.84 ^{ns}	1.23	132	32.88 ^{ns}	0.55	199	32.21 ^{ns}	0.42	129	32.75 ^{ns}	0.59	*ns*
MUFA	13	29.16 ^{ns}	0.85	132	27.41 ^{ns}	0.41	199	27.92 ^{ns}	0.36	129	28.73 ^{ns}	0.38	*ns*
PUFA	13	40.01 ^{ns}	1.24	132	39.71 ^{ns}	0.60	199	39.87 ^{ns}	0.49	129	38.52 ^{ns}	0.60	*ns*
LC (n-6) PUFA	13	36.00 ^{ns}	1.28	132	35.54 ^{ns}	0.54	199	35.76 ^{ns}	0.45	129	34.86 ^{ns}	0.56	*ns*
LC (n-3) PUFA	13	4.01 ^{ns}	0.75	132	4.17 ^{ns}	0.20	199	4.12 ^{ns}	0.14	129	3.66 ^{ns}	0.15	*ns*
Dietary Fatty Acids													
TotalFat	13	69.89 ^{ns}	6.15	130	83.11 ^{ns}	2.55	182	84.16 ^{ns}	2.34	124	90.17 ^{ns}	2.86	*ns*
TotalFat%	13	33.60 ^{ab}	1.93	130	34.95 ^{a}	0.65	182	36.37 ^{ab}	0.51	124	37.68 ^{b}	0.65	*p=0.001*
SAFA	13	24.93 ^{ns}	2.22	130	30.65 ^{ns}	1.09	182	28.61 ^{ns}	0.86	124	29.69 ^{ns}	1.24	*ns*
SAFA%	13	12.19 ^{ns}	1.00	129	12.81 ^{ns}	0.31	182	12.33 ^{ns}	0.22	124	12.17 ^{ns}	0.27	*ns*
MUFA	13	24.38 ^{a}	2.95	130	28.49 ^{a}	1.03	182	31.74 ^{ab}	1.04	124	35.60 ^{b}	1.35	*p=0.000*
MUFA%	13	11.72 ^{ab}	1.18	130	12.07 ^{a}	0.36	182	13.81 ^{b}	0.35	124	15.03 ^{b}	0.48	*p=0.000*
PUFA	13	11.72 ^{ns}	1.84	130	13.58 ^{ns}	0.65	182	13.74 ^{ns}	0.55	124	13.29 ^{ns}	0.58	*ns*
PUFA%	13	5.52 ^{ns}	0.60	130	5.67 ^{ns}	0.22	182	5.87 ^{ns}	0.16	124	5.56 ^{ns}	0.19	*ns*
LC (n-3) PUFA	6	1.44 ^{ns}	0.44	81	1.52 ^{ns}	0.13	125	1.66 ^{ns}	0.13	100	1.31 ^{ns}	0.09	*ns*
LC (n-6) PUFA	6	6.89 ^{ns}	1.63	81	9.49 ^{ns}	0.72	125	9.48 ^{ns}	0.57	100	8.81 ^{ns}	0.53	*ns*
C8:0 (Caprylic acid)	5	0.19 ^{ns}	0.05	48	0.23 ^{ns}	0.02	67	0.20 ^{ns}	0.01	55	0.24 ^{ns}	0.03	*ns*
C10:0 (Capric acid)	6	0.38 ^{ns}	0.09	72	0.56 ^{ns}	0.04	95	0.51 ^{ns}	0.03	63	0.52 ^{ns}	0.06	*ns*
C12:0 (Lauric acid)	10	1.20 ^{ns}	0.39	92	1.21 ^{ns}	0.09	113	1.25 ^{ns}	0.08	67	1.07 ^{ns}	0.12	*ns*
C14:0 (Mysteric acid)	10	2.19 ^{ns}	0.34	101	2.69 ^{ns}	0.15	143	2.40 ^{ns}	0.11	104	2.49 ^{ns}	0.21	*ns*
C16:0 (Palmitic acid)	10	10.80 ^{ns}	1.40	101	13.92 ^{ns}	0.59	143	13.55 ^{ns}	0.48	104	14.48 ^{ns}	0.69	*ns*
C16:1 (Palmitoleic acid)	10	0.93 ^{ns}	0.17	101	1.18 ^{ns}	0.06	143	1.29 ^{ns}	0.06	104	1.52 ^{ns}	0.08	*ns*
C18:0 (Stearic acid)	10	4.77 ^{ns}	0.53	101	6.56 ^{ns}	0.33	143	6.28 ^{ns}	0.28	104	6.76 ^{ns}	0.48	*ns*
C18:1 (Oleic acid)	10	19.02 ^{ns}	3.30	101	23.05 ^{ns}	1.13	143	26.02 ^{ns}	1.21	104	30.30 ^{ns}	1.51	*ns*
C18:2:6 (LA)	12	8.60 ^{ns}	1.86	125	10.50 ^{ns}	0.60	164	10.08 ^{ns}	0.49	116	9.26 ^{ns}	0.49	*ns*
C18:3: (ALA)	12	1.13 ^{ns}	0.21	125	1.28 ^{ns}	0.07	164	1.20 ^{ns}	0.07	116	1.08 ^{ns}	0.07	*ns*
C20:4:6 (AA)	6	0.08 ^{ns}	0.03	72	0.12 ^{ns}	0.01	95	0.11 ^{ns}	0.01	63	0.13 ^{ns}	0.01	*ns*
C20:5 (EPA)	8	0.18 ^{ns}	0.09	105	0.12 ^{ns}	0.02	146	0.16 ^{ns}	0.02	112	0.12 ^{ns}	0.02	*ns*
C22:6 (DHA)	8	0.31 ^{ns}	0.15	105	0.18 ^{ns}	0.03	146	0.26 ^{ns}	0.03	112	0.19 ^{ns}	0.03	*ns*
trans Fatty Acids	7	1.66 ^{ns}	0.58	68	2.35 ^{ns}	0.25	75	2.36 ^{ns}	0.25	54	2.35 ^{ns}	0.29	*ns*
Anthropometry													
Body Weight (Kg)	13	68.96 ^{a}	3.86	130	81.27 ^{b}	0.82	183	93.22 ^{c}	0.82	124	101.60 ^{d}	1.37	*p=0.000*
Height (m)	13	104.50 ^{ns}	23.51	130	117.10 ^{ns}	6.93	183	113.20 ^{ns}	5.88	124	106.72 ^{ns}	7.06	*ns*
BMR	13	5.90 ^{a}	0.30	130	6.77 ^{b}	0.08	183	7.35 ^{c}	0.08	121	7.53 ^{d}	0.11	*p=0.000*

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{ns} (non significant); ^{a,b,c}... denotes significance across age categories (when 2 superscripts contain the same letter there is no signifcant difference between 2 of the 4 BMI categories)

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids);

caprylic acid (C8:0); capric acid (C10:0), lauric acid (C12:0); mysteric acid (C14:0); palmitic acid (C16:0); palmitoleic acid (C16:1); Stearic acid (18:0); Oleic acid (18:1); Linoleic acid (18:2:6); Arachidonic Acid (20:4:6); EPA (20:5:3); DHA (22:6:3); BMR (Basal Metabolic Rate)

4.5 DISCUSSION

In 1988 Gerald M Reaven highlighted the existence of a syndrome due to the escalating incidence of the several metabolic disorders in unison, including glucose intolerance, hyperinsulinemia, dyslipidemia and hypertension. This condition was named *'Syndrome X'* and was associated with heart threatening deformities generally found in IR individuals. Today, almost 20 years later from first documented, the condition is more commonly referred to as the MetS and this is also associated with the presence with a pro-inflammatory state, hypercoagulability and microalbuminuria (Anzai & Hayashida, 2007). It is well recognised that the MetS is strongly associated with T2DM and is positively correlated with CVD. Epidemiological studies have shown that individuals with the MetS have a 3-5 fold increase in cardiovascular (CV) mortality (Isomaa *et al*, 2001; Ford *et al*, 2005). The MetS is thought to have a genetic basis, but environmental factors, particularly obesity, sedentary living and a diet of energy-dense, SFA-rich foods are largely implicated in the cause of the MetS (Stone & Schmeltz, 2007). Therefore, the importance of lifestyle modification for the prevention of T2DM and CVD is necessary. A low-SFA diet (≤ 30% total calories) is considered to be the healthy choice of both primary and secondary prevention of CVD. Additionally, MUFA-rich diets have been recommended to individuals with T2DM (Giugliano & Esposiko, 2008; Mustad *et al*, 2006). Furthermore, the American Heart Association recommends increasing intakes of LC n-3 PUFA to approximately 1g/d of EPA plus DHA to reduce the risk of CVD in high-risk individuals (Covington, 2004).

Early epidemiological studies reported that total fat intake was positively associated with the risk of T2DM (West & Kalbfleish, 1971). These effects were partly mediated by the influence of fat intake on weight gain, since fat-rich foods are generally energy-dense, irrespective of the type of fat. Dietary FAs are classified as SFA, MUFA and PUFA (n-6 and n-3 FA) on the basis of their chemical structure. High levels of palmitic acid (C16:0) and low levels of LA (C18:2:6) and a proportionally higher level of palmitoleic acid (C16:1:7) are characteristic of individuals with the IR and MetS (Warensjö *et al*, 2006*a*). The majority of cholesterol in the body is esterified into free fatty acids (FFA) in the form of cholesterol esters and they form lipid droplets in cells as storage sites for cholesterol and FA (Raatz *et al*, 2001). FA composition in serum CE's mirrors to a certain extent the dietary FA pattern of about 6 - 8 weeks prior. Fasting plasma has been deemed a comparable FA biomarker to WAT and as plasma is an easy biomarker to collect, process and store, it was used as the primary biomarker for this LIPGENE cross-sectional study.

The mechanisms by which dietary fat consumption can influence the development of T2DM are indeed linked with insulin sensitivity. Animal studies have clearly shown that a high-fat diet, particularly a SFA-rich diet impairs insulin sensitivity (Storlien *et al*, 1991; Barnea *et al*, 2006). Furthermore, cross-sectional studies in humans that a high intake of SFA may contribute to the development of obesity (Astrup *et al.* 2000; Warensjö *et al*, 2006*b*)

The euglycaemia hyperinsulinaemic clamp is considered the *'gold-standard'* technique for measuring insulin sensitivity (DeFronzo *et al*, 1979; Monzillo & Hamdy, 2003; Wallace & Matthews, 2002). However, this study employed the IVGTT technique to measure insulin sensitivity in the MetS volunteers. The IVGTT technique involves an injection 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 & Bergman, 1986). Beard *et al* (1986) proposed a modified protocol which requires an infusion of tolbutamide 20 mins after the infusion of glucose at baseline. This is to ensure an adequate endogenous plasma insulin response which is critical for determining insulin sensitivity using minimal (MINMOD) modelling. The MINMOD Millennium® is a soft-ware package that computes estimates of S_I, S_G and β-cell function for each individual profile by curve fitting techniques (Bergman *et al*, 1979; Pacini & Bergman, 1986; Boston *et al*, 2003).

The objective of this cross-sectional study was to determine if a link exists between plasma and dietary FAs, inflammation and insulin sensitivity in the MetS. It did not, however reveal a strong association between plasma FAs, inflammatory markers and insulin sensitivity markers. Nonetheless, when individuals were categorized into tertiles of S_I, those with lower insulin sensitivity had a notably higher intake of dietary TFA. TFA are unsaturated FA that are formed during the industrial process of partial biohydrogenation or are formed naturally in the rumen of dairy cows such as conjugated linoleic acid (CLA). Two main isomers of CLA exist, *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) isomer and *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA) and inconsistencies exist in the studies which examined the isomer specific effects of CLA supplementation on insulin sensitivity and inflammation (Choi & Song, 2005). A study examining the effect of the *t*10, *c*12-CLA isomer on insulin sensitivity was investigated on obese, MetS males and showed an increase in insulin sensitivity (Risérus *et al*, 2002). Within the same research group, the *c*9, *t*11-CLA isomer alone showed a decrease in insulin sensitivity and an increased lipid perioxidation in obese middle aged men at high risk for CVD (Risérus *et al*, 2004*c*). It is important to investigate whether low amounts of TFA consumed during long periods may promote IR, which eventually may have clinically relevant effects on

diabetes risk. Another cross-sectional study showed that those in the higher tertile for TFA, had a 73% increase in plasma CRP concentrations and a 10% increase in sICAM-1 and sVCAM-1 levels (Lopez-Garcia *et al*, 2004).

It has become increasingly evident that inflammation correlates positively with endothelial dysfunction and IR, and very clear results has been found in those with the MetS (Guigliano *et al*, 2004). Endothelial dysfunction is associated with several vascular conditions such as hypertension, hyperlipidemia and T2DM. IR is known to be present in all these conditions. Cytokines such as sICAM-1 and sVCAM-1 are classical markers of endothelial dysfunction and these are secreted from the WAT. Thus, increased AT mass is associated with alterations in cytokine production (De Carvalho *et al*, 2006). Observational data from this study showed an increase in plasma sICAM-1 concentrations with lower insulin sensitivity. Substantially higher concentrations of plasma PAI-1 and tPA were also associated with lower insulin sensitivity and higher $HOMA_{IR}$ and unsurprisingly, body weight and BMI was notably higher in the low insulin sensitivity cohort, Similar results were noted in the Framingham Offspring Study where circulating PAI-1 concentrations were strongly associated with the development of MetS (Ingelsson *et al*, 2007). Several *in vitro* experiments have shown that FA varying in degrees of saturation increases the production of PAI-1 (Nilsson *et al*, 1998; Karikó *et al*, 1995; Banfi *et al*, 1997), whereas unsaturated FA does not (Nilsson *et al*, 1998; Chen *et al*, 2002). It is not fully understood how insulin sensitivity exerts its effects on PAI-1, it has been suggested that insulin sensitivity may exert its effect through elevated insulin levels and insulin sensitivity may also act on PAI-1 production via insulin propeptides. Individuals with IR and T2DM have an increased amount of proinsulin relative to insulin levels. Proinsulin is the precursor of insulin and is converted into insulin and c-peptide and stored in vacuoles forming the secretory granulae (Steiner *et al*, 1996). In cross-sectional studies, proinsulin or the proinsulin to insulin ratio have been considered as a marker for impaired β-cell function (Mykkänen *et al*, 1999). Analysis of proinsulin was not completed as part of this LIPGENE cross-sectional study.

A number of surrogate indices for insulin sensitivity have been developed such as $HOMA_{IR}$ and more recently the revised QUICKI, both are mathematical models and both have correlated positively with hyperinsulinemic-euglycemic clamp and minimal MINMOD Millennium® techinque (Chen *et al*, 2005*b*; Katsuki *et al*, 2005; Brady *et al*, 2004*a*; Katsuki *et al*, 2001). $HOMA_{IR}$ is mathematical modelling of the normal homeostatis balance of insulin

and glucose providing equations for the estimations of S_I and β-cell function (Matthews *et al*, 1985). In this study, those in the higher $HOMA_{IR}$ category were noted to have markedly higher concentrations of leptin. A very recent study was performed to explore the relationship between serum level of leptin and the components of MetS in 345 mid-aged Chinese individuals. Results showed that serum leptin levels increased with the prevalence of MetS. The study concluded that hyperleptinaemia could possibly be a novel component of MetS (Ding *et al*, 2007). In the LIPGENE cross-sectional study those with a high $HOMA_{IR}$ index consumed particularly more total MUFA and oleic acid (C18:1), suggesting that MUFA may decrease insulin sensitivity levels.

Cytokine biology can be modulated by anti-inflammatory drugs, recombinant cytokine receptors antagonists and nutrients. Among the nutrients, fats have an immense potential for modulating cytokine biology (Grimble & Tappia, 1998). Resistin is a recently identified 12kDa cytokine, with a proposed role in obesity and IR, both of which are key features of the MetS. In rodents, resistin is almost exclusively expressed in WAT, whereas human resistin expression is primarily from monocytes or macrophages (Lehrke *et al*, 2004; Savage *et al,* 2001; Steppan & Lazar, 2004). It has been postulated that resistin mediates IR, but the role of resistin in human biology still remains uncertain and has been suggested to be limited to rodents (Lago *et al*, 2007*b*). Obese mice treated with thiazolidinediones (TZD) showed a notable decrease in resistin concentrations and improved insulin sensitivity. Moreover, neutralisation of resistin activity by an injection of antibodies against resistin decreases blood glucose levels and improves insulin sensitivity in obese, IR mice. Additionally, decreased resistin concentrations were in turn rapidly increased after insulin infusion in the obese mice and this observation suggests that insulin may modulate its own activity through the regulation of resistin (Kim *et al*, 2001). Several studies in IR humans reported no link between obesity, IR and resistin concentrations (Hasegawa *et al*, 2005; Iqbal *et al*, 2005; Utzschneider *et al*, 2005; Chen *et al*, 2005*a*). In contrast, a recent cross-sectional trial on 135 patients with T2DM showed positively strong correlations with IR and factors linked to obesity and inflammation (Mojiminiyi & Abdella, 2007). These results are in line with another study that found resistin concentrations to be elevated in patients with T2DM and was associated with obesity and IR (Al-Harithy & Al-Ghamdi, 2005). Observational data from this study shows that resistin concentrations were lower in INSERM, France compared to all other centres. In accordance to tertiles of resistin concentrations, fasting glucose and c-peptide levels were elevated in those volunteers with higher resistin concentrations compared to those with lower resistin levels.

Additionally, in this study, numerous inflammatory cytokines, coagulation markers and oxidative stress markers were higher in those with high resistin concentrations compared to those with lower resistin levels. Interestingly, plasma caprylic acid (C8:0) and TFA were higher in those with lower resistin, however both dietary fatty demonstrated significantly inverse correlations with resistin. These results suggest that prudent dietary patterns of the 'Mediterranean-style diet', in particular, a diet rich in MUFA may be potential therapy for reducing a pro-inflammatory state. Moreover, these results provide more support to the debate that higher resistin concentrations may orchestrate IR and inflammation in humans with the MetS.

AT secretes a large number of physiologically active peptides that often share properties with cytokines. A more recently discovered cytokine is adiponectin, also referred to as 30kDa adipocyte complement-related protein (Acrp30) and gelatin-binding protein-28 (GBP28) (Capeau, 2007; Scherer *et al*, 1995). It is secreted in copious amounts from WAT and is present in relatively high concentrations in circulation. Previously, low adiponectin concentrations have been associated with obesity, dyslipidemia, essential hypertension, T2DM and CVD (Yang *et al*, 2001; Matsubara *et al*, 2003; Baratta *et al*, 2004; Adamczak *et al*, 2003; Weyer *et al*, 2001*a*; Kojima *et al*, 2005). More recently, it has been suggested that hypoadiponectinemia may contribute to the development of inflammatory disease by promoting the phagcytosis of apoptotic cells and the accumulation of apoptopic debris is known to cause inflammation and immune system dysfunction (Tan *et al*, 2004). It is also known to inhibit the adhesion of monocytes to endothelial cells (EC), reducing the synthesis of cell adhesion molecules (CAM) and TNFα and reducing NF-κB levels (Funahasti *et al*, 1999). As dietary FA may influence the expression of adipokines, adiponectin concentrations were observed in this study. Cross-sectionally, it was noted that TCD had the highest concentrations of plasma adiponectin compared to the other EU centres and females had remarkably higher levels compared to males. Furthermore, those with higher concentrations of plasma adiponectin were found to have increased S_I and lower c-peptide concentrations. Plasma myristic acid (C14:0), EPA (C20:5:3) and LC n-3 PUFA were noted to be higher in those individuals with lower adiponectin levels. Whereas, dietary intake to total SFA, MUFA and PUFA as well as LC n-3 PUFA and LC n-6 PUFA were noted to be higher in those with lower levels of adiponectin.

Another cross-sectional evaluation was performed to determine whether adherence to a 'Mediterranean-type diet' is associated with higher plasma adiponectin concentrations as part

of the Nurses' Health Study. The Mediterranean diet is characterised by a MUFA-rich, low SFA diet. The results of the study found that adiponectin concentrations were increased in those who followed the habitual 'Mediterranean-style' diet (Mantzoros *et al*, 2006). Another recent study reported lower adiponectin concentrations after 8 weeks supplementing with ALA (C18:3:3) among overweight individuals (Nelson *et al*, 2007). However, an ALA-rich diet demonstrated anti-inflammatory effects *in vitro* by inhibiting IL-6 and TNFα expression in peripheral blood mononuclear cells (PBMC) (Zhao *et al,* 2007). An animal study demonstrated that 3 months EPA feeding increases adiponectin in the genetically obese *ob/ob* mouse model (Itoh *et al*, 2007). Similar results were demonstrated in the *ob/ob* mice in *Chapter 3* of this thesis, where natural and synthetic sources of beef enriched *c*9, *t*11-CLA isomer increase adiponectin concentrations compared to the LA diet (C18:2:6). These results provide an important insight into its therapeutic implication of the obesity-related metabolic sequelae as not only is the quantity of dietary fat important but also the composition of the dietary fat consumed.

CRP is an acute phase reactant synthesized by the liver and is released into the bloodstream as a natural reaction to infection, fever or other injury (Ozdogan *et al*, 2007). High concentrations of CRP correlate with adiposity, the presence of CVD and risk of acute CVD events (Rutter *et al*, 2008). Increased plasma CRP levels are strong independent predictors of future myocardial infarction (MI), stroke and microalbumuria, a marker of impaired kidney function (Nettleton & Katz 2005). Elevated levels of CRP are known to predict the development of T2DM and CVD; in fact all 5 characteristics of the MetS are associated with increased CRP levels (Ridker *et al*, 2003). A study carried out in 3 different centres in Belgium, Italy and England, CRP levels were found to be higher in females compared to males and CRP correlated strongly with BMI (kg/m^2) in females and waist circumference in males (Arcari *et al*, 2007). Similar results were noted in subjects with MetS who showed higher levels of CRP and the main determinant of the CRP elevation was waist circumference (Nakamura *et al*, 2007). Observational data from this study shows that females have higher concentrations of CRP compared to males, and those with a BMI >30 have increased CRP concentrations compared to the leaner MetS volunteers. Furthermore, those with lower CRP levels had higher levels of plasma myristic acid (C14:0), palmitoleic acid (C16:1), oleic acid (C18:1) and total SFA levels. Dietary fat and antioxidants are known for their immune-modulating actions, however their reported effects on CRP concentrations have been inconsistent. A study on overweight individuals concluded that adopting the

'Mediterranean diet' in combination with medium physical activity seems to reduce the likelihood of having high CRP levels by 72% (Pitsavos *et al,* 2007). Likewise, another study found that a Mediterranean-rich diet, high in MUFA and low in SFA decreased endothelial markers sICAM-1 and sVCAM-1 as well as IL-6 and CRP concentrations in individuals at high risk for CVD (Salas-Salvadó *et al*, 2007). Contrary to this, no association was noted between CRP levels and dietary intake of total calories, total SFA, MUFA, PUFA, LC n-3 PUFA and LC n-6 PUFA in patients with high cholesterol levels (Fredrikson *et al*, 2004). Inflammatory data from the LIPGENE cross-sectional study showed that fibrinogen and leptin concentrations were higher in those with elevated CRP levels, suggesting that increased CRP concentrations may be linked with increased fibrinolysis.

Ratios of SFA/MUFA and SFA/PUFA (saturated fatty acids/unsaturated fatty acids) were investigated in this cross-sectional study. The main findings of this analysis were found in for SFA/MUFA levels, where results show that c-peptide and AIRg were higher in those with a high SFA/MUFA levels, suggesting that they are more IR compared to those with a lower SFA/MUFA levels. However, inflammatory mediators such as CRP, leptin and adiponectin concentrations were noted to be higher in those with lower SFA/MUFA levels. On the contrary, IL-6 concentrations were observed to be elevated in those with high SFA/MUFA compared to those with lower SFA/MUFA levels.

Observational data in this study showed that TNFα and adiponectin were positively associated with insulin sensitivity, whereas BMI, plasma tPA concentration and alcohol intake were negatively associated with insulin sensitivity independent of age, gender and BMI. Risérus *et al* (2007) also found that BMI was the single strongest predictor of insulin sensitivity, followed by physical activity, HDL-c, SFA and socioeconomic status. In a study on 153 MetS males, adiponectin concentrations were lower and leptin concentrations were higher compared to non-MetS patients. In addition, both adiponectin and leptin were predictors of insulin sensitivity independent of age and BMI (Gannagé-Yared *et al,* 2006).

Binary logistic regression analysis was performed to determine the independent predictors of insulin sensitivity. The main outcomes show that those individuals with lower insulin sensitivity compared to those with medium insulin sensitivity and those with higher insulin sensitivity, UoR, UO, INSERM, HURS-UCO and UU were positive predictors of insulin sensitivity as well as alcohol intake, tPA and sICAM-1 concentrations.

Healthful diets that avoid excess energy intake and reduce SFA consumption are a key component of lifestyle management in T2DM (Nettleton & Katz, 2005). However fats are

vital in the diet, as their functional role is manifold whereby they provides high energy, essential FA, a vehicle to transport fat soluble vitamins, nutrients and anti-oxidants, flavour and satiety value. In addition to fat, the quality of FA consumed plays considerably different roles in body metabolism. The FA profile of T2DM diet was improved by substitution MUFA and PUFA for SFA and increasing the consumption of LC n-3 PUFA in 3 cross-over design studies (Parillo *et al*, 1992; Madigan *et al,* 2000; Perez-Jiménez *et al*, 2001).

This study was of a cross-sectional nature aimed to investigate the interaction between plasma and dietary FA composition and inflammation on insulin sensitivity. The unique feature about this observational study was that a relatively large cohort of MetS volunteers were recruited within 8 EU countries. Cross-sectional studies have advantages whereby they are relatively inexpensive and time efficient and they can be useful for public health planning, understanding disease aetiology and for the generation of hypotheses and many outcomes and risk factors can be assessed (Levin, 2006). On the contrary, limitations occurs with cross-sectional studies as they are only a snapshot and the situation may provide differing results if another time-frame had been chosen and they have been previously associated with bias. Therefore, it is important to follow-up cross-sectional studies with a longer intervention type-study using the same cohort. This LIPGENE cross-sectional study was followed-up by conducting a 12 week dietary intervention study involving the same 8 EU centres and MetS cohort. This dietary intervention study as described in detail in *Chapter 5* of this thesis.

There are other shortcomings in the present study, which include the type of biomarker used to analyse FA composition. Many types of biomarkers exist that have many potential functions in nutritional epidemiology studies. Moreover, many studies have used FA biomarkers to assess compliance in short-term dietary intervention studies and habitual diets in observational studies, however little information is known on the reliability and comparability of these measures (Baylin & Campos, 2006). Plasma was used in this investigation for the analysis of circulating FAs, cytokines, coagulation markers and markers of endothelial dynfunction. However, plasma CEs or AT have been documented as the biomarkers of choice, because of there stability over time under conditions of energy balance and they are ideal measures for longer-term dietary intakes of exogenous FA (Arab & Akbar, 2002). The FA composition of serum lipid esters CEs and phospholipids, mirror dietary FA intake over the previous few weeks and also reflects endogenous FA metabolism. This may be a possible reason as to why Warensjö *et al* (2006*b*) found FA composition to be substantially different between subjects with or without MetS and how Aldámiz-Echevarría *et al* (2007)

demonstrated a positive association between insulinemia or $HOMA_{IR}$ index and AA content in AT.

In addition to the limitations, S_I is a crucial parameter of glucose metabolism. The gold standard method for the determination of insulin sensitivity is the euglycaemic hyperinsulinaemic clamp from which indices of insulin sensitivity can be derived. The MINMOD® Millennium can also derive insulin sensitivity, however insulin sensitivity does not account for the dynamics of insulin action, e.g. how fast or slow insulin action reaches its plateau value (Pillonetto *et al*, 2006). Surrogate indices can also be derived from one-off fasting specimens, in particular $HOMA_{IR}$. These indices lend themselves for use in large population studies where a relatively simple, inexpensive assessment is necessary. However, these tests suffer limitations, including poor precision. The major drawback of $HOMA_{IR}$ is that when the glucose or insulin concentration increases, the value is overestimated. $HOMA_{IR}$ is not linear over wide ranges of insulin sensitivity in man, the primary input data need to be robust, and the data need to be interpreted carefully (Borai *et al*, 2007; Wallace *et al*, 2004).

In conclusion, overweight and obesity are associated with an increased risk of such metabolic abnormalities as dyslipidemia, hypertension or T2DM and CVD, common features of the MetS. Initially, IR or hyperinsulinemia was suggested as the origin of these abnormalities. More recent studies indicate that adipokines have an important role in obesity-associated metabolic complications, and suggest that chronically elevated local or systemic concentrations of adipokines contribute to the development of complications associated with obesity and MetS (Bulló *et al*, 2007). Past studies have proven that it is possible to achieve primary prevention of T2DM by means of intensive interventions. Therefore, appropriate strategies to identify and analyse high-risk individuals in a simple and inexpensive way are urgently warranted (Parillo & Riccardi, 2004).

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Chapter 5

To determine the efficacy of reducing dietary SFA, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS

5.1 ABSTRACT

Background: The metabolic syndrome (MetS) defines a clustering of metabolic irregularities which are associated with a high risk for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). Previous dietary intervention studies have shown positive influences of all characteristics associated with the MetS, through the modification of habitual dietary fat intake.

Aim: The aim of this study was to determine the efficacy of reducing dietary saturated fatty acids (SFA) consumption, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS.

Design: 417 free-living subjects with the MetS were randomly assigned to one of the following diets: Diet A: High-fat (38%), SFA-rich diet (***HSFA***), Diet B: High-fat (38%), monounsaturated fatty acids (MUFA)-rich diet (***HMUFA***), Diet C: Low-fat (28%), high complex carbohydrate diet (***LFHCC***), Diet D: Low-fat (28%), low complex carbohydrate diet & 1.24grams per day (g/d) long chain (LC) polyunsaturated fatty acids (***LFn-3PUFA***) for 12-weeks (weeks). An intravenous glucose tolerance test (IVGTT) was performed and fasting bloods samples were harvested at the beginning and immediately after the 12-week (week) intervention.

Results: Dietary fat modification had no significant effects on markers of insulin sensitivity, inflammation or oxidative stress. However, substantially modifications were noted for lipoprotein concentrations, the majority of these being with the LFn-3PUFA diet. When MetS volunteers were stratified according to tertiles of CRP concentrations, no significant differences was demonstrated for insulin sensitivity status. Linear regression analysis performed on this cohort showed that glucose effectiveness (S_G), fasting glucose, soluble intercellular adhesion molecule-1 (sICAM-1) and Uppsala University (UU), Sweden were associated with lower insulin sensitivity, irrespective of dietary treatment.

Conclusions: Although no impact was demonstrated on insulin sensitivity, inflammation and lipid peroxidation by dietary fat modification, positive changes were demonstrated for lipoprotein concentrations. Therefore, more long-term studies are required to elucidate the hypothesis that diet and lifestyle should be the first line of treatment for the MetS.

5.2 INTRODUCTION

In 1988 the existence of a syndrome coined '*Syndrome X*', describing a clustering of metabolic disorders including glucose intolerance, hyperinsulinemia, dyslipidemia and hypertension was defined (Reaven, 1988). A few years later in 1993, Björntrop presented strong evidence in which he powerfully demonstrated that abdominal obesity is an integral characteristic of '*Syndrome X*'. In the same article, it was stated that this syndrome be titled the MetS, due to its metabolic nature (Björntrop, 1993). Therefore, the MetS has been defined as a multi-component disorder consisting of a combination of metabolic abnormalities associated with an increased risk of developing T2DM, CVD and atherosclerosis (Assmann *et al*, 2007). Moreover, these metabolic abnormalities include insulin resistance (IR), central obesity, particularly increased levels of visceral abdominal fat, low high-density lipoprotein cholesterol (HDL-c) concentrations, high triacylglycerol (TAG) levels, hypertension and urinary microalbuminuria (Lorenzo *et al*, 2007). In the proposed definitions by the world health organisation (WHO) and European group for the study of insulin resistance (EGIR), IR is included as *the* key underlying feature to the MetS (Saely *et al*, 2007; Koehler *et al*, 2007). IR is defined as a state in which normal levels of insulin produce a subnormal biological response. The tissues of the body have a reduced sensitivity to the action of insulin and therefore, greater than normal concentrations of insulin are required to elicit a quantitatively normal response (Fonseca *et al*, 2005). As a result hyperinsulinemia develops, which is associated with increased CVD risk as well as the risk of developing overt diabetes mellitus (Kirwan & del Aguila 2003; Rader, 2007).

Obesity is associated with chronic low-grade inflammation and has been identified as the key aetiological condition that predisposes to IR and the development of T2DM (Herder *et al,* 2007). Inflammation is linked with various diseases and has been suggested to be part of the MetS (Ridker *et al*, 2003). Currently, obesity is a major public health challenge and is stated as the second leading cause of preventable death in the USA (Aronne, 2002). In the MetS, intra-abdominal visceral fat accumulation has been shown to play a key role in the development of a variety of metabolic and circulatory disorders through the dysregulation of adipokines (Moller & Kaufman, 2005; Zeyda *et al*, 2007). Adipokines are involved in several processes like inflammation, regulation of energy balance and appetite, insulin sensitivity, vascular homeostatis, lipid metabolism and angiogenesis (Trayhurn *et al*, 2006). Epidemiological and clinical investigations have shown strong and consistent relationships between markers of inflammation and a risk of future CVD events (Ridker, 2003). Markers of

oxidative stress are also positively correlated with body fat percentage. Oxidative stress is an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to tissue damage. The most valuable of the currently available biomarkers of lipid peroxidation in the human body are the isoprostanes, which are specific products arising from the peroxidation of unsaturated fatty acid (FA) residues in lipids (Basu, 2004). Increased markers of lipid peroxidation have been observed in smokers and are known to contribute to a number of human pathologies including atherosclerosis (Helmersson *et al*, 2005; Pratico *et al*, 1997; Lusis, 2000) hypercholesterolaemia (Reilly *et al*, 1998; Davi *et al*, 1999; Wilson *et al*, 1999), IR and diabetes-related complications (Davi *et al*, 1999; Riserús *et al*, 2002). A dietary study supplemented with LC n-3 PUFA study demonstrated an outstanding decrease in isoprostanes, yet prostaglandins remain unaffected (Nälsén *et al*, 2006). Contrary to this, a dietary study investigating the impact of whole grains, showed no effect of insulin sensitivity or markers of lipid peroxidation. However, two recent weight-loss trials in healthy obese subjects showed a notable decrease in inflammatory markers and markers of oxidative stress (Boujoulia *et al*, 2006; Crujeiras *et al*, 2007).

It is without hesitation that the existence of the MetS is increasing at an alarming rate, with approximately 25% or 47 million adult Americans residents showing clear signs of this condition (Jolliffe & Janssen, 2007; Spinler, 2006). By 2010, it has been estimated that approximately 31 million Europeans will require treatment for T2DM and its related complications (Zimmet *et al*, 2001). The grim reality is that this condition is having a detrimental impact on healthcare and social welfare costs. The total costs of obesity or the EU was estimated to be approximately €32,800 x 10^6 per year (Fry & Finlay, 2005) Thus, it is expected that the prevalence of the MetS will increase markedly unless effective public health strategies are enforced (Shaw *et al*, 2005). The obesity epidemic is now seen as an increasing public health concern that requires strategic action by both governmental and non-governmental organisations (Skidmore & Yarnell, 2004). However, it seems unlikely that this epidemic can be reversed in the short-term and despite immense public health attention, few intervention studies of adequate population size and duration have been performed to date (Riccardi *et al*, 2003).

An effective approach to alleviate the prevalence of this epidemic may be to focus on the importance of dietary therapy. The KANWU study included 162 healthy individuals who participated for 3 months and results of this study indicated that insulin sensitivity was significantly impaired by the high SFA-rich diet, yet remained unaffected by the MUFA-rich

diet (Vessby *et al*, 2001). Similar results have been reported by Summers *et al* (2002) where a PUFA-rich diet increased insulin sensitivity compared to a diet rich in SFA. Furthermore, Pérez-Jiménez *et al* (2001) conducted a cross-over study in healthy young individuals and found that substituting carbohydrates and MUFA for SFA improved insulin sensitivity.

It is without doubt, that a profound understanding of the root causes is crucial if strategies for the prevention and treatment of obesity are to be developed. Therefore, longer-term research, investigating environmental factors, such as diet and lifestyle modifications in the prevention and treatment of the MetS and on the best ways to promote lifestyle changes are necessary. In the short-time efforts to curb obesity and overweight such as increasing physical activity and improving compliance of current dietary recommendations should be continued. (Riccardi *et al*, 2004; Laaksonen *et al*, 2004).

Healthful diets that avoid excess energy intake and reduce SFA consumption may be instrumental in alleviating the current worldwide obesity epidemic (Stone & Schmeltz, 2007; Hu *et al*, 2007). Dietary FAs are known to modify lipoprotein metabolism based on their wholesale degree of saturation. The FA profile of healthful T2DM diets can be improved by substituting MUFA and PUFA for SFA and increasing the consumption of LC n-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found mainly in oily/fatty fish (Nettleton & Katz, 2005). In a study where rats were fed LC n-3 PUFA rich in EPA and DHA, IR was prevented in muscle by decreasing SFA content and maintaining expression and translocation of GLUT4 receptors in the liver by sustaining the inhibition of hepatic glucose production (Carpentier *et al*, 2006*a*). Laaksonen *et al* (2002) showed that individuals with a high proportion of plasma linoleic acid (LA) (C18:2:6) had a lower risk for developing T2DM. These findings are in line with dietary epidemiological studies which indicate that individuals with a low proportion of LA or vegetable oil in their diet have an increased risk of developing T2DM (Feskens, 2001).

In recent years, several studies on MetS subjects have investigated the impact of dietary treatment combined with physical activity. In a diet and exercise intervention study carried out on 31 male subjects, 15 with MetS, 12 with T2DM, the remaining 4 with >1 MetS criteria and 5 subjects had coronary artery disease (CAD), subjects were placed a high-fibre (>40g/d), low-fat diet and undertook 45-60 mins of daily aerobic exercise for 21d. Results showed extraordinary reductions in body mass index (BMI), serum lipids and lipid ratios, fasting glucose, insulin and $HOMA_{IR}$, urinary isoprostanes and inflammatory cytokines ($p<0.05$). Interestingly, at post-intervention 9 of the 15 were no longer positive for the MetS.

Thus, this study concluded that diet and lifestyle modifications may ameliorate CAD risk factors in men with the MetS (Roberts *et al*, 2005). Another diet and exercise trial for 8 weeks was completed on obese humans (BMI > 33kg/m^2), who also had >1 characteristic of the MetS. Subjects were either assigned to a low-fat (30%, low-SFA, high-MUFA, PUFA and LC n-3 PUFA), high-complex carbohydrate isocaloric diet or to a moderate exercise regime, (45 mins 3 times per week). Although weight-loss was not intended, results demonstrated a small reduction in BMI within the dietary group only ($p<0.05$). Overall results highlighted that both interventions reduced fasting insulin concentrations ($p<0.05$). However, a significant reduction in skeletal muscle TNFα was noted after the dietary intervention only ($p<0.05$) which was independent of the change in body weight. Furthermore, skeletal muscle glucose transporter4 (GLUT4) levels were increased indicating an improvement in insulin sensitivity within the exercise group only. This study also concluded that diet and exercise modifications combined may alleviate CVD risk factors (Ferrier *et al*, 2004).

LIPGENE is a 5 year (2004-2009), trans-EU project entitled *Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis*. The objective of the study was to apply an integrated approach to examine how variations in the composition of dietary fats interact with common genetic variations to influence the development of the MetS (Buttriss and Nugent, 2005). LIPGENE comprises six different workpackages, all at various different stages of completion. This thesis focuses on the human dietary intervention study, involving 8 EU centres. The aim of the human dietary intervention study was to determine the efficacy of reducing dietary SFA consumption, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS (Tierney *et al*, 2007). The objective was to create four isoenergetic diets which differed greatly in dietary fat composition. The approach was designed to cause minimal disruption to the normal dietary habits of the participants and was to be appropriate for use in all EU countries. Shaw *et al* (2007) describes in detail the food exchange model approaches used to achieve the dietary targets, dietary advice given during the intervention and the monitoring of study compliance, all initial aspects that were achieved successfully. Although, the TCD cohort recruited only 55 MetS subjects, this was combined with the 417 subjects that completed the study. Fasting blood samples were harvested before an IVGTT was performed at pre- and post-intervention and all inflammatory markers, endothelial dysfunction markers and plasma FA were analysed within the TCD centre.

5.3 RESEARCH DESIGN AND METHODS

The LIPGENE human dietary intervention study was a randomised, controlled trial approved by the local ethics committees at each of the 8 clinical intervention centres, all of which conformed to the Helsinki Declaration of 1975 as revised in 1983. The study was registered with The US National Library of Medicine Clinical Trials registry (NCT00429195). The Ethics Committee of the St James's Hospital under the Federated Dublin Voluntary Hospitals Amalgamation Act, 1961 granted approval for the study to be conducted for the TCD cohort. Written informed consent was obtained from all volunteers prior to commencing the study. 15,593 individuals were screened of which 417 with the MetS completed the dietary intervention study across the 8 EU research centres; Trinity College Dublin (TCD), University of Reading (UoR), University of Oslo (UO), INSERM, U476 Marseille, France, University of Maastricht (UM), Córdoba (HURS-UCO), Poland (JUMC) and Uppsala University (UU) described in *Table 2.7.* Within the TCD cohort, the volunteers were recruited primarily from personnel in TCD, out-patients of the Endocrinology Departments of Adelaide and Meath Hospital, Dublin incorporating the National Children's Hospital (AMNCH), Tallaght, Dublin 24 and Loughlinstown Hospital, Co. Dublin and employees from a local taxi-firm and the general public.

Table 5.1 Baseline screening criteria adapted from the NCEP-ATP III (Assmann *et al*, 2007) and used by LIPGENE Human Dietary Intervention Study to diagnose the MetS.

Criteria	Biochemical/Anthropometric marker	Concentrations
1	Fasting glucose concentration	5.5 - 7.0 mmol/L
2	Serum TAG concentration	≥ 1.5 mmol/l
3	Serum HDL-c concentration	< 1.0 mmol/l (male), < 1.3mmol/l (female)
4	Blood Pressure	130/85 mmHg
5	Waist girth	>102cm (male), > 88cm (female)

HDL-c, High density lipoprotein - cholesterol; **TAG**: Tricylglycerol; **mmol/L**: millimoles/Litre; **mmHG**: millimetre of mercury; **cm**: centimetres

5.3.1 Collection of Anthropometric, bioelectrical impedance analysis and blood pressure measurements

All anthropometric, bioelectrical impedance analysis and blood pressure measurements were taken as previously detailed in *Section 2.8 to Section 2.9.*

5.3.2 LIPGENE human dietary intervention treatments

Volunteers were randomly stratified to one of four dietary treatments for an intervention period of 12 weeks, as described in *Table 5.2*. The randomisation process was based on age, gender and fasting plasma glucose concentrations. An equal number of subjects were allocated to each dietary group at each centre using the MINIM (Minimisation Programme for Allocating patients to Clinical Trials, Dept of Clinical Epidemiology, The London Hospital Medical College, UK) randomisation programme. The volunteer's habitual diets were manipulated according to a dietary food exchange model (Shaw *et al*, 2007). Briefly, the food exchange model was developed based on the National Diet and Nutrition Survey (NDNS) of UK adults (aged 19-64 years) and the National Food Survey (DEFRA, 2000, Henderson, 2003) and the North South Ireland Food Consumption Survey (adults aged 18-64 years) (Harrington, 2001). The food exchange model was designed to ensure the dietary FA composition of the four diets was achieved in a free-living population across Europe. Unilever Best Foods (Vlaardingen Unilever Foods, The Netherlands) produced several foods (margarines, cooking fats, baking fats, mayonnaise, salad dressings and biscuits) with varying consistency of each food product. Study foods were delivered by the investigators or subjects collected them from the study centre throughout the 12 week study period. Lipid Nutrition, Looders Crooklan (Wormerveer, The Netherlands) provided the 1.24g LC n-3 PUFA (Marinol ^{TM} C-38) and placebo high-oleic acid sunflower seed oil (HOSO) supplement containing high concentrations of oleic acid (C18:1), the composition of which is presented in *Table 5.3*. Each volunteer received his or her capsules on day 0 (pre-intervention clinical investigation day) of the study. A capsule count was taken at the end of the study period (week 12) and volunteers were asked to note down any capsules missed or lost during the 12 weeks.

An intensive dietary assessment schedule was implemented. Volunteers kept a 3-day weighed food dietary record (two weekdays and one weekend day) to assess pre-intervention dietary habitual intake. This assessment formed the basis for dietary fat modification in order to achieve the dietary intervention targets using the intervention foods, without a significant (<2% body weight change) alteration in body weight. Dietary advice was given to each

volunteer using the dietary intake data gained at the pre-intervention dietary assessment and modified in order to achieve their target intervention diet. Two further weighed 3-day food dietary records were completed at week 6 and 12 to assess dietary intake mid- and post-intervention. Compliance was promoted and assessed by regular contact with each volunteer by 24-hour recalls and food use questionnaires, every two weeks of the trial.

A 3-day weighed food diary was completed prior to commencing the LIPGENE cross-sectional study. The volunteers were instructed to include 2 week days (Monday to Thursday) and 1 weekend day (Saturday or Sunday). Friday was not included as there was much ambiguity as to whether it is considered a week day or weekend day. The food was weighed using calibrated Soehnle Vita Food Scales (Soehnle-Waagen GmbH & Co, Germany). Volunteers were asked to include food packaging and home made recipes when possible. Various dietary analysis programs were used across the 8 EU centres to ensure culturally specific foods were included (**TCD**: WISP version 3.0; **UoR**: Food Base version 3.1; **UO**: Kostberegningssystems (nutrient calculation software developed in the Department of Nutrition, UO; **INSERM**: Nutrilog; **UM**: Komeet & Orion software; **HURS-UCO**: DietSource version 2.0; **JUMC**: Dietetian software; **UU**: MATs connected to the Swedish Board of Food Administration database for foods).

Body weight was measured pre-, mid- and post-intervention at week 0, 6 and 12 to ensure isocaloric dietary compliance. The level of physical activity, alcohol consumption and smoking habits did not deviate throughout the intervention period.

Table 5.2: Description and compositional details of the LIPGENE dietary treatments

LIPGENE Human Dietary Intervention Treatments

	Diet A	Diet B	Diet C	Diet D
Diet Description	Control – high-fat, SFA-rich diet	High-fat, MUFA-rich diet	Low-fat, high complex carbohydrate diet	Low-fat, high complex carbohydrate diet & 1.24g (n-3) LC-PUFA
Abbreviation	**HSFA**	**HMUFA**	**LFHCC**	**LFn-3PUFA**
Total Energy %	38	38	28	28
SFA %	16	8	8	8
MUFA %	12	20	11	11
PUFA %	6	6	6	6
Placebo †	NA	NA	HOSO	NA
1g/d (n-3) LC-PUFA %	NA	NA	NA	1.24g/d n-3 PUFA

SFA: (Saturated Fatty Acids); **MUFA:** (Monounsaturated Fatty Acids); **PUFA:** (Polyunsaturated Fatty Acids); **1.24g/d LC n-3 PUFA:** (1.24 gram/day Long Chain n-3 PUFA Marinol™ C-38, concentrated natural fish oil with a high content of EPA and DHA); **Placebo** (HOSO: High oleic acid (C18:1) sunflower seed oil supplement); **NA**: Non applicable.

Table 5.3: Fatty acid composition and tocopherol content of the LC n-3 PUFA and placebo HOSO supplement

	LC n-3 PUFA	Placebo HOSO
Description:	Marinol™ C-38 is a concentrated natural fish oil with a high content of EPA and DHA	High Oleic Acid Sunflower Seed Oil supplement
Fatty Acid Composition	w/w %	w/w %
C14:0	5	0
C16:0	10.5	3.5
C16:1	4.5	0
C17:0	0.2	0
C18:0	2.5	3.4
C18:1	8.0	79.2
C18:2	1.0	11.8
C18:3	0.5	0.2
C20:0	0.2	0.3
C20:1	1.1	0.3
C22:0	0.2	0.9
C24:0	0.2	0.3
C20:5	24	-
C22:6	17.5	-
Total EPA and DHA	38*	-
Mixed Natural Tocopherol	3.0mg	3.0mg

*38% is the standard minimum value of EPA and DHA that is assured in all batches of the product.

5.3.3 Clinical investigations and collection of bloods samples

Following diagnosis of the MetS, volunteers were invited to participate in the LIPGENE human dietary intervention study. Across the EU, 473 volunteers accepted invitation to take part in this 12 week dietary study. Within TCD, volunteers attending the Diabetes Day Centre, AMNCH, Tallaght Hospital after observation of a 12 hrs overnight fast (no food or drink, except water), avoiding smoking tobacco on the morning of each visit, abstaining from alcohol 12 hrs proceeding to the blood sample donation and avoiding vigorous exercise on the day prior to the clinical investigation (week 0 and week 12). Volunteers were asked to donate the mid-portion of the first urine void prior to attending the clinic. On arrival to the clinic, a fasting blood sample was drawn, prior to undergoing IVGTT lasting 180 mins as described by Bergman *et al*, 1985. In brief, a bolus of 50% glucose solution (Phoenix Pharma Ltd, Gloucester, UK) (0.3g/kg BW) was infused into cannula 1 over a 1 min period and this was followed by 20mls of saline. 20 mins later, a dose of insulin was infused into the same cannula (0.03U/kg BW). Meanwhile, blood samples from cannula 2 occurred at the following intervals over the 3 hrs period: -5, 0, 2, 4, 8, 19, 22, 30, 40, 70, 90 and 180 mins. At each time point real-time blood glucose monitoring was performed using a gluco-trend machine to confirm that the volunteer's blood glucose was not excessively low (<2.0mmol/l). If blood glucose dropped below this level, the IVGTT was discontinued and an isotonic high glucose drink was administered immediately. The blood tubes were inverted 8 to 10 times to ensure that the coagulant was mixed thoroughly. Subsequently, blood samples from EDTA, sodium citrate and fluoride oxalate tubes were placed on ice for 30 mins to allow for clotting, whilst serum and serum separator vaccunainer tubes were left at room temperature (RT) to allow for clotting. Following this, blood tubes were centrifuged and plasma or serum was harvested immediately, both blood and urine samples were stored at -70°C until further analysis.

5.3.4 Determination of human inflammatory markers and coagulation markers

Human high sensitivity C-reactive protein (hsCRP), (interleukin) IL-6, tumour necrosis factor α (TNFα), sICAM-1, soluble vascular adhesion molecule-1 (sVCAM-1), resistin, total adiponectin and leptin, concentrations were measured by on a basis on solid phase sandwich enzyme linked immunosorbent assay (ELISA) as described in *Section 2.12* and *Table 2.14* displays the coefficience of variance (CVs) achieved for each parameters. Plasma tissue plasminogen activator (tPA) and PAI-1 concentrations were determined using the

commercially available kits (ASSERACHROM® tPA, ASSERACHROM® PAI-1, respectively) by enzyme immunoassay (EIA) procedure by ELISA (Diagnostica Stago, France) as described in *Section 2.13.* Fibrinogen clotting activity was measured by using an automated clotting assay as described in *Section 2.13.1.*

5.3.5 Determination of human urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$

As a marker of oxidative stress urinary samples were analysed from free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$ F2-isoprostane) without any extraction by a radioimmunoassay, as described in *Section 2.14.* Lipid peroxidaiton enzyme 15-keto-dihydro-prostaglandin $F_{2\alpha}$ (15-keto-dihydro-$PGF_{2\alpha}$ F2α-prostaglandin) was analysed without any extraction by a radioimmunoassay as described in *Section 2.14.*

5.3.6 Determination of human insulin and c-peptide and glucose concentrations

Serum insulin and c-peptide concentrations were measured using a solid phase, two site fluoroimmunometric assays (AutoDELFIA Insulin kit, Wallac Oy, Finland and AutoDELFIA C-peptide kit, Wallac Oy, Finland) (*Section 2.15*).

5.3.7 Determination of human plasma fatty acids composition

Plasma FA composition was determined according to the Bligh and Dyer method as described in *Section 2.16* (Bligh & Dyer, 1959). A more detailed description of these FAs is presented in *Table 2.15.*

5.3.8 Determination of human HDL, LDL, total cholesterol, TAG, NEFA and apolipoprotein concentrations

The IL Test™ HDL-c Kit (Instrumentation Laboratories, Warrington, UK) was used for direct quantification of HDL-c. LDL-c was isolated from serum using LDL-c reagent (Randox, Laboratories Ltd., Co. Antrim) (*Section 2.17.1* and *Section 2.17.2*, respectively). Quantitation of total cholesterol, LDL-c and TRL from defrosted plasma and supernatant samples was performed using the IL Test™ Cholesterol Kit (Instrumentation Laboratories, Warrington, UK) on the ILAB (*Section 2.17.3*). Quantitation of plasma TAG and non-esterified fatty acids (NEFA) was performed using the IL Test™ TAG Kit (Instrumentation Laboratories, Warrington, UK) (*Section 2.17.4* and *Section 2.17.5*). Plasma apolipoproteins (Apo) AI, B and

E concentrations were measured using an immunonephelometric methods on a BN Prospec® System with commercial kits (Dade Behring, Deerfield, USA) (*Section 2.17.6*).

5.3.9 Statistical Power, data preparation and statistical analysis

The statistical power of the LIPGENE dietary intervention study was based on insulin sensitivity as the primary biomarker of the MetS. Insulin sensitivity was assessed by an IVGTT wherein insulin sensitivity (S_I) was measured by the MINMOD minimal modeling programme. The power of the study was calculated with the ability to detect at least 1 unit difference at a standard deviation (SD) of 2, between diets. The power calculation was based on the assumption that 15% difference in insulin sensitivity was physiologically important and that this difference can be detected at a significance level of 0.05 and a power of 0.8. Thus, a minimum of 98 subjects per dietary group would be required to detect significance differences. The group size was increased to 120 per group, to account for an anticipated 20% drop-out rate.

All statistical analysis one-way ANOVA, correlation analysis, group comparisons and linear regression analysis was conducted using SPSS® for Windows™, version 14.0 (SPSS Inc, Chicago, IL, USA). A detailed outline of all the data preparation and statistical analysis is presented in *Section 2.18.1* and *Section 2.18.2.* Results are presented as means ± standard error of the mean (SEM) unless otherwise stated.

Age and change of body weight are continuous variables, therefore, they were categorised for *post-hoc* analysis. Age categories were defined into three groups: 35-50 years, 50-65 years and 65-70 years. Change in body weight (BW) was categorised into 5 categories: Lost 4-8Kg, Lost 2-4Kg, Lost 0-2Kg, Gained 0-2Kg and Gained 2-5Kg.

IVGTT definitions: Homeostasis model assessment-insulin resistance ($HOMA_{IR}$) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) according to the equation [1/(log $insulin_0$ + log $glucose_0$ + log $NEFA_0$)] (Roche, 2004). Insulin and glucose results from the IVGTT were entered into MINMOD Millennium, a programme based on Bergman's minimal model which enables the estimation of 2 key indices of glucose/insulin dynamics: insulin sensitivity (S_I) and glucose effectiveness (S_G). Additional analysis from the MINMOD modelling include, acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from

the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (Zero) (G0) were all calculated by the Bergman MINMOD Millennium® (Boston *et al*, 2003).

5.4 RESULTS

5.4.1 Main findings of the LIPGENE human dietary intervention study

LIPGENE human dietary intervention study set out to determine the effects of dietary fat modification on multiple markers on the MetS. The main outcomes of this study are detailed in *Section 5.4.2-5.4.8.* Additional analysis was performed in order to investigate any potential novel findings as presented in this chapter. All data is presented in mean ± SEM

5.4.2 Effects of dietary fat modification on markers of inflammation, coagulation and lipid peroxidation

Table 5.4 presents the effects of the dietary intervention on a range of classical inflammatory markers and indices of lipid peroxidation and coagulation markers related to the MetS according to the dietary treatments. Results showed no significant effect of dietary fat modification on the change in these biomarkers between diets from pre- to post-intervention. However, a RM-ANOVA demonstrated a significant diet*time*gender*centre interaction for resistin only.

The effect of dietary fat modification on the change in inflammatory markers following the dietary intervention was also determined. The change intervention data was determined by the post-intervention concentration minus the pre-intervention concentration and included the following markers: plasma CRP, IL-6, TNFα, sICAM-1, sVCAM-1, resistin, adiponectin, leptin and urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ concentrations. The data presented in *Table 5.5* showed that there was no notable effect of dietary fat modification on the change in these biomarkers between diets. This analysis was important to carry out as it takes into account the baseline status on inflammation for subjects with the MetS, as well as the nature and degree of response after the 12 week dietary intervention. Furthermore, the analysis is less complex and therefore a one-way ANOVA was employed to demonstrate that there was no significant effect of dietary fat modification on plasma inflammatory mediators.

Table 5.4: Adjusted Means(±SEM) on the effects of dietary fat modification on inflammatory markers, oxidative stress markers and coagulation markers for the completers Pre and Post-Intervention across the four dietary treatments (n=417)

	HSFA Diet A				HMUFA Diet B				LFHCC Diet C				LFn-3PUFA Diet D			
	Pre		Post		Pre		Post		Pre		Post		Pre		Post	
	n=100				n=111				n=106				n=100			
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CRP (mg/L)	5.00	0.42	4.85	0.39	4.82	0.40	4.60	0.37	5.54	0.41	5.27	0.38	5.10	0.42	4.80	0.39
IL-6 (pg/mL)	4.57	0.42	4.56	0.41	5.69	0.40	4.81	0.39	4.76	0.40	4.99	0.41	4.99	0.42	4.53	0.41
TNFα (pg/mL)	5.68	0.49	5.13	0.41	5.44	0.47	4.86	0.39	4.77	0.47	4.23	0.40	4.90	0.49	4.71	0.41
sICAM-1 (ng/mL)	286.27	7.45	284.90	7.53	287.32	7.07	289.20	7.14	287.35	7.20	286.63	7.28	279.34	7.41	280.75	7.49
sVCAM-1 (ng/mL)	601.12	17.06	609.69	18.15	591.18	16.18	608.86	17.22	632.70	16.48	625.60	17.54	583.29	16.97	597.70	18.06
Resistin (pg/mL)	10452.40	503.13	10114.70	551.70	10082.77	477.31	9709.70	523.40	10088.80	486.24	10582.88	533.20	9985.42	503.13	9965.88	551.73
Adiponectin (μg/mL)	3.76	0.25	3.89	0.25	3.70	0.23	3.66	0.24	3.82	0.24	4.20	0.24	3.45	0.24	3.79	0.25
Leptin (ng/mL)	22.43	2.11	21.87	2.14	21.95	1.98	22.45	2.01	20.04	2.02	18.99	2.05	23.80	2.07	21.96	2.10
8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	0.49	0.02	0.58	0.05	0.44	0.01	0.45	0.04	0.48	0.02	0.48	0.04	0.47	0.02	0.50	0.05
15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	0.17	0.01	0.17	0.01	0.17	0.01	0.16	0.01	0.17	0.01	0.16	0.01	0.18	0.01	0.18	0.01
PAI-1 (ng/mL)	53.47	3.68	54.17	3.58	51.75	3.49	51.38	3.40	58.68	3.55	55.34	3.46	51.07	3.68	58.17	3.58
tPA (ng/mL)	8.86	0.41	8.63	0.42	8.30	0.39	8.44	0.40	8.64	0.40	8.02	0.41	8.77	0.41	8.51	0.42
Fibrinogen (mg/dL)	335.13	8.54	322.60	7.43	327.25	8.14	312.95	7.08	327.81	8.26	328.02	7.18	325.85	8.54	317.94	7.43

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

CRP (C-Reactive Protein); **IL-6** (Interleukin-6), **TNFα** (Tumour Necrosis Factor-a), **sICAM-1** (soluble Intracellular Adhesion Molecule-1); **sVCAM-1** (soluble Vascular Adhesion Molecule);

8-Iso-PGF_{2a} (8-iso-prostaglandin F_2alpha); **15-keto-PGF_{2a}** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine); **PAI-1** (Plasminogen Activator Inhibitor-1); **tPA** (tissue Plasminogen Activator); **ng/mL** (nanograms per millilitre); **mg/dL** (milligrams per deciliter)
Diet A High-fat,high-SFA diet (HSFA); **Diet B High-Fat, MUFA-rich diet** (HMUFA); **Diet C** Low-fat, high complex carbohydrate diet (LFHCC); **Diet D** Low-fat, high complex carbohydrate & 1.24 LC n-3 PUFA (LFn-3PUFA)

Table 5.5: *The Mean (±SEM) on the change from pre- to post-intervention for the inflammatory and oxidative stress parameters*

	HSFA		HMUFA		LFHCC		LFn-3PUFA		
	Diet A n=99		**Diet B n=110**		**Diet C n=106**		**Diet D n=100**		***ANOVA***
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	
Change in CRP (mg/L)	-0.15	0.29	-0.23	0.36	-0.27	0.47	-0.3	0.37	*ns*
Change in IL-6 (pg/mL)	0	0.4	-0.88	0.4	0.33	0.39	-0.46	0.41	*ns*
Change in TNFα (pg/mL)	-0.55	0.68	-0.58	0.41	-0.54	0.29	-0.19	0.4	*ns*
Change in sICAM-1 (ng/mL)	-1.37	5.28	1.89	6.18	-0.72	4.92	1.41	5.02	*ns*
Change in sVCAM-1 (ng/mL)	8.57	10.56	17.68	11.46	-7.09	8.99	14.41	9.61	*ns*
Change in Resistin (pg/mL)	-337.7	324.08	-373.06	304.38	494.06	358.44	-19.55	331.07	*ns*
Change in Adiponectin (μg/mL)	0.14	0.13	-0.03	0.24	0.38	0.2	0.34	0.17	*ns*
Change in 8-iso-$PGF_{2\alpha}$ (mmol/mmol crea)	0.01	0.02	0.01	0.02	0.08	0.08	0.02	0.02	*ns*
Change in 15-keto-$PGF_{2\alpha}$ (mmol/mmol crea)	0	0	0	0.01	-0.01	0	-0.01	0.01	*ns*

Values present the adjusted Mean ± SEM (Standard Error of the Mean); ns (non significant)

CRP (C-Reactive Protein); **IL-6** (Interleukin-6), **TNF**α (Tumour Necrosis Factor-a),

sICAM-1 (soluble Intercellular Adhesion Molecule -1), **sVCAM-1** (souble Vascular Adhesion Molecule-1)

8-Iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F_2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F_2alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

Diet A High-fat,high-SFA diet (HSFA); **Diet B High-Fat, MUFA-rich diet** (HMUFA); **Diet C** Low-fat, high complex carbohydrate diet (LFHCC);

Diet D Low-fat, high complex carbohydrate & 1.24 LC n-3 PUFA (LFn-3PUFA)

ANOVA (Analysis of Variance); **Change value for each variable** (post-intervention value minus pre-intervention value)

5.4.3 The effects of dietary fat modification of metabolic markers of insulin sensitivity

Insulin sensitivity (S_I) was measured pre- and post-intervention by an IVGTT for all subjects in each participating EU centre. Analysis of results was completed by Bergman's Minimal Model Analysis Software (MINMOD) Millennium package (Boston *et al*, 2003). Homeostasis model assessment-insulin resistance ($HOMA_{IR}$) was calculated as fasting glucose (mg/dl) multiplied by fasting insulin (μU/ml) divided by 22.5 (Matthews *et al*, 1985). Quantitative insulin sensitivity check index (QUICKI) was calculated as $1/[\log(I_0) + \log(G_0)]$, where I = insulin and G = glucose (Katz *et al*, 2000). Insulin sensitivity (S_I), glucose effectiveness (S_G), acute insulin response to glucose (AIRg), disposition index (DI), removal rate of insulin from the interstitial space (P2), movement of circulating insulin to interstitial space (P3) and distribution of glucose to time 0 (G0) were all calculated by the Bergman MINMOD Millennium (Boston *et al*, 2003). *Table 5.6* presents the results for the IVGTT parameters across the dietary treatments, RM-ANOVA demonstrated a significant diet*time interaction for insulin/c-peptide ratio only, whereby concentrations were decreased at post-intervention for the LFn-3PUFA diet ($p<0.0005$).

5.4.4 Effects of dietary fat modification of plasma fatty acids composition

Table 5.7 presents the effects of dietary fat modification on plasma FAs across the dietary treatments. RM-ANOVA demonstrated a diet*time interaction for oleic acid (C18:1), whereby concentrations are greatly reduced at post-intervention within HSFA and LFn-3PUFA diets ($p<0.0005$). Contrary to this, concentrations were immensely increased within HMUFA at post-intervention ($p<0.0005$). Concentrations of EPA (20:5:3) were also significantly altered following dietary fat modification, whereby within the HSFA, LFHCC and LFn-3PUFA diets concentrations were increased at post-intervention ($p<0.0005$). Moreover, dietary fat modification had a significant influence on plasma FA, DHA (22:6:3), wherein post-intervention concentrations within LFHCC and LFn-3PUFA diets were significantly increased ($p<0.0005$). Concentrations of MUFA were also altered by dietary treatment, namely the HSFA and LFn-3PUFA groups, which were substantially decreased at post-intervention ($p<0.0005$) and finally for LC n-3 PUFA, concentrations in all four diets were increased following dietary fat modification ($p<0.0005$).

Table 5.6: The Means (±SEM) on the effects of dietary fat modification on the parameters from the IVGTT MINMOD® modelling process.

	HSFA Diet A						HMUFA Diet B						LFHCC Diet C						LFn-3PUFA Diet D					
	Pre			Post			Pre			Post			Pre			Post			Pre			Post		
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
S_I (mU/L)-1min-1	77	3.09	0.19	73	2.89	0.18	94	2.89	0.17	94	2.89	0.16	83	2.75	0.18	84	2.79	0.17	77	2.58	0.19	77	2.53	0.18
S_G (min-1)	77	0.016	0.001	74	0.015	0.001	94	0.016	0.001	94	0.016	0.001	84	0.016	0.001	84	0.017	0.001	78	0.015	0.001	78	0.017	0.001
AIRg (mUL^{-1} min^{-1})	77	388.62	42.08	74	398.31	44.04	94	359.35	37.33	94	383.80	39.08	84	365.53	39.49	84	388.66	41.34	78	385.75	40.99	78	378.75	42.90
DI	77	955.96	84.00	74	994.03	94.36	94	955.33	74.53	94	957.50	83.72	84	814.73	78.84	84	913.38	88.57	78	879.87	81.82	78	921.61	91.91
G0 (mg/dl^{-1})	77	297.46	4.74	74	299.63	10.49	94	297.83	4.21	94	300.71	9.31	84	296.09	4.45	84	295.76	9.85	78	297.67	4.62	78	313.00	10.22
P2 (min^{-1})	77	0.037	0.004	74	0.043	0.015	94	0.045	0.003	94	0.047	0.013	84	0.043	0.003	84	0.053	0.014	78	0.044	0.003	78	0.070	0.014
P3 (mU/L) min^{-2}	77	0.00	0.00	74	0.00	0.00	94	0.00	0.00	94	0.00	0.00	84	0.00	0.00	84	0.00	0.00	78	0.00	0.00	78	0.00	0.00
Fasting Glucose (mmol/l)	100	5.96	0.09	97	6.00	0.11	110	5.90	0.08	110	5.97	0.10	105	5.94	0.08	105	5.81	0.10	96	6.02	0.09	96	6.16	0.11
Fasting Insulin (uIU/ml)	100	9.83	0.54	97	10.05	0.59	110	9.86	0.51	110	9.20	0.56	105	10.31	0.52	105	10.22	0.57	96	10.22	0.55	96	9.60	0.60
$HOMA_{IR}$	100	2.65	0.16	97	2.82	0.19	110	2.59	0.15	110	2.45	0.18	105	2.75	0.15	105	2.66	0.18	96	2.77	0.16	96	2.63	0.19
QUICKI	100	0.67	0.04	97	0.61	0.02	110	0.60	0.04	110	0.60	0.01	105	0.54	0.04	105	0.61	0.02	96	0.59	0.04	96	0.61	0.02
FPIR (uIU/ml)	98	40.77	4.10	96	42.05	4.32	106	40.25	3.87	109	41.77	4.09	104	38.69	3.99	100	42.23	4.21	96	39.31	4.05	96	38.82	4.28
C-peptide (ng/ml)	99	2.67	0.09	96	2.82	0.10	109	2.54	0.09	108	2.68	0.10	105	2.68	0.09	105	2.78	0.10	95	2.64	0.09	95	2.81	0.11
Insulin/c-peptide ratio	99	3.67	0.16	96	$3.38^{\dagger}$	0.15	109	3.86	0.15	108	$3.33^{\dagger}$	0.14	105	3.81	0.15	105	$3.61^{\dagger}$	0.14	95	3.89	0.16	95	$3.32^{\dagger}$	0.15

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

† significant time*diet interaction $p<0.0005$, post compared to pre-intervention in that diet, not all IVGTT procedures were deemed and modelled successful, therefore n numbers vary

S_I, insulin sensitivity; **S_G**, glucose effectiveness; **AIRg**, acute insulin response to glucose; **DI**, disposition index ($AIRg*S_I$); **G0**, distributed glucose at time 0min;

P2 (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space); **$HOMA_{IR}$** (Homeostatis Model Assessement-Insulin Resistance); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **FPIR** (First Phase Insulin Response)

Diet A High-fat,high-SFA diet (HSFA); **Diet B High-Fat, MUFA-rich diet** (HMUFA); **Diet C** Low-fat, high complex carbohydrate diet (LFHCC); **Diet D** Low-fat, high complex carbohydrate & 1.24 LC n-3 PUFA (LFn-3PUFA)

Table 5.7: The Means(±SEM) on the effects of dietary fat modification on Plasma Fatty Acids (%)

	HSFA				HMUFA				LFHCC				LFn-3PUFA			
	Diet A				Diet B				Diet C				Diet D			
	Pre		Post		Pre		Post		Pre		Post		Pre		Post	
%	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
C14:0 (Mysteric acid)	1.90	0.10	2.66	0.14	1.85	0.10	2.56	0.14	1.90	0.10	2.53	0.14	1.97	0.10	2.62	0.14
C16:0 (Palmitic acid)	26.66	0.50	25.31	0.42	26.14	0.48	24.57	0.40	27.03	0.48	25.00	0.40	26.19	0.50	24.51	0.42
C16:1 (Palmitoleic acid)	1.29	0.10	1.26	0.11	1.50	0.10	1.37	0.10	1.29	0.10	1.25	0.10	1.45	0.10	1.34	0.11
C18:0 (stearic acid)	4.21	0.21	3.92	0.19	4.17	0.20	3.81	0.18	4.08	0.20	3.51	0.18	4.00	0.21	3.67	0.19
C18:1 (oleic acid)	26.81	1.45	25.16 †	0.42	26.25	0.43	27.19 †	0.40	26.06	0.43	26.01	0.41	26.92	0.45	25.64 †	0.43
C18:2:6) (LA)	27.51	0.55	28.47	0.54	27.64	0.53	27.31	0.52	27.58	0.53	27.95	0.52	27.33	0.56	27.59	0.55
C20:4:6)	6.66	0.24	7.52	0.20	7.07	0.23	7.37	0.20	6.63	0.23	7.36	0.20	6.95	0.24	6.91	0.21
C20:5:3 (EPA)	0.82	0.09	0.97 a†	0.09	0.98	0.09	1.01 a	0.09	1.01	0.09	1.13 a†	0.09	0.98	0.09	1.95 b†	0.09
C22:6:3 (DHA)	2.04	0.12	2.31 a	0.16	2.20	0.12	2.45 a	0.16	2.34	0.12	2.86 ab†	0.16	2.12	0.12	3.25 b†	0.17
SFA	32.77	0.65	31.89	0.50	32.16	0.62	30.94	0.48	33.01	0.62	31.04	0.48	32.16	0.65	30.80	0.51
MUFA	28.32	0.48	26.56 a†	0.45	27.94	0.46	28.74 b	0.43	27.57	0.47	27.44 ab	0.44	28.59	0.49	27.20 a†	0.46
PUFA	38.92	0.70	41.55	0.56	39.90	0.67	40.32	0.53	39.42	0.67	41.52	0.53	39.26	0.71	41.99	0.56
LC (n-6) PUFA	35.27	0.64	37.29	0.55	35.88	0.61	35.95	0.53	35.23	0.62	36.46	0.53	35.37	0.65	35.63	0.56
LC (n-3) PUFA	3.65	0.20	4.27 a†	0.22	4.03	0.19	4.38 ab	0.21	4.19	0.19	5.06 b†	0.21	3.89	0.20	6.37 c†	0.22

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

ns (non significant); †significant time*diet interaction p<0.0005, post- compared to pre-intervention;

[abc...] denotes significance across diet groups (when two superscripts contain the same letter there is no significant difference between two of the four diets)

Diet A High-fat,high-SFA diet (HSFA); **Diet B High-Fat, MUFA-rich diet** (HMUFA); **Diet C** Low-fat, high complex carbohydrate diet (LFHCC); **Diet D** Low-fat, high complex carbohydrate & 1.24 LC n-3 PUFA (LFn-3PUFA)

Mysteric Acid (14:0); Palmitic Acid (16:0); Palmitoleic Acid (16:1); Stearic Acid (18:0); Oleic Acid (18:1); Linoleic Acid (18:2:6); Arachidonic Acid (20:4:6); EPA (20:5:3); DHA (22:6:3)

5.4.5 Demographics and anthropometric measurement of the volunteers' pre- and post-human dietary intervention

Anthropometric measurements were recorded at pre- and post intervention for all participating subjects. *Table 5.8* presents the anthropometric data across the dietary treatments, the mean age was 54.91, 54.62, 54.70 and 55.39 years, for the HSFA, HMUFA, LFHCC and LFn-3PUFA diets, respectively. RM-ANOVA demonstrated a significant diet*time interaction for body weight ($p<0.0005$). On further analysis this significance was noted within the LFHCC diet, whereby post-intervention body weight was significantly reduced. In contrast, body weight was not altered over time in the other diet groups. Hence, given the importance of body weight as a determinant of IR and AT derived inflammatory markers, it was included within the RM-ANOVA as a covariate.

5.4.6 Effects of dietary fat modification on plasma lipid and lipoprotein concentrations

RM-ANOVA demonstrate several significant diet*time interactions for the effects of dietary fat modification on lipid and lipoprotein metabolism pre- to post-intervention. Plasma TAG concentrations showed a significant diet*time interaction (P=0.006), whereby plasma TAG concentrations were remarkably reduced following HSFA diet and LFn-3PUFA diet (p=0.018 and p=0.005, respectively). Additionally, plasma NEFA concentrations showed a diet*time interaction which approached significance (p=0.061). *Post-hoc* analysis showed that there was a great reduction in plasma NEFA concentrations following the LFn-3PUFA diet post intervention (p=0.006). Plasma total HDL-c concentrations showed a significant diet*time interaction (p=0.005), whereby HDL-c levels were vastly increased by high-fat diets, HSFA and HMUFA post-intervention (p=0.0005 and p=0.006, respectively). When total plasma cholesterol to HDL-c concentrations were expressed as a ratio (TC/HDL-cholesterol) another significant time*diet interaction was observed (P=0.005), wherein the plasma TC/HDL-cholesterol concentrations ratio was reduced following the HSFA and the HMUFA diets (p=0.0005) but increased by the LFn-3PUFA diet (p=0.0005). TAG-rich lipoprotein (TRL)/TAG concentrations also showed a significant time*diet interaction (p=0.003), this was explained by reduction in TRL:TAG levels following the LFn-3PUFA diet only (p=0.032) (*Data not shown*).

Table 5.8: Adjusted Means (±SEM) of age and anthropometric variables across the four dietary intervention groups, Pre and Post-intervention (n=417)

	HSFA Diet A n=100				HMUFA Diet B n=111				LFHCC Diet C n=106				LFn-3PUFA Diet D n=100			
	Pre		Post		Pre		Post		Pre		Post		Pre		Post	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Age (years)	54.91	0.86			54.62	0.83			54.70	0.91			55.39	0.96		
Height (m)	1.68	0.01			1.68	0.01			1.68	0.01			1.68	0.01		
BodyWeight (Kg)	89.91	1.44	89.70	1.43	91.17	1.35	90.94	1.35	91.96	1.38	91.03†	1.38	91.20	1.43	90.45†	1.43
BMI (kg/m^2)	31.81	0.43	31.74	0.43	32.44	0.41	32.37	0.41	32.51	0.42	32.18	0.42	32.42	0.43	32.16	0.43
Waist circumference (cm)	105.21	1.11	104.52	1.09	105.92	1.05	106.11	1.03	106.95	1.07	105.24	1.05	106.42	1.11	105.51	1.09
Hip circumference (cm)	112.57	1.00	111.52	1.02	112.03	0.94	111.84	0.95	113.50	0.96	112.31	0.95	111.97	1.00	111.08	1.01
Diastolic BP (mmHg)	85.94	0.91	84.72	0.94	86.03	0.85	84.76	0.88	84.90	0.87	83.57	0.90	85.57	0.90	82.68	0.93
Systolic BP (mmHg)	137.66	1.55	134.24	1.50	138.93	1.46	134.64	1.41	138.44	1.49	134.55	1.44	137.63	1.55	132.85	1.49
Body Fat (%)	37.24	0.90	3.88	0.90	37.13	0.83	36.41	0.83	37.17	0.86	36.58	0.86	36.70	0.90	36.58	0.90
Body Fat (Kg)	33.46	0.96	33.22	0.95	33.61	0.89	32.85	0.88	33.54	0.92	32.76	0.91	33.46	0.96	33.13	0.95
Lean Body Mass(%)	61.16	1.01	61.28	1.00	60.70	0.94	61.34	0.93	60.75	0.96	60.67	0.96	61.71	1.01	61.33	1.00
Lean Mass Body (Kg)	55.18	1.42	55.15	1.42	55.44	1.32	55.96	1.32	54.98	1.36	55.05	1.36	56.72	1.42	56.02	1.42
Body Water (%)	44.32	0.78	44.70	0.76	44.44	0.73	44.92	0.71	43.93	0.74	44.24	0.72	44.51	0.77	44.58	0.75
Body Water (Litres)	39.78	1.15	39.69	1.12	39.77	1.05	40.40	1.03	40.50	1.08	40.15	1.06	40.89	1.13	40.71	1.10
BIA - Impedance (KHz)	529.20	10.46	531.51	9.76	524.82	10.04	520.15	9.37	536.37	9.97	527.19	9.30	510.46	10.39	513.92	9.69

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

ns (non significant); † significant time*diet interaction $p < 0.0005$, post- compared to pre-intervention; BMI (kg/m2), Body Mass Index; BP, blood pressure, BIA (Bioelectircal Impedance)

cm (centimeters), **KHz** (Kilohertz), **mmHG** (millimeter of mercury)

Diet A High-fat,high-SFA diet (HSFA); **Diet B High-Fat, MUFA-rich diet** (HMUFA); **Diet C** Low-fat, high complex carbohydrate diet (LFHCC); **Diet D** Low-fat, high complex carbohydrate & 1.24 LC n-3 PUFA (LFn-3PUFA)

5.4.7 Effects of dietary fat modification on plasma apoprotein concentrations

The effects of dietary fat modification on plasma and lipoprotein apoprotein concentrations pre- to post-intervention show that neither plasma ApoA1 nor Apo B were considerably altered by dietary fat modification, as defined by a significant time*diet interaction. Additionally, plasma ApoCII, ApoE nor TRL ApoB concentrations did not show a significant time*diet interaction after dietary fat modification. However, plasma ApoCIII demonstrated a significant time*diet interaction (p=0.034), whereby levels were greatly reduced in HSFA and LFn-3PUFA (p=0.014 and p=0.006, respectively) (*Data not shown*).

5.4.8 Anthropometric measurements, inflammatory and oxidative stress markers according to LIPGENE partners

Although no significant main effect was noted for the metabolic markers of inflammation and oxidative stress, further analysis demonstrated a number of interesting observations, particularly with respect to EU centre specific interactions, gender, age and body weight. Therefore, a number of complimentary tables have been generated to present the data as follows. *Table 5.9* presents data for the inflammatory and oxidative stress markers across the participating EU centres involved in the dietary intervention study.

5.4.9 CRP concentrations according to LIPGENE partners

CRP is an acute phase reactant synthesized by the liver. Elevated levels of CRP are now known to predict the development of T2DM and CVD, in fact all 5 characteristics of the MetS are associated with increased CRP concentrations (Ridker *et al*, 2003). No time*diet, time*age or time*change in body weight interactions were noted for plasma CRP concentrations. However, a time*centre and time*gender interaction was observed for plasma CRP concentrations. In terms of the differences between centres for CRP, a one-way ANOVA demonstrated that CRP concentrations were substantially lower at pre-dietary intervention within the UoR, UO, UM and UU cohorts compared to the HURS-UCO cohort ($p<0.05$) as shown in *Table 5.9*. Additionally, baseline CRP concentrations were significantly higher in the TCD cohort as compared with the UO cohort ($p<0.05$). No noteworthy differences between centres were noted for CRP post-dietary intervention concentrations. Plasma CRP concentrations were observed to be significantly higher in females compared to males ($p<0.001$).

5.4.10 IL-6 concentrations according to LIPGENE partners

IL-6 is a pro-inflammatory cytokine secreted from many cell types including AT. This cytokine is largely associated with the presence of the MetS and CVD as it is believed to be a regulator of the hepatic acute phase response (Fernández-Real & Ricart, 2006). In the case of IL-6 concentrations, no time*diet, time*gender or time*age interactions were noted for plasma IL-6 concentrations. However, a significant time*centre difference were observed. At pre-intervention, UoR and UO subjects had appreciably higher mean plasma IL-6 concentrations compared with TCD, INSERM, UM, HURS-UCO, JUMC and UU ($p<0.01$) as displayed in *Table 5.9.* The mean value was highest for UoR and lowest in the TCD cohort (7.97pg/mL and 3.57pg/mL, respectively). Following the dietary intervention, IL-6 concentrations remain considerably higher in the UoR and UO cohorts compared with the TCD, INSERM, HURS-UCO, JUMC and UU cohorts ($p<0.0005$ and $p<0.05$, respectively). Moreover, HURS-UCO and JUMC had the lowest post-intervention levels of circulating IL-6 compared to all other partners ($p<0.0001$).

5.4.11 TNFα concentrations according to LIPGENE partners

TNFα is a cytokine that is over-produced in immune cells of obese AT. Discovered in 1975, its expression is positively correlated with the degree of obesity and hyperinsulinemia (Carswell *et al*, 1975). No time*diet, time*gender or time*age interactions were noted for plasma TNFα concentrations. However, plasma TNFα concentrations demonstrated a significant time*centre difference. At pre-intervention, the UU cohort had the highest circulating plasma TNFα concentrations and this was remarkably greater compared to the lowest levels found in UM ($p<0.0005$). Plasma TNFα concentrations in the UM cohort were considerably lower compared to the TCD, UoR, UO and JUMC cohorts at pre-intervention ($p<0.0001$) (*Table 5.9*). Following dietary intervention, UO had the highest plasma TNFα concentrations, whereas INSERM had the lowest levels ($p<0.0005$). Although, UU had the highest concentrations pre-intervention, interestingly at post-intervention the UU cohort had extensively lower concentrations compared to UoR, UO and JUMC cohorts ($p<0.01$).

5.4.12 sICAM-1 concentrations according to LIPGENE partners

Soluble intercellular adhesion molecule (sICAM-1) is a member of the immunoglobulin (Ig) superfamily and is now considered one of the prototypic markers of inflammation and levels

have been demonstrated to be increased in response to the *western-style* diet (Blankenberg *et al*, 2003). No time*diet, time*gender or time*age interactions were noted for plasma sICAM-1 concentrations. However, RM-ANOVA demonstrated a significant time*centre effect. Pre- and post-dietary intervention sICAM-1 concentrations were highest in the TCD cohort and lowest for the UU cohort ($p<0.0005$) (*Table 5.9*). The UU cohort also had considerably lower sICAM-1 concentrations were compared with UoR, UO, HURS-UCO and JUMC cohorts pre- and post-intervention ($p<0.01$). Moreover, plasma sICAM-1 concentrations for the UM cohort were much lower at pre- and post-intervention compared to the TCD, HURS-UCO and JUMC cohorts ($p<0.05$).

5.4.13 sVCAM-1 concentrations according to LIPGENE partners

Soluble vascular cell adhesion molecule-1 (sVCAM-1) is generated during inflammation and can alter lymphocyte functions (Becker *et al*, 2002). No time*diet or time*gender interactions were noted for plasma sVCAM-1 concentrations within this study. However, RM-ANOVA demonstrated a time*centre and time*age effect for plasma sVCAM-1 concentrations. With respect to the time*centre interaction, it was noted that within UO, INSERM and JUMC cohorts, the sVCAM-1 concentrations increased considerably at post-intervention compared to pre-invention concentrations ($p<0.05$) (*Table 5.9*).

Plasma sVCAM-1 concentrations were significantly altered by age, wherein pre-intervention sVCAM-1 concentrations were significantly higher in the 65 – 71 age category compared to the 35-50 age and 50 – 65 age categories ($p<0.001$ and $p<0.05$, respectively)

5.4.14 Resistin concentrations according to LIPGENE partners

Analysis of resistin concentrations was important as this novel hormone secreted by adipocytes has a proposed link with obesity, IR and T2DM (Mojiminiyi & Abdella, 2007; Al-Harity & Al-Ghamdi, 2005). In regards to the circulating resistin concentrations, RM-ANOVA revealed a significant time*gender*centre*diet interaction ($p<0.05$) only. This observation related to HMUFA diet for the HURS-UCO and UU cohorts whereby resistin concentrations were substantially reduced following 12 weeks dietary intervention ($p<0.05$). For the HURS-UCO cohort the reduction in plasma resistin concentrations was identified in males only (9805.4 mg/L v's 8143.80 mg/L) and within the UU cohort the significant decrease was noted in females only (10350.7 mg/L v's 6838.50 mg/L).

5.4.15 Adiponectin concentrations according to LIPGENE partners

In current years, adiponectin has been postulated to have anti-inflammatory effects, interceded in part, by inhibiting TNFα production in macrophages (Whitehead *et al*, 2006). Previous studies have reported that higher circulating adiponectin levels are associated with improved glycaemic control, lipid profile and inflammatory status (Mantzoros *et al*, 2005; Lu *et al,* 2006; Saely *et al*, 2007). RM-ANOVA model demonstrated no time*diet or time*gender interactions for plasma adiponectin concentrations. However, a significant time*centre interaction was noted for total adiponectin concentrations at pre- and post-intervention. Results reveal that circulating levels of total adiponectin were highest for the TCD cohort compared with all other participating centres pre- and post-intervention ($p<0.0005$) (*Table 5.9*). Additionally, UoR levels were considerably higher compared with UO and UM pre and post-intervention ($p<0.0005$). At pre-intervention the UM cohort had the lowest circulating adiponectin concentrations which was noticeably different compared to the other participating centres ($p<0.001$).

5.4.16 Leptin concentrations according to LIPGENE partners

The discovery of leptin has provided a potential strategy to alleviate the obesity epidemic as it is involved in the long-term regulation of food intake, body weight and energy expenditure. Leptin is also associated with S_I through promoting FA oxidation and reducing ectopic fat accumulation in non-adipose (Steinberger *et al*, 2003; Guerre-Millo, 2004). No time*diet or time*age interactions were noted for plasma adiponectin concentrations. However, RM-ANOVA showed a noteworthy time*centre and time*gender difference for plasma leptin concentrations ($p<0.0005$). HURS-UCO plasma leptin concentrations were much higher pre- and post-intervention compared with TCD, UoR, UO, INSERM, UM and UU ($p<0.0005$). Plasma leptin concentrations for the JUMC cohort were markedly higher compared with UM, UoR and INSERM after dietary intervention only ($p<0.05$) (*Table 5.9*).

An independent sample *t*-test showed that plasma leptin levels were significantly higher in females as compared to males for both pre- and post-intervention ($p<0.001$)

5.4.17 Urinary 8-iso-$PGF_{2\alpha}$ concentrations according to LIPGENE partners

Analysis of urinary 8-iso-prostaglandin F2alpha (8-iso-$PGF_{2\alpha}$) was carried out as a biomarker of lipid peroxidation. No time*diet, time*age or time*gender interactions were noted for

urinary 8-iso-$PGF_{2\alpha}$ concentrations. However, RM-ANOVA demonstrated that a time*centre and time*change in body weight difference exists. At pre-intervention, the TCD cohort had the highest 8-iso-$PGF_{2\alpha}$ isoprostane concentrations whereas the INSERM cohort had the lowest concentrations ($p<0.05$) (*Table 5.9*). At post-intervention, the TCD cohort remained as having the highest levels of 8-iso-$PGF_{2\alpha}$ isoprostanes, whereas the UoR cohort had the lowest concentrations ($p<0.05$). The JUMC cohort had extensively higher concentrations compared to UoR, UO and HURS-UCO ($p<0.05$).

Body weight was divided into five classes: lost 4-8kg, lost 2-4kg, lost 2-0kg, gained 0-2kg, gained 2 – 5kg. Results showed that those individuals who gained between 0-2kg were noted to have significantly higher levels of 8-isoPGF2α concentrations at post-dietary intervention compared to pre-dietary intervention ($p<0.05$)

5.4.18 Urinary 15-keto-dihydro-$PGF_{2\alpha}$ concentrations according to LIPGENE partners

Analysis of 15-keto-dihydro-prostaglandin F2alpha (15-keto-dihydro-$PGF_{2\alpha}$) was performed as a biomarker of enzymatically cyclooxygenase (COX)-mediated inflammation. No time*diet, time*age, time*gender or time*change in body weight interactions were noted for urinary 15-keto-dihydro-$PGF_{2\alpha}$ concentrations. However, RM-ANOVA demonstrated that a noteworthy time*centre interaction exists at pre- and post-intervention. The UM cohort have the highest concentrations of 15-keto-dihydro-$PGF_{2\alpha}$, whereas HURS-UCO have the lowest concentrations of 15-keto-dihydro-$PGF_{2\alpha}$ at pre- and post-intervention ($p<0.01$) (*Table 5.9*). The UM cohort also have significantly higher concentrations of 15-keto-dihydro-$PGF_{2\alpha}$ compared to UoR ($p<0.05$) and TCD have higher concentrations compared to HURS-UCO cohort ($p<0.05$).

Table 5.9: The Mean(±SEM) on the effects of dietary fat modification on the inflammatory markers across the 8 centres involved in LIPGENE (n=417)

	TCD n=55		UoR n=58		UO n=54		INSERM n=38		UM n=49		HURS-UCO n=74		JUMC n=43		UU n=46	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Pre CRP (mg/L)	6.25 ^{ac}	0.65	4.21 ^{ab}	0.46	3.90 ^{b}	0.47	4.82 ^{abc}	0.54	4.33 ^{ab}	0.57	7.00 ^{c}	0.60	5.23 ^{abc}	0.51	4.20 ^{ab}	0.53
Post CRP (mg/L)	5.01	0.51	4.66	0.42	4.43	0.48	4.33	0.65	3.99	0.41	6.22	0.61	5.74	0.53	4.09	0.45
Pre IL-6 (pg/mL)	3.57 ^{a}	0.48	8.48 ^{bc}	0.72	7.03 ^{c}	0.61	4.03 ^{a}	0.43	4.78 ^{abc}	0.32	3.67 ^{a}	0.28	3.74 ^{a}	0.39	4.60 ^{a}	0.80
Post IL-6 (pg/mL)	3.63 ^{a}	0.48	7.97 ^{bc}	0.82	6.54 ^{c}	0.58	3.68 ^{a}	0.30	4.82 ^{abc}	0.34	3.44 ^{a}	0.31	3.96 ^{a}	0.47	3.72 ^{a}	0.58
Pre TNFα (pg/mL)	4.57 ^{a}	0.21	4.96 ^{a}	0.24	6.12 ^{b}	0.30	4.23 ^{cd}	1.46	3.85 ^{ce}	1.21	4.95 ^{abe}	0.30	5.30 ^{ab}	0.45	7.44 ^{abd}	0.87
Post TNFα (pg/mL)	4.51 ^{ad}	0.20	5.38 ^{ab}	0.34	5.93 ^{b}	0.32	3.05 ^{c}	0.69	3.18 ^{ce}	1.09	5.36 ^{abe}	0.45	5.98 ^{ab}	0.63	3.58 ^{cd}	0.45
Pre sICAM-1 (ng/mL)	310.22 ^{a}	10.26	292.03 ^{ab}	7.87	296.07 ^{ad}	11.81	265.12 ^{bcde}	12.25	253.20 ^{bcd}	7.66	306.16 ^{a}	7.46	304.20 ^{ae}	13.00	232.97 ^{c}	7.64
Post sICAM-1 (ng/mL)	315.79 ^{a}	11.30	294.76 ^{ab}	8.02	289.29 ^{abc}	11.04	268.34 ^{bcag}	12.42	247.95 ^{cf}	7.01	312.05 ^{a}	9.10	295.40 ^{ag}	10.18	233.67 ^{def}	6.68
Pre sVCAM-1 (ng/mL)	597.13	22.37	649.63	25.22	601.16 †	23.22	612.17 †	26.44	601.89	20.35	601.20	17.75	581.84 †	34.52	565.00	21.65
Post sVCAM-1 (ng/mL)	607.74	24.32	640.28	22.28	643.67	22.64	677.97	33.75	607.92	21.29	579.35	19.13	607.73	35.97	543.43	22.75
Pre Resistin (pg/mL)	11366.53	588.77	11495.93	488.93	12628.80	1011.90	5319.24	381.42	10126.45	548.30	9987.15	471.67	9429.21	727.78	8961.41	718.16
Post Resistin (pg/mL)	11328.96	536.77	10747.07	553.37	13253.13	1120.71	5375.58	420.29	10294.06	601.18	9474.01	438.39	10662.58	1065.67	8353.81	738.72
Pre Adiponectin (µg/mL)	5.86 ^{a}	0.41	3.81 ^{b}	0.18	3.35 ^{cd}	0.52	3.32 ^{bc}	0.29	2.08 ^{d}	0.19	3.93 ^{b}	0.22	3.81 ^{b}	0.23	2.80 ^{c}	0.16
Post Adiponectin (µg/mL)	6.46 ^{a}	0.49	3.50 ^{bc}	0.16	3.48 ^{bc}	0.48	3.41 ^{bc}	0.24	2.93 ^{b}	0.24	4.02 ^{c}	0.21	3.81 ^{bc}	0.27	2.90 ^{b}	0.21
Pre Leptin (ng/mL)	20.14 ^{a}	2.19	15.65 ^{a}	1.97	16.38 ^{a}	1.67	16.57 ^{a}	2.48	16.13 ^{a}	2.98	39.89 ^{b}	3.38	24.07 ^{a}	2.53	18.88 ^{a}	1.92
Post Leptin (ng/mL)	20.42 ^{abc}	2.38	15.73 ^{b}	2.15	18.29 ^{abc}	3.24	13.81 ^{b}	1.88	12.37 ^{b}	2.33	37.80 ^{d}	3.12	25.70 ^{cd}	2.67	18.21 ^{bc}	1.98
Pre 8-iso-PGF2α (mmol/mmol crea)	0.53 ^{a}	0.02	0.44 ^{a}	0.02	0.45 ^{a}	0.02	0.43 ^{b}	0.02	0.49 ^{a}	0.02	0.47 ^{a}	0.02	0.50 ^{a}	0.03	0.45 ^{a}	0.02
Post 8-iso-PGF2α (mmol/mmol crea)	0.68 ^{a}	0.15	0.44 ^{b}	0.02	0.45 ^{b}	0.03	0.46 ^{b}	0.03	0.52 ^{b}	0.02	0.45 ^{b}	0.02	0.55 ^{a}	0.03	0.47 ^{b}	0.02
Pre 15-keto-PGF2α (mmol/mmol crea)	0.18	0.01	0.16	0.01	0.16	0.01	0.16	0.01	0.19	0.01	0.16	0.01	0.17	0.01	0.18	0.01
Post 15-keto-PGF2α (mmol/mmol crea)	0.18 ^{ac}	0.01	0.15 ^{ab}	0.01	0.16 ^{abc}	0.01	0.15 ^{abc}	0.01	0.19 ^{c}	0.01	0.15 ^{b}	0.01	0.19 ^{abc}	0.02	0.17 ^{abc}	0.01

Values present the adjusted Mean ± SEM (Standard Error of the Mean);

^{a,b,c}... denctes significance across centres (when 2 superscripts contain the same letter there is no significant difference between 2 of the 8 categories); ;

CRP (C-Reactive Protein); **IL-6** (Interleukin-6), **TNFα** (Tumour Necrosis Factor-α), **sICAM-1** (soluble Intracellular Adhesion Molecule-1); **sVCAM-1** (soluble Vascular Adhesion Molecule);

8-Iso-PGF$_{2\alpha}$ (8-iso-prostaglandin F$_2$alpha); **15-keto-PGF$_{2\alpha}$** (15-keto-prostaglandin-F$_2$alpha); **mmol/mmol crea** (millimole per millimole of creatinine)

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Oslo); **INSERM**, Marseille, France; **UM** (University of Maastricht); **JUMC**, Krakow, Poland; **UU** (Uppsala University)

5.4.19 Univariate correlations analysis were performed to determine the relationship between plasma and dietary fatty acids and anthropometric measurements

Correlations were performed to investigate the strength and direction of the relationship between two continuous variables. Pearson's product moment correlations or Spearman's Rank Order non-parametric correlation analysis was used to assess univariate associations between post-intervention inflammatory markers and plasma FAs, dietary FAs and anthropometric measurements, irrespective and according to dietary treatment (*Table 5.10 to Table 5.12a*).

5.4.20 Univariate correlations to determine factors influencing plasma and dietary fatty acids in the metabolic syndrome

When plasma and dietary FAs were correlated with the inflammatory biomarkers, yet again the majority of associations were very weak. However, when plasma FA were correlated with resistin a number of strong relationships emerged for plasma palmitic acid levels (C16:0) (r=0.315, P=0.0005) and plasma SFA levels (r=0.254, P=0.0005) and two strong inverse relationships were demonstrated for plasma oleic acid (C18:1) concentrations (r=-0.263, P=0.0005) and plasma MUFA levels (r=-0.257, P=0.05) (*Table 5.10*). On analysis per dietary treatment, plasma palmitic acid levels (C16:0) and SFA was strong and positive across the four dietary treatments (HSFA diet: r=0.335, P=0.001; HMUFA diet: r=0.293, P=0.05; LFHCC diet: r=0.293, P=0.05; LFn-3PUFA: r=0.356, P=0.0005). Furthermore, oleic acid (C18:1) and MUFA were strongly and inversely correlated with resistin within the HSFA, HMUFA and LFn-3PUFA diets (r=-0.315, P=0.05, r=-0.331, P=0.0005 and r=-0.286, P=0.0005, respectively) (*Table 5.10a*).

When dietary FAs were correlated with inflammatory markers, only adiponectin emerged as having a strong relationship with the dietary FAs (*Table 5.11*). Dietary correlations show that adiponectin was strongly and inversely correlated with palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), LA (C18:2:6), γ-LA (C18:3:6), PUFA, LC n-3 PUFA and LC n-6 PUFA, irrespective of diet. According to diet, the majority of the powerful correlations were seen with LFHCC and LFn-3PUFA diets (*Table 5.11a*).

Table 5.10: Correlation matrix showing the significant and non-significant relationships between inflammatory and oxidative stress markers v's plasma fatty acids, irrespective of dietary treatment **(n=417)**

Post Parameters	C14:0		C16:0		C16:1		C18:0		C18:1		C18:2:6		C20:4:6		C20:5:3		C22:6:3		SFA		MUFA		PUFA		n-6		n-3	
	r		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*	
CRP	-0.010	ns	-0.028	ns	0.073	ns	0.052	ns	0.003	ns	-0.033	ns	0.001	ns	-0.047	ns	0.027	ns	-0.029	ns	0.021	ns	-0.009	ns	-0.020	ns	0.028	ns
IL-6	0.006	ns	0.095	*	0.139	*	-0.111	*	-0.088	ns	0.024	ns	0.013	ns	0.019	ns	-0.064	ns	0.064	ns	-0.036	ns	0.019	ns	0.020	ns	-0.034	ns
TNFα	-0.003	ns	0.073	ns	0.046	ns	0.030	ns	-0.030	ns	-0.120	*	0.017	ns	0.034	ns	0.135	*	0.061	ns	-0.012	ns	-0.055	ns	-0.102	*	0.103	*
sICAM-1	0.044	ns	0.060	ns	0.076	ns	-0.092	ns	0.068	ns	-0.058	ns	-0.029	ns	-0.133	*	0.014	ns	0.025	ns	0.083	ns	-0.088	ns	-0.071		-0.007	ns
sVCAM-1	-0.110	*	0.136	*	0.116	*	-0.264	***	-0.084	ns	0.116	*	0.016	ns	-0.041	ns	-0.101	*	-0.010	ns	-0.046	ns	0.069	ns	0.086	ns	-0.083	ns
Resistin	-0.033	ns	0.315	***	-0.008	ns	-0.033	ns	-0.263	***	-0.027	ns	-0.052	ns	0.001	ns	0.040	ns	0.254	***	-0.257	***	-0.013	ns	-0.048	ns	0.063	ns
Adiponectin	0.109	*	0.022	ns	0.040	ns	0.200	***	-0.121	*	-0.074	ns	-0.047	ns	-0.011	ns	-0.035	ns	0.092	ns	-0.108	*	-0.065	ns	-0.071	ns	0.004	ns
Leptin	-0.032	ns	-0.074	ns	0.086	ns	-0.014	ns	0.123	*	-0.011	ns	-0.022	ns	-0.058	ns	0.102	*	-0.081	ns	0.126	*	-0.028	ns	-0.036	ns	0.047	ns
8-iso-$PGF_{2\alpha}$	-0.042	ns	-0.015	ns	-0.069	ns	-0.067	ns	-0.013	ns	0.059	ns	-0.049	ns	0.011	ns	0.031	ns	-0.065	ns	-0.025	ns	0.059	ns	0.038	ns	0.045	ns
15Keto-$PGF_{2\alpha}$	-0.008	ns	0.034	ns	-0.014	ns	-0.012	ns	-0.040	ns	0.041	ns	-0.079	ns	-0.049	ns	-0.085	ns	0.013	ns	-0.049	ns	-0.017	ns	0.006	ns	-0.067	ns

ns (non significant) * ($p<0.05$), ** ($p<0.001$), *** ($p<0.0005$), **hsCRP** (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6), **TNFα** (Tumor Necrosis Factor-α), **sICAM-1** (soluble Intracellular Adhesion Molecule-1) & **sVCAM-1** (soluble Vascular Adhesion Molecule-1)

8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F2alpha); **15-keto-$PGF_{2\alpha}$** (15-keto-prostaglandin-F2alpha)

C14:0 (Mysteric Acid); **C16:0** (Palmitic Acid); **C16:1** (Palmitoleic Acid); **C18:0** (Stearic Acid); **C18:1** (Oleic Acid); **C18:2:6** (Linoleic Acid); **C20:4:6** (Arachidonic Acid); **C20:5:6** (EPA); **C22:6:3** (DHA)

SFA (Saturated Fatty Acids); **MUFA** (Monounsaturated Fatty Acids); **PUFA** (Polyunsaturated Fatty Acids)

Table 5.10a: Correlations between inflammatory biomarkers and plasma fatty acids at post-dietary intervention, according to dietary tretment

Table 5.10a (1): Corrrelations between post-intervention IL-6 and plasma fatty acids across the dietary treatments

IL-6								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C16:1	0.085	ns	0.177	ns	0.232	*	0.045	ns
C18:0	-0.098	ns	-0.134	ns	0.058	ns	-0.254	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.10a (2): Corrrelations between post-intervention TNFa and plasma fatty acids across the dietary treatments

TNFα								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C18:2 (n-6)	-0.219	*	-0.023	ns	-0.002	ns	-0.273	*
C22:6 (n-3)	0.009	ns	0.110	ns	0.115	ns	0.286	*
(n-6)	-0.154	ns	-0.021	ns	-0.039	ns	-0.219	*
(n-3)	-0.033	ns	0.116	ns	0.141	ns	0.172	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005),

Table 5.10a (3): Corrrelations between post-intervention sICAM-1 and plasma fatty acids, across the dietary treatments

sICAM-1								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C20:5 (n-3)	-0.038	ns	-0.142	ns	-0.214	*	-0.203	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.10a (4): Corrrelations between post-intervention sVCAM-1 and plasma fatty acids, across the dietary treatments

sVCAM-1								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C14:0	-0.009	ns	-0.174	ns	-0.107	ns	-0.139	ns
C16:0	0.062	ns	0.280	*	-0.008	ns	0.217	*
C16:1	0.001	ns	0.089	ns	0.201	*	0.200	*
C18:0	-0.311	*	-0.312	*	-0.233	*	-0.169	ns
C18:2 (n-6)	0.184	ns	0.151	ns	0.089	ns	0.003	ns
C22:6 (n-3)	-0.070	ns	-0.155	ns	-0.036	ns	-0.146	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.10a (5): Corrrelations between post-intervention Resistin and plasma fatty acids, across the dietary treatments

Resistin								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C16:0	0.335	**	0.293	*	0.259	*	0.356	***
C18:1	-0.326	**	-0.334	***	-0.107	ns	-0.325	**
SFA	0.340	**	0.267	*	0.195	*	0.238	*
MUFA	-0.315	*	-0.331	***	-0.113	ns	-0.286	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.10a (6): Corrrelations between post-intervention Adiponectin and plasma fatty acids, across the dietary treatments

Adiponectin								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C14:0	-0.170	ns	0.243	*	0.176	ns	0.128	ns
C18:0	0.151	ns	0.184	*	0.372	***	0.112	ns
C18:1	-0.096	ns	-0.180	ns	-0.116	ns	-0.027	ns
MUFA	-0.072	ns	-0.182	ns	-0.144	ns	0.020	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.10a (7) Corrrelations between post-intervention Leptin and plasma fatty acids across the dietary treatments

Leptin								
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C18:1	0.084	ns	0.083	ns	0.117	ns	0.148	ns
MUFA	0.095	ns	0.083	ns	0.132	ns	0.155	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11: Correlation matrix displaying the significant and non-significant relationships between the inflammatory and oxidative stress markers v's dietary fatty acids, irrespective of any dietary treatment (n=417)

Post Parameters	Total fat		Total Fat A		C8:0 g		C10:0 g		C12:0 g		C14:0 g		C16:0 g		C16:1 g		C18:0 g		C18:1 g		C18:2 (n-6) g		C18:3 (n-3) g		C20:4 (n-6) g		C20:5 (n-3) g		C22:6 (n-3) g		Total En		SAFA g		MUFA g		PUFA g		n-3 g		n-6 g	
	r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r		r	
CRP	0.022	ns	0.015	ns	-0.083	ns	-0.006	ns	-0.011	ns	-0.069	ns	-0.009	ns	0.018	ns	-0.022	ns	0.023	ns	-0.049	ns	-0.067	ns	0.029	ns	-0.018	ns	-0.020	ns	-0.049	ns	-0.045	ns	-0.040	ns	-0.075	ns	0.003	ns	0.013	ns
IL-6	-0.113	*	-0.097	ns	-0.212	*	-0.142	*	-0.158	*	-0.008	ns	-0.091	ns	-0.006	ns	-0.051	ns	-0.057	ns	0.040	ns	-0.013	ns	0.180	*	-0.012	ns	0.055	ns	-0.113	ns	-0.087	ns	-0.127	*	-0.064	ns	0.099	ns	0.039	ns
TNFα	0.000	ns	0.002	ns	0.064	ns	0.117	ns	0.043	ns	0.060	ns	0.065	ns	0.053	ns	0.062	ns	0.000	ns	-0.035	ns	-0.095	ns	0.067	ns	0.121	*	0.149	**	0.000	ns	0.013	ns	-0.036	ns	-0.021	ns	0.047	ns	0.082	ns
sICAM-1	-0.110	*	-0.074	ns	-0.033	ns	-0.022	ns	-0.121	ns	-0.110	*	-0.119	*	-0.088	ns	-0.084	ns	-0.124	ns	-0.156	*	-0.173	**	0.002	ns	0.029	ns	-0.013	ns	-0.110	*	-0.090	ns	-0.102	*	-0.104	*	-0.059	ns	-0.051	ns
sVCAM-1	-0.104	*	-0.007	ns	-0.145	ns	-0.120	ns	-0.123	*	-0.051	ns	-0.162	*	-0.184	**	-0.117	*	-0.100	ns	-0.013	ns	0.021	ns	-0.121	ns	-0.047	ns	-0.035	ns	-0.104	*	-0.067	ns	-0.123	*	-0.022	ns	0.039	ns	0.018	ns
Resistin	-0.036	ns	0.017	ns	-0.147	ns	-0.065	ns	-0.070	ns	0.004	ns	-0.039	ns	0.022	ns	-0.059	ns	-0.093	ns	-0.002	ns	-0.067	ns	0.064	ns	-0.006	ns	0.052	ns	-0.036	ns	-0.008	ns	-0.068	ns	0.030	ns	-0.089	ns	-0.064	ns
Adiponectin	-0.146	*	-0.129	*	-0.055	ns	-0.071	ns	-0.143	*	-0.104	ns	-0.194	***	-0.192	***	-0.202	***	-0.218	***	-0.279	***	-0.289	***	-0.205	*	-0.101	ns	-0.108	*	-0.129	*	-0.114	*	-0.146	*	-0.189	***	-0.292	***	-0.284	***
Leptin	-0.161	**	0.028	ns	-0.046	ns	-0.118	ns	-0.134	*	-0.179	**	-0.130	*	-0.056	ns	-0.131	*	-0.101	ns	-0.243	***	-0.139	*	-0.162	*	0.000	ns	-0.001	ns	-0.161	**	-0.139	*	-0.115	*	-0.203	***	-0.116	ns	-0.150	*
8-iso-$PGF_{2\alpha}$	-0.032	ns	-0.012	ns	0.083	ns	0.088	ns	0.073	ns	0.116	*	0.080	ns	-0.023	ns	0.074	ns	-0.025	ns	-0.034	ns	-0.020	ns	-0.031	ns	-0.051	ns	-0.044	ns	-0.020	ns	0.010	ns	-0.051	ns	-0.047	ns	-0.043	ns	-0.024	ns
15Kelo-$PGF_{2\alpha}$	-0.048	ns	-0.028	ns	-0.050	ns	-0.067	ns	-0.034	ns	0.012	ns	-0.045	ns	-0.087	ns	-0.017	ns	-0.079	ns	-0.038	ns	-0.057	ns	-0.096	ns	0.052	ns	-0.104	ns	-0.028	ns	-0.028	ns	-0.076	ns	-0.017	ns	-0.091	ns	-0.056	ns

ns (non significant) * (p<0.05), ** (p<0.001), *** (p<0.0005), **hsCRP** (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6), **TNFα** (Tumour Necrosis Factor-α), **sICAM-1** (soluble Intracellular Adhesion Molecule-1) &
sVCAM-1 (soluble Vascular Adhesion Molecule-1); **g** (grams); **BMR** (Basal Metabolic Rate); **Total En** (Total Energy); **SFA** (Saturated Fatty Acids); **MUFA** (Monounsaturated fatty Acids); **PUFA** (Polyunsaturated Fatty Acids); **%En** (percentage energy)
8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F2alpha) ; **15-keto-$PGF_{2\alpha}$** (15-kelo-prostaglandin-F2alpha)
C8:0 (Caprylic Acid); **C10:0** (Capric Acid); **C12:0** (Lauric Acid); **C14:0** (Mysteric Acid); **C16:0** (Palmitic Acid); **C16:1** (Palmitoleic Acid); **C18:0** (Stearic Acid); **C18:1** (Oleic Acid); **C18:2:6** (Linoleic Acid); **C20:4:6** (Arachidonic Acid); **C20:5:6** (EPA); **C22:6:3** (DHA)

Table 5.11a: Correlations between inflammatory biomarkers v's dietary fatty acids at post-dietary intervention, according to dietary treatment

Table 5.11a (1): Correlations between post-intervention IL-6 and dietary fatty acids, across the dietary treatments

	IL-6							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C8:0	0.218	ns	0.216	ns	-0.101	ns	-0.160	ns
C10:0	0.477	**	0.299	*	-0.054	ns	-0.048	ns
C12:0	-0.193	ns	-0.017	ns	-0.323	*	-0.127	ns
C20:4:6	0.201	ns	0.285	*	0.166		0.109	ns
Total Fat	-0.147	ns	-0.001	ns	-0.206	*	-0.156	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11a (2): Correlations between post-intervention TNFa and dietary fatty acids, across the dietary treatments

	TNFα							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C20:5:3	0.186	ns	0.106	ns	-0.025	ns	0.259	*
C22:6:3	0.213	ns	0.135	ns	0.023	ns	0.245	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11a (3): Correlations between post-intervention sICAM-1 and dietary fatty acids, across the dietary treatments

	sICAM-1							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C18:3:3	-0.128	ns	-0.190	ns	-0.198	ns	-0.138	ns
MUFA	-0.077	ns	-0.036	ns	-0.187	ns	-0.051	ns
PUFA	-0.083	ns	-0.018	ns	-0.161	ns	-0.078	ns
Total Fat	-0.128	ns	0.010	ns	-0.170	ns	-0.096	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11a (4): Correlations between post-intervention sVCAM and dietary fatty acids, across the dietary treatments

	sVCAM-1							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
C12:0	-0.166	ns	-0.156	ns	0.004	ns	0.064	ns
C16:0	-0.224	ns	-0.187	ns	-0.203	ns	-0.069	ns
C16:1	-0.147	ns	-0.166	ns	-0.218	ns	-0.182	ns
C18:0	-0.119		-0.179	ns	-0.178	ns	0.033	ns
MUFA	-0.218	*	-0.091	ns	-0.230	*	-0.081	ns
Total Fat	-0.165	ns	-0.069	ns	-0.199	*	-0.040	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11a (5): Correlations between post-intervention Adiponectin and dietary fatty acids, across the dietary treatments

	Adiponectin							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Total Fat_A	-0.102	ns	-0.206	*	-0.185	ns	-0.105	ns
MUFA	-0.009	ns	-0.124	ns	-0.136	ns	-0.284	*
SAFA	-0.119	ns	-0.078	ns	-0.194	*	-0.123	ns
PUFA	-0.092	ns	-0.124	ns	-0.206	*	-0.287	*
PUFA % En	-0.121	ns	-0.230	*	-0.203	*	-0.213	*
n-3	-0.286	*	-0.216	ns	-0.334	*	-0.311	*
n-6	-0.264	*	-0.277	*	-0.248	*	-0.351	*
MUFA % En	0.065	ns	-0.214	*	-0.104	ns	-0.175	ns
Total Fat	-0.046	ns	-0.068	ns	-0.190	ns	-0.245	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.11a (6): Correlations between post-intervention Leptin and dietary fatty acids, across the dietary treatments

	Leptin							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Total Fat	-0.291	*	-0.093	ns	-0.167	ns	-0.272	*
Total Fat_A	0.000	ns	0.184	ns	-0.089	ns	0.003	ns
MUFA	-0.243	*	-0.061	ns	-0.117	ns	-0.254	*
SAFA	-0.336	**	-0.087	ns	-0.155	ns	-0.262	*
PUFA	-0.180	ns	-0.215	*	-0.148	ns	-0.315	*
n-6	-0.109	ns	-0.199	ns	-0.133	ns	-0.220	ns
C12:0	-0.382	*	-0.193	ns	-0.032	ns	-0.115	ns
C14:0	-0.243	*	-0.313	*	-0.274	*	-0.188	ns
C18:0	-0.177		-0.284	*	-0.144	ns	-0.138	ns
C18:2:6	-0.237	*	-0.350	**	-0.181	ns	-0.260	*
C18:3:3	-0.161	ns	-0.118	ns	-0.117	ns	-0.150	ns
C20:4:6	-0.041	ns	-0.255	ns	-0.179	ns	-0.148	ns
Total Energy	-0.271	**	-0.155	ns	-0.102	ns	-0.299	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

5.4.21 Univariate correlations to determine factors influencing anthropometric measurements in the metabolic syndrome

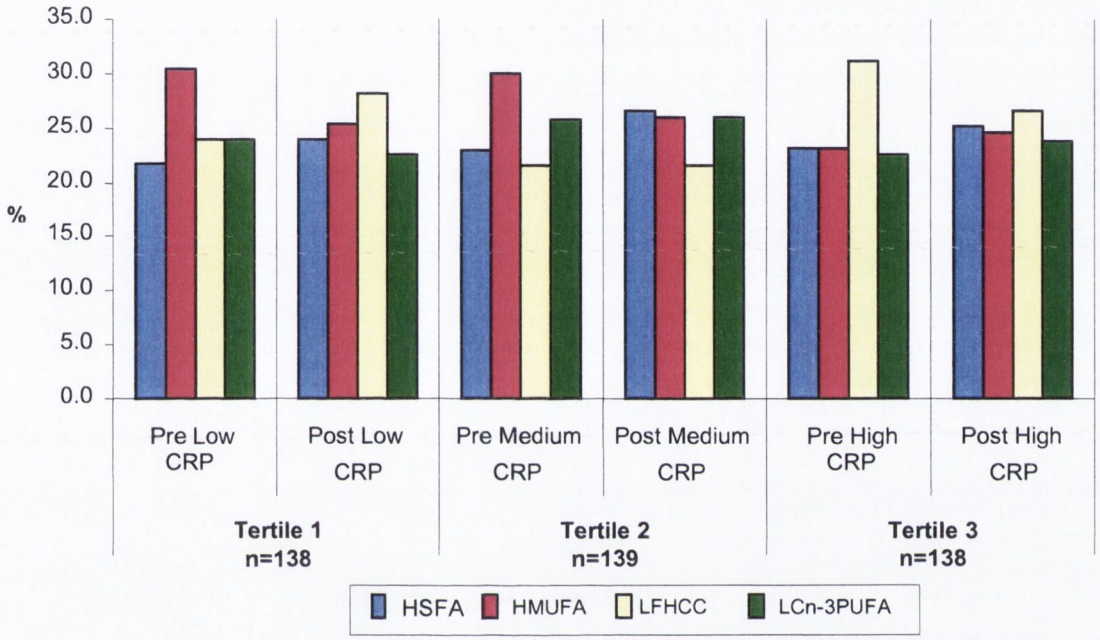
On analysis with anthropometric measurements, stronger relationships were identified; however these strong correlations were all associated with post-intervention leptin concentrations (*Table 5.12)*. These positive relationships identified were waist circumference (r=0.227, P=0.001), hip circumference (r=0.587, P=0.0005), body fat percentage (r=0.638, P=0.0005) and body fat (r=0.613, P=0.0005) Contrary to this, several very strong negative relationships were also identified, namely lean body mass percentage (r=-0.650, P=0.0005) and lean body mass kg (r=-0.356, P=0.0005). *Table 5.12a* presents the many correlations identified across the four dietary treatments, the more interesting significances being for waist circumference that was found to be strongly correlated within the HSFA, HMUFA and LFHCC diets (r=0.312, P=0.001; r=0.222, P=0.05 and r=0.202, P=0.05, respectively). Hip circumference, body fat percentage and body fat were positively and strongly correlated with leptin across the four dietary treatments. On the other hand, lean body mass kg, lean body mass percentage and body water percentage were very powerfully and negatively correlated with leptin across the dietary treatments.

5.4.22 Ranking of volunteers according to their pre-intervention CRP concentrations as a method to defining inflammatory status

As a method of characterizing the cohort according to inflammatory status, the data was subdivided according to pre-intervention CRP concentrations. This created low, medium and high tertiles of pre-intervention concentrations in the ranges of between 0.09-2.64mg/L, 2.65-4.38mg/L and ≥4.39 mg/L, respectively. Within the low, medium and high CRP tertiles, there were 138, 139 and 138 subjects in each group, respectively. Cross-tabulation analysis was performed to demonstrate that no significant differences exist for the number of individuals assigned to the four dietary treatments across the CRP tertiles as displayed in *Figure 5.2*. Therefore, from the outset of this analysis no bias was created. RM-ANOVA was used to determine noteworthy effects of the pre-intervention CRP tertiles. Diet and pre-intervention CRP tertiles were included as between subject factors. Statistical differences between all interactions (time*PreCRPtertiles, time*diet*PreCRPtertiles) were assessed by *post hoc* analysis and Bonferroni adjustments of the P values were accepted. One-way ANOVA analysis was performed to compare the variance across the CRP tertiles on the inflammatory

data, IVGTT parameters, irrespective and according to diet as presented in *Table 5.13* and *Table 5.14.*

Figure 5.2: Frequency of volunteers randomised per dietary at pre and post-intervention according to the pre-intervention CRP tertiles



5.4.23 Associations between pre-intervention CRP tertiles and inflammatory markers

Table 5.13 displays the data for the inflammatory markers across the pre-intervention CRP tertiles irrespective of dietary treatment, no statistical differences were noted when a RM-ANOVA. When a one-way ANOVA were performed, pre-intervention and post-intervention IL-6 levels were found to be greatly increased in the low CRP tertile compared to the medium and high CRP tertiles. Additionally, the pre- and post-intervention leptin concentrations were found to be significantly higher in the medium and high CRP tertiles compared to the low-CRP tertile.

5.4.24 Associations between pre-intervention CRP tertiles and IVGTT markers

Table 5.14 displays the data for the IVGTT parameters across the pre-intervention CRP tertiles irrespective of dietary treatment, no statistical differences were noted when a RM-ANOVA or a one-way ANOVA were performed. When CRP was divided into tertiles, no statistically differences were noted for the IVGTT parameters across the diets within the low, medium and high pre-CRP tertiles.

Table 5.12: Correlation matrix showing the significant and non-significant relationships between inflammatory and oxidative stress markers v's anthropometric measurements, irrespective of dietary treatment (n=417)

Post Parameters	Body Weight		Waist		Hip		Diastolic BP		Systolic BP		Body Fat%		Body FatKg		Lean BodyMass %		Lean BodyMassKg		Body Water %		Body Water Ltrs		BIA Impedance		BMR	
	r		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*		*r*	
CRP	0.006	ns	-0.037		-0.030	ns	-0.034	ns	-0.093	ns	-0.075	ns	-0.033	ns	0.116	*	0.063	ns	0.099	ns	0.082	ns	0.006	ns	-0.008	ns
IL6	0.023	ns	0.006		-0.042	ns	-0.004	ns	-0.018	ns	-0.055	ns	-0.041	ns	0.135	*	0.098	ns	0.131	*	0.081	ns	0.174	*	0.028	ns
TNFa	0.086	ns	0.094		0.107	*	0.125	*	0.082	ns	0.027	ns	0.052	ns	-0.060	ns	0.040	ns	-0.003	ns	0.066	ns	0.183	**	0.032	ns
sICAM-1	0.140	**	0.140	**	0.142	ns	0.088	ns	0.078	ns	-0.013	ns	0.046	ns	-0.012	ns	0.007	ns	-0.025	ns	0.005	ns	0.092	ns	0.031	ns
sVCAM-1	-0.046	ns	0.008		0.008	ns	0.025	ns	0.019	ns	0.079	ns	0.054	ns	-0.036	ns	-0.059	ns	-0.012	ns	-0.035	ns	0.061	ns	-0.087	ns
Resistin	0.052	ns	0.040		0.082	ns	0.031	ns	-0.023	ns	0.006	ns	0.020	ns	-0.004	ns	0.060	ns	0.017	ns	0.053	ns	0.028	ns	0.046	ns
Adiponectin	-0.086	ns	-0.114	*	0.150	*	-0.097	*	-0.080	ns	0.093	ns	0.024	ns	-0.153	*	-0.167	**	-0.168	**	-0.176	**	0.067	ns	-0.115	*
Leptin	0.085	ns	0.227	**	0.587	***	0.047	ns	0.032	ns	0.638	***	0.613	***	-0.650	***	-0.356	***	-0.665	***	-0.367	***	0.194	**	-0.243	***
8-iso-$PGF_{2\alpha}$	-0.037	ns	-0.029		0.031	ns	0.043	ns	0.070	ns	0.038	ns	0.024	ns	-0.090	ns	-0.060	ns	-0.095	ns	-0.067	ns	-0.035	ns	-0.048	ns
15Keto-$PGF_{2\alpha}$	-0.059	ns	-0.052		-0.023	ns	-0.028	ns	-0.028	ns	0.072	ns	0.034	ns	-0.040	ns	-0.065	ns	-0.031	ns	-0.041	ns	-0.043	ns	-0.012	ns

ns (non significant) * ($p<0.05$), ** ($p<0.001$), *** ($p<0.0005$), **hsCRP** (high sensitivity C-Reactive Protein); **IL-6** (Interleukin-6), **TNF**α (Tumour Necrosis Factor-α), **sICAM-1** (soluble Intracellular Adhesion Molecule-1) & **sVCAM-1**(soluble Vascular Adhesion Molecule-1); **BIA** (Bioelectrical Impedance); **BP** (Blood Pressure); **Ltrs** (Litres)

8-Iso-$PGF_{2\alpha}$ (8-iso-prostaglandin F2alpha); 15-keto-$PGF_{2\alpha}$ (15-keto-prostaglandin-F2alpha)

Table 5.12a: Correlations between inflammatory and anthropometric measurements at post-dietary intervention, according to dietary treatment

Table 5.12a (1): Correlations between post-intervention hsCRP and anthropometic measurements, across the dietary treatments

	CRP							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Lean Body Mass %	0.029	ns	0.118	ns	0.111	ns	0.228	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.12a (2): Correlations between post-intervention IL-6 and anthropometic measurements, across the dietary treatments

	IL-6							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Lean Body Mass %	-0.006	ns	0.127	ns	0.264	*	0.130	ns
Body Water %	-0.004	ns	0.204	*	0.274	*	0.037	ns
BIA - Impedance	0.159	ns	0.226	*	0.226	*	0.098	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.12a (3): Correlation between post-intervention TNFα and anthropometic measurements, across the dietary treatments

	TNFα							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Hip Circumference	0.110	ns	0.005	ns	0.093	ns	0.227	*
Diastolic Blood Pressure	0.011	ns	0.281	*	0.020	ns	0.195	*
BIA- Impedance	0.214	ns	0.323	*	0.104	ns	0.072	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.12a (4): Correlations between post-intervention sICAM-1 and anthropometic measurements, across the dietary treatments

	sICAM-1							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Body Weight	0.202	*	-0.008	ns	0.104	ns	-0.071	ns
Waist Circumference	0.202	*	0.026	ns	0.237	*	0.086	ns

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.12a (5): Correlations between post-intervention Adiponectin and anthropometic measurements, across the dietary treatments

	Adiponectin							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Waist Circumference	0.031	ns	-0.150	ns	-0.021	ns	-0.268	*
Hip Circumference	0.143	ns	0.065	ns	0.257	*	0.162	ns
Diasytolic Blood Pressure	0.013	ns	-0.134	ns	-0.153	ns	-0.075	ns
Lean Body Mass %	-0.063	ns	-0.086	ns	-0.201	*	-0.256	*
Lean Body Mass Kg	-0.101	ns	-0.176	ns	-0.150	ns	-0.243	*
Body Water %	-0.105	ns	-0.151	ns	-0.177	ns	-0.245	*
Body Water	-0.164	ns	-0.213	ns	-0.114	ns	-0.233	*
Body Water Litres	-0.164	ns	-0.213	*	-0.114	ns	-0.233	*

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.12a (6): Correlations between post-intervention Leptin and anthropometic measurements, across the dietary treatments

	Leptin							
Post Parameters	**Diet A**		**Diet B**		**Diet C**		**Diet D**	
	r		*r*		*r*		*r*	
Waist	0.312	**	0.222	*	0.202	*	0.161	ns
Hip	0.722	**	0.560	**	0.537	**	0.550	**
Body Fat %	0.675	**	0.616	**	0.579	**	0.681	**
Lean Body Mass Kg	-0.406	**	-0.268	**	-0.295	**	-0.482	**
Lean Body Mass %	-0.764	**	-0.604	**	-0.586	**	-0.671	**
Body Fat Kg	0.649	**	0.616	**	0.555	**	0.614	**
Body Water %	-0.748	**	-0.601	**	-0.606	**	-0.706	**
Body Water Litres	-0.435	**	-0.300	**	-0.284	**	-0.457	**
BIA - Impedance	0.272	**	0.175	ns	0.128	ns	0.269	*
BMR	-0.268	*	-0.172	ns	-0.177	ns	-0.357	**

ns (non significant),* (p<0.05), ** (p<0.001), *** (p<0.0005)

Table 5.13: Mean(±SEM) of the inflammatory markers according to the hsCRP tertiles irrespective of diet

	Tertile 1: Low CRP n=138		Tertile 2: Medium CRP n=139		Tertile 3: High CRP n=138		*Statistical Tests*	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**RM-ANOVA**	***ANOVA***
Pre CRP	1.43 [a]	0.06	4.06 [b]	0.08	9.86 [c]	0.32	[ns]	*p=0.0005*
Post CRP	2.52 [a]	0.20	4.50 [b]	0.21	7.62 [c]	0.38	[ns]	*p=0.0005*
Pre IL-6	5.90 [a]	0.38	4.52 [b]	0.35	4.63 [b]	0.32	[ns]	*p=0.008*
Post IL-6	5.66 [a]	0.44	4.13 [b]	0.28	4.48 [b]	0.29	[ns]	*p=0.035*
Pre TNFα	5.14 [ns]	0.49	5.67 [ns]	0.46	4.70 [ns]	0.25	[ns]	*† ns*
Post TNFα	4.34 [a]	0.42	5.08 [b]	0.31	4.76 [ab]	0.31	[ns]	*† p=0.013*
Pre sICAM-1	275.18 [a]	6.16	283.45 [ab]	6.04	296.84 [b]	6.55	[ns]	*p=0.04*
Post sICAM-1	277.60 [ns]	6.28	283.01 [ns]	6.00	295.86 [ns]	6.70	[ns]	*ns*
Pre sVCAM-1	598.03 [ns]	15.50	589.08 [ns]	14.23	619.76 [ns]	13.58	[ns]	*ns*
Post sVCAM-1	610.84 [ns]	15.60	585.58 [ns]	14.54	635.71 [ns]	15.63	[ns]	*ns*
Pre Resistin	9607.78 [ns]	424.14	9970.63 [ns]	432.72	10827.19 [ns]	412.92	[ns]	*† ns*
Post Resistin	9901.76 [ab]	489.59	9721.32 [b]	504.77	10657.86 [a]	395.33	[ns]	*† p = 0.012*
Pre Adiponectin	3.45 [ns]	0.21	3.61 [ns]	0.16	3.99 [ns]	0.24	[ns]	*† ns*
Post Adiponectin	3.53 [ns]	0.15	3.89 [ns]	0.19	4.23 [ns]	0.28	[ns]	*† ns*
Pre Leptin	14.41 [a]	1.21	23.46 [b]	1.66	28.25 [b]	2.09	[ns]	*p=0.0005*
Post Leptin	13.46 [a]	1.15	23.90 [b]	1.98	26.88 [b]	1.94	[ns]	*p=0.0005*

[a,b,c]... denotes significance across centres (when 2 superscripts contain the same letter there is no significant difference between 2 of 8 categories); **†** NPara Kruskal-Wallis ; **ANOVA** (Analysis of Variance); [ns] (non significant)

CRP (C-reactive protein); **IL-6** (Interleukin-6); **TNFα** (Tumour Necrosis Factor-α);

sICAM-1 (soluble Adhesion Molecule-1); **sVCAM-1** (soluble Vascular Adhesion Molecule-1)

Tertile Ranges: Low CRP tertile (0.09 - 2.69mg/L); **Medium CRP tertile** (2.65 - 4.38mg/L); **High CRP tertile** (≥4.39mg/L)

Table 5.14: Mean(±SEM) of the IVGTT parameters according to the hsCRP tertiles, irrespective of diet

	Tertile 1: Low CRP n=137		Tertile 2: Medium CRP n=138		Tertile 3: High CRP n=134		*Statistical Tests*	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	***RM-ANOVA***	***ANOVA***
Pre S_I	2.96	0.18	2.79	0.18	2.87	0.17	*ns*	*ns*
Post S_I	3.14	0.29	2.68	0.13	2.83	0.15	*ns*	*ns*
Pre S_G	0.02	0.00	0.02	0.00	0.02	0.00	*ns*	*† ns*
Post S_G	0.02	0.00	0.02	0.00	0.02	0.00	*ns*	*† ns*
Pre AIRg	386.19	30.15	401.02	42.42	328.88	26.95	*ns*	*† ns*
Post AIRg	407.23	32.42	395.79	44.26	360.60	29.17	*ns*	*† ns*
Pre DI	927.50	67.79	908.90	68.27	847.76	69.31	*ns*	*ns*
Post DI	1054.06	91.58	891.46	71.20	905.35	68.42	*ns*	*ns*
Pre G0	292.07	3.59	301.82	3.61	298.27	4.32	*ns*	*† ns*
Post G0	299.79	4.24	313.36	13.46	292.73	3.56	*ns*	*† ns*
Pre P2	0.04	0.00	0.04	0.00	0.04	0.00	*ns*	*† ns*
Post P2	0.04	0.00	0.07	0.02	0.05	0.01	*ns*	*† ns*
Pre P3	0.00	0.00	0.00	0.00	0.00	0.00	*ns*	*† ns*
Post P3	0.00	0.00	0.00	0.00	0.00	0.00	*ns*	*† ns*
Pre Fasting Glucose	5.97	0.07	5.89	0.07	6.02	0.07	*ns*	*ns*
Post Fasting Glucose	6.01	0.10	5.97	0.09	5.95	0.08	*ns*	*ns*
Pre Fasting Insulin	10.18	0.48	9.78	0.45	10.19	0.44	*ns*	*† ns*
Post Fasting Insulin	9.86	0.55	9.44	0.51	9.97	0.44	*ns*	*† ns*
Pre $HOMA_{IR}$	2.74	0.15	2.57	0.12	2.75	0.13	*ns*	*† ns*
Post $HOMA_{IR}$	2.69	0.19	2.54	0.15	2.66	0.13	*ns*	*† ns*
Pre QUICKI	0.59	0.01	0.57	0.04	0.64	0.05	*ns*	*† ns*
Post QUICKI	0.61	0.01	0.63	0.01	0.58	0.02	*ns*	*† ns*
Pre FPIR	40.72	2.84	43.58	4.35	34.04	2.69	*ns*	*† ns*
Post FPIR	41.85	3.03	42.76	4.52	37.96	3.06	*ns*	*† ns*

† NPara Krausal Wallis; **ns** (non significant); **ANOVA** (Analysis of Variance); **CRP** (C-reactive protein)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI));

G0 (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **P3** (Movement of circulating insulin to interstitial space);

HOMA-IR (Homeostatis Model Assessment); **QUICKI** (Quantitiative Insulin Sensitive Check Index); **FPIR** (First Phase Insulin Response)

Tertile Ranges: Low CRP tertile (0.09 - 2.69mg/L); **Medium CRP tertile** (2.65 - 4.38mg/L); **High CRP tertile** (≥4.39mg/L)

5.4.25 Linear regression analysis to determine the predictors of post-intervention insulin sensitivity (S_I), irrespective of dietary treatment

Table 5.15 presents the independent relationship of each variable to S_I measured at post-intervention, using univariate and multivariate regression analysis. Although many variables were significant predictors of post-intervention S_I, some failed to be of note in subsequent multiple linear regression analysis, controlling for age, gender and post-intervention body weight. In particular, pre-intervention G0, pre- and post-intervention body fat (kg), post-intervention systolic blood pressure, post-intervention TRL TAG and total HDL, pre-intervention plasma palmitic acid (C16:0), BMR, total fat, total MUFA, caprylic acid (C8:0), oleic acid (C18:1), starch and pre-intervention BMR . Interestingly, diet had no predictive impact on S_I measured at post-intervention.

Table 5.16 shows seven separate models for which multiple linear regression analysis was performed on each, using the dependent variable S_I post-intervention. In the IVGTT parameters model, pre-intervention S_G, DI, G0, QUICKI, Revised QUICKI and AIRg and post-intervention QUICKI were shown to be the strongest significant predictors of S_I post-intervention. Increasing S_G and DI pre-intervention were inversely associated with S_I post-intervention and increasing G0, Revised QUICKI, AIRg pre-intervention and QUICKI pre- and post-intervention were positively associated with S_I post-intervention.

In the anthropometric model, increasing body weight, waist circumference and percentage body water post-intervention were inversely associated with S_I post-intervention ($p<0.05$). Increasing hip circumference was an important positive predictor of post-intervention S_I ($p<0.05$). In the coagulation factors model, post-intervention body weight and tPA concentrations were considerable predictors, negatively associated with post-intervention S_I. In the inflammatory markers model, increasing age, post-intervention body weight and sICAM-1 and in the lipid markers model, increasing age, post-intervention body weight and pre-intervention NEFA concentrations were negatively and significantly associated with S_I post-intervention ($p<0.05$). In the plasma FA model, plasma EPA (C20:5:3) pre-intervention was the only important predictor, positively associated with S_I post-intervention. Finally, from the dietary variables model, from the 3-day weighed food diaries pre- and post-intervention, body weight and TFA post-intervention and starch pre-intervention were the only significant predictors ($p<0.05$).

Table 5.17 illustrates the main predictors of S_I post-intervention. The model had a high adjusted r^2 (99.8%), signifying an excellent fit for the population. G0 and S_G pre-intervention were the strongest predictors for S_I post-intervention. Increasing G0 was a positive predictor whilst increasing S_G showed an inverse association with S_I post-intervention. G0 post intervention was a significant albeit a weak predictor of S_I post-intervention Additionally, and as expected increasing QUICKI values pre- and post-intervention and Revised QUICKI pre-intervention were associated with an increase in post-intervention S_I values. Volunteers in the UoR, UM and UU cohorts were associated with a lower S_I post-intervention, compared to volunteers in TCD ($p<0.05$). In contrast, volunteers in the UO, HURS-UCO and JUMC cohorts were associated with an increase in S_I post-intervention. Increasing age and body weight post-intervention were negatively associated with S_I at post-intervention. Sex was a important predictor of S_I at post-intervention in the LIPGENE study. Other strong positive and major predictors in the final model were DI post-intervention, fasting insulin, AIRg and plasma concentrations of EPA (C20:5:3) pre-intervention. However, DI pre-intervention and fasting insulin post-intervention were negatively associated with S_I post-intervention. Other IVGTT parameters inversely associated with S_I at post-intervention were P2 and fasting glucose, pre-and post-intervention. Non-smokers compared to smokers and those volunteers that participate in sporting activities compared to those who do not, were significant negative predictors of S_I at post-intervention. From the dietary variables, TFA (g/d) were negatively associated with S_I post-intervention.

5.4.26 Linear regression analysis to determine the predictors of post-intervention S_I, according to dietary treatment

Table 5.18 presents the results of the final models with the main predictors of S_I post-intervention, in each of the four dietary groups, controlling for age, gender and body weight post-intervention. The same process was applied as above (*Tables 1 and 2 of the model process not shown*). However, some variables that were not significant in the prediction of S_I post-intervention, irrespective of diet, became noteworthy when assessed according to diet. For example, tPA concentration post-intervention was a weak, but positive predictor of S_I post-intervention, irrespective of diet. Interestingly, concentrations of tPA at pre-intervention were inversely related to S_I post-intervention in HSFA diet. Also in the HSFA diet, FPIR and insulin/c-peptide ratio post-intervention, were negatively associated with S_I post-intervention. While Arachidonic acid (AA, C20:4:6) was the only plasma FA positively predicting S_I post-

intervention in HSFA diet. In both the analysis irrespective of diet and in the HSFA, both fasting glucose pre-intervention was negatively and G0 post-intervention was positively associated with S_I post-intervention. This model also had a high adjusted r^2 (74.3%).

In both the analysis, irrespective of diet and according to the HMUFA diet, DI was a strong positive predictor of S_I, while post-intervention G0 showed an inverse association with S_I. FPIR pre-and post-intervention was negatively associated with S_I post-intervention whereas S_I pre-intervention and volunteers in the JUMC cohort were associated with an increase in S_I post-intervention as compared to volunteers in the TCD cohort, in the HMUFA diet. The adjusted r^2 of the model was 0.61.

Interestingly, increasing body weight negatively associated with S_I post-intervention was only apparent in the final prediction model of the LFHCC diet. In addition, post-intervention DI and pre-intervention revised QUICKI were positively associated with S_I post-intervention in the LFHCC diet. In the previous steps of the final process model, volunteers with a total fat intake >35.98% pre-intervention showed an inverse association with S_I post-intervention and this was also a predictor of S_I post-intervention in the final model. However, when entered univariately and multivariately, controlling for age, gender and body weight post-intervention, volunteers with a total fat intake <35.98% pre-intervention were positively assocatied with post-intervention S_I. When this was entered with the other important predictors at the next stage, the significance were retained, however, the direction of the influence changed to a negative association which was maintained in the final prediction model. The dietary variables in the LFHCC diet demonstrate that energy from CHO and fibre intake was positively and negatively associated with S_I post-intervention, respectively. The adjusted r^2 of the model for the LFHCC diet was 0.72.

Finally, the main predictors for S_I post-intervention in the LFn-3PUFA diet were decreasing age, P2 and FPIR post-intervention and insulin/c-peptide ratio pre-intervention. Increasing DI, QUICKI values and adiponectin concentration post-intervention were positively associated with S_I post-intervention. In the LFn-3PUFA diet, the volunteers who do not participate in sport compared to who do demonstrated a negative predictor of S_I post-intervention.

Table 5.15: Model 1 showing the relationship of each of the individual variables to S_I measured at post-intervention using univariate and multivariate (controlling for age, gender & post body weight) linear regression analysis

	Post S_I					Post S_I					Post S_I			
	Univariate		Multivariate (age, gender & body weight)			Univariate		Multivariate (age, gender & body weight)			Univariate		Multivariate (age, gender & body weight)	
	β	sig	β	sig		β	sig	β	sig		β	sig	β	sig
Age (years)	+	0.88	na	na	**Inflammatory markers**					Pre PUFA (%En)	+	0.94	+	0.50
Gender (male/female)	+	0.00	na	na	Pre CRP (mg/L)	-	0.03	-	0.13	Pre n-3 (g/d)	+	0.78	+	0.10
Ethnicity (Caucasian/Asian)					Pre IL-6 (pg/ml)	+	0.43	+	0.39	Pre n-6 (g/d)	-	0.78	+	0.44
TCD v UoR	+	0.76	+	0.52	PreTNFα (pg/ml)	+	0.31	+	0.20	Pre C8:0 (g/d)	-	0.13	-	0.32
TCD v UO	+	0.40	+	0.24	Pre sICAM-1(ng/ml)	-	0.01	-	0.01	Pre C10:0 (g/d)	-	0.84	+	0.56
TCD v INSERM	+	0.44	+	0.92	Pre sVCAM-1(ng/ml)	-	0.53	-	0.52	Pre C12:0 (g/d)	+	0.28	+	0.07
TCD v UM	+	0.89	+	0.54	Pre Resistin (pg/mL)	-	0.30	-	0.37	Pre C14:0 (g/d)	+	0.50	+	0.07
TCD v HURS-UCO	-	0.24	-	0.29	Pre Adiponectin (mg/L)	+	0.06	+	0.12	Pre C16:0 (g/d)	-	0.70	+	0.17
TCD v JUMC	+	0.23	+	0.08	Pre Leptin (ng/ml)	-	0.02	-	0.02	Pre C16:1 (g/d)	-	0.36	+	0.46
TCD v UU	+	0.10	+	0.22	Post CRP (mg/L)	-	0.23	-	0.31	Pre C18 (g/d)	-	0.66	+	0.36
Change of Body Weight (kg)	-	0.14	-	0.14	Post IL-6 (pg/ml)	-	0.81	-	0.87	Pre C18:1 (g/d)	-	0.07	-	0.60
DIETARY DATA					Post TNFα (pg/ml)	-	0.40	-	0.91	Pre C18:2:6 (g/d)	-	0.90	+	0.28
Diet A v Diet B	+	0.98	+	0.84	Post sICAM-1 (ng/ml)	-	0.00	-	0.00	Pre C18:3:3 (g/d)	+	0.69	+	0.20
Diet A v Diet C	-	0.61	-	0.73	Post sVCAM-1 (ng/ml)	-	0.35	-	0.29	Pre C20:4:6 (g/d)	-	0.90	+	0.34
Diet A v Diet D	-	0.19	-	0.22	Post Resistin (pg/mL)	-	0.18	-	0.24	Pre C20:5:3 (g/d)	-	0.71	+	0.62
Pre-intervention Biochemistry					Post Adiponectin (mg/L)	+	0.03	+	0.08	Pre C22:6:3 (g/d)	-	0.91	+	0.42
Pre S_I (mU/L)-1min-1	+	0.00	+	0.00	Post Leptin (ng/ml)	-	0.01	-	0.01	Pre *trans* FA (g/d)	-	0.40	-	0.53
Pre $S_{I\ min^{-1}}$	+	0.01	+	0.02	**Post Biochemistry**					Pre CHO (g/d)	-	0.48	+	0.43
Pre AIRg mU/L^{-1} min^{-1}	-	0.00	-	0.00	Pre TAG (mmol/L)	-	0.02	-	0.01	Pre CHO (%En)	+	0.37	+	0.67
Pre DI	+	0.00	+	0.00	Pre Cholesterol (mmol/L)	+	0.38	+	0.98	Pre Starch (g/d)	-	0.01	-	0.28
Pre G0 mg/dl^{-1}	-	0.07	-	0.31	Pre NEFA (μmol/L)	-	0.07	-	0.01	Pre Total Sugars (g/d)	-	0.45	-	0.83
Pre P2 min^{-1}	+	0.01	+	0.13	Pre TRL TAG (mmol/L)	-	0.05	-	0.11	Pre Protein (g/d)	-	0.21	+	0.66
Pre P3 (mU/L) min^{-2}	+	0.00	+	0.00	Pre TRL-c (mmol/L)	-	0.39	-	0.42	Pre Protein (%En)	-	0.82	-	0.61
Pre Fasting Glucose (mmol/l)	-	0.00	-	0.00	Pre LDL-c (mmol/L)	+	0.52	+	0.71	Pre Alcohol (g/d)	+	0.42	+	0.03
Pre Fasting Insulin(uIU/ml)	-	0.00	-	0.00	Pre Total HDL-c (mmol/L)	+	0.00	+	0.08	Pre Alcohol (%En)	+	0.23	+	0.01
Pre $HOMA_{IR}$	-	0.00	-	0.00	Pre Apo A1(g/L)	+	0.17	+	0.79	Pre Fibre (g)	+	0.93	+	0.31
Pre QUICKI	+	0.00	+	0.00	Pre Apo B (g/L)	-	0.44	-	0.40	Pre Vitamin E (mg/d)	-	0.20	-	0.67
Pre FPIR (uIU/ml)	-	0.00	-	0.00	Pre Apo CII (mg/L)	-	0.21	-	0.08	Post BMR (MJ/d)	-	0.00	+	0.54
Post $S_{I\ min^{-1}}$	+	0.90	-	0.84	Pre Apo CIII (mg/L)	-	0.09	-	0.02	Post Total Energy (MJ)	-	0.22	+	0.79
Post AIRg mU/L^{-1} min^{-1}	-	0.00	-	0.00	Pre Apo E (mg/L)	-	0.29	-	0.12	Post Total fat (g/d)	-	0.21	+	0.97
Post DI	+	0.00	+	0.00	Pre Apo B48 (mg/L)	+	0.79	+	0.77	Post Total fat (%En)	-	0.75	-	0.92
Post G0 mg/dl^{-1}	-	0.00	-	0.00	Pre TRL ApoB (mg/L)	+	0.18	+	0.06	Post SAFA (g/d)	-	0.61	+	0.70
Post P2 min^{-1}	-	0.01	-	0.01	Post TAG (mmol/L)	-	0.01	-	0.02	Post SAFA (%En)	+	0.58	+	0.52
Post P3 (mU/L) min^{-2}	+	0.00	+	0.00	Post Cholesterol (mmol/L)	+	0.93	-	0.55	Post MUFA (g/d)	-	0.26	+	0.99
Post Fasting Glucose (mmol/l)	-	0.00	-	0.00	Post NEFA (μmol/L)	-	0.30	-	0.08	Post MUFA (%En)	-	0.78	-	0.79
Post Fasting Insulin(uIU/ml)	-	0.00	-	0.00	Post TRL TAG (mmol/L)	-	0.06	-	0.17	Post PUFA (g/d)	-	0.41	+	0.88
Post $HOMA_{IR}$	-	0.00	-	0.00	Post TRL-c (mmol/L)	-	0.49	-	0.70	Post PUFA (%En)	-	0.90	+	0.95
Post QUICKI	+	0.00	+	0.00	Post LDL-c (mmol/L)	-	0.37	-	0.37	Post LC n-3 PUFA (g/d)	+	0.96	+	0.52
Post FPIR (uIU/ml)	-	0.00	-	0.00	Post Total HDL-c (mmol/L)	+	0.01	+	0.16	Post LC n-6 PUFA (g/d)	-	0.53	+	0.76
Pre C-peptide ng/ml	-	0.00	-	0.00	Post Apo A1(g/L)	+	0.15	+	0.74	Post C8 (g/d)	+	0.83	+	0.94
Post C-peptide ng/ml	-	0.00	-	0.00	Post Apo B (g/L)	-	0.32	-	0.21	Post C10 (g/d)	-	0.88	+	1.00
Pre Insulin/c-peptide	-	0.00	-	0.00	Post Apo CII (mg/L)	-	0.40	-	0.25	Post C12 (g/d)	+	0.41	+	0.44
Post Insulin/c-peptide	-	0.00	-	0.00	Post Apo CIII (mg/L)	-	0.09	-	0.02	Post C14 (g/d)	+	0.34	+	0.12
Lifestyle Factors					Post Apo E (mg/L)	-	0.24	-	0.10	Post C16 (g/d)	-	0.82	+	0.34
Smoke (Yes v no)	-	0.07	-	0.07	Post Apo B48 (mg/L)	+	0.95	+	0.77	Post C18:1 (g/d)	-	0.52	+	0.47
Play sports (Yes v No)	-	0.05	-	0.03	Post TRL ApoB (mg/L)	+	0.43	+	0.26	Post C18 (g/d)	-	0.93	+	0.30
Pre Revised QUICKI	+	0.00	+	0.00	**Post Fatty Acids**					Post C18:1 (g/d)	+	0.93	+	0.30
Post Revised QUICKI	+	0.00	+	0.00	Pre C14:0 (%)	-	0.55	+	0.88	Post C18:2:6 (g/d)	+	0.99	+	0.19
Pre Total Fat >35.98% Total En	-	0.85	-	0.83	Pre C16:0 (%)	-	0.12	-	0.26	Post C18:3:3 (g/d)	+	0.80	+	0.37
Pre Total Fat <35.98% Total En	+	0.87	+	0.80	Pre C16:1(%)	+	0.83	+	0.70	Post C20:4:6 (g/d)	-	0.90	+	0.45
Anthropometric measurements					Pre C18:0 (%)	-	0.80	-	0.48	Post C20:5:3 (g/d)	+	0.80	+	0.52
Height (m)	-	0.47	+	0.00	Pre C18:1 (%)	-	0.03	-	0.03	Post C22:6:3 (g/d)	+	0.39	+	0.22
Pre Body weight (Kg)	-	0.00	+	0.13	Pre C18:2:6 (%)	+	0.01	+	0.05	Post *trans* FA (g/d)	-	0.17	-	0.07
Pre Waist circumference(cm)	-	0.00	-	0.01	Pre C20:4:6 (%)	+	0.39	+	0.31	Post CHO (g/d)	-	0.67	+	0.57
Pre Hip circumference (cm)	-	0.01	+	0.42	Pre C20:5:3 (%)	+	0.02	+	0.01	Post CHO (%En)	+	0.26	+	0.66
Pre Diastolic BP (mmHg)	-	0.24	+	0.94	Pre C22:6:3 (%)	+	0.71	+	0.82	Post Starch (g/d)	-	0.12	-	0.41
Pre Systolic BP (mmHg)	-	0.31	-	0.59	Pre SFA (%)	-	0.17	-	0.29	Post total Sugars (g/d)	-	0.57	-	0.46
Pre Body Fat (%)	-	0.67	-	0.40	Pre MUFA (%)	-	0.06	-	0.06	Post Protein (g/d)	-	0.05	-	0.57
Pre Body Fat (Kg)	-	0.00	-	0.86	Pre PUFA (%)	+	0.01	+	0.02	Post Protein (%En)	-	0.13	-	0.21
Pre Lean Body Mass (%)	+	0.39	+	0.00	Pre LC n-6 PUFA (%)	+	0.02	+	0.04	Post Alcohol (g/d)	+	0.88	+	0.28
Pre Lean Mass Body (Kg)	-	0.00	+	0.00	Pre LC n-3 PUFA (%)	+	0.12	+	0.05	Post Alcohol (%En)	+	0.99	+	0.46
Pre Body Water (%)	+	0.96	+	0.12	Post C14:0 (%)	-	0.06	-	0.10	Post Fibre (g)	+	0.36	+	0.10
Pre Body Water (Litres)	-	0.11	+	0.00	Post C16:0 (%)	-	0.07	-	0.13	Post Vitamin E (mg/d)	-	0.72	+	0.51
Pre Impedance (KHz)	+	0.01	+	0.74	Post C16:1 (%)	+	0.27	+	0.39					
Post Body weight(Kg)	-	0.00	na	na	Post C18:0 (%)	-	0.52	-	0.17					
Post Waist circumference (cm)	-	0.00	-	0.00	Post C18:1 (%)	-	0.37	-	0.59					
Post Hip circumference (cm)	-	0.00	+	0.06	Post C18:2:6 (%)	+	0.06	+	0.07					
Post Diastolic BP(mmHg)	-	0.00	-	0.05	Post C20:4:6 (%)	+	0.04	+	0.04					
Post Systolic BP(mmHg)	-	0.01	-	0.22	Post C20:5:3 (%)	+	0.36	+	0.51					
Post Body Fat (%)	+	0.97	-	0.80	Post C22:6:3 (%)	-	0.39	-	0.53					
Post Body Fat(Kg)	-	0.00	-	0.84	Post SFA (%)	-	0.03	-	0.03					
Post Lean Body Mass(%)	+	0.60	+	0.00	Post MUFA (%)	-	0.59	-	0.80					
Post Lean Mass Body(Kg)	-	0.00	+	0.00	Post PUFA (%)	+	0.02	+	0.03					
Post Body Water(%)	+	0.62	+	0.01	Post LC n-6 PUFA (%)	+	0.01	+	0.02					
Post Body Water (Litres)	-	0.01	+	0.00	Post LC n-3 PUFA (%)	-	0.73	-	0.78					
Post Impedance(KHz)	+	0.00	+	0.17	**Resting Energy Expenditure**									
Pre BMI (kg/m^2)	-	0.00	-	0.00	Pre BMR (MJ/day)	-	0.00	+	0.24					
Post BMI (kg/m^2)	-	0.00	-	0.00	Pre Total Energy (MJ)	-	0.23	+	0.36					
Pre PAI-1 ng/ml	-	0.00	-	0.00	Pre Total fat (g/day)	-	0.09	+	0.93					
Haemostatic Biochemistry					Pre Total fat (%Energy)	-	0.14	-	0.14					
Pre tPA ng/ml	-	0.00	-	0.00	Pre SAFA (g/day)	-	0.82	+	0.18					
Pre Fibrinogen mg/dl	+	0.93	+	0.83	Pre SAFA (%Energy)	+	0.37	+	0.39					
Post PAI-1 ng/ml	-	0.00	-	0.00	Pre MUFA (g/day)	-	0.02	-	0.30					
Post tPA ng/ml	-	0.00	-	0.00	Pre MUFA (%Energy)	-	0.05	-	0.05					
Post Fibrinogen mg/dl	-	0.79	+	0.96	Pre PUFA (g/day)	-	0.50	+	0.41					

*, non applicable, Sig.=Significance value, β= Beta co-efficient. ^Categorical variables with ≥3 categories for which dummy variables were created

p=0.00 = p<0.001

pre= pre-intervention; post=post-intervention

Table 5.16: Multiple linear regression predictors of S_I measured at post-intervention in LIPGENE volunteers, analysis of seven different regression models

	Post S_I			
			95% CI	
	β	*sig*	Lower	Upper
Model 1 [redacted]				
Age (years)	-0.13	0.00	-0.030	-0.016
Gender (male/female)	0.16	0.00	0.376	0.648
Post Body weight (Kg)	-0.33	0.00	-0.042	-0.032
Change of Body weight (kg)	0.02	0.30	-0.014	0.046
Smoke (Yes v no)	-0.20	0.00	-0.847	-0.589
Play sports (Yes v No)	-0.05	0.01	-0.253	-0.038
[redacted]				
TCD v UoR	0.14	0.00	0.403	0.820
TCD v UO	0.26	0.00	0.980	1.450
TCD v INSERM	0.08	0.00	0.176	0.643
TCD v UM	0.01	0.60	-0.160	0.277
TCD v HURS-UCO	0.08	0.00	0.140	0.535
TCD v JUMC	0.17	0.00	0.632	1.077
TCD v UU	-0.24	0.00	-1.458	-0.993
[redacted] (r^2=0.915, r^2=0.923)†				
Pre $S_{G\ min}^{-1}$	-0.80	0.00	-208.599	-173.979
Pre DI	-0.61	0.00	-0.001	-0.001
Post DI	0.42	0.00	0.001	0.001
Pre G0 mg/dl $^{-1}$	0.82	0.00	0.029	0.034
Post G0 mg/dl $^{-1}$	0.06	0.00	0.000	0.002
Pre P2 min^{-1}	-0.38	0.00	-21.981	-17.742
Post P2 min^{-1}	-0.08	0.00	-1.444	-0.519
Pre Fasting Glucose (mmol/l)	-0.10	0.00	-0.295	-0.089
Post Fasting Glucose (mmol/l	-0.22	0.00	-0.393	-0.247
Pre QUICKI	0.68	0.00	2.473	2.829
Pre AIRg mUL^{-1} min^{-1}	0.48	0.00	0.002	0.003
Post AIRg mUL^{-1} min^{-1}	-0.15	0.00	-0.001	0.000
Pre Revised QUICKI	0.58	0.00	42.950	51.075
Post Revised QUICKI	0.64	0.00	6.172	7.232
Pre Fasting Insulin(uIU/ml)	0.13	0.00	0.019	0.059
Post Fasting Insulin(uIU/ml)	-0.20	0.00	-0.070	-0.037
Pre C-peptide ng/ml	-0.06	0.06	-0.203	0.005
Post C-peptide ng/ml	0.04	0.21	-0.033	0.152
†variables: Pre P3, Post P3, Pre SI, Pre $HOMA_{IR}$, Post $HOMA_{IR}$, Post RQUICKI, Pre insulin to c-peptide ratio, Post insulin to c-peptide ratio, Pre and Post First Phase Insulin Response were ren from model due to their confounding effects				
Model 2 [redacted] (r^2=0.234, r^2=0.283)¥				
Age (years)	-0.01	0.84	-0.027	0.022
Gender (male/female)	0.12	0.38	-0.465	1.213
Post Body weight(Kg)	-1.31	0.00	-0.230	-0.060
Pre Waist circumference (cm)	0.03	0.80	-0.029	0.038
Pre BIA - Impedance(KHz)	-0.07	0.54	-0.005	0.003
Post Waist circumference(cm	-0.34	0.02	-0.091	-0.008
Post Hip circumference (cm)	0.03	0.88	-0.053	0.062
Post Diastolic BP(mmHg)	-0.09	0.21	-0.038	0.008
Post Systolic BP(mmHg)	0.01	0.85	-0.013	0.016
Post Body Fat(Kg)	0.07	0.59	-0.033	0.057
Post Body Water(%)	-0.78	0.00	-0.300	-0.057
Post BIA - Impedance(KHz)	0.11	0.42	-0.003	0.007
Post BMI (kg/m^2)	-0.11	0.51	-0.157	0.078
Pre Hip circumference (cm)	0.27	0.15	-0.015	0.100
Post Body Water (Litres)	1.77	0.00	0.131	0.446
¥variables: Height, Pre body weight, Pre lean body mass (%) and (kg), Pre body water (%) and (L), Post lean body mass (%) and (kg), pre body fat (kg) and Pre BMI, were removed from model due to their confounding effects				
Model 3 [redacted] r^2=0.173, r^2=0.191)				
Age (years)	-0.06	0.26	-0.030	0.008
Gender (male/female)	0.01	0.81	-0.320	0.410
Post Body weight(Kg)	-0.26	0.00	-0.043	-0.015
Pre PAI-1 ng/ml	-0.08	0.24	-0.009	0.002
Pre tPA ng/ml	-0.04	0.60	-0.076	0.044
Post PAI-1 ng/ml	-0.05	0.45	-0.008	0.004
Post tPA ng/ml	-0.19	0.02	-0.131	-0.012

	Post S_I			
			95% CI	
	β	*sig*	Lower	Upper
Model 4 [redacted] r^2=0.147, r^2=0.173)				
Age (years)	-0.10	0.07	-0.037	0.001
Gender (male/female)	0.11	0.13	-0.106	0.788
Post Body weight (Kg)	-0.28	0.00	-0.046	-0.016
Pre CRP (mg/L)	-0.07	0.20	-0.066	0.014
Pre sICAM-1(ng/ml)	0.00	0.99	-0.003	0.003
Pre Adiponectin (mg/L)	0.04	0.59	-0.065	0.114
Pre Leptin (ng/ml)	-0.04	0.68	-0.016	0.010
Post sICAM-1 (ng/ml)	-0.14	0.06	-0.006	0.000
Post Adiponectin (mg/L)	0.07	0.31	-0.042	0.131
Post Leptin (ng/ml)	-0.10	0.27	-0.020	0.006
Model 5 [redacted] r^2=0.174, r^2=0.218)				
Age (years)	-0.14	0.01	-0.043	-0.005
Gender (male/female)	0.07	0.29	-0.182	0.610
Post Body weight (Kg)	-0.34	0.00	-0.051	-0.024
Pre TAG (mmol/L)	-0.11	0.49	-0.752	0.364
Pre NEFA (*μ*mol/L)	-0.14	0.03	-0.002	0.000
Pre TRL TAG (mmol/L)	-0.04	0.80	-0.824	0.637
Pre Total HDL-c (mmol/L)	0.14	0.17	-0.350	1.941
Pre Apo CII (mg/L)	0.04	0.66	-0.011	0.018
Pre Apo CIII (mg/L)	-0.13	0.33	-0.012	0.004
Pre Apo E (mg/L)	0.07	0.47	-0.012	0.026
Pre TRL ApoB (mg/L)	0.28	0.00	0.005	0.014
Post TAG (mmol/L)	0.00	0.97	-0.526	0.508
Post NEFA (*μ*mol/L)	-0.03	0.62	-0.001	0.001
Post TRL TAG (mmol/L)	0.03	0.79	-0.565	0.743
Post Total HDL-c (mmol/L)	0.02	0.86	-1.024	1.224
Post Apo CIII (mg/L)	-0.06	0.60	-0.009	0.005
Post Apo E (mg/L)	-0.10	0.30	-0.027	0.008
Model 6 [redacted] r^2=0.137, r^2=0.172)§				
Age (years)	-0.14	0.02	-0.044	-0.004
Gender (male/female)	0.02	0.77	-0.317	0.427
Post Body weight (Kg)	-0.36	0.00	-0.053	-0.026
Pre C20:5:3 (%)	0.20	0.04	0.011	0.698
Pre MUFA (%)	-0.08	0.25	-0.071	0.018
Pre LC n-6 PUFA (%)	0.00	0.95	-0.032	0.034
Pre LC n-3 PUFA (%)	-0.07	0.52	-0.215	0.108
Post C14:0 (%)	-0.06	0.50	-0.241	0.117
Post C16:0 (%)	-0.03	0.82	-0.127	0.100
Post C20:4:6 (%)	0.08	0.15	-0.023	0.151
Post SFA (%)	-0.08	0.60	-0.123	0.072
Post PUFA (%)	-0.16	0.29	-0.128	0.038
Post LC n-6 PUFA (%)	0.13	0.38	-0.045	0.120
§variables: Pre C16:0 (%), Pre C18:1 (%), Pre C18:2:6 (%), Post C18:2:6 (%) and Pre PUFA (%), were removed from model due to their confounding effects				
Model 7 [redacted] r^2=0.133, r^2=0.247)¶				
Age (years)	-0.15	0.11	-0.057	0.006
Gender (male/female)	0.01	0.95	-0.633	0.672
Post Body weight (Kg)	-0.37	0.00	-0.063	-0.019
Pre Total fat (g/d)	0.32	0.16	-0.006	0.037
Pre Total fat (%En)	-0.36	0.15	-0.179	0.029
Pre MUFA (g/d)	0.02	0.89	-0.099	0.114
Pre n-3 (g/d)	0.00	0.99	-0.286	0.289
Pre C12:0 (g/d)	0.20	0.14	-0.118	0.821
Pre C14:0 (g/d)	0.03	0.85	-0.232	0.282
Pre Starch (g/d)	-0.38	0.03	-0.019	-0.001
Pre Alcohol (g/d)	0.07	0.77	-0.044	0.060
Pre Alcohol (%En)	0.02	0.94	-0.175	0.187
Post C14:0 (g/d)	0.11	0.26	-0.082	0.304
Post *trans* fatty acids (g/d)	-0.18	0.04	-0.376	-0.008
Post Starch (g/d)	-0.06	0.69	-0.010	0.007
Post Protein (g/d)	-0.07	0.67	-0.020	0.013
Post Protein (%En)	0.01	0.94	-0.094	0.101
Post Fibre (g)	0.04	0.66	-0.029	0.046
¶variables: Pre BMR, Post BMR, Pre C8 (g/day), Pre C18:1 (g/day), Pre MUFA (g/day), were removed from the model due to their confounding effects				

Sig. significance value, *β*= Beta co-efficient, CI = confidence intervals. Each model controlled for sex, age and body weight at post-intervention
*Categorical variables with ≥8 categories for which dummy variables were created. p=0.00 = p<0.001
pre= pre-intervention; post=post-intervention

Table 5.17: Multiple linear regression model of variables predicting increasing S_I at post-intervention in LIPGENE volunteers

	S_I *Irrespective of diet*			
			95% CI	
	β	*sig*	Lower	Upper
Age (years)	-0.14	0.00	-0.026	-0.023
Gender (male/female)	0.28	0.00	0.846	0.918
Post Body weight (Kg)	-0.35	0.00	-0.040	-0.037
Pre $S_{G\ min}^{-1}$	-0.90	0.00	-219.591	-213.623
Pre DI	-0.69	0.00	-0.002	-0.001
Pre G0 mg/dl $^{-1}$	0.92	0.00	0.035	0.036
Pre P2 min^{-1}	-0.44	0.00	-23.232	-22.496
Pre Fasting Glucose (mmol/l)	-0.15	0.00	-0.302	-0.267
Pre QUICKI	0.78	0.00	3.003	3.066
Post AIRg mUL^{-1} min^{-1}	-0.01	0.08	0.000	0.000
Post DI	0.37	0.00	0.001	0.001
Post G0 mg/dl $^{-1}$	0.09	0.00	0.001	0.002
Post P2 min^{-1}	-0.08	0.00	-1.124	-0.967
Post Fasting Glucose (mmol/l)	-0.20	0.00	-0.303	-0.278
Post QUICKI	0.73	0.00	7.620	7.808
Smoke (Yes v no)	-0.26	0.00	-0.987	-0.941
Play sports (Yes v No)	-0.05	0.00	-0.178	-0.141
Pre Revised QUICKI	0.64	0.00	51.333	52.995
LIPGENE Partners*				
TCD v UoR	-0.02	0.00	-0.140	-0.056
TCD v UO	0.15	0.00	0.655	0.737
TCD v INSERM	0.00	0.78	-0.048	0.036
TCD v UM	-0.11	0.00	-0.587	-0.504
TCD v HURS-UCO	0.16	0.00	0.615	0.687
TCD v JUMC	0.06	0.00	0.286	0.369
TCD v UU	-0.40	0.00	-2.036	-1.950
Pre Fasting Insulin (uIU/ml)	0.12	0.00	0.032	0.038
Pre AIRg mUL^{-1} min^{-1}	0.51	0.00	0.002	0.002
Post Fasting Insulin (uIU/ml)	-0.19	0.00	-0.053	-0.048
Post Waist circumference(cm)	-0.01	0.20	-0.003	0.001
Post Body water (%)	0.08	0.00	0.015	0.020
Post tPA (ng/ml)	0.05	0.00	0.016	0.021
Post sICAM-1 (ng/ml)	-0.20	0.00	-0.004	-0.004
Pre NEFA (μmol/L)	0.00	0.88	0.000	0.000
Pre TRL ApoB (mg/L)	0.26	0.00	0.009	0.009
Pre plasma C20:5:3 (%)	0.12	0.00	0.196	0.220
Post *trans* fatty acids (g/d)	-0.18	0.04	-0.376	-0.008

S_G (Glucose Effectiveness); **AIR_g** (Acute Insulin response to Glucose); **DI** (Disposition Index (AIRg*SI)); **G0** (Distribution of Glucose at Time 0); **P2** (Removal rate of Insulin from the Interstitial space); **QUICKI** (Quantitiative Insulin Sensitive Check Index);sICAM-1 (soluble Adhesion Molecule-1); **tPA** (tissue Plasminogen activator); **NEFA** (Non esterifief fatty acids); **TRL ApoB** (Triglyceride rich lipoprotein Apolipoprotein B) **C20:5:3** (Eicosapentaeoic Acid, EPA); **UoR** (University of Reading); **UO** (University of Olso); I**NSERM**, France; **UM** (University of Maastricht); **HURS-UCO** (Cordoba, Spain); **JUMC**, Poland; **UU** (Uppsala University, Sweden)

Table 5.18: Multiple linear regression model of variables predicting increasing S_I at post-intervention in the LIPGENE volunteers, according to the dietary treatments

Adjusted r^2=0.743, r^2=0.835	**Post S_I - Diet A (HSFA)**			
	Multivariate *(controlling for age, gender, body weight)*			
			95% CI	
	β	*sig*	Lower	Upper
Age (years)	0.03	0.72	-0.030	0.043
Gender (male/female)	-0.11	0.27	-1.101	0.312
Post Body weight(Kg)	-0.09	0.56	-0.052	0.028
Pre $S_{G\ min}^{-1}$	0.12	0.21	-19.902	85.595
Pre DI	0.18	0.26	0.000	0.001
Pre Fasting Glucose (mmol/l)	-0.17	0.08	-0.786	0.052
Post G0 mg/dl $^{-1}$	0.38	0.01	0.005	0.027
Post Fasting Glucose (mmol/l)	-0.14	0.35	-0.805	0.289
Post FPIR (ulU/ml)	-0.36	0.01	-0.021	-0.002
Pre c-Peptide ng/ml	0.14	0.30	-0.247	0.773
Post c-Peptide ng/ml	-0.14	0.35	-0.714	0.259
Post Insulin/c-peptide ratio	-0.27	0.01	-0.638	-0.106
Smoke (Yes v no)	-0.26	0.00	-1.859	-0.405
Pre BIA - Impedance(KHz)	0.06	0.59	-0.003	0.006
Post Waist circumference(cm)	-0.10	0.52	-0.065	0.034
Pre tPA (ng/ml)	-0.41	0.00	-0.276	-0.100
Post TRL TAG (mmol/L)	0.07	0.45	-0.358	0.790
Post Apo E (mg/L)	0.01	0.89	-0.014	0.016
Post plamsa C20:4:6 (%)	0.24	0.00	0.056	0.285
Pre S_I (mU/L)-1min-1	0.15	0.21	-0.080	0.346

	Post S_I - Diet B (HMUFA)			
	Multivariate *(controlling for age, gender, body weight)*			
			95% CI	
	β	*sig*	Lower	Upper
Age (years)	-0.14	0.16	-0.063	0.011
Gender (male/female)	-0.03	0.74	-0.723	0.516
Post Body Weight(Kg)	-0.15	0.18	-0.045	0.009
LIPGENE Partners*				
TCD v UoR	0.03	0.74	-0.736	1.025
TCD v UoR	0.09	0.33	-0.445	1.292
TCD v INSERM	0.03	0.78	-0.909	1.212
TCD v UM	0.01	0.92	-1.010	1.121
TCD v HURS-UCO	0.01	0.92	-0.796	0.881
TCD v JUMC	0.24	0.01	0.241	2.128
Pre FPIR (ulU/ml)	-0.34	0.02	-0.032	-0.003
Post DI	0.76	0.00	0.001	0.002
Post G0 mg/dl $^{-1}$	-0.26	0.01	-0.018	-0.002
Post FPIR (ulU/ml)	-0.53	0.00	-0.039	-0.011
Pre S_I (mU/L)-1min-1	0.20	0.04	0.013	0.452
Pre C22:6(n-3) (g/d)	0.13	0.13	-0.191	1.463

Adjusted r^2=0.724, r^2=0.801	**Post S_I - Diet C (LFHCC)**			
	Multivariate *(controlling for age, gender, body weight)*			
			95% CI	
	β	*sig*	Lower	Upper
Age (years)	-0.10	0.21	-0.042	0.010
Gender (male/female)	0.06	0.51	-0.357	0.703
Post Body weight(Kg)	-0.21	0.06	-0.039	0.000
LIPGENE Partners*				
TCD v UoR	-0.09	0.31	-1.107	0.354
TCD v UoR	0.08	0.38	-0.409	1.058
TCD v INSERM	-0.09	0.24	-1.299	0.328
TCD v HURS-UCO	0.06	0.52	-0.439	0.859
TCD v JUMC	0.09	0.30	-0.415	1.310
TCD v UU	0.03	0.76	-0.705	0.959
Post AIRg mUL^{-1} min^{-1}	-0.57	0.00	-0.003	-0.001
Post DI	0.40	0.00	0.000	0.001
Pre Revised QUICKI	0.40	0.00	13.511	36.172
Pre Total Fat >35.98% Total En	-0.33	0.01	-1.734	-0.247
Pre Total Fat <35.98% Total En	-0.23	0.05	-1.380	-0.011
Pre Fibrinogen mg/dl	0.04	0.59	-0.002	0.003
Pre Adiponectin (mg/L)	-0.09	0.29	-0.133	0.040
Pre plasma C20:5:3 (%)	0.14	0.11	-0.051	0.481
Pre C16:0 (g/d)	0.01	0.91	-0.040	0.045
Pre CHO (%En)	0.30	0.00	0.024	0.092
Pre Fibre (g)	-0.23	0.02	-0.068	-0.007

Adjusted r^2=0.780, r^2=0.822	**Post S_I - Diet D (LCn-3PUFA)**			
	Multivariate *(controlling for age, gender, body weight)*			
			95% CI	
	β	*sig*	Lower	Upper
Age	-0.27	0.00	-0.056	-0.018
Gender (male/female)	-0.02	0.80	-0.384	0.296
Post Body weight (Kg)	-0.10	0.38	-0.033	0.013
Post $S_{G\ min}^{-1}$	-0.23	0.12	-52.101	6.137
Post DI	0.69	0.00	0.001	0.001
Post G0 mg/dl $^{-1}$	0.24	0.12	-0.001	0.004
Post P2 min^{-1}	-0.20	0.00	-2.275	-0.437
Post QUICKI	0.57	0.00	4.904	9.233
Pre Insulin/C-peptide ratio	-0.32	0.00	-0.334	-0.143
Post Insulin/C-peptide ratio	0.16	0.08	-0.024	0.393
Play sports (Yes v No)	-0.13	0.04	0.012	0.684
Post Waist circumference(cm)	0.12	0.30	-0.014	0.046
Post Adiponectin (mg/L)	0.15	0.01	0.019	0.165
Post FPIR (ulU/ml)	-0.67	0.00	-0.034	-0.021

Models controlled for sex, age and body weight at post-intervention.
sig - significance value, *β*= Beta co-efficient, CI = confidence intervals.
pre= pre-intervention; post=post-intervention
Pre QUICKI excluded due to its confounding effects on the model
Pre C10:0 (g/day) and Pre Starch (g/day), TCD v UU excluded due to its confounding effects on the model
Pre C10:0 (g/day) and Pre Total Sugars (g/day) and Post Trans Fatty Acids (g/day) excluded due to its confounding effects on the model
* Categorical variables of 3 categories or more, for which dummy variables were created

5.4.27 Linear regression analysis to determine the predictors of post-intervention IL-6 concentrations, irrespective of dietary treatment

Linear regression analysis was completed in order to investigate the predictors of increasing IL-6 concentrations at post-intervention. The same process was applied as above, univariately and multivariately (*Tables 1 and 2 of the model process not shown*). This analysis was conducted irrespective of dietary treatment. All variables that reached statistical significance in model 1 and 2 were carried forward in a stepwise manner. *Table 5.19* illustrates the main predictor(s) of post-intervention IL-6 within the LIPGENE study cohort, irrespective of diet. UoR and pre-intervention IL-6 concentrations were demonstrated as the main positive predictors of post-intervention IL-6 concentrations, irrespective of dietary treatment (β=0.16 and β=0.42, respectively).

5.4.28 Linear regression analysis to determine the predictors of post-intervention IL-6 concentrations, according to dietary treatment

Table 5.20 presents linear regression to show the relationship between the inflammatory data, IVGTT parameters, anthropometric data, dietary and plasma FAs and lipid data using univariate and multivariate models against post-intervention IL-6, with the HSFA, HMUFA, LFHCC and LFn-3PUFA diets. The data was inputted in a univariate style and subsequently in a multivariate manner, controlling for age, gender and body weight *(Tables 1 and 2 of the model process not shown).* For the HSFA diet, plasma IL-6 concentrations emerged as the only significant predictor for post-intervention IL-6 concentrations from the results of the final model (β=0.40). For the HMUFA diet, pre-intervention IL-6 and BIA were found to notably predict post-intervention IL-6 (β=0.42 and β=0.26, respectively). In the case of the LFHCC diet, post-intervention lean body mass and body water as well as energy intake from MUFA were found to substantially predict post-intervention IL-6 concentrations. Finally, for the LFn-3PUFA diet, those subjects in the UoR were found to inversely predict post-intervention IL-6 concentrations only (β=-0.33).

5.4.29 Linear regression analysis to determine the predictors of post-intervention adiponectin concentrations, irrespective of dietary treatment

Table 5.21 presents linear regression to show the relationship between the inflammatory data, IVGTT parameters, anthropometric data, dietary and plasma FAs and lipid data using univariate and multivariate models against post-intervention adiponectin irrespective of diet

(*Tables 1 and 2 of the model process not shown*). When significant variables were carried forward to the final model to predict increasing adiponectin at post-intervention irrespective of dietary treatment, no powerful variables emerged.

5.4.30 Linear regression analysis to determine the predictors of post-intervention adiponectin concentrations, according to dietary treatment

Table 5.22 presents linear regression to show the relationship between the inflammatory data, IVGTT parameters, anthropometric data, dietary and plasma FAs and lipid data using univariate and multivariate models against post-intervention adiponectin, with the HSFA, HMUFA, LFHCC and LFn-3PUFA diets. A similar approach was implemented across the dietary treatments, the data was inputted in a univariate style and subsequently in a multivariate manner, controlling for age, gender and body weight *(Tables 1 and 2 of the model process not shown).* The results of the overall final model show that again pre-adiponectin concentrations and pre-intervention G0 concentrations were very strong predictors of post-adiponectin concentrations within the HSFA diet ($p<0.05$). For the HMUFA diet, when all noteworthy variables were brought forward to the final stage of multiple linear regression analysis, several overall predictors were found ($p<0.05$). Within the LIPGENE partners, UoR, UO, INSERM, HURS-UCO, JUMC and UU as well as pre-intervention IL-6 were all noted to be inverse predictors of post-adiponectin concentrations. Whereas, pre-intervention IL-6 and adiponectin concentrations were positive predictors of post-intervention adiponectin concentrations (β=0.31 and β=0.39, respectively). For the LFHCC and LFn-3PUFA diets, when the significance results were brought forward to the final model, no significance was noted for predictors of post adiponectin concentrations.

5.4.31 Linear regression analysis to determine the predictors of post-intervention resistin concentrations, irrespective of dietary treatment

Multiple linear regression was preformed as before to investigate the predictive potential of variables for increasing resistin concentration at post-intervention (*Tables 1 and 2 of the model process not shown*). Multivariate analysis within the final model demonstrated a high r^2 (>60%), signifying an excellent fit for the population, pre-resistin and pre-G0 were noted to be positively strong significant predictors of post-resistin irrespective of diet (β=0.75 and β=0.15, respectively) (*Table 5.23*).

Table 5.19: *Multiple linear regression model of variables predicting increase IL-6 at post-intervention in LIPGENE volunteers (n=417)*

		Post IL-6 Irrespecive of Diet			
		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI Lower	Upper
LIPGENE Partners	TCD v UoR	0.16	0.03	0.23	3.49
	TCD v UO	0.08	0.27	-0.73	2.57
Inflammatory Data	Pre CRP (mg/L)	-0.05	0.42	-0.17	0.07
	Pre IL-6 (pg/mL)	0.42	0.00	0.29	0.54
IVGTT Data	Pre DI	-0.01	0.85	0.00	0.00
	Pre G0 (mg/dl $^{-1}$)	-0.04	0.48	-0.02	0.01
	Post G0 (mg/dl -1)	-0.01	0.81	-0.01	0.00
	Post Fasting Insulin (uIU/ml)	-0.03	0.60	-0.10	0.06
Dietary Fatty Acids	Pre PUFAg (% Energy)	0.16	0.14	-0.03	0.21
	Pre n-6 (g/day)	-0.16	0.12	-0.26	0.03
	Post Vitamin E (mg/d)	0.00	0.96	-0.12	0.13
Plasma Fatty Acids	Pre C16:0 (%)	0.15	0.09	-0.02	0.28
	Pre C20:4:6 (%)	-0.01	0.88	-0.29	0.25
	Pre PUFA (%)	0.14	0.18	-0.04	0.21
	Pre C16:1 (%)	0.10	0.14	-0.14	0.97
Lipids Data	Pre ApoA1(g/L)	0.07	0.27	-0.99	3.50
	Post LDL-c (mmol/L)	-0.04	0.48	-0.65	0.31
	Smokers (Yes V No)	-0.06	0.31	-1.75	0.56

UoR (University of Reading); **UO** (University of Olso); **CRP** (C-reactive protein) ; **IL-6** (Interleukin-6);
TNFα (Tumour Necrosis Factor-α); **sICAM-1** (soluble Adhesion Molecule-1); DI (Disposition Index (AIRg*SI));
G0 (Distribution of Glucose at Time 0); palmitic acid (**C16:0**); palmitoleic acid **(C16:1)**;
Arachidonic Acid (AA, **20:4:6**); **PUFA** (Polyunsaturated Fatty Acids); **LDL-c** (Low density lipoprotein cholesterol);
ApoA1 (Apolipoprotein A1)

Table 5.20: Multiple linear regression models of variables predicting increasing IL-6 at post-intervention in LIPGENE volunteers, according to the dietary treatments

Adjusted r^2 = .112 r^2 = .473		**Post IL-6 - Diet A (HSFA)**			
		Multivariate *(controlling for age, gender, body weight)*			
		β	*sig*	95% CI	
				Lower	Upper
Inflammatory Data	Pre IL-6 (pg/mL)	0.41	0.01	0.13	0.79
	Pre TNFα (pg/mL)	-0.09	0.52	-0.57	0.29
Plasma Fatty acids	Pre C20:4:6 %	0.09	0.55	-6.30	11.75

Adjusted r^2 = .294 r^2 =.380		**Post IL-6 - Diet B (HMUFA)**			
		Multivariate *(controlling for age, gender, body weight)*			
		β	*sig*	95% CI	
				Lower	Upper
Inflammatory Data	Pre CRP (mg/L)	0.09	0.42	-0.13	0.30
	Pre IL-6 (pg/mL)	0.42	0.00	0.18	0.59
IVGTT Parameters	Pre Fasting Insulin (uIU/ml)	-0.11	0.38	-0.29	0.11
Anthropometric Data	Pre BIA - Impedance (KHz)	0.26	0.05	0.00	0.02
Plasma Fatty Acids	Pre C18:2:6 (%)	0.13	0.22	-0.07	0.30
Lipid Data	Post LDL-c (mmol/L)	-0.05	0.69	-0.95	0.63

Adjusted r^2 =.134 r^2 =.326		**Post IL-6 - Diet C (LFHCC)**			
		Multivariate *(controlling for age, gender, body weight)*			
		β	*sig*	95% CI	
				Lower	Upper
Inflammatory data	Pre CRP (mg/L)	0.02	0.89	-0.10	0.12
	Pre IL-6 (pg/mL)	-0.02	0.83	-0.16	0.13
	Pre sVCAM-1 (ng/mL)	0.10	0.39	0.00	0.00
	Pre Resistin (mg/L)	-0.06	0.59	0.00	0.00
IVGTT Parameters	Post Fasting Glucose (mmol/L)	0.14	0.20	-0.18	0.86
	Pre Diastolic BP (mmHG)	0.08	0.48	-0.04	0.07
Anthropometric Data	Post Lean Body Mass (Kg)	2.53	0.01	0.09	0.73
	Post Body Water (Litres)	-2.38	0.03	-0.98	-0.05
	Pre Body Weight (Kg)	0.86	0.35	-0.14	0.39
Dietary Fatty Aicds	Pre MUFA (g/day)	0.05	0.80	-0.04	0.06
	Pre MUFA (%Energy)	-0.38	0.06	-0.29	0.01
Plasma Fatty Acids	Pre C16:1 (%)	-0.07	0.63	-0.89	0.54
	Pre C20:4:6 (%)	0.08	0.52	-0.15	0.30
	Post C16:1	0.14	0.37	-0.47	1.25
	Post SFA	0.05	0.71	-0.11	0.16
Lipid Data	Pre LDL-c (mmol/L)	-0.02	0.91	-0.79	0.71
	Post LDL-c (mmol/L)	0.02	0.90	-0.67	0.76

Adjusted r^2 = -.034 r^2 = .124		**Post IL-6 - Diet D (LCn-3PUFA)**			
		Multivariate *(controlling for age, gender, body weight)*			
		β	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v Reading	-0.33	0.07	-5.35	0.23
	TCD v Oslo	-0.14	0.37	-4.16	1.59
Inflammatory Data	Pre IL-6 (pg/mL)	0.06	0.69	-0.17	0.26
IVGTT Parameters	Post G0 (mg/dl -1)	0.05	0.77	0.00	0.01
Anthropometric Data	Pre Body Water (Litres)	-0.10	0.68	-0.18	0.12
Lipid Data	Pre ApoE (mg/L)	0.08	0.58	-0.06	0.10

Table 5.21 *Multiple linear regression models of LIPGENE partners, inflammatory data, IVGTT data, dietary and plasma fatty acids and lipid data predicting increasing post-intervention Adiponectin concentrations, irrespective of dietary treatment*

		Post Adiponectin - Irrespective of Diet			
		Multivariate *(controlling for age, gender, body weight)*			
Adjusted r^2 =.045 r^2:		*β*	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v UoR	-0.08	0.59	-2.75	1.56
	TCD v UO	-0.06	0.67	-2.69	1.73
	TCD v INSERM	-0.13	0.30	-3.28	1.02
	TCD v UM	0.11	0.44	-1.31	2.96
	TCD v HURS-UCO	-0.02	0.88	-1.91	1.64
	TCD v JUMC	-0.04	0.74	-2.56	1.82
	TCD v UU	-0.10	0.46	-2.90	1.31
Inflammatory Data	Pre Adiponectin (mg/L)	0.04	0.71	-0.19	0.28
IVGTT Data	Pre S_I (mU/L)-1min-1	0.05	0.66	-0.23	0.36
	Pre $S_{G\ min}^{-1}$	0.01	0.93	-76.88	83.57
	Pre QUICKI	0.04	0.69	-1.11	1.66
Dietary Fatty Acids	Pre n-3 (g/day)	0.01	0.90	-0.26	0.29
	Pre C8:0 (g/day)	-0.11	0.61	-7.88	4.64
	Pre C14:0 (g/day)	0.08	0.70	-0.51	0.76
	Pre C18:2:6 (g/day)	-0.07	0.58	-0.12	0.07
Lipid Data	Pre TRL ApoB (mg/L)	0.12	0.29	-0.01	0.02

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interrval.

This model controlled for age, gender and body weight

TCD (Trinity College Dublin); UoR (University of Reading); **UO** (University of Olso); I**NSERM**, France;

UM (University of Maastricht); **HURS-UCO** (Cordoba, Spain); **JUMC**, Poland; **UU** (Uppsala University, Sweden)

S_I (Insulin Sensitivity); **S_G** (Glucose Effectiveness); **QUICKI** (Quantitiative Insulin Sensitive Check Index);

caprylic acid **(C8:0)**; mysteric acid **(C14:0)**; Linoleic Acid (LA, **18:2:6**); **TRL ApoB** (Triglyceride rich lipoprotein Apolipoprotein B)

Table 5.22: Multiple linear regression models of variables predicting increasing Adiponectin at post-intervention in LIPGENE volunteers, according to the dietary treatments

Adjusted r^2 = .622 r^2 =0.678		Post Adiponectin - Diet A (HSFA)			
		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v UoR	-0.14	0.10	-1.84	0.17
	TCD v UO	-0.05	0.52	-1.48	0.75
Inflammatory Data	Pre Adiponectin (mg/L)	0.72	0.00	0.57	0.94
IVGTT Data	Pre G0 (mg/dl -1)	0.30	0.01	0.01	0.03
Dietary Fatty Acids	Pre n-6 (g/day)	-0.02	0.87	-0.11	0.10
	Pre C18:1 (g/day)	0.06	0.76	-0.08	0.11
	Pre C18 (g/day)	-0.16	0.33	-0.30	0.10
	Post Alcohol (g/day)	0.02	0.74	-0.01	0.02
Plasma Fatty Acids	Pre C20:5 (n-3) (%)	-0.02	0.79	-0.56	0.43
	Post C16:1 (%)	0.09	0.32	-0.18	0.55
	Post C20:5 (n-3) (%)	-0.12	0.19	-0.68	0.14

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval.
This model controlled for age, gender and body weight

Adjusted r^2 = 0.410 r^2 = 0.563		Post Adiponectin - Diet B (HMUFA)			
		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v UoR	-0.34	0.02	-3.97	-0.29
	TCD v UO	-0.43	0.01	-4.58	-0.75
	TCD v INSERM	-0.37	0.01	-4.41	-0.81
	TCD v Maastricht	-0.20	0.14	-3.19	0.47
	TCD v HURS-UCO	-0.24	0.07	-2.95	0.13
	TCD v JUMC	-0.24	0.10	-3.31	0.31
	TCD v Uppsala	-0.44	0.00	-4.79	-0.92
Inflammatory data	Pre IL-6 (pg/mL)	0.31	0.02	0.02	0.25
	Pre TNFα (pg/mL)	-0.12	0.21	-0.11	0.03
	Pre Adiponectin (mg/L)	0.39	0.00	0.15	0.46
	Post IL-6 (pg/mL)	-0.26	0.03	-0.26	-0.02
IVGTT Data	Pre $S_{G\ min}^{-1}$	-0.05	0.64	-97.16	59.79
Plasma Fatty Acids	Post C14:0 (%)	0.06	0.65	-0.26	0.41
	Post C18:0 (%)	0.04	0.68	-0.20	0.30
	Post C20:5 (n-3) (%)	-0.06	0.63	-0.76	0.46
Lipid Data	Post Apo B48 (mg/L)	-0.08	0.45	-0.77	0.34
	Post ApoE (mg/L)	-0.02	0.85	-0.04	0.03

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interrval.
This model controlled for age, gender and body weight

Adjusted r^2 = -.270 r^2 =.-043		Post Adiponectin - Diet C (LFHCC)			
		Multivariate (controlling for age. gender, body weight)			
		β	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v UoR	0.15	0.42	-1.03	2.43
	TCD v HURS-UCO	0.00	0.98	-1.13	1.16
Inflammatory Data	Pre TNFα (pg/mL)	-0.11	0.54	-0.39	0.21
	Pre Adiponectin (mg/L)	-0.16	0.51	-0.43	0.22
IVGTT Data	Pre S_i (mU/L)-1min-1	0.13	0.49	-0.24	0.49
	Pre $S_{G\ min}^{-1}$	0.27	0.27	-53.54	185.47
	Pre QUICKI	0.27	0.25	-4.61	16.93
Dietary Fatty Acids	Pre C12 (g/day)	-0.03	0.88	-1.04	0.90
	Post n-3 (g/day)	-0.30	0.21	-1.09	0.25
Plasma Fatty Acids	Post C18:1 (%)	0.01	0.97	-0.37	0.38

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interrval.
This model controlled for age, gender and body weight

Adjusted r^2 = -.073 r^2 =.134		Post Adiponectin - Diet D (LCn-3PUFA)			
		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI	
				Lower	Upper
LIPGENE Partners	TCD v HURS-UCO	-0.13	0.46	-2.31	1.05
Inflammatory Data	Pre Adiponectin (mg/L)	-0.01	0.96	-0.30	0.28
Anthropometric Data	Post Lean Body Mass (Kg)	0.43	0.18	-0.03	0.15
	Post Body Water (%)	-0.42	0.15	-0.29	0.05
	Post Impedance (KHz)	-0.07	0.75	-0.01	0.01
Plasma Fatty Acids	Post C16:1 (%)	0.07	0.63	-0.40	0.65
Lipid Data	Pre Cholesterol (mmol/L)	0.13	0.43	-0.40	0.94
	Pre TRL TAG-c (mmol/L)	-0.23	0.21	-1.99	0.44
	Pre Apo B48 (mg/L)	0.20	0.24	-0.37	1.43

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interrval.
This model controlled for age, gender and body weight

Table 5.23: *Multiple linear regression models of LIPGENE partners, inflammatory data, IVGTT data, dietary and plasma fatty acids, and lipoprotein concentrations predicting increasing post-intervention Resistin concentrations, irrespective of dietary treatment*

Adjusted r^2 =.647 r^2 =.672		**Post Resistin - Irrespectve of Diet**			
		Multivariate			
		(controlling for age, gender, body weight)			
		β	*sig*	95% CI	
				Lower	Upper
Inflammatory Data	Pre CRP (mg/L)	-0.07	0.18	-225.11	41.42
	Pre Resistin (pg/mL)	0.75	0.00	0.73	0.92
	Post CRP (mg/L)	0.07	0.16	-40.56	247.70
IVGTT Data	Post G0 (mg/dl $^{-1}$)	0.15	0.00	3.70	14.07
Dietary Fatty Acids	Pre Total Sugars (g)	0.05	0.24	-3.75	15.16
Plasma Fatty Acids	Pre C16:0 (%)	0.05	0.32	-50.92	155.34
Lipid Data	Pre TAG-c (mmol/L)	-0.10	0.38	-1877.82	720.26
	Pre TRL TAG-c (mmol/L)	0.09	0.39	-1005.40	2593.73
	Pre ApoB48 (mg/L)	0.03	0.66	-790.82	1252.59
	Post TAG-c (mmol/L)	0.04	0.61	-688.62	1173.78
	Post Total HDL-c (mmol/L)	0.00	0.98	-1772.62	1726.71
	Post ApoB48 (mg/L)	-0.04	0.59	-1353.03	775.34

sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval.

This model controlled for age, gender and body weight

CRP (C-reactive protein); **G0** (Distribution of Glucose at Time 0); palmitic acid (**C16:0**); **TAG-c** (Triglyceride Cholesterol); **TRL TAG-c** (Triglyceride rich lipoprotein cholesterol); **ApoB48** (Apolipoprotein B48); **HDL-c** (High Density Lipoprotein Cholesterol)

5.4.32 Linear regression analysis to determine the predictors of post-intervention resistin, according to dietary treatment

Table 5.24 illustrates the predictors of post-intervention resistin concentrations investigated across the dietary treatments. A similar approach was adopted as before, univariately and multivariately (*Tables 1 and 2 of the model process not shown*). On analysis within the final model for the HSFA diet, three predictors were noted to be significant with pre-intervention CRP concentrations being inversely and post-intervention plasma CRP and pre-intervention resistin concentrations positively important predictors of post-intervention resistin concentrations at post-intervention. In regards to the HMUFA diet, within the final model, only post-intervention sVCAM-1 and pre-intervention ApoCIII were the overall predictors of post-intervention resistin, both were inversely and strongly associated (β = -0.60 and β = -0.64, respectively).

In respect to the LFHCC diet, no significance variables were noted in the final model, However, for the LCn-3PUFA, when significant results were analysed within the final model, three positive significant results emerged, including pre-resistin, post-IL-6 and post-P2 (β = 0.73, β = 0.17 and β = 0.26, respectively).

Table 5.24: Multiple linear regression models of variables predicting increasing Resistin at post-intervention in LIPGENE volunteers, according to the dietary treatments

		Post Resistin - Diet A (HSFA)			
Adjusted r^2 =.687 r^2 =.709		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI Lower	Upper
Inflammatory Data	Pre CRP (mg/L)	-0.19	0.03	-498.27	-31.44
	Pre Resistin (pg/mL)	0.81	0.00	0.82	1.10
	Post CRP (mg/L)	0.24	0.00	119.06	617.46
Lipid Data	Post Cholesterol (mmol/L)	0.09	0.14	-180.20	1247.83

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval.
This model controlled for age, gender and body weight

		Post Resistin - Diet B (HMUFA)			
Adjusted r^2 = -.226 r^2 =.559		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI Lower	Upper
Inflammatory Data	Pre sVCAM-1 (ng/mL)	-0.60	0.01	-33.13	-4.46
	Pre Resistin (mg/L)	0.10	0.66	-0.40	0.61
IVGTT Data	Post P2 min^{-1}	-0.17	0.45	-161559.24	75483.35
Anthropometric Data	Pre Waist circumference (cm)	-0.04	0.90	-264.93	235.19
Dietary Fatty Acids	Pre C8:0 (g/day)	-0.08	0.86	-29904.21	25108.71
	Pre C12:0 (g/day)	0.40	0.32	-3697.86	10758.60
Plasma Fatty Acids	Post SAFA (%Energy)	0.16	0.46	-140.61	296.53
Lipid Data	Post ApoA1 (g/L)	-0.38	0.33	-26158.21	9378.45
	Pre ApoCIII (mg/L)	-0.64	0.02	-108.17	-9.96
	Pre ApoB48 (mg/L)	0.06	0.74	-2823.70	3880.93
	Post Total HDL-c (mmol/L)	0.59	0.18	-5179.93	26296.00

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval.
This model controlled for age, gender and body weight

		Post Resistin - Diet C (LFHCC)			
Adjusted r^2 =-0.30 r^2 =.069		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI Lower	Upper
LIPGENE Partner	TCD v INSERM	0.08	0.51	-3144.69	6228.64
Inflammatory Data	Pre TNF*α* (pg/mL)	0.12	0.34	-197.05	569.96
	Pre sVCAM-1 (ng/mL)	-0.16	0.17	-10.45	1.89
IVGTT Data	Post $S_{G\ min}{}^{-1}$	-0.09	0.49	-230989.58	111042.64
	Post P2 min^{-1}	0.10	0.40	-4523.95	11333.49

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval
This model controlled for age, gender and body weight

		Post Resistin - Diet D (LCn-3PUFA)			
Adjusted r^2 =.672 r^2 =.720		Multivariate (controlling for age, gender, body weight)			
		β	*sig*	95% CI Lower	Upper
Inflammtory Data	Pre Resistin (mg/L)	0.73	0.00	0.74	1.15
	Post IL-6 (pg/mL)	0.17	0.04	15.80	475.15
IVGTT Data	Post S_I (mU/L)-1min-1	-0.01	0.93	-594.83	547.94
	Post P2 min^{-1}	0.26	0.02	1184.66	13318.08
	Post P3 (mU/L) min^{-2}	-0.13	0.20	-159410507	34646826
Plasma Fatty Acids	Pre C16:0 (%)	-0.02	0.81	-228.88	179.02
Lipid Data	Pre TRL Apo B (mg/L)	-0.06	0.48	-29.35	13.94

Sig = Significance value, *β* = Beta co-efficient, CI = Confidence interval.
This model controlled for age, gender and body weight

5.5 DISCUSSION

The overall aim of the LIPGENE human dietary intervention study was to examine the efficacy of reducing dietary SFA consumption, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS, in particular S_I, inflammatory status, markers of oxidative stress, lipoprotein metabolism, anthropometric measurements and hypertension. The key objective to accomplish this aim was to create four diets varying significantly in overall fat intake, by creating one diet higher in SFA content compared to all other diets, by creating another diet higher in MUFA content compared with the three other diets and by creating a diet to investigate the effects of LC n-3 PUFA supplementation compared to a placebo, based on a low-fat background diet. A central minimisation procedure was used to randomise 417 volunteers to one of four study diets. Although, these diets differed in dietary fat quantity and quality they remained isoenergetic. The HSFA and HMUFA diets were designed to provide 38% energy intake from fat; the HSFA diet was designed to provide ~16%E as SFA, whereas, the HMUFA was designed to provide ~20% energy as MUFA. The LFHCC and LFn-3PUFA were low-fat diets and were designed to provide 28% energy from fat with the LFn-3PUFA including a 1.24g/day supplement of EPA and DHA (Shaw *et al*, 2007). The effects of LC n-3 PUFA supplementation was investigated due to the proven positive effects of lipid profiles, inflammatory status and insulin sensitivity (Guebre-Egziabher *et al*, 2007; Neschen *et al*, 2007).

The results of the food exchange model used to achieve the composition of the diets proved that all dietary targets were achieved successfully. Compliance and under-reporting were assessed using Schofield equations to estimate BMR and the Goldberg cut-off for energy intake (EI): basal metabolic rate (BMR) of 1.05 was used to identify under-reporters. All volunteers on the LFHCC and LFn-3PUFA diets reported ≥85% compliance to the capsules. Therefore, at the outset of this LIPGENE human dietary intervention study, the dietary compositional objectives were largely achieved (Shaw *et al*, 2007).

At the end of the 12 week intervention period results showed that although not anticipated, body weight was significantly reduced in the LFHCC cohort ($p<0.0005$), however no significant differences were noted for the other treatment groups. It is probable that this reducing in weight was simply due to the volunteers consuming a low-fat diet for a 12 week period. An unintended significant weight-loss was also noted by Ferrier *et al* (2004), who conducted a diet and exercise study on humans, however, significant results from this study were found to be independent of weight-loss.

The dietary fat content and FA composition, as well as FA composition in serum lipids, have been well documented as indicators for CHD and MetS (Kotseva *et al*, 2007; Vessby, 2003). Several studies have underlined the importance of fat quality in the diet for the development of IR and other diseases related to the MetS (Vessby, 2003; Lovejoy *et al*, 2002; Summers *et al*, 2002; Perez-Jiménez *et al,* 2001). Within this study the effects of dietary fat modification were investigated on plasma FAs by GC analysis and interestingly, results showed several main dietary interactions, whereby oleic acid (C18:1) levels were significantly lower within HSFA and LFn-3PUFA diets, contrary to this, oleic acid (C18:1) was significantly increased within HMUFA as anticipated. Similarly, significant reductions were noted for MUFA (%) concentrations within the HSFA and LFn-3PUFA diets. There results are unsurprising, considering that the nutrient composition of the exchangeable foods provided to the volunteers were lower in levels of plasma oleic acid (C18:1) and energy form MUFA for the HSFA and LFn-3PUFA groups compared to high complex carbohydrate, HMUFA diet as described by Shaw *et al* (2007). Additionally, plasma EPA levels were increased within HSFA, LFHCC and LFn-3PUFA, whereas levels of plasma DHA were increased in LFHCC and LFn-3PUFA only. Again, these results are expected for LFHCC and LFn-3PUFA diets, due to the LC n-3 PUFA fish-oil supplement. Additionally, results illustrated that plasma long china (LC) n-3 PUFA levels were significantly increased across the four dietary treatments.

Markers of insulin sensitivity were assessed by the IVGTT procedure (Bergman *et al*, 1979), which was carried out at pre- and post-intervention. Interestingly, results of the dietary fat modification demonstrated a significant decrease for insulin/c-peptide ratio at post-intervention ($p<0.0005$). However, no other effects were noted for insulin sensitivity parameters. The insulin/c-peptide ratio is an important indicator of insulin production, hepatic insulin extraction and clearance. Additionally, c-peptide concentrations are considered a more reliable reflection of pancreatic β-cell function and hence insulin secretion than insulin concentrations alone. This may be because c-peptide has a 2-5 times longer half-life than insulin and higher concentrations may persist in the peripheral circulation (Chen *et al,* 1999). Banks *et al* (2007) found that insulin/c-peptide ratio correlates positively with the percentage of body fat. On the other hand, Jiang *et al* (1996) found that obese individuals have lower insulin/c-peptide concentrations compared to lean controls suggesting that the pancreas is producing significantly less insulin. Therefore, dietary fat modification may decrease insulin secretion and increase hepatic insulin clearance.

Dietary fat modification was also the principal aim of the KANWU study as it set out to determine whether dietary fat modifications could improve insulin action in 162 healthy individuals, age 30-65 years. Similar to LIPGENE, volunteers were chosen at random to receive a controlled, isoenergetic diet, containing a high proportion of SFA or MUFA for 3 months. Results of this study indicated that insulin sensitivity was significantly impaired on the SFA-rich diet, but no change was demonstrated for the MUFA-rich diet. A second assignment to fish oil supplements (3.6g/d n-3 FAs) or placebo was completed, but results showed no improvement in insulin sensitivity (Vessby *et al*, 2001). Contrary to these findings, the effects of 3 weight maintenance diets with different macronutrient composition on carbohydrate, lipid metabolism and insulin levels was investigated on IR subjects in a cross-over design for 28 days. No effects on body weight were noted within the 3 diets and results demonstrated that insulin sensitivity was improved on the MUFA-rich diet as indicated by a lower $HOMA_{IR}$ score compared to the SFA-rich and CHO-rich diets. Furthermore, after ingestion of breakfast meal rich in virgin olive-oil, postprandial glucose and insulin concentrations decreased and HDL-c and GLP-1 concentrations increased in MUFA and SFA rich diets compared to the CHO-rich diet (Paniagua *et al*, 2007).

Inflammation is one of the most important developing areas in obesity biology as the obese state is characteristized by chronic low-grade inflammation (Trayhurn, 2007). The main basis for this view is that circulating levels of several markers of inflammation, such as IL-6, IL-18, CRP and PAI-1 are elevated in obesity and reduced with weight-loss (Yudkin *et al*, 1999; Festa *et al*, 2001; Esposito *et al*; 2003; Chiellni *et al*, 2004). It has been postulated that T2DM is a manifestation of the inflammatory host response (Creely, 2007). Therefore, several pro-inflammatory cytokines and an adipokine thought to possess anti-inflammatory properties namely adiponectin, were analysed to investigate the effects of dietary fat of inflammatory status in the MetS. After dietary treatment, no significant main effect was demonstrated for the influence of dietary fat modification on the following inflammatory markers: plasma CRP, IL-6, TNFα, sICAM-1, sVCAM-1, resistin, adiponectin and leptin concentrations. However a significant time*centre*gender*diet interaction revealed that plasma resistin concentrations were significantly higher for males in the HMUFA diet in HURS-UCO, whereas a significant decrease was demonstrated for females in the HMUFA diet in UU, Sweden ($p<0.05$).

Another study investigated the impact of an isocaloric weight-maintenance diet for 12 months on inflammation and lipoprotein concentrations. During the first phase of the study, subject's habitual fat energy intake was reduced from 35% to 15% in a stepwise manner over

4 months, in order to prevent rapid weight-loss. During the second phase of 8 months, the subjects followed a diet of 15% energy from fat, under *ad libitum*, free-living conditions. Results showed that the isocaloric / weight-maintenance diet observed unfavourable effects whereby adiponectin concentrations were significantly decreased ($p<0.05$) and TAG concentrations significantly increased at post-intervention ($p<0.01$). Contrary to this, the *ad libitum* diet caused 6kg weight-loss, significantly decreased plasma CRP ($p<0.01$), decreased HDL-c serum amyloid levels ($p<0.001$), increased plasma adiponectin concentrations and decreased TAG levels at post-intervention ($p<0.05$). This study concluded that a combination of low-fat, high-carbohydrate diet and weight-loss may be beneficial to lipoprotein metabolism and inflammatory status (Kasim-Karakas *et al*, 2006). Conflicting results were found when LC n-6 PUFA administered to Indian Asian men age 35 – 70 years for 6 weeks did not modulate the effects of fish-oil supplementation on lipoprotein concentrations or measures of S_I (Brady *et al*, 2004*b*). Krebs *et al* (2006) conducted a randomised study on 116, overweight IR women for 24 weeks to test the effects of the consumption of LC n-3 PUFA and weight-loss on CVD risk factors. Subjects were randomised to weight-loss with LC n-3 PUFA supplementation, weight-loss alone or to the control group, weight-maintenance only. Significant weight-loss was seen in weight-loss & LC n-3 PUFA group and the weight-loss alone group compared to the controls and the weight-loss showed significant improvements in S_I, lipid profile and inflammatory status. It was concluded that weight-loss improved risk factors for CVD with some added benefits of LC n-3 PUFA on TAG and adiponectin levels. The impact of weight-loss alone was investigated on the MetS, after a moderate decline in body weight (6.5%), through the consumption of very low-calorie diet (600-800kcal/d) for 4 weeks, significant reductions were noted in diastolic BP, glucose, TAG and TC concentrations ($p<0.0001$). Moreover, BMI was also found to be significantly reduced at post-intervention ($p<0.001$) (Case *et al*, 2002).

From previous reports of isoenergic, weight-maintenance studies versus those investigating low-fat diets combined with weight-loss, it is apparent that the hypocaloric diets combined weight-loss illustrates more significantly positive results. To further investigate this statement, a study was performed to examine the effects of exercise training on inflammatory and systemic oxidative stress markers in overweight children. No change in body weight was noted at post-intervention and no significant alterations were illustrated for CRP, IL-6, TNFα, adiponectin, leptin and 8-iso-$PGF_{2\alpha}$ concentrations. The study concluded that the negative

results may be due to the lack of weight-loss and changes in body composition as they both may be the main mechanisms responsible for improving inflammatory and oxidative stress status (Kelly *et al*, 2007).

Within the LIPGENE study, significant findings were noted for the inflammatory markers according to gender, whereby females were found to have significantly higher concentrations of plasma CRP and plasma leptin concentrations compared to the male volunteers at pre- and post-intervention ($p<0.001$). Comparable results have been noted in recent studies in regards to plasma CRP concentrations been higher in females compared to males (Chapidize *et al*, 2007; Rutter *et al*, 2004; Nakaniski *et al*, 2005). In this respect, it is important to consider the potential confounding influence of hormone replacement therapy (HRT) in women, as administration of HRT has been demonstrated to increase circulating CRP concentrations (Manning *et al*, 2002) and in a very recent study HRT was found to increase CRP concentrations in females by 121% after 1 year (Cooper *et al*, 2007). Unfortunately, HRT data was not recorded for this study. In another study, CRP concentrations were noted to be highly correlated with smokers compared to non-smokers (Block *et al*, 2004), however, no significant relationship was noted to plasma CRP concentrations and cigarette smoking data within this study. In regards to the high leptin concentrations found in female LIPGENE volunteers, in a study on Saudi women, leptin, glucose and insulin concentrations were all significantly higher in obese and overweight females compared to lean controls. Leptin concentrations were also markedly elevated with increasing BMI (Daghestani *et al*, 2007). Another study concluded that leptin concentrations are associated with adiposity and hip circumference (Al-Daghri *et al*, 2007). Interestingly, this study showed very strong positive correlations between leptin concentrations against hip and waist circumference as well as body fat (%) and body fat (kg), irrespective of diet and across the four dietary treatments (*Table 5.21* and *Table 5.21a*, respectively). To date, several studies have demonstrated that leptin concentrations are modulated through the actions on the muscle sympathetic nerve activity (MSNA) (Lambert *et al*, 2007; Shen *et al*, 2007). A consequence of increased MSNA activity is a decrease in femoral blood flow and conductance, an increase in femoral vascular resistance and a lower oxygen demand (Dinenno *et al*, 1999). The sympathetic nerve is known to regulate energy balance by decreasing food intake and increasing resting metabolic rate (RMR) and has been reported to be possibly conducive to the development of a MetS (Tentolouris *et al*, 2006). Additionally, it has also been postulated that an increase in the sympathetic vasoconstrictor activity and a reduction in skeletal muscular

blood flow could contribute to the development of IR (Julius *et al*, 1992). A low-fat diet and exercise trial for 4 months on obese women, showed significant reduction in body weight and subsequently, a significant decreased activity in MSNA was observed, this study concluded that this provides convincing evidence for a low-fat diet and weight-loss, a non-pharmological therapy for human obesity (Tonacio *et al*, 2006). Another weight-loss study performed on MetS subjects, fed a hypocaloric diet (26% fat, 22% protein, 51% CHO and 100mmol/d sodium) for 3 months, demonstrated significant body weight-loss and subsequent improvements in all MetS criteria, as well as significant reduction in MSNA activity and increased insulin sensitivity (Straznicky *et al*, 2005).

The LIPGENE study also demonstrated a significant increase in sVCAM-1 concentrations at pre-intervention in the 65-71 age category compared to the 50 – 64 years category ($p<0.05$) and the 35 – 49 years category. These finding are in line with other studies that suggest sVCAM-1 concentrations to be significantly positively correlated with age (Miles *et al*, 2008; Morisaki *et al*, 1997). It has been reported that oxidative stress increases with aging as this may cause an increase in endothelial markers. However, no significant increases were noted for urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-$PGF_{2\alpha}$ in the LIPGENE volunteers when assessed according to age. In addition, no significant difference was observed when analysis was completed on the change in body weight categories. It may be probable that this increase in age may be due to the wide range of ages studies in this trans-EU cohort or because they are over-nourished and have decreased physically activitiy than their younger counterparts.

Within the LIPGENE study, numerous significant effects were observed across the EU participating centres, intriguingly, a trend was noted for the Scandinavian countries, Oslo, Norway (UO) and Uppsala, Sweden (UU) to have lower circulating levels of inflammatory and oxidative stress markers compared to the Irish (TCD) and English (UoR) plasma concentrations. For example, at pre-intervention UO had the lowest levels of plasma CRP and IL-6 concentrations and at pre- and post-intervention UU had the lowest levels of sICAM-1 and sVCAM-1, whereas TCD had the highest levels of sICAM-1 and 8-iso-$PGF_{2\alpha}$ at pre- and post-intervention and UoR had the highest levels of sVCAM-1 at pre-intervention only. Contrary to this trend, TCD had the highest levels of plasma adiponectin concentrations at pre- and post-intervention, whereas UU had the lowest levels of adiponectin at post-intervention only. Additionally, UO had the highest levels of resistin at pre- and post-intervention. Correlation analysis shows no significant relationship for CRP and IL-6 as noted with UO, however, interestingly, a strong positive significant relationship was noted for

sICAM-1 and sVCAM-1 as seen in UU. As for TCD, no significant relationship was noted for sICAM-1 and 8-iso-$PGF_{2\alpha}$ concentrations. Interestingly, UO had the highest dietary intakes of EPA and DHA, which might explain their decrease in plasma CRP and IL-6 concentrations. On the other hand, UO did not demonstrate increased insulin sensitivity or a more desirable lipoprotein profile when compared to the other centres at pre-intervention. In regards to the southern EU participating centres, HURS-UCO, Córdoba, Spain and INSERM, Marseille, France, HURS-UCO had the highest levels of plasma CRP and plasma leptin concentrations at pre- and post-intervention, which were very strong correlated after analysis. INSERM, at the lowest levels of plasma TNFα at post-intervention and the lowest levels of plasma resistin at pre- and post-intervention and again a strong correlation emerged after analysis.

After a 3-day dietary intervention was performed in T2DM subjects in Norway, who were advised to increase consumption of fibre and decrease habitual SFA consumption, results showed significant decreases in TC ($p<0.005$), leptin concentrations ($p<0.005$) and significant increases in adiponectin concentrations (p=0.024) (Mostad *et al*, 2004). A study, based on the traditional Swedish diet combined with very a low intensity exercise regime for 4 weeks found body weight to be unaffected, yet peripheral insulin sensitivity was improved (Andersson *et al*, 1998).

Because oxidative stress and inflammation may play a role in the pathophysiology of obesity-related diseases, the effects of dietary fat modification were investigated on biomarkers of nonenzymatic and enzymatic lipid perioxidation by measuring urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$ concentrations. Again, no significant main dietary effect was noted for both urinary markers. Opposing these findings, the KANWU study investigated the effects of 3 months supplementation of LC n-3 PUFA on plasma 8-iso-$PGF_{2\alpha}$ and 15-dihydro-$PGF_{2\alpha}$ in healthy humans. Results showed a significant decrease in plasma 8-iso-$PGF_{2\alpha}$ (p=0.015) however, similarly plasma 15-keto-dihydro-$PGF_{2\alpha}$ was unaffected (Nälsén *et al*, 2006). Several other studies have reported significantly reduced effects after n-3 FA supplementation on markers of lipid peroxidation (Higdon *et al*, 2000). Within the LIPGENE study, significant effects across the EU centres emerged. As mentioned previously, TCD was noted to have the highest circulating levels of 8-iso-$PGF_{2\alpha}$ at pre- and post-intervention. In respect to urinary 15-keto-dihydro-$PGF_{2\alpha}$, UM was noted to have the highest circulating levels pre- and post-intervention, whereas UoR had the lowest levels at pre-intervention only and HURS-UCO had the lowest levels at post-intervention only. Although, the LIPGENE human

dietary intervention study set out to be isocaloric, with two low-fat diets involved this quest proved difficult and a significant change in body weight was noted within the LFHCC diet, therefore, to investigate the effects of this change in body weight, five categories were defined; lost 4-8kg, lost 2-4kg, lost 0-2kg, gained 0-2kg and gained 2-5kg. A significant effect was discovered for those volunteers who gained 0-2kg body weight showing a significant increase in urinary 8-iso-$PGF_{2\alpha}$ concentrations ($p<0.001$). When urinary 8-iso-$PGF_{2\alpha}$ and 15-keto- dihydro-$PGF_{2\alpha}$ were correlated with each other a very strong relationship was noted, however when they were both correlated with cigarette smoking data, a very weak positive relationship was noted for urinary 8-iso-$PGF_{2\alpha}$ and smoking data irrespective of dietary treatment. Interestingly, a strong inverse relationship was noted for plasma IL-6 concentrations and smokers in this study, which is contrary to the positive relationship for plasma IL-6 and smoking demonstrated by Chan *et al* (2002).

CRP is an acute phase reactant synthesized by the liver under the control of IL-6 and other pro-inflammatory cytokines (Li & Fang, 2004). High concentrations of CRP correlate with adiposity and the presence and risk factors of CVD (Joveli *et al,* 2005). CRP concentrations have also be noted to been elevated in the obese, IR state are are known to predict the development of T2DM, in fact, all 5 characteristics of the MetS are associated with increased CRP levels (Florez *et al*, 2006; González *et al*, 2006; Ridker *et al*, 2003). Therefore, within this study pre-intervention CRP concentration were divided into tertiles ranging from low, medium to high circulating CPR levels. The aim was to test the hypothesis that concentrations of CRP are associated with inflammatory status and risk factors for CVD events. Results of this analysis demonstrated no significant associations across the CRP tertiles with IVGTT parameters and anthropometric data. However, within the lipoprotein data irrespective of diet, pre- and post-intervention NEFA and ApoCII concentrations were significantly higher in the medium CRP tertile compared to the low CRP tertile, additionally TRL ApoB concentrations were significantly higher in medium compared to the low CRP tertile at post-intervention only. When the medium tertile was investigated according to diet, total cholesterol and ApoB concentrations at post-intervention were significantly higher within HSFA diet compared to the other dietary treatments. In regards to the CRP tertiles across the inflammatory data irrespective of diet, concentrations of CRP and leptin were significantly increased at pre- and post-intervention as well as pre-intervention sICAM-1 concentrations and post-intervention resistin concentrations. Contrary to this, plasma IL-6 concentrations

were significantly higher in the low CRP tertile compared to the medium and high tertiles at pre- and post-intervention. However, the data suggests that those in the high CRP tertile have increased inflammatory status compared to those in the low and medium tertiles, whereas those in the medium tertile have increased risk of CVD events due to the increase lipoprotein concentrations demonstrated.

Generally, within this study the univariate correlations performed tended to be rather weak with the exception of some. This study aimed to investigate the independent predictors of insulin sensitivity in a large trans-EU population. Additionally, it sought to investigate the predictors of circulating levels of IL-6, resistin and adiponectin concentrations irrespective and according to diet. Linear regression analysis data was performed and results for S_I, show that a multitude of significant predictors emerged. Unsurprisingly, increasing age and body weight strongly predict decreasing S_I. Novel findings of this analysis, includes increasing G0, which was a positive predictor, whereas S_G was inversely associated with S_I. However, P2, glucose effectiveness, fasting glucose, sICAM-1 and UU, Sweden were all associated with lower S_I, irrespective of dietary treatment. When assessed according to diet, G0 and plasma FA, AA (C20:4:6) were found to strongly predict increasing S_I within HSFA, whereas insulin/c-peptide ratio, smoking, tPA concentrations, FPIR and fasting glucose predict decreasing S_I. Novel findings with the HMUFA diet, include FPIR at pre- and post-intervention and G0 predicts decreasing S_I. Within the low fat LFHCC, fat intake greater than 36% and fibre intake predict decreasing S_I and within low-fat and LC n-3 PUFA diet, novel observations include insulin/c-peptide and volunteers who did not take part in sporting activities predicted decreasing S_I. In a long-term study to investigate the predictors of S_I, BMI was the strongest predictor of S_I, followed by physical activity, HDL-c, SFA and socioeconomic status (Risérus *et al*, 2007).

It has been reported that increasing IL-6 concentrations are an independent long-term predictor of death or heart failure (Kavsak *et al*, 2007, Ravaglia *et al*, 2006). Therefore, analysis was completed to determine the possible variables predicting post-intervention IL-6 concentrations within this study. Results show that UoR and pre-IL-6 concentrations are the only predictors of increase post-intervention IL-6, irrespective of diet. These results are unsurprising, given that UoR had the highest levels of IL-6 across the EU countries. In relations to dietary treatment, pre-intervention IL-6 concentrations were the only significant predictors of increasing IL-6 with HSFA and HMUFA diets, no significant predictors were noted for the LFHCC and LFn-3PUFA diets.

Recent studies have been conducted to investigate the possible predictors of adiponectin concentrations (Sobngwi *et al*, 2007, Galler *et al*, 2007). Results of some studies show that the main predictors of the MetS were low adiponectin concentrations (Johnson *et al,* 2007). Additionally, adiponectin levels were found to be the main predictors in organ damage in children (Litwin *et al*, 2007). With that knowledge, possible predictors of adiponectin were analysed within the LIPGENE dietary intervention study. The interesting finding appeared for the HMUFA diet, where UoR, UO, INSERM, HURS-UCO, JUMC and UU compared to TCD were found to decrease levels of adiponectin at post-intervention. Again, these results are unsurprising given that TCD had the highest levels of adiponectin pre- and post-intervention. Additionally, pre-intervention IL-6 concentrations were found to increase adiponectin concentration, yet post-intervention IL-6 was found to decrease adiponectin concentrations. Increasing, TNFα at pre-intervention was also observed as a decreasing predictor of adiponectin concentrations.

Resistin, also found in inflammatory zone 3 (F1ZZ3) or adipocyte-secreted factor (ADSF) is a novel hormone secreted by adipoctye cells. High concentrations of plasma resistin have a proposed link with IR by antagonizing insulin action and modulating one or more steps in the insulin-signalling pathway (Steppan *et al*, 2001; Steppan *et al*, 2002). Therefore, analysis of potential predictors of resistin was completed within the LIPGENE human dietary intervention study. Novel results showed that G0 predicts increasing resistin concentrations at post-intervention, irrespective of diet. According to diet, original results show that pre-intervention CRP predicts decreasing resistin concentrations, whereas post-intervention CRP predicts increasing resistin with HSFA diet. Within the HMUFA diet, post-intervention sVCAM-1 strongly predicts a decrease in resistin, which can be confirmed by the correlation analysis that highlights a significant positive relationship between both cytokines ($r=0.246$, $P=0.05$). No significant results were noted for the LFHCC diet, however for the LFn-3PUFAd diet, post-intervention IL-6 and P2 strongly predicts increasing resistin levels.

The pleiotrophic effects of LC n-3 PUFA may contribute to decreasing the burden of the MetS, such as modulating inflammation, platelet activation, endothelial function and hypertension (Carpentier *et al*, 2006*b*). Therefore, within this dietary intervention study, the effects of dietary fat modification on lipoprotein concentrations were investigated and results showed several significant interactions. Interestingly, the majority of these significant effects were noted within the HSFA diet and LC n-3 PUFA diet as concentrations of plasma TAG, plasma ApoA1 and plasma ApoCIII were all significantly decreased in both diets. NEFA and

TRL TAG concentrations were also noted to be significantly decreased within LC n-3 PUFA diet only. Additionally, HDL-c concentrations were significantly increased within HSFA, HMUFA and LC n-3 PUFA. However, no significant effect was demonstrated for concentrations of total cholesterol, LDL-c, ApoA1, ApoB ApoCII, ApoB48 or TRL ApoB. Although studies comparing the effect of both major LC n-3 PUFA are limited, however, DHA appears at least as efficient as EPA in correcting several risk factors for CVD events. These positive effects are likely mediated through the activity of transcription factors, such as NF-κB relating to expression of genes involved in lipid oxidation and synthesis. A number of mechanisms through which LC n-3 PUFA may exert their effects have been identified. Yet the most frequent proposed mechanism relates to the displacement of LC n-6 PUFA AA in the phospholipids bilayer and a subsequent shunting of eicosanoid production towards a less bioactive phenotype (Grimm *et al*, 1994). The use of LC n-3 PUFA should be considered in more global strategies including changes in lifestyle, such as adhering to a healthy Mediterranean type of diet and practicing regular physical exercise (Carpentier *et al*, 2006*b*). A study on Wistar rats investigated the effects of LC n-3 PUFA on the MetS abnormalities, baseline results showed that the MetS rats had a significant increase in body weight, ($p<0.001$), hypertension ($p<0.001$), serum insulin concentrations ($p<0.01$), lipoprotein concentrations ($p<0.0001$) and serum TNFα concentrations ($p<0.001$). After this, the rats were randomly assigned to receive a corn-canola diet (7.5%) or 7.5% LC n-3 PUFA and both groups received 30% sucrose whilst on the diets for 6 weeks to maintained MetS status. Results of the LC n-3 PUFA treatment showed that MetS rats had significant reductions in hypertension levels ($p<0.0001$), serum insulin ($p<0.001$) and lipoprotein concentrations ($p<0.0001$), however no change was observed for the serum TNFα concentrations in the rats fed the LC n-3 PUFA diet. This study concluded that fish-oil administration reversed the alteration of the MetS parameters and hypertension, despite having no effect on inflammatory status and adiposity (Aguilera *et al*, 2004). Consistent results have been found in human studies investigating the effects of LC n-3 PUFA on lipoprotein metabolism, most notably in the reduction of plasma TAG concentrations (Putadechakum *et al*, 2005; Sanders *et al*, 1997; Hau *et al*, 1996) this could possibly be due to the decreased VLDL production (Roche & Gibney, 1999).

In conclusion, given the pathophysiological importance of IR, the potential impact of dietary FA, observational studies assessing fatty acid composition in serum or tissues suggest that IR is associated with relatively high intakes of saturated fat (e.g. palmitic acid) and low intakes of polyunsaturated fat (e.g. linoleic acid). These findings are supported by recent clinical data, whereby substituting saturated fat with unsaturated fat seems to have beneficial effects on insulin sensitivity, although the clinical significance of modifying fat quality alone is still unclear (Risérus, 2008). Intervention studies have also reported that lifestyle changes can reduce body weight, blood pressure and serum TAG, increase HDL-c and lower the risk of progression from impaired fasting glucose to T2DM by 58% (Tuomilehto *et al*, 2001; Knowler *et al*, 2002). This is the first trans-EU study, involving such a diversity of countries in terms of habitual dietary fat intake and lifestyle and to including a large population size to investigate the potential benefits of modifying dietary fat, in terms of quantity and quality of markers associated with the MetS. The study concluded that dietary fat modification does not alter S_I, inflammatory status and markers of oxidative stress associated with the MetS.

Despite the unaltered insulin sensitivity and inflammatory status, lipoprotein concentrations were significantly reduced within the LFn-3PUFA diet, namely TAG, ApoAI, ApoCIII, NEFA and TRL TAG concentrations, suggesting that this diet may be advantageous in alleviating the risks for CVD. These positive effects are in concurrence with a recent study that noted the reversal of dyslipidemia after LC n-3 PUFA administration (Lombardo *et al*, 2007). This evidence for the potential benefits of LC n-3 PUFA supplementation is promising, yet inconclusive and further large scale dietary studies are required to elucidate this hypothesis. Many previous studies have proclaimed positive results with weight-loss relating to the reversal of the characteristics of the MetS as illustrated throughout this report. Therefore, with future studies investigating the effects of dietary fat modification on the MetS, a modified diet combining weight-loss compared to modified diet alone may be of interest. Additionally, it may be of interest to conduct a randomised cross-over study on the same study cohort to investigate the effects of dietary fat modification on markers of insulin sensitivity and inflammation similar to the study design by Pérez-Jiménez *et al* (2004).

It has been reported that shared environmental components and genetics almost certainly contribute to the development of the MetS (Roche *et al*, 2005). Although, this report solely investigates the effects of environmental factors, genetic analysis for the LIPGENE volunteers is ongoing and will be presented in the near future.

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Ms Audrey Tierney (TCD) – tPA, PAI-1 and Fibrinogen analysis and insulin modelling by MINMOD Millennium®.

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Ms Olfa Helal – apoprotein analysis

Ms Jane Ferguson (UCD) – Leptin analysis and GC analysis

Ms Eva Sejby & Ms Barbro Simu (UU) – analysis of urinary 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro $PGF_{2\alpha}$

Chapter 6

General Discussion

The metabolic syndrome (MetS) describes a constellation of metabolic risk factors including dyslipidemia, hypertension, impaired glucose tolerance (IGT) and central obesity. This clustering of risk factors is thought to be responsible for much of the excess cardiovascular disease (CVD) morbidity among overweight and obese patients and those with type 2 diabetes mellitus (T2DM). Current research has reported that the MetS is also associated with a proinflammatory/prothrombotic state that may include elevated levels of C-reactive protein (CRP), endothelial dysfunction, hyperfibrinogenemia, increase levels of plasminogen activator inhibitor-1 (PAI-1) and a shift towards small, dense particles of low-density lipoprotein (LDL) cholesterol. The etiology of the MetS has not been established definitively, hence the title *metabolic syndrome* and not *metabolic disease*. One hypothesis presumes that the primary cause is insulin resistance (IR), while the second hypothesis is the dramatic increase in abdominal obesity. To date, it is unknown which hypothesis precedes the other and has been questioned as *which is the chicken and which is the egg*?

However, it is well established is that all criteria of this syndrome are potentially modifiable with lifestyle modification and changes in one's lifestyle should always be the first treatment of choice for the MetS (Case *et al*, 2002). Intervention studies have concluded that lifestyle changes can reduce body weight, hypertension and serum tricylglycerol (TAG) concentrations as well as increasing high-density lipoprotein (HDL) cholesterol and lower the risk of progression from IFG to T2DM by 58% (Tuomilehto *et al*, 2001; Knowler *et al*, 2002).

No single diet is currently recommended for patients with the MetS, therefore, it has been recommended that physicians focus on individual patients' specific metabolic alterations when offering dietary advice (Szapary *et al*, 2002). An effective approach to alleviate the prevalence of the MetS may be to focus on the importance of dietary therapy and in particular the quality rather than the quantity of dietary fats being consumed. Dietary fatty acids (FAs) represent one of the most important energy sources for the human body and excess FA can induce obesity and IR. Early epidemiological studies reported that total fat intake was positively associated with the risk of diabetes (West & Kalbfliesh, 1971). It has been reported that saturated fatty acids (SFA) induces IR by altering insulin signalling (Le Marchand-Brustel *et al*, 2003). The KANWU study showed that insulin sensitivity was significantly impaired by the high SFA-rich diet, yet remained unaffected by the monounsaturated fatty acid (MUFA) diet in healthy individuals (Vessby *et al*, 2001). Similar results have been reported by Summers *et al* (2002) whereby a polyunsaturated fatty acid (PUFA) diet increased insulin sensitivity compared to a diet rich in SFA. Furthermore, Pérez-Jiménez *et al* (2001) conducted

a cross-over study in healthy young individuals and found that substituting carbohydrates and MUFA for SFA improved insulin sensitivity. Additionally, CVD mortality was reported to be significantly reduced when a high intake of PUFA and LA were consumed for 15 years (Laaksonen *et al*, 2005).

Conjugated linoleic acid (CLA) is a PUFA found naturally occurring in foods from ruminant sources. Several isomers of CLA exist with *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) and *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA) having received the most attention due to their potential anti-diabetic (Eyjolfson *et al*, 2004), anti-inflammatory (Yu *et al*, 2002) and chemoprotective properties (Belury, 1995) demonstrated *in vivo* and *in vitro* models. CLA is found naturally in the lipid portion of ruminant meat and dairy products and is now under the spotlight to be considered a functional food (Toomey *et al*, 2006). A substantial amount of evidence has been reported on the distinct physiological effects exerted by the CLA isomers. Although, the *t*10, *c*12-CLA isomer is responsible for a reduction in body fat in animal feeding studies, emerging evidence suggests that this isomer also exerts a pro-diabetic effect in both humans and mice (Roche *et al*, 2002; Risérus *et al*, 2002). For this reason, this thesis explored the beneficial relationship between natural *c*9, *t*11-CLA enriched beef, synthetically produced *c*9, *t*11-CLA and *trans* Vaccenic Acid (TVA) on body composition and metabolic and molecular markers of the MetS, including a significant improvement in insulin sensitivity, inflammatory status and lipoprotein metabolism in male ob/ob mice after a 28 day intervention as reported in *Chapter 3*. TVA (11-*trans* octadecenoic acid) is a major *trans* monoene found in ruminant fat and can be endogenously converted to *c*9, *t*11-CLA by Δ9-desaturase (Griinari *et al*, 2000). No significant findings were noted for the TVA diet within this study, yet a trend was observed for the TVA diet to decrease total body weight in the *ob/ob* mice. It was postulated that the total amount of TVA contained in the foods was simply inadequate to bioconvert to CLA, hence the null effects on insulin sensitivity, inflammation and lipoprotein metabolism. Despite no significant findings, it may be of interest to re-investigate the effects of TVA in the future using an increased supplemented amount of TVA in a longer term investigation.

As stated previously, the MetS is now associated with an ongoing sub-acute pro-inflammatory state (Ghanim *et al*, 2004; Dandona *et al*, 2004; Roche *et al*, 2005). Obesity is associated with chronic low grade inflammation of the white adipose tissue (WAT). WAT is now seen as a highly dynamic organ, not only is it the physiological site for energy storage as lipids, but it is a secretory site for several hormones collectively termed 'adipocytokines' (Trayhurn & Wood, 2004). Results show that after intervention, anti-inflammatory effects

were observed for both the natural *c*9, *t*11-CLA diet and the synthetic *c*9, *t*11-CLA diet, whereby serum concentrations of IL-6 were significantly decreased and concentrations of the anti-inflammatory cytokine, serum adiponectin was significantly increased in both the *c*9, *t*11-CLA diets. Additionally, anti-diabetic effects were demonstrated within this study whereby significant reductions were noted for plasma glucose concentrations as well as a decreased $HOMA_{IR}$ and an improved revised QUICKI was noted after natural *c*9, *t*11-CLA and synthetic *c*9, *t*11-CLA feeding.

In recent years a number of studies have examined the effect of CLA on animal models of atherosclerosis. Atherosclerosis is one of the primary causes of death in the world. Disturbances in tricylglycerol (TAG) metabolism are one of the major abnormalities associated with the MetS, which in turn leads to the progression of atherosclerosis (Alipour *et al*, 2007). This study found that both plasma non-esterified fatty acids (NEFA) and TAG concentrations were significantly decreased following both the natural and synthetic *c*9, *t*11-CLA diets compared to the control. On the other hand, total cholesterol was significantly increased in both these diets, compared to the control diet, which may be due to the high percentage of PUFA contained in the diets.

To our knowledge this is the first study to compare the effects of feeding either natural beef derived *c*9, *t*11-CLA or synthetically prepared *c*9, *t*11-CLA on markers on body composition, insulin sensitivity, inflammatory status and lipid metabolism. The results of the study are very promising in terms of the MetS, as body weight gain was less, insulin sensitivity, inflammatory status and blood lipids were all significantly improved on a diet containing *c*9, *t*11-CLA in both the natural and synthetic form. These results have important implications for human nutrition and the food industry as they support the use of CLA as a functional food. At the present time, attempts in several countries are being made to enrich the amount of *c*9, *t*11-CLA in foods by altering cattle feeding. A major limitation of this present study was its short-term duration and it was conducted in rodents. Therefore, future epidemiological studies together with larger long-term studies could help answer the important question of whether relatively small, but apparently potent, amount of dietary CLA is effectively safe with regard to the MetS and CVD risk in humans.

CLA occurs naturally in ruminant meats, pasteurised cheeses, and dairy products and therefore is a dietary constituent for many Europeans and Americans. However health organisations continue to recommend that we limit out intake of these animal products. Thus as we decrease our consumption of animal fats, CLA consumption also decreases. Therefore,

continued controlled intervention studies in humans are needed to further explore the effects of varying amounts of CLA on beneficial effects to health.

To date there is no medical reason for humans to consume CLA as supplements. Yet, results from studies have prompted the natural supplement industry to market CLA to humans, with the promotional literature stating that body fat can be reduced by consuming CLA. One preliminary clinical study performed by Pharmanutrients indicated that after a 90 day period, CLA consumption reduced body fat by an average of 20%. With minimal information available regarding both the mode of action of CLA in reducing animal fat and regarding the action upon humans, more basic information is necessary to protect consumers from possible detrimental effects of the *t*10, *c*12-CLA isomer in particular (Brodie *et al*, 1999).

Chapter 4 was a cross-sectional/observational study carried out at one time-point to determine the interaction between plasma FAs and markers of inflammation, endothelial dysfunction, coagulation and oxidative stress on IR in the MetS volunteers recruited across the 8 participating EU centres. This study was part of the LIPGENE 'Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis' 5-year (2004-2009) intervention, set out to apply an integrated approach to examine how variation in the composition of dietary fats interact with common genetic variations to influence the development of the MetS (Buttriss & Nugent, 2005).

Observational data from this study showed an increase in plasma sICAM-1 concentrations with lower insulin sensitivity. Significantly higher concentrations of plasma PAI-1 and tPA were also associated with lower insulin sensitivity and higher $HOMA_{IR}$ and unsurprisingly, body weight and BMI were significantly higher in the low insulin sensitivity cohort. Interestingly, this cross-sectional study showed that when individuals were categorized into tertiles of insulin sensitivity, those with lower insulin sensitivity had a significantly higher intake of dietary *trans* Fatty Acids (TFA). TFA are unsaturated FA that are formed during the industrial process of partial biohydrogenation or are formed naturally in the rumen of diary cows such as CLA. Additionally, those in the higher $HOMA_{IR}$ category were noted to have significantly higher concentrations of leptin. Results from another study showed that serum leptin levels increased with the prevalence of the MetS. In the LIPGENE cross-sectional study those with a high $HOMA_{IR}$ index consumed significantly more total MUFA and oleic acid (C18:1), suggesting that MUFA may decrease insulin sensitivity levels. Cross-sectionally, it was noted that TCD had the highest concentrations of plasma adiponectin compared to the other EU centres and females had significantly higher levels compared to males. Furthermore,

those with higher concentrations of plasma adiponectin were found to have increased S_I and lower c-peptide concentrations. Plasma myristic acid (C14:0), EPA (C20:5:3) and LC n-3 PUFA were noted to be higher in those individuals with lower adiponectin levels. Whereas, dietary intake to total SFA, MUFA and PUFA as well as LC n-3 PUFA and LC n-6 PUFA were noted to be higher in those with lower levels of adiponectin.

As previously demonstrated females had higher CRP concentrations compared to males and those with BMI >30 have increased CRP concentrations compared to leaner MetS volunteers. Interestingly after regression analysis, TNFα and adiponectin concentrations were positively associated with insulin sensitivity, whereas BMI, tPA and alcohol intake were negatively associated with insulin sensitivity. Binary logistic regression analysis was performed to determine the independent predictors of insulin sensitivity. The main outcomes of the binary regression analysis showed that those MetS volunteers living in UoR, UO, INSERM, HURS-UCO and UU were positive predictors of low versus high insulin sensitivity as well as alcohol intake, tPA and sICAM-1 concentrations. Furthermore, inflammatory data demonstrated that plasma CRP, sICAM-1 and leptin concentrations were highest in HURS-UCO, Cordoba, Spain. Whereas, plasma concentrations of IL-6 and fibrinogen were lowest in UO and plasma sVCAM-1 levels were lowest in UU. This is very interesting information given that INSERM, HURS-UCO comes under the so-called *Mediterranean diet* which is characterised by a MUFA-rich, low SFA diet and UO and UU follow the habitual Scandinavian diet, which have often reported lower rates of CVD due to the higher intake of PUFA.

Past studies have proven that it is possible to achieve primary prevention of T2DM by means of intensive interventions. Therefore, appropriate strategies to identify and analyse high-risk individuals in a simple and inexpensive way are urgently warranted. Long-term further research investigating environmental factors, such as diet and lifestyle modifications in the prevention and treatment of the MetS and on the best ways to promote lifestyle changes are necessary. The findings outlined in *Chapter 4* were of a cross-sectional nature and the unique feature about this study was the recruitment of a relatively large cohort of MetS volunteers within 8 EU countries. Cross-sectional studies are advantageous, in that they are relatively inexpensive and time efficient. However, limitations occur with cross-sectional studies as they are only a snapshot of the population and the situation may provide differing results if another timeframe had been chosen and it must also be remembered that they have been previously associated with bias.

With these limitations in mind, *Chapter 5* examined the aim of this study which was to determining the efficacy of reducing dietary SFA consumption, by altering the quality and reducing the quantity of dietary fats on risk factors associated with the MetS. 417 subjects with the MetS were randomly assigned to one of the following isoenergetic diets: Diet A: High-fat, SFA-rich diet (HSFA), Diet B: High-fat, MUFA-rich diet (HMUFA), Diet C: Low-fat, low complex carbohydrate diet (LFHCC), Diet D: Low-fat, low complex carbohydrate diet & 1.24g/d LC PUFA (LFn-3PUFA) for 12 weeks. An IVGTT was performed and fasting bloods samples were harvested at the beginning and immediately after the 12 weeks intervention. From the outset of the study, it was demonstrated by the food exchange model used to achieve the composition of the diets for each individual, that all dietary targets were achieved successfully.

Several previous studies have underlined the importance of fat quality in the diet for the development of IR and other diseases related to the MetS (Vessby, 2003; Lovejoy *et al*, 2002; Summers *et al*, 2002; Perez-Jiménez *et al,* 2001). However, results from the LIPGENE dietary intervention study show that dietary fat modification had no significant effects on markers of insulin sensitivity, inflammation or oxidative stress. However a significant time*centre*gender*diet revealed that plasma resistin concentrations were significantly higher for males in the HMUFA diet in HURS-UCO, whereas a significant decrease was demonstrated for females in the HMUFA diet in UU ($p<0.05$). Furthermore, significant modifications were noted for lipoprotein concentrations, the majority of these being with the LFn-3PUFA diet.

From previous reports of isoenergic, weight-maintenance studies versus those investigating low-fat diets combined with weight-loss, it is apparent that the hypocaloric diets combined weight-loss illustrates more significantly positive results. A study investigated the impact of an isocaloric weight-maintenance diet for 12 months on inflammation and lipoprotein concentrations. During the first phase of the study, subject's habitual fat energy intake was reduced from 35% -15% in a stepwise manner over 4 months, in order to prevent rapid weight-loss. During the second phase of 8 months, the subjects followed a diet of 15% energy diet, under *ad libitum*, free-living conditions. Results showed that the isocaloric / weight-maintenance diet showed unfavourable effects whereby adiponectin concentrations were significantly decreased ($p<0.05$) and TAG concentrations significantly increased at post-intervention ($p<0.01$). Contrary to this, the *ad libitum* diet caused 6kg weight-loss, significantly decreased plasma CRP ($p<0.01$), decreased HDL-c serum amyloid levels

($p<0.001$), increased plasma adiponectin concentrations and decreased TAG levels at post-intervention ($p<0.05$). This study concluded that a combination of low-fat, high-carbohydrate diet and weight-loss may be beneficial to lipoprotein metabolism and inflammatory status (Kasim-Karakas *et al*, 2006). Krebs *et al* (2006) conducted a randomised study on 116, overweight IR women for 24 weeks to test the effects the consumption of LC n-3 PUFA and weight-loss on CVD risk factors. Subjects were randomised to weight-loss with LC n-3 PUFA supplementation, weight-loss alone or to the control group, weight-maintenance only. Significant weight-loss was seen in weight-loss & LC n-3 PUFA group and the weight-loss alone group compared to the controls & the weight-loss showed significant improvements in insulin sensitivity, lipid profile and inflammatory status. It was concluded that weight-loss improved risk factors for CVD with some added benefits of LC n-3 PUFA on TAG and adiponectin levels. The impact of weight-loss alone was investigated on the MetS, after a moderate decline in body weight (6.5%), through the consumption of very low-calorie diet (600-800kcal/d) for 4 weeks, significant reductions were noted in diastolic BP, glucose, TAG and TC concentrations ($p<0.0001$). Moreover, BMI was also found to be significantly reduced at post-intervention ($p<0.001$) (Case *et al*, 2002). The findings of the LIPGENE study suggests that improvements in dietary fat quality alone as not enough to demonstrate improvements in inflammatory status and insulin sensitivity, for future studies improvements in dietary fat quality should be combined with weight-loss therapy.

LIPGENE is the largest study sample of MetS volunteers recruited over several different countries, this takes into account different habitual diets and ethnic backgrounds. The study also involved a vast range of ages, BMI categories and both genders. However, the major limitations of this study was that is was of for 12 week duration and employed the minimal model Intravenous Glucose Tolerance Test (IVGTT) to measure S_I in the MetS volunteers instead of the 'gold-standard' euglycaemia hyperinsulinaemic clamp technique for measuring insulin sensitivity. The thesis explored the effects of environmental factors such as dietary fat modification on inflammation and insulin sensitivity. However, a genetic component in the pathophysiology of IR and the MetS certainly exists and lifestyle change may not be enough to reduce CVD risk. The MetS volunteers recruited as part of the LIPGENE dietary intervention study were also genotyped for genetic analysis related to insulin sensitivity and inflammation. This information will be published in the near future and will hopefully provide more light on the complex relationship between inflammation and IR in the MetS.

Overall, this thesis demonstrates that CLA is indeed a significantly metabolically active fatty acid and mice are particularly sensitive to the *c*9, *t*11-CLA isomer. However, a major limitation occurs as the *ob/ob* mice are homogeneous by nature, whereas the MetS cohort used in the Human Dietary Intervention study are heterogeneous by nature. Previous research on CLA in humans is not in complete agreement and therefore, it remains to be seen if the results found in the *ob/ob* mice can be reproduced in humans. Longer-term health aspects and efficacy of CLA in human's warrants future research and monitoring as CLA-supplemented food products enter the market. The LIPGENE dietary intervention study represents the largest pan-EU intervention study to date. Results of the cross-sectional analysis were unable to demonstrate an association between plasma and dietary FAs on inflammatory status and insulin sensitivity. Nor was the human dietary intervention study able to prove alterations in inflammation and insulin sensitivity in response to intake of varying intake of FAs. Nevertheless, long-term studies, perhaps using the euglycaemia hyperinsulinaemic clamp to measure insulin sensitivity and cholesterol esters to measure FAs composition are needed to identify and understand the relation between fatty acid metabolism, inflammatory stress and insulin sensitivity.

Chapter 7
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