A Randomised, Double blind, Crossover Study of the Effects of CLA isomers on Inflammation, Body composition, Metabolic profiles and Vascular function in Subjects with the Metabolic Syndrome

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A dissertation submitted to Cardiff University in candidature for the degree of

Doctor of Medicine

2013

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SUMMARY

BACKGROUND

A variety of individual and population based approaches to prevention and management of obesity are required and there is an increasing interest in functional foods and natriceuticals. Conjugated linoleic acid (CLA) has generated enormous interest in this context due to their ability to modulate PPAR's and the associated metabolic processes.

AIMS & METHODS

This study aimed to investigate the effects of CLA supplementation in a randomised, double blind crossover trial on inflammation, vascular function and metabolic profiles in subjects with the metabolic syndrome.

RESULTS

This study utilised a cross-over design to investigate any differences in outcomes using 9,11 CLA isomer in comparison to the CLA mixture. The results revealed no significant differences at baseline between the two groups and a relatively small number of significant outcomes (peripheral SBP, aPWV, HDL-cholesterol, adiponectin, platelet aggregation and HOMA B). As there was a lack of substantial differences in the two treatments and an inconsistency in specifying these differences to a specific isomer, further analysis was undertaken combining the data from both groups and comparing the outcomes at baseline and at the end of the treatment using a paired't' test. Results from this sub analysis showed a significant increase in circulating sRAGE levels ('p'=0.002) along with reductions in aortic PWV ('p'=0.003) and markers of endothelial and platelet function. There were no changes in body composition or insulin sensitivity although adiponectin levels were elevated ('p'=0.003) and resistin levels decreased markedly ('p'<0.005) following the treatment.

CONCLUSIONS

Dietary supplementation with CLA seems to produce a number of vascular benefits which are noted to be independent of its metabolic effects and mediated possibly by attenuating inflammation through changes in circulatory sRAGE. This is of considerable clinical relevance and may open new perspectives and offer effective strategies to reduce the CVD burden in the metabolic syndrome.

ACKNOWLEDGEMENTS

The study was conducted at the Clinical Research Facility, University Hospital of Wales, Cardiff. The work would not have been possible without the support of many people. As only a few can be mentioned I offer my sincere gratitude to all who have helped.

First and foremost I would like to express my gratitude to my supervisor Dr Aled Rees for the valuable advice, generous support and supervision provided during the crucial stages of this project. I would also like to thank my other supervisor, Dr Marc Evans and Professor Colin Dayan for their guidance at various stages of this project.

I am grateful to Dr Keith Morris (Department of Applied Sciences, Cardiff Metropolitan University) for his endless help and advice throughout the course of this project. I would also like to thank Dr Sam Rice, Dr Neera Agarwal and Dr Manish Khanolkar for their help with vascular, platelet studies and support throughout this project. I would like to thank Kate Craig and her team for their help with patient care at the Clinical Research facility in Cardiff.

I would like to express my gratitude to all the patients who volunteered to participate in this study, as this project would not have been possible without their help.

Lastly, I would like to thank my wife Bharati and my sons Praneet and Vineet for their immense patience and understanding throughout the course of this project and to you I dedicate this thesis.

Abbreviations

ATP III	Third Adult treatment Panel
AHA	American Heart Association
Aus Diab Study	Australian Diabetes Obesity and Lifestyle Study
Aix	Augmentation Index
ADP	Adenosine diphosphate
AGEs	Advanced Glycation End products
ARB	Angiotensin receptor blockers
ACS	Acetyl Coenzyme A synthetase
ALE	Advanced lipoxidation products
BOLD	Bariatric Outcomes Longitudinal Database
BMI	Body Mass Index
BP	Blood pressure
BD	twice a day
CHD	Coronary heart Disease
CVD	Cardiovascular Disease
CRP	C - reactive protein
CLA	Conjugated linoleic acid
Co A	coenzyme A
^{0}C	degree centigrade
cm	centimetre
CV	coefficient of variance
DPS	Diabetes Prevention Study

DREAM	Diabetes Reduction Assessment with Ramipril and Rosiglitazone medication Study
EGIR	The European Group for the Study of Insulin Resistance
EASD	European Association for the Study of Diabetes
ET-1	Endothelin -1
eNOS	endothelial nitric oxide synthetase
ELISA	Enzyme Linked Immunosorbant Assay
ECG	Electrocardiogram
FFA	Free Fatty Acids
FPG	Fasting Plasma Glucose
FPI	Fasting Plasma Insulin
gm	grams
HbA1c	Glycated Haemoglobin
HDL-C	High density Lipoprotein- Cholesterol
hs CRP	high sensitivity C- Reactive protein
HOMA-IR	Homeostatic Model Assessment- insulin resistance
HCL	Hydrochloric Acid
IL-6	Interleukin-6
IL-1	Interleukin-1
IL-8	Interleukin-8
ICAM-1	Intercellular adhesion molecules
IR	Insulin Resistance
IGT	Impaired Glucose Tolerance
IFG	Impaired Fasting Glucose

IDF	International Diabetes Federation
IHD	ischaemic heart disease
Ig E	Immunoglobulin E
IMP	investigational medicinal product
Kg	Kilograms
LDL	Low Density lipoprotein
MAP	Mitogen activated protein
MAP-K	Mitogen activated protein- Kinase
MCP-1	monocyte chemo attractant protein-1
MRFIT	Multiple Risk Factor Intervention Trial
MI	Myocardial Infarction
MHRA	Medicines and Healthcare Products Regulatory Authority
m^2	square meter
mmol/l	millimoles/litre
mg	milligrams
mL	millilitres
M	Molar
mm Hg	millimetre of mercury
NEFA	Non Esterified Fatty Acids
NF-κB	Nuclear Factor kappa Beta
NHLBI	National Heart, Lung and Blood institute
NCEP -ATP III	National Cholesterol Education Programme Third Adult Treatment Panel

NHANES	The National Health and Nutrition Examination Survey
NO	Nitric oxide
ng	nanograms
OGTT	Oral Glucose Tolerance Test
PKC	Protein Kinase C
PPAR- α	Peroxisome proliferator activated receptor- alpha
PPAR- β/δ	Peroxisome proliferator activated receptor - beta/ delta
PPAR- γ	Peroxisome proliferator activated receptors - gamma
PUFA	polyunsaturated fatty acids
PAI-1	Plasminogen activator inhibitor-1
PWV	Pulse Wave velocity
aPWV	Aortic pulse wave velocity
bPWV	Brachial pulse wave velocity
PI3-K	Phosphoinositide - 3 – Kinase
PG	Prostaglandin
PBS	Phosphate buffered solution
pg	picograms
PRP	Platelet rich plasma
RAGE	Receptor for advanced glycation end products
sRAGE	Soluble Receptor for advanced glycation end products
ROS	Reactive oxygen species
sVCAM	soluble Vascular adhesion molecules
sICAM	soluble intercellular adhesion molecules
SA-A	serum amyloid associated protein

SR-A	scavenger receptor pathway
TG	Triglycerides
TZDS	Thiazolidenediones
TRIPOD	Troglitazone in prevention of Diabetes
t-PA	tissue plasminogen activator
TXA2	Thromboxane A2
TNF α	Tumour necrosis factor- alpha
T2DM	Type 2 diabetes mellitus
VCAM	Vascular cell adhesion molecules
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
VLDL	Very low-density lipoprotein
WHO	World Health Organisation
WOSCOPS	West of Scotland Coronary Prevention Study

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Chapter 1 - General Introduction

1.1 Historical Overview - The Metabolic Syndrome

The Metabolic syndrome was first described more than a century ago. Eskil Kylin, a Swedish physician, recognised that high blood pressure, dysglycaemia and elevated uric acid levels frequently appeared in patients at the same time in the early part of the 20th century (1). He hypothesised that this syndrome arose from metabolic abnormalities related to the development of diabetes and / or cardiovascular disease and led to an increase in the incidence of coronary heart disease (CHD) in the middle of the 20th century with an associated increase in morbidity and mortality. The concept of risk factors was developed to combat this clinical problem with the hope that identification and treatment of causal risk factors would lead to a reduction in the incidence of cardiovascular disease (CVD).

Such clustering, however did not receive further attention until 1988, when Gerald Reaven introduced the concept of insulin resistance in his Banting lecture under the name of ‘syndrome X’ (2). The following year, Kaplan described four important features in the development of type 2 diabetes and CVD i.e. upper-body obesity, glucose intolerance, hypertriglyceridemia and hypertension, and called them ‘the deadly quartet’ (3) (Figure1.1).

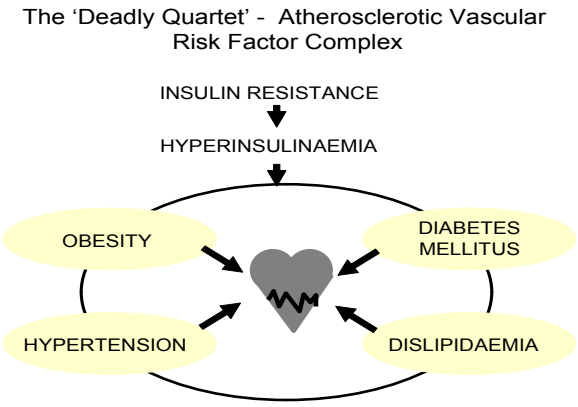

Figure 1.1: The Metabolic syndrome: The 'Deadly Quartet'

(Adapted from Kaplan *et al*, Arch Intern Med. 1989; 149(7):1514-1520)

The otherwise named 'insulin resistance syndrome' represents similar features **(Figure 1.2)** consisting of clusters of cardiovascular disease risk factors and their relationship to insulin resistance (4).

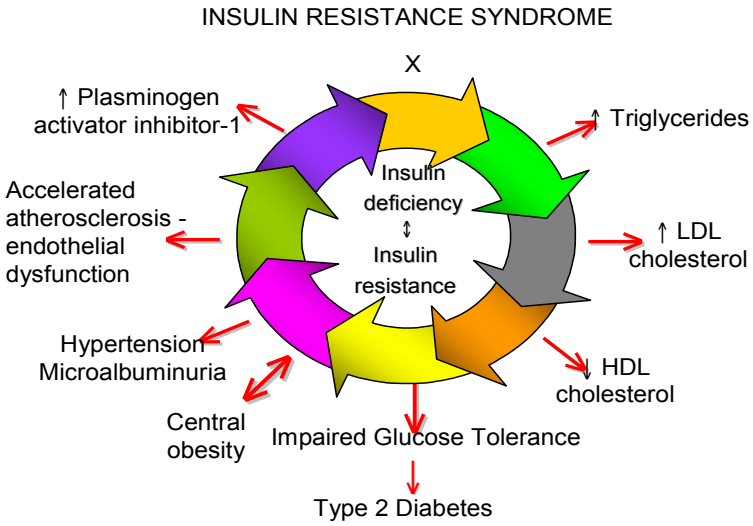

Figure 1.2: Insulin resistance syndrome and clusters of cardiovascular risk factors (Adapted from Hafner *et al*. Diabetes 1992*)*

1.2 Definition of the metabolic syndrome

The term 'metabolic syndrome' was introduced later, and slightly different definitions were proposed by the World Health Organization (WHO) (5) and the Third Report of National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III) (6). The WHO definition of metabolic syndrome was written by diabetologists with the core feature of glucose intolerance **(Table 1.1)**.

1.2.1 WHO definition of the metabolic syndrome

Table 1.1: The 1999 World Health Organization definition of the metabolic syndrome.

Glucose intolerance, Impaired glucose tolerance (IGT) or diabetes and/or insulin resistance[a] together with two or more of the following:
• Raised arterial pressure ≥ 140/90 mmHg
• Raised plasma TG (≥1.7 mmol/l) and/or low HDL-cholesterol (<0.9 mmol/l men; <1.0 mmol/l women)
• Central obesity (males: waist to hip ratio >0.90; females: waist to hip ratio
>0.85) and/or BMI >30 kg/m²
• Microalbuminuria (urinary albumin excretion rate ≥ 20g/min or albumin:creatinine ratio ≥ 30mg/g)
[a]Insulin resistance measured under hyperinsulinaemic euglycaemic conditions, glucose uptake below lowest quartile for background population under investigation.

1.2.2 The European Group for the study of Insulin Resistance (EGIR) definition for the metabolic syndrome

The European Group for the study of Insulin Resistance (EGIR) proposed a modified version of the WHO definition to be used in non- diabetic subjects. Both these definitions included a measure of insulin resistance which was not practical for use in large populations. The EGIR included fasting insulin to estimate insulin resistance and replaced impaired glucose tolerance (IGT) with impaired fasting glycaemia (IFG) **(Table 1.2)** (7).

Table 1.2: EGIR Definition of the metabolic syndrome

Fasting hyperinsulinaemia (highest 25%) and at least two of the following criteria:
• Fasting plasma glucose ≥6.1 mmol/l (excluding diabetes)
• Blood pressure ≥140/90 mmHg or treated for hypertension
• Triglycerides >2.0 mmol/l or HDL-cholesterol <1.0 mmol/l or treated for dyslipidemia
• Waist circumference ≥94 cm in men and ≥80 cm in women

Over the years the definition of the metabolic syndrome based on WHO guidelines or the ATP III criteria has been used in many publications.

1.2.3 The 2001 ATP III (Adult Treatment Panel III) definition of the metabolic syndrome

Table 1.3: NCEP ATP III Definition of the metabolic syndrome

Three or more of the following five risk factors:	
• Central obesity (waist circumference)	
Men	> 102 cm
Women	> 88 cm
• Triglycerides	≥ 1.7 mmol/l
• HDL-cholesterol	
Men	< 1.03 mmol/l
Women	< 1.29 mmol/l
• Blood pressure	≥ 130/ ≥ 85 mmHg
• Fasting glucose	≥ 5.6 mmol/l

In 2001, the USA National Cholesterol Education Program (NCEP) Adult treatment panel III (ATP III) comprising mainly of cardiologists and lipidologists, provided a definition of the metabolic syndrome, with a follow – up in 2004, replacing the glucose tolerance test with fasting glucose **(Table 1.3)**. This definition gained more acceptance as it is easy to use in clinical practice, as well as in epidemiological studies and clinical trials.

The major difference between NCEP ATP III definition and that provided by the WHO is that the WHO definition includes insulin resistance / hyperglycaemia as a categorical component, while it is one of the five optional risk factors in the NCEP ATP III definition. The NCEP ATP III criteria also recognised the association between the above components of the metabolic syndrome and both pro-inflammatory and pro-thrombotic states although these are not required for defining the metabolic syndrome.

Table 1.4: Summary of abnormalities that can be present in the metabolic syndrome

Abdominal Obesity	Elevated Uric Acid	Elevated free fatty acids
Insulin resistance (IR) / Hyperglycaemia	Pro inflammatory state	
Dyslipidaemia	Pro thrombotic state	
Hypertension	Endothelial Dysfunction	

The international Diabetes Federation (IDF) added its definition to the literature in 2005, placing more emphasis on central obesity as the core measurement and building upon the less glucocentric criteria of the NCEP ATP III. Ethnic-specific cut - off points were also incorporated into the definition, further increasing its practical utility (8).

1.2.4 International Diabetes federation (IDF) definition of the metabolic syndrome

Table 1.5: IDF definition of the metabolic syndrome

• Central obesity: waist circumference (Europid men: ≥94 cm;Europid women: ≥80 cm)**
PLUS at least two of the following components:
• Raised TG level: ≥ 1.7 mmol/l OR on treatment for this abnormality
• Reduced HDL-cholesterol: < 1.03 mmol/l in men; < 1.29 mmol/l in women or on specific treatment for this lipid abnormality
• Raised blood pressure: systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg or treatment of previously diagnosed hypertension
• Raised fasting plasma glucose ≥ 5.6 mmol/l OR previously diagnosed type 2 diabetes (If above 5.6 mmol/l, OGTT is strongly recommended but is not necessary to define presence of the syndrome)
***Waist circumference: with ethnicity specific values for other group*

The new IDF definition for the metabolic syndrome resolved the controversies and inconsistencies of previous definitions and eventually provides a standard definition which could be used across research and clinical groups.

The American Heart Association / National Heart, Lung and Blood institute (NHLBI) revised the glucose criterion in the NCEP ATP III definition in 2005 (9).Looking at all four groups (WHO 1999, ATP III, EGIR and IDF) of diagnostic criteria for metabolic syndrome, it can be concluded that waist

circumference / obesity, dyslipidaemia, hypertension and glucose intolerance are the main metabolic abnormalities with different values suggested for certain components **(Table 1.6)**.

Table 1.6: Similarities and differences between diagnostic criteria for the metabolic syndrome

Component	Similarities	Differences
Waist circumference (cm)	IDF, EGIR (men ≥94, women ≥80)	ATP III (men >102, women >88) WHO (BMI ≥30 kg/m²)
Blood pressure (mmHg)	IDF, ATP III (≥130/85)	EGIR, WHO (≥140/90)
Triglycerides (mmol/l)	IDF, ATP III, WHO (≥1.7)	EGIR (>2.0)
HDL-C (mmol/l)	IDF, ATP III (men <1.03, women <1.29)	EGIR (HDL-c <1.0) WHO (men <0.9, women <1.0)
Plasma glucose (mmol/l)	EGIR, ATP III (FPG ≥6.1 mmol/l)	IDF (FPG ≥5.6 mmol/l) WHO (any glucose intolerance) High fasting insulin (EGIR)
Others	-	Microalbuminuria (WHO)

Controversy exists regarding the usefulness of the concept of the metabolic syndrome, specifically whether the concept of the syndrome is at all useful to individuals, clinicians, researchers or policy makers and, if so, how the syndrome should be defined (Gale *et al* 2005 & 2008, Kahn *et al* 2005, Alberti *et al* 2008, Borch-Johnson & Wareham *et al* 2010).

There is little doubt that the individual components of the metabolic syndrome cluster together and that this clustering of components is associated with an increased risk of both diabetes and cardiovascular disease. Thus the metabolic syndrome has considerable utility in placing the emphasis on the importance of ectopic fat location, allowing appropriate lifestyle changes to be encouraged and identification and treatment of other components where appropriate. The presence of the metabolic syndrome should also highlight the need for estimating an individual's absolute cardiovascular risk with appropriate treatment if the cardiovascular risk is greater than the threshold set for the population. Furthermore, identification of the presence of the metabolic syndrome heightens awareness for both the individual and clinicians of the risk of associated diseases and trigger an active strategy focused on life style to attenuate the effects of risk factors and associated diseases.

A further revision of the criteria was thus presented in 2009 in a consensus statement from the IDF, NHLBI, the World Health Federation, the International Atherosclerosis Society and the American Heart Association (AHA) (10) (Table 1.7).

Table 1.7 Consensus statement 2009 criteria (Alberti, Eckel, Grundy, Zimmet, Fruchart *et al*)

Central obesity: waist circumference according to population and country specific definitions: (≥ 102 cm (North American men) ≥ 88cms (North American women) ≥94 cm (Europid men) ≥80 cm (All women other than North Americans)
•Raised TG level: ≥ 1.7 mmol/l OR on treatment for this abnormality
•Reduced HDL-cholesterol: < 1.03 mmol/l in men; < 1.29 mmol/l in women or on specific treatment for this lipid abnormality
•Raised blood pressure: systolic BP ≥ 130 mmHg or diastolic BP ≥ 85 mmHg or treatment of previously diagnosed hypertension
• Raised fasting plasma glucose ≥ 5.6 mmol/l OR previously diagnosed type 2 diabetes

The European Association for the study of Diabetes (EASD) and the American Diabetes Association (ADA) did not contributed to the consensus statement as a result of polarisation of views between different professional groups.

1.3 Epidemiology

With increases in obesity and an ageing population, the metabolic syndrome is a growing problem worldwide. However, describing the global burden of the metabolic syndrome can be challenging and the prevalence rates have varied according to the definition used and population studied. Cut off points for each feature of the syndrome are likely to differ between populations in terms of their clinical risks. Furthermore, there are limited data for certain populations and for the young and elderly, as most of the studies were based in developed countries or urban areas of developing countries.

Ethnicity, age and gender clearly have a great impact on the prevalence of the metabolic syndrome, and this variation may be due to differences in genetic susceptibility, diet, obesity, and intrauterine development. The difference in prevalence rates for the metabolic syndrome using WHO, EGIR and ATP III is demonstrated from a large Australian Diabetes, Obesity and Lifestyle study (Aus Diab study) (11) and a Finnish study (12).

Using both WHO and ATP III criteria, Ford and Giles reported approximately 20% of individuals were classified as having the syndrome by WHO criteria but not by the ATP III criteria, and vice versa (13). Meigs et al. (14) have shown using WHO and ATP III criteria that the prevalence of the metabolic syndrome is different between men and women, even among a high risk population for the metabolic syndrome such as Mexican-Americans.

The prevalence of the metabolic syndrome increases with age. For example, in the National Health and Nutrition Examination Survey (NHANES III) in the U.S, the overall prevalence of the metabolic syndrome among adults aged 20 years and over was 24% whilst in those aged more than 50 years old the prevalence was 44% (15). There are ethnic variations with South Asians in particular tending to have higher intra abdominal fat at lower BMIs making them more susceptible to type 2 diabetes, hypertension and CHD (36): for example, in 1988, Mckeigue et al showed a higher prevalence of type 2 diabetes and the metabolic syndrome in Bangladeshies compared with Europeans in East London (16). These findings were replicated in a larger population based survey of 4858 men and women - the Southall and Brent studies in West and Northwest London (17). Furthermore, migrant African Caribbeans who participated in the above study had higher prevalence rates of hypertension and type 2 diabetes.

Another study in Asian subjects reported increased prevalence of the metabolic syndrome using the IDF definition (46.3%) compared with ATP III criteria (30.5%), and the prevalence was higher in women than in men (18).

In South Korea, although central obesity was only 0.2% in men and 27.3% in women respectively (lower than in the United States), the prevalence of the metabolic syndrome was similar to the North Americans. According to the ATP III criteria, 29.4% of Korean subjects above 40 years of age have the metabolic syndrome (19).

Most recent studies apply the new IDF definition for diagnosing the metabolic syndrome. Harzallah *et al*. reported a higher prevalence of the metabolic syndrome amongst an Arab population using IDF criteria (45.5%) compared with WHO (28.7%) and ATP III (24.3%).The prevalence was also significantly higher in women than in men (20). Of 9669 subjects in Greece, the age-adjusted prevalence of the metabolic syndrome was 24.5% (ATP III) which increased to 43.4% using the new IDF definition (21). Another study in Asian subjects reported increased prevalence of the metabolic syndrome using the IDF definition (46.3%) compared with ATP III criteria (30.5%), and the prevalence was higher in women than in men (22).

Applying the new IDF criteria clearly results in an increase in the prevalence of the metabolic syndrome, and this may have significant implications for public health. The higher rate could be due to the predominant focus on central obesity which is measured by waist circumference. In the Finnish Kuopio Ischemic Heart Study, the prevalence of the metabolic syndrome varied between 9% and 14% depending on whether ATP III or the WHO criteria were used.

Whilst the use of different definitions of the metabolic syndrome makes comparisons across studies and ethnic groups difficult, there is no doubt that profound ethnic differences exist in the prevalence and clustering of cardio metabolic risk factors.

The prevalence of the metabolic syndrome varies approximately from 10% to 40% amongst different populations with a tendency to increase with age. The US National Health and Nutrition Examination Survey (NHANES III) reported an age - adjusted prevalence of 24% amongst more than 20,000 adults, with the numbers increasing significantly with age (23)

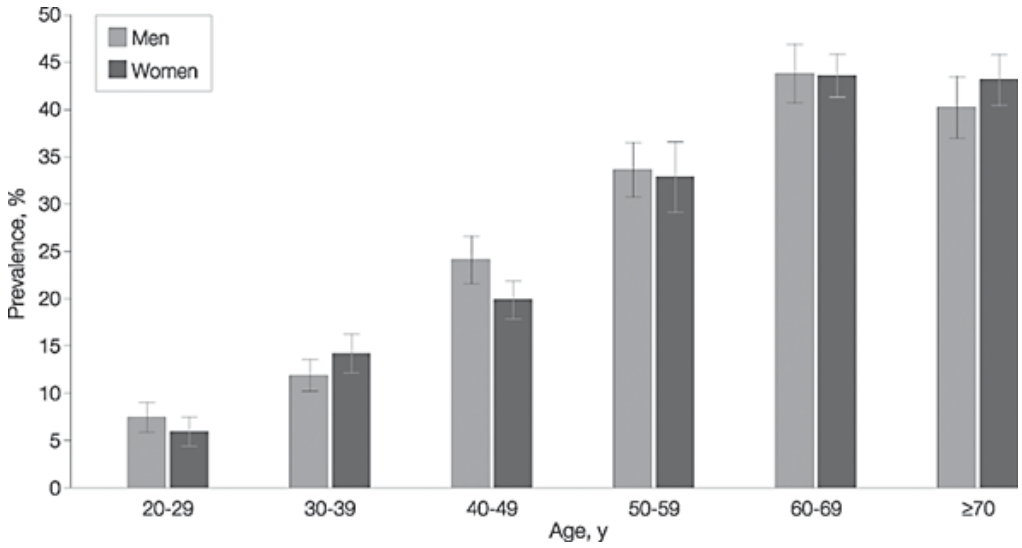

Figure 1.3 - Age-Specific Prevalence of the Metabolic Syndrome among 8814 US Adults Aged at Least 20 Years, by Sex, National Health and Nutrition Examination Survey III, 1988-1994 (Adapted from Earl S. Ford, Wayne H. Giles, William H. Dietz *JAMA.* 2002;287(3):356-359).

Diet and physical activity can also have an effect on the prevalence of insulin resistance and the metabolic syndrome independently of obesity.

In the Framingham Offspring Study the authors reported that whole grain intake was associated with a lower prevalence of the metabolic syndrome and inversely associated with insulin resistance (24). The Whitehall II study of civil servants in Britain showed a similar association between insulin resistance, moderate physical exercise and prevalence of the metabolic syndrome (25).

1.4 Aetiology of the metabolic syndrome

The complex pathogenesis of the metabolic syndrome and its key components is still to be elucidated completely. It is likely that both genetic and environmental factors are involved in the development of the metabolic syndrome with a primary focus on insulin resistance and visceral obesity. Other factors associated with an increased risk of the metabolic syndrome in NHANES III included postmenopausal status, smoking, poor diet and physical inactivity (23).

Various hypotheses have been proposed for the development of the metabolic syndrome.

1.4.1 The Thrifty Genotype Hypothesis

Neel proposed this hypothesis more than three decades ago to explain the widespread prevalence of insulin resistance and type 2 diabetes in modern society. He hypothesised that insulin resistance / hyperinsulinemia conferred a survival advantage during evolution and provided a protective mechanism to humans during periods of famine, as it would minimise caloric loss during times of famine and facilitated fat storage during times of plenty (26). It has

also been suggested that selective insulin resistance within the muscles facilitated energy storage within the liver and adipose tissue. However, in an environment of persistent calorie excess these characteristics would predispose to obesity, the metabolic syndrome and type 2 diabetes (27).

1.4.2 Reaven / Cahill Hypothesis

Cahill & Reaven proposed that insulin resistance limited protein / muscle breakdown thus allowing hunting to continue rather than as a means to minimise calorie loss for survival (28).

1.4.3 Barker's Hypothesis – The foetal origins of adult disease

Using data from the county of Hertfordshire, where detailed records were kept by midwives since 1911, Barker suggested that insulin resistance, type 2 diabetes and the metabolic syndrome seen in adults are the results of an adverse intrauterine and neonatal environment and are therefore manifestations of a thrifty phenotype. Barker suggested that a thrifty genotype might be induced by malnutrition during fetal and early life. In particular, intrauterine growth restriction leading to low birth weight may be associated with an increased risk in adulthood of insulin resistance, glucose intolerance, type 2 diabetes and dyslipidaemia (figure 1.4). He showed that the prevalence of the metabolic syndrome reduced progressively from 30% to 6% as birth weight rose from less than 2.5 to more than 4.1 kg (figure 1.5 & 1.6). This has led to the hypothesis that these diseases are 'programmed' in utero in response to an adverse environment.

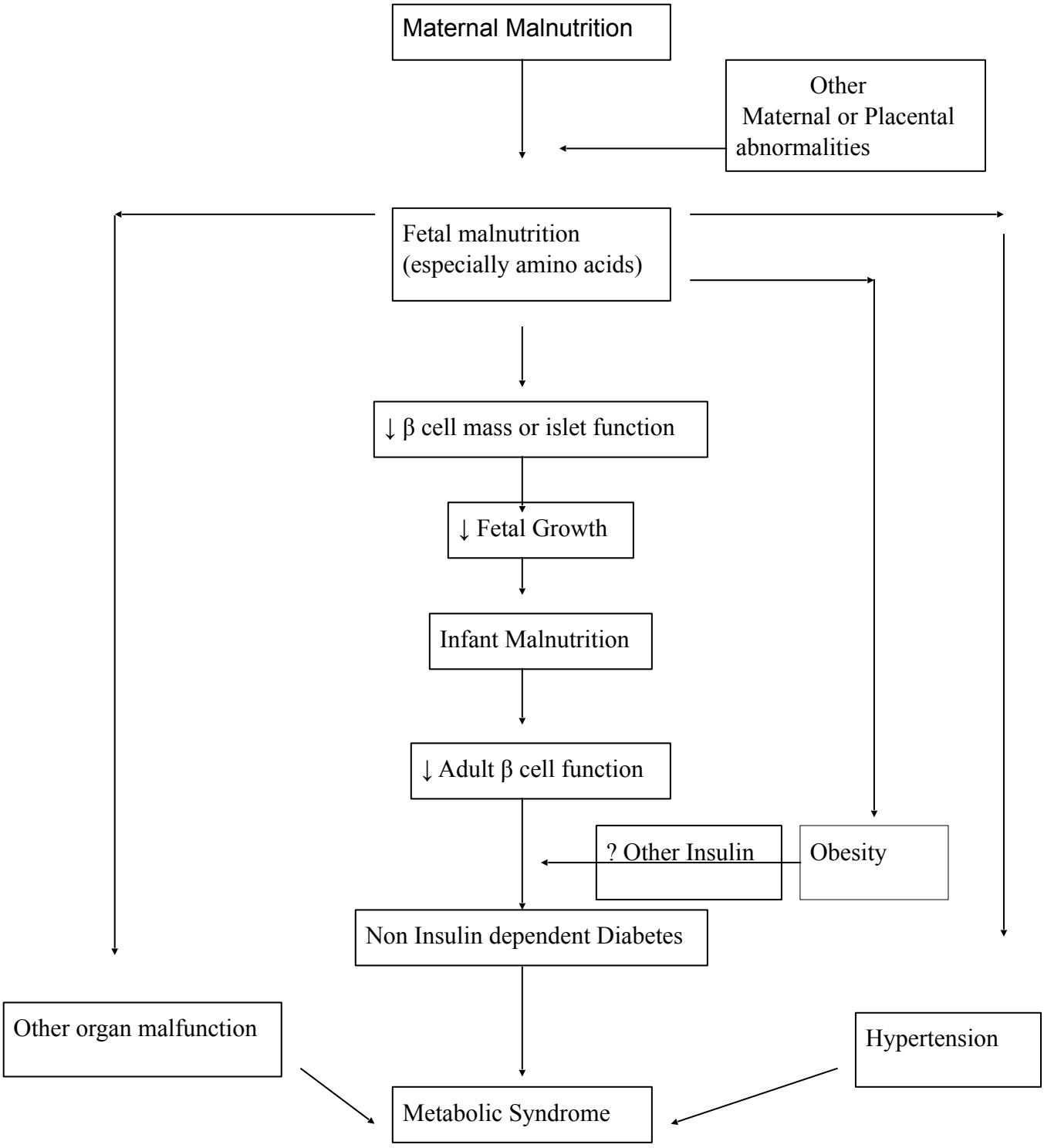

Figure 1.4 Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. (Adapted from DJP, Hales CN, Fall CHD, Osmond C, Phipps K, Clark PMS. *Diabetologia,* 1993; 36: 62–7).

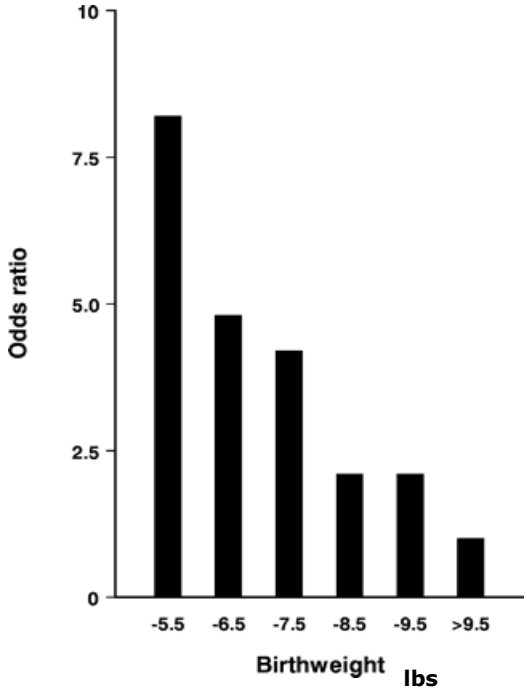

Figure 1.5 Odds ratio for impaired glucose tolerance or type 2 diabetes according to birth weight among 370 men aged 64 years born in Hertfordshire (adjusted for BMI).

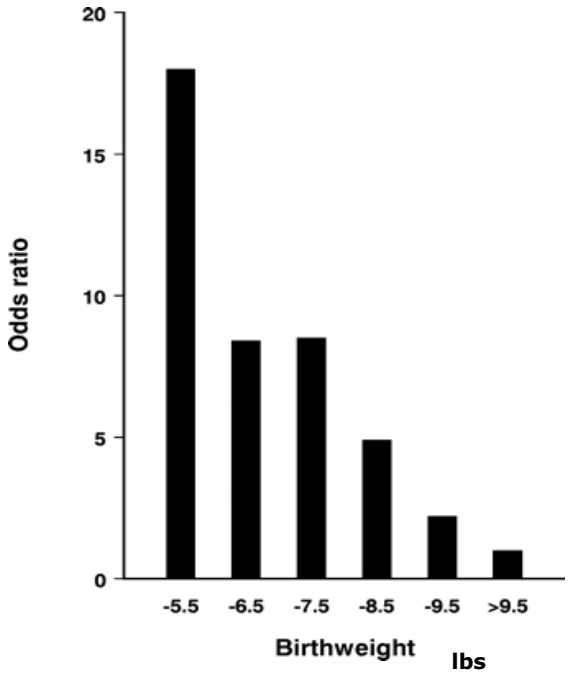

Figure 1.6 Odds ratio for the metabolic syndrome according to birth weight among 407 men born in Hertfordshire (adjusted for adult body mass index).

(Adapted from The thrifty phenotype hypothesis, CN Hales, DJP Barker, *Br Med Bull* (2001) 60 (1): 5-20).

1.4.4 The ‘Common Soil’ Hypothesis

Stern suggested that atherosclerosis and type 2 diabetes share many of the same genetic and environmental antecedents and therefore should be considered as a consequence of the metabolic syndrome, i.e. from a common soil (30). Thus, fetal programming effects with resultant thriftiness and hence the propensity to develop obesity as a consequence of modern day life may result in obesity and complex metabolic disease. Alternatively, central obesity with reduced insulin action and inflammation may be an essential early step resulting in clustering of various metabolic risk factors for the development of diabetes and cardiovascular disease.

However, there is a continuing debate as to the relative importance of genetic versus environmental factors in determining foetal growth and subsequent adult susceptibility to type 2 diabetes and the metabolic syndrome (29).

1.5 Pathogenesis of the Metabolic Syndrome

Insulin resistance and central obesity are believed to be underlying factors causing the metabolic syndrome.

1.5.1 Insulin Resistance and the Metabolic Syndrome

There is clear evidence supporting a major role for insulin resistance in the development of the metabolic syndrome.

Insulin resistance, the associated hyperinsulinaemia and hyperglycemia, body fat redistribution and adipocyte cytokine changes (adipokines), dyslipidaemia, haemostatic alterations, low grade inflammation and high blood pressure may all lead to the development of atherosclerotic cardiovascular disease. The exact aetiology of insulin resistance is not completely understood, although it is believed to be genetically predetermined and exacerbated by physical inactivity, obesity and aging.

1.5.2 Obesity, adipocytokine dysregulation and the metabolic syndrome

Increasing central adiposity is associated with an increased risk of morbidity and mortality (31). Even in people of nearly normal weight, increased central adiposity may increase the risk for a wide range of chronic conditions including type 2 diabetes, hypertension, dyslipidaemia, gall bladder disease, osteoarthritis and in women, breast cancer. In one study, waist circumference, but not BMI, was a predictor for developing hypertension, dyslipidaemia and the metabolic syndrome (32, 33), while in a second it was a predictor of myocardial infarction (34).

Adipose tissue was historically considered to be a passive depot for the storage of excess calories. During fasting, adipocytes through lipolysis break down triglycerides with release of fatty acids to meet the energy needs of the body. However, during the post prandial state the balance shifts back toward lipogenesis. More recently, adipose tissue has been shown to function as an

active metabolic organ capable of producing various chemical cytokines such as interleukin – 6 (IL-6), tumour necrosis factor alpha (TNFα) and other adipokines including leptin, adiponectin and plasminogen activator inhibitor (PAI-1) all of which, among other things, may be important for the development of the metabolic syndrome.

The location of the excess fat plays an important role, with intra abdominal / visceral fat playing a critical role in determining insulin sensitivity and atherogenic lipid profile. Intra - abdominal adipose tissue is more sensitive to free fatty acid (FFA) mobilisation stimuli than subcutaneous fat with insulin being the principal inhibitor of lipolysis. The FFAs mobilised from adipose tissue are mainly taken up by the liver and esterified into triglycerides and incorporated into very low density lipoproteins (VLDL).The triglyceride rich VLDL particles are metabolised by lipolysis with delivery of fatty acids to peripheral cells. This process is finely regulated and the rate of FFA mobilisation is in tune with the energy needs of the body.

Excessive mobilisation of FFA from the expanded adipose tissue in patients with the metabolic syndrome raises plasma levels of FFA. High levels of FFA decrease peripheral glucose uptake due to the Randle effect (the reciprocal uptake of glucose and FFA in tissues). In addition, elevation of FFA increases glucose output from the liver by stimulating gluconeogenesis and glycogenolysis.

The pro inflammatory cytokine TNF-α has been shown to be an important regulator of insulin sensitivity while IL-6 is believed to be the main driver of C reactive protein (CRP) release from the hepatocytes. IL-6 has also been shown to impair insulin signalling in mouse hepatocytes (135,136).

Adipocytes secrete leptin in response to nutritional status and adipose tissue mass. Leptin is predominantly secreted by subcutaneous adipose tissue and plays an important role in appetite regulation and energy expenditure through its effects on hypothalamic leptin receptors. Resistin is another adipocytokine that has been implicated in the pathogenesis of type 2 diabetes and its complications (137).

Visceral obesity is also associated with a reduction in adiponectin levels and thus a loss of its anti inflammatory, anti diabetic and anti atherosclerotic properties (35). Over time, insulin resistance is associated with more components of the metabolic syndrome, including increased plasminogen activator inhibitor-1 (PAI-1) mediated thrombotic tendency, hypertension, hyperuricemia and a characteristic dyslipidaemia.

Adipose derived secretory proteins, collectively referred to as adipokines, may thus have an important role in the development of insulin resistance, appetite, inflammatory responses and thrombosis in overweight subjects with visceral obesity.

1.6 Clinical implications of the Metabolic Syndrome

As stated previously, subjects with the metabolic syndrome are at increased risk for the development of type 2 diabetes and cardiovascular events (12, 15).

Increasing evidence suggests that the progression of insulin resistance to type 2 diabetes parallels the progression of endothelial dysfunction to atherosclerosis **(Figure 1.7).**

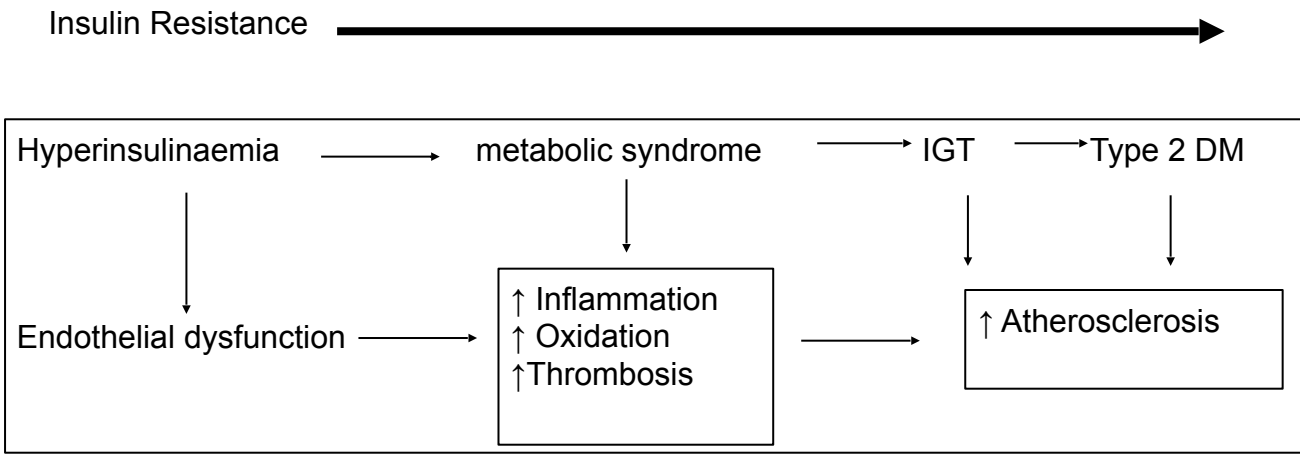

Figure 1.7 (Adapted from Hsueh and Law, *Am J Cardiol*. 2003; 92:3J–9J)

It is increasingly clear that impaired Insulin sensitivity is not confined to the major tissues that regulate glucose metabolism (skeletal muscle, liver and adipose tissue), but also present in cells that play an important role in cardiovascular regulation i.e. vascular endothelium, platelets, macrophages and smooth muscle cells.

1.6.1 Metabolic Syndrome and Cardiovascular disease – Overview

Each of the components of the metabolic syndrome is associated with an increased risk of CVD but in combination they increase the risk several fold.

The Paris Prospective Study and the Interheart studies showed central obesity as measured by waist-hip ratio or waist circumference to be a better predictor of cardiovascular disease and mortality than BMI, and suggested that such measurements should replace BMI as an indicator of obesity (49, 50). Many studies have shown that patients diagnosed with the metabolic syndrome, by either the ATP III or WHO definition (or by their modifications), have more prevalent CVD or are at greater risk of developing it and this excess risk remained after adjustment for other conventional risk factors (138-145). In the Framingham study, the metabolic syndrome alone predicts about 25% of new CVD cases (141, 147). Stern *et al* estimates that individuals with the metabolic syndrome are twice as likely to die of cardiovascular complications compared to those without (146). This association was however highlighted much earlier by Gerald Reaven who, in 1988, reported abnormalities of blood pressure regulation and lipid abnormalities in insulin resistant individuals and proposed that clustering of these risk factors accounted for the development of type 2 diabetes and cardiovascular disease (2). Subsequently alterations in the fluid and cellular phases of both thrombotic and inflammatory processes have been shown to be associated with insulin resistance with the entire process being driven by obesity.

1.6.2 Atherothrombosis is defined as atherosclerotic plaque disruption with superimposed thrombosis formation leading to cardiovascular events including myocardial infarction, stroke and peripheral vascular disease. At the molecular level, atherosclerosis is a multi-step process involving the interaction of many different complex cascades. These include endothelial function, inflammation, lipoprotein metabolism, coagulation and oxidative stress. (Figure 1.8)

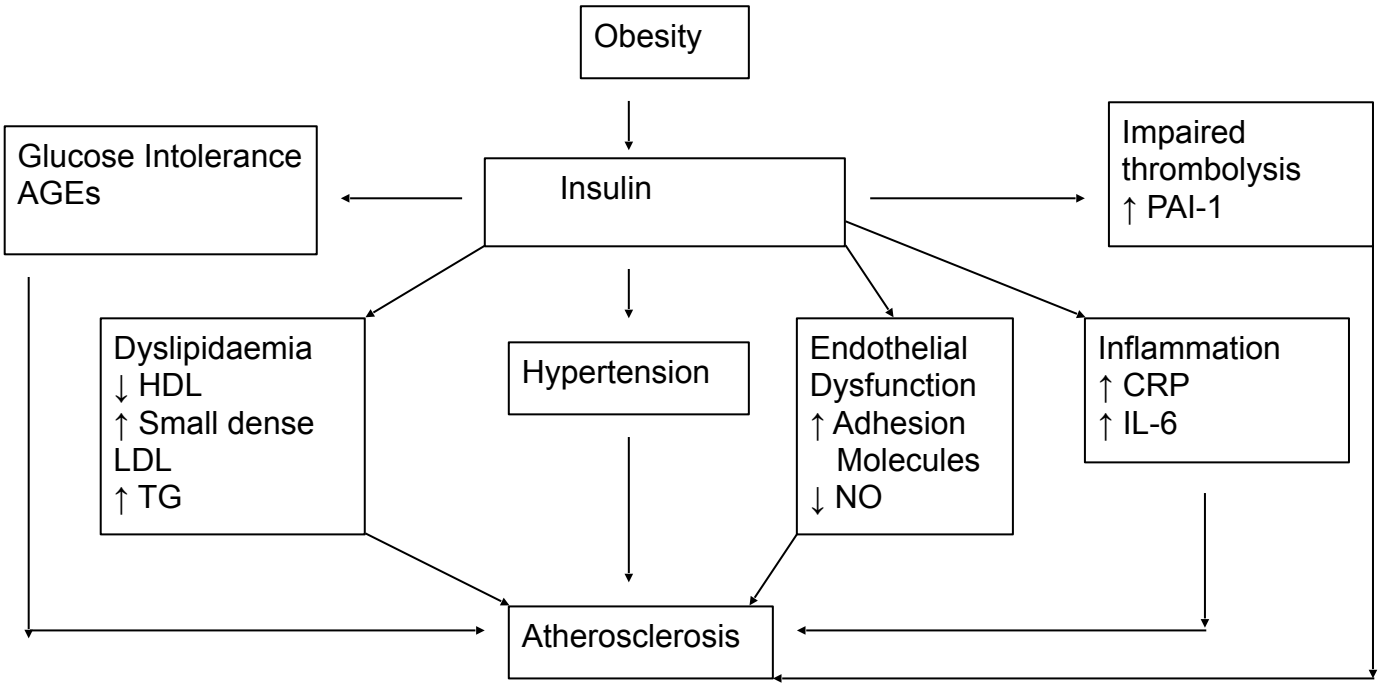

Figure 1.8 Association of Insulin resistance with cardiovascular risk factors and Atherosclerosis (Adapted from Macfarlane et al *JCEM*; 2001:86; 713).

Considerable ‘cross talk’ exists between these processes, resulting in a complex ‘cause or consequence’ phenomenon with positive feedback on one another. It is difficult to unravel the impact that these processes have on one another, and is crucial to our understanding of the pathophysiology of atherosclerosis in patients with the metabolic syndrome.

1.6.3 Endothelial Dysfunction

Besides providing a physical barrier between the vessel wall and lumen, the endothelium secretes a number of mediators that regulate vessel tone, platelet aggregation, coagulation and fibrinolysis (table 1.8).

Table 1.8 - Vasoactive substances and their effect on the endothelium

Vasodilators	**Vasoconstrictors**
Nitric oxide	Angiotensin II
Endothelium-derived hyperpolarising factor	Endothelin
C-type natriuretic peptide	
Kinins	

The most important of these mediators is nitric oxide (NO) which is a major vasodilator with multiple vascular protective effects. These include inhibition of vascular smooth muscle cell (VSMC) growth and migration, monocyte adhesion, inflammation and oxidation, platelet aggregation and thrombosis.

In contrast, angiotensin II induces expression of adhesion molecules like intercellular adhesion molecules (ICAM-1) and vascular adhesion molecules (VCAM-1) which enhance the adhesion of monocytes to the endothelial surface, stimulates platelet aggregation and thrombosis and also induces inflammation and oxidative stress through the generation of superoxide radicals.

During this process of endothelial activation, soluble forms of cell adhesion molecules are shed into the circulation such as sVCAM-1, sICAM-1 and sE-selectin. Increased levels of these soluble cell adhesion molecules have been detected in plasma of subjects with CVD, T2DM and IR (148) and have been associated with increased risk of CVD mortality in subjects with T2DM (149). This has lead to a wider use of soluble cell adhesion molecules as a surrogate marker of disease activity in CVD. Endothelial dysfunction is an early step in the atherogenic process in patients with the metabolic syndrome with insulin resistance playing an important role and insulin itself potentially contributing to vascular damage.

Insulin causes vasodilatation through a direct effect on blood vessels mediated by endothelium-derived nitric oxide (NO). Insulin increases endothelial NO production through induction of eNOS gene expression that is mediated by the phosphoinositide 3-kinase (PI3 kinase) signalling pathway (150) (figure 1.9)

PI3-kinase activation also regulates glucose uptake into insulin-dependent target tissues, such as skeletal muscle, heart and adipose tissue. Therefore a defect in this pathway impairs glucose uptake as well as insulin mediated endothelial vasodilatation (151).Vascular insulin signalling is also capable of activating potentially proatherogenic mechanisms such as induction of endothelin-1 (ET1) expression, mediated primarily by the Mitogen-activated protein (MAP) kinase signalling pathway (figure 1.9)

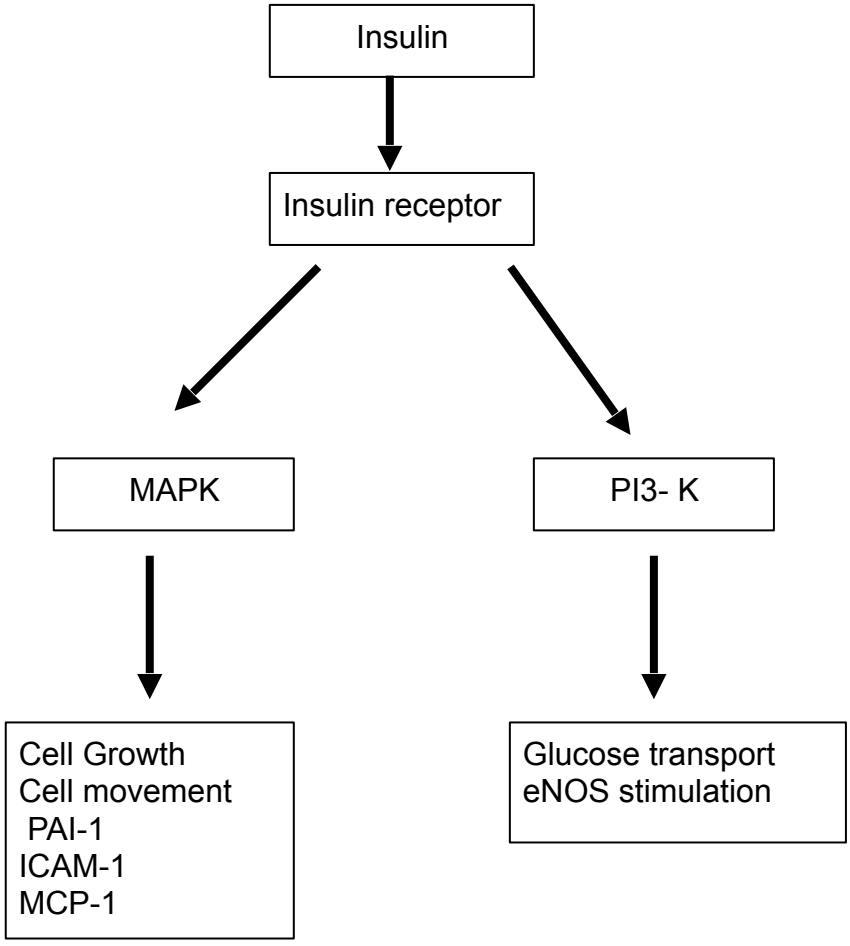

Figure 1.9 - Insulin action mediated by the PI3 kinase and MAP kinase signalling pathways

In the insulin resistant state, there seems to be a 'selective resistance' to the potential anti-atherosclerotic mechanisms mediated by the PI3 kinase pathway, whilst leaving certain pro atherosclerotic pathways mediated by MAP kinase unchecked (152).

Interventions to reverse endothelial dysfunction

As described previously clustering of both traditional and novel CVD risk factors in subjects with the metabolic syndrome initiates a chronic inflammatory process accompanied by loss of vasodilator and antithrombotic factors with resultant increase in vasoconstrictor and prothrombotic activity. Interventions that are known to reduce cardiovascular risk such as treatment with statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or Peroxisome Proliferator-activated Receptor (PPAR) ligands, as discussed below have been shown to improve endothelial dysfunction.

A sedentary lifestyle is associated visceral adiposity, increased inflammation, oxidative stress and endothelial dysfunction. In contrast exercise has been shown to improve endothelial function and this is believed to be mediated in large part through increased NO bioavailability (326,327,328,329).

Diets low in saturated fat and n-3 fatty acids have been shown to improve endothelial dependent vasodilatation (330,331).Studies of combinations of antioxidants such as vitamin C, vitamin E and β- carotene have provided mixed results (332-336).

Treatment with Statins has been shown to improve reduce cardiovascular risk and reverse endothelial function (335-339). Apart from lowering cholesterol, statins have been shown to directly enhance expression and activation of the endothelial form of NO synthetase (337-340). Furthermore statins have also been shown to protect against the adverse effects of inflammation on the vasculature (341,342). Similarly ACE inhibitors and angiotensin receptor blockade also improves endothelial function and reduce endothelial inflammation and oxidative stress by inhibiting NADP oxidase activity and breakdown of bradykinin (343 -347).

Conjugated linoleic acid, a naturally occurring PPAR α & γ ligand can reduce cardiovascular risk and improve endothelial function through their effects on BP, body composition, inflammation and insulin sensitivity.

1.6.4 Arterial Stiffness

The arterial wall consists of the outer adventitia and the connective tissue, the smooth muscle and elastin containing media which determines the elasticity, and the inner endothelium which plays an important role in regulating the vascular tone. Arteries are compliant elastic structures and serve to buffer the pressure changes resulting from intermittent ventricular ejection into the aorta. They transform the pulsatile flow generated by cardiac contraction into a continuous flow of blood in the periphery by absorbing a proportion of energy in systole, and releasing it in diastole. The elasticity of the proximal large arteries is largely the result of the high elastin to collagen ratio within the media of the vessel wall. Arterial stiffness is a

term that characterises the reduced ability of arteries to expand and contract with cardiac pulsation and relaxation and can therefore arise as a consequence of structural changes within the vessel, for example secondary to age related elastin fibre degeneration, as well as disease states such as diabetes and hypertension affecting the functional properties of the arterial wall, which is largely dependent on the endothelium (153).

1.6.4.1 Arterial pressure wave form

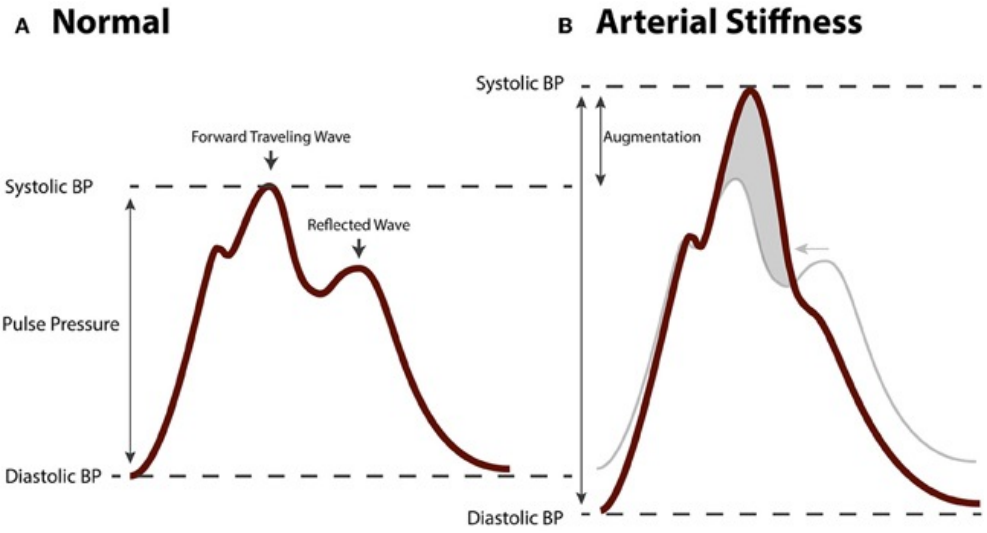

Figure 1.10 Hemodynamic changes in arterial stiffening. (A) Aortic blood pressure waveform of a healthy, normotensive person. The forwards travelling wave precedes the (backwards travelling) reflected wave. **(B)** Aortic pressure waveform of a person with arterial stiffness. Due to increased pulse wave velocity, the forward travelling wave and reflected wave are summated leading to augmented pulse pressure (Reproduced from *Front. Genet.* 13 December 2012)

The arterial pressure wave form is a composite representation of the pressure waves generated during ventricular systole (1st waveform peak) which then travels through the circulation until resistance is encountered at the peripheries when it is reflected back upon itself in diastole. The 2nd waveform thus represents a summation of reflected waves. Augmentation index is defined as the difference between the first and second systolic peaks of the central pressure waveforms, expressed as a percentage of the central pulse pressure (Figure 1.10).

With increasing arterial stiffness the speed at which the left ventricular ejection pressure wave travels through the arterial circulation increases, thus resulting in an earlier return of the reflected wave from the peripheries. If the reflected wave continues to return less in diastole and more in systole itself, this can augment the late systolic pressure on the left ventricle and reduce the coronary perfusion during diastole with resultant risk of IHD, cerebrovascular disease (due to increased central systolic pressure) and cardiac failure (as a result of increased left ventricular load).

Arterial stiffness has been shown to be independently predictive of cardiovascular mortality in a number of disease states including glucose intolerance / diabetes, hypertension and renal impairment (154-156). There is also evidence of a positive correlation between arterial stiffness, high sensitivity c-reactive protein (hs CRP) (157) and declining endothelial function (158).

A number of studies have also demonstrated an association between these indices of arterial stiffness and cardiovascular risk factors in subjects with the metabolic syndrome (156) and smokers (157). These studies illustrate the importance of arterial stiffness in predicting cardiovascular risk.

Arterial stiffness can be assessed by measuring a number of factors including central BP, augmentation index (Aix) and pulse wave velocity (PWV). There are various invasive and non invasive methodologies to measure arterial stiffness and these are discussed in detail in chapter 2.

Interventions to reduce arterial stiffness have been shown to be associated with improved survival (159).

1.6.5 Hypertension, the metabolic Syndrome and CVD risk

Several mechanisms (Figure 1.11) have been postulated to explain the association between insulin resistance and elevated blood pressure. Insulin resistance and its associated hyperinsulinaemia can lead to increased renal sodium reabsorption through its effects on membrane - ion transport system and by stimulating sympathetic nervous system activity (44, 45). Hypertension could also be due to a reduction in synthesis of vasodilatory prostaglandins with a relative vasoconstrictive effect (46).

Hypertension is closely associated with obesity and insulin resistance. Persistently elevated blood pressure is a risk factor for the development of CHD and underlying atherosclerosis (42, 43).

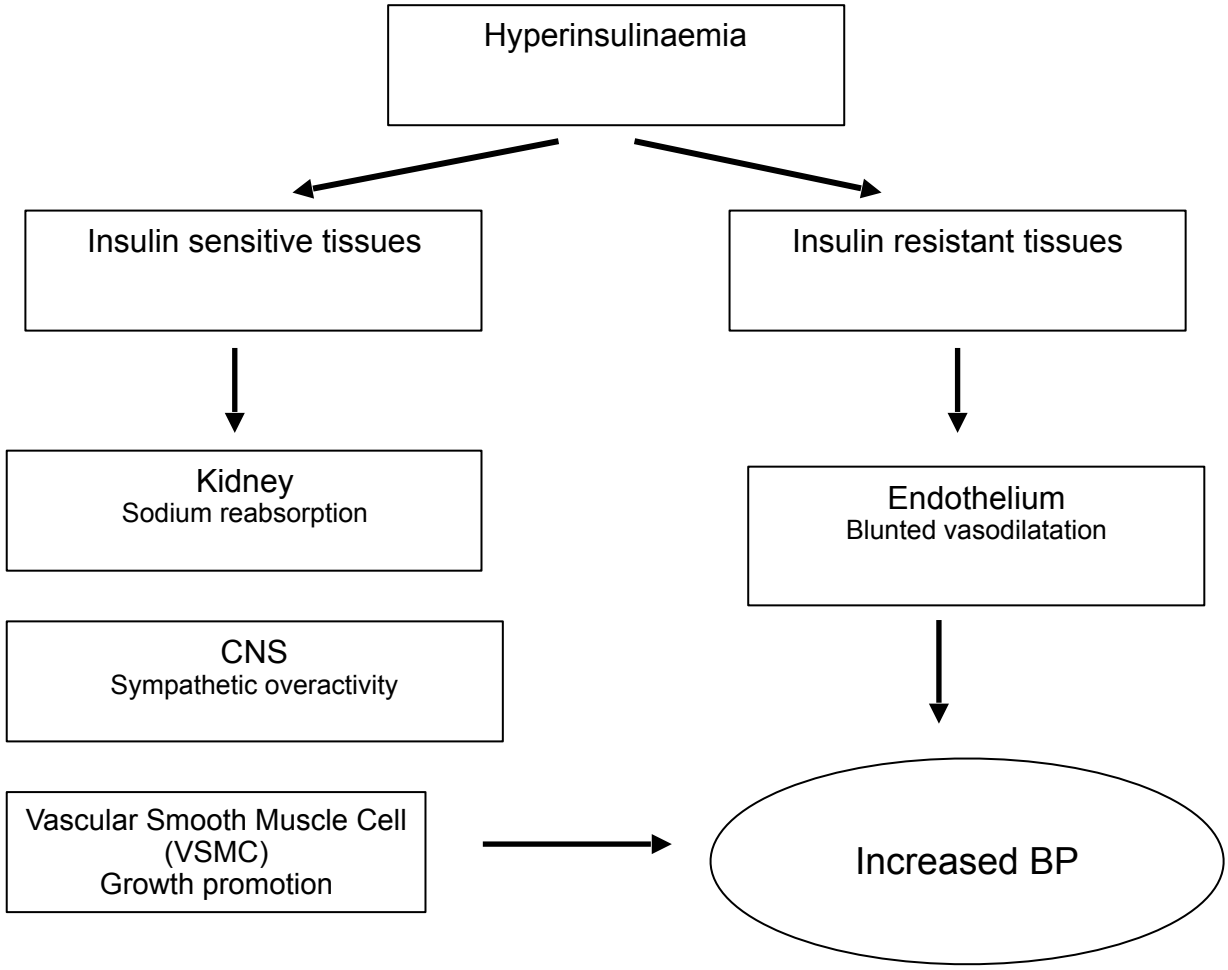

Figure 1.11 - Pathogenesis of hypertension in the hyperinsulinaemic state.

1.6.6 Metabolic Dyslipidaemia and CVD

Lipid abnormalities play a critical role in the development of atherosclerosis. Patients with the metabolic syndrome typically have a dyslipidaemia characterised by high triglycerides and reduced levels of cardio protective HDL-C levels. They also have a preponderance of small, dense and highly atherogenic LDL particles as they are more likely to form oxidised LDL (37).

As discussed previously, visceral obesity gives rise to excess free fatty acids (FFAs) which increase hepatic TG synthesis and the production of Apo B-containing, triglyceride rich VLDL(38).Triglycerides are responsible for an associated re modelling of HDL by a process of reverse cholesterol transport and esterification leading to a reduction in the cholesterol content of HDL. (39). High triglyceride levels are associated with a two fold increase in the risk of CHD after adjustment for other conventional risk factors (40). Likewise, low levels of HDL-C have been shown to be an independent risk factor for CVD (41).

1.6.7 Inflammation and the metabolic syndrome

The metabolic syndrome is believed to be associated with low - grade systemic inflammation. Many of the adipokines whose expressions are altered during obesity lead to inflammation and can promote insulin resistance, endothelial dysfunction and ultimately atherosclerosis (160) (Figure 1.12).

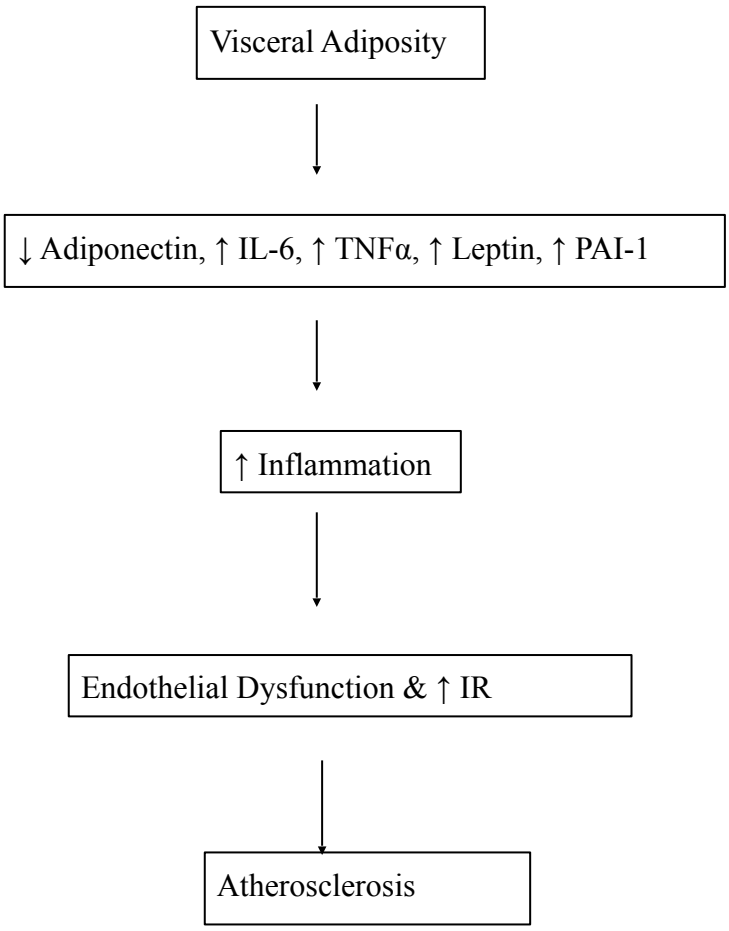

Figure 1.12 Adipocytokines, Inflammation & Atherosclerosis

Cytokines, in particular IL-1, IL-6 and TNFα are the main inducers of the acute phase response. Several studies have shown IL-6 to be the main mediator of the inflammatory response.

Interlukin-6 (IL-6) is secreted by a number of cells including activated macrophages and lymphocytes and approximately 25-30% of systemic IL-6 is derived from the adipose tissue.IL-6 may play an important role in atherogenesis and diabetes by virtue of its metabolic effects on reducing insulin sensitivity, endothelial effects on inducing adhesion molecules, and procoagulant effects by increasing hepatic synthesis of fibrinogen and by its direct effect on platelet activation (161).

Tumour necrosis factor alpha (TNF-α) similarly, is another pro-inflammatory cytokine secreted by monocytes-macrophages, endothelial cells and to a large extent by the adipocytes. It is an important regulator of insulin sensitivity. However, the best characterised marker and mediator of CVD & inflammation is c-reactive protein (CRP).

1.6.7.1 CRP

CRP is an acute phase reactant and a marker of inflammation. It is synthesised in the liver and its production is stimulated by cytokines, mainly IL-6 and tumour necrosis factor alpha (TNF-α). Although standard assays for CRP lack the sensitivity to determine the levels of inflammation within the normal range, the availability of high sensitivity CRP assays has overcome this hurdle. Several studies have indicated that elevated levels of CRP are a strong indicator of future CVD risk in healthy subjects (162).

In a cohort of 22000 healthy middle aged men, those with CRP levels in the highest quartile had a 2 fold increase in the risk of stroke or peripheral vascular disease and a 3 fold increase in the risk of myocardial infarction (163). Also, the role of CRP as a marker of vascular risk is consistent across different study populations, such as smokers in the Multiple Risk Factor Intervention Trial (MRFIT) (165), elderly subjects in the Cardiovascular Health Study (166) and postmenopausal subjects in the Women's Health Study (164).

Furthermore, studies have also demonstrated an association between CRP and all cause mortality (167). Interestingly, data from the Women's Heart Study has identified elevated levels of CRP to predict the development of type 2 diabetes in women and this concept of CRP being recognised as an independent predictor of risk for the development of diabetes is further strengthened by the West of Scotland Coronary Prevention Study (WOSCOPS), wherein raised CRP was shown to predict the development of diabetes in middle aged men (168).

Taken together, these studies provide evidence to support the role of inflammation in the pathogenesis of atherosclerosis and diabetes. This would suggest that anti-inflammatory agents could play an important role in the prevention and treatment of atherosclerosis in general and, in particular in patients with diabetes and the metabolic syndrome.

1.6.7.2 Inflammation & Atherothrombosis - Overview

Among the earliest steps in atherogenesis is the induction on the endothelial surface of adhesion molecules such as vascular cell adhesion molecule (V-CAM-1), intercellular adhesion molecule (ICAM-1), and selectins (sE-selectin, sP-selectin) which allows circulating inflammatory cells such as monocytes and T-lymphocytes to enter the vessel wall through a process of diapedesis. This process is also promoted by monocyte chemo attractant protein-1(MCP-1) and interlukin-8 (IL-8) (169,170).

Once resident in the arterial wall, monocytes differentiate into macrophages and this is facilitated by macrophage colony stimulating factor (M-CSF). Subsequently, these macrophages ingest lipids from oxidised LDL via the scavenger receptor pathway (CD-36, SR-A) and develop into lipid-laden foam cells (171). Macrophages also provide signals for T lymphocytes, to elaborate inflammatory cytokines such as interferon-γ, TNF-α, and IL-1 leading to further activation and propagation of the atherosclerotic process. They also inhibit vascular smooth-muscle cell production of collagen and other extracellular matrix components of the fibrous cap, Furthermore the lipid laden foam cells which are enriched in tissue factor also elaborate matrix metalloproteinases that degrade the collagen thus weakening the structure that separates the highly coagulable necrotic lipid core from the circulating coagulation system resulting in thrombus formation and acute coronary syndromes (169-173). Thus, monocytes / macrophages are critical in all phases of atherosclerosis.

Similarly, once resident in the intima, T lymphocytes are activated by antigens such as oxidised LDL and heat shock proteins. Upon activation, the T lymphocytes can produce cytokines, notably CD154, which is capable of binding to CD40 ligand on macrophages leading to platelet activation (184).

1.6.8 The role of platelets in atherothrombosis

Platelets play an important role in physiological homeostasis and provide a first line defence against injury to the endothelium. Following endothelial injury, circulating platelets adhere to the endothelium as a result of interaction between glycoprotein 1b/V/IX, a receptor complex expressed on the surface of the platelets and von Willebrand factor (vWF) which is an important ligand for glycoprotein 1b. Bound platelets release a variety of pro thrombotic factors including adenosine diphosphate (ADP), thrombin, thromboxane A2 (TXA2) and epinephrine which sustain and amplify the initial platelet response leading to the formation of a platelet plug. This process is mediated by glycoprotein IIb/IIIa which binds several adhesive substrates including fibrinogen and acts as the main receptor for platelet adhesion. The interaction between glycoprotein IIb/IIIa and fibrinogen represents a key event in thrombus formation (174). More recently, it has been shown that this aspect of platelet aggregation is supported by CD 40 ligand. CD40 ligand interacts with the CD40 receptor on monocytes / macrophages, leading to induction of tissue factor, a potent procoagulant,

which contributes to the thrombogenicity of the lipid core and when the circulating blood contacts the lipid core, a thrombus form (175).

1.6.8.1 CD40

CD40 has been implicated as an important signal connecting inflammation to the thrombotic elements of atherosclerotic pathology (176).

CD40 ligand (CD40L) is a transmembrane protein with a structural similarity to TNF-α that, upon engagement with its receptor CD40, elicits a series of inflammatory and pro thrombotic responses including over expression of adhesion proteins, chemokines, metalloproteinases and tissue factor (174-176). Several cells express CD40L including lymphocytes and cells of the vascular system such as endothelial cells, smooth muscle cells, monocytes and platelets (174-175).

Platelets express CD40L on their surface upon stimulation with agonists such as thrombin and collagen; CD40L is then cleaved and circulates as soluble CD40L (sCD40L) which binds to the glycoprotein IIb/IIIa complex, a process which increases the stability of platelet aggregates and leads to further inflammatory processes within the vessel wall. It is calculated that more than 95% of circulating sCD40L is of platelet origin and is an indicator of platelet activation (177). Recent studies demonstrated that sCD40L levels are elevated in patients at risk of CVD and in those with acute coronary syndrome suggesting that it may represent a new marker of atherosclerotic

progression (176-184). Experiments in animal models of atherosclerosis suggest that interruption of many of these pathways such as adhesion molecules, CD40/CD40 ligand, and chemokines can limit atherosclerotic lesion formation (181-184). Thus enhanced platelet activation may contribute to the pathogenesis of atherothrombotic complications of the metabolic syndrome.

Platelets have been shown to retain a functional insulin receptor capable of insulin binding and auto phosphorylation (185). Insulin is thought to reduce platelet response to various aggregants such as ADP, collagen, thrombin, arachidonate and platelet activating factor, and thus is believed to possess anti-platelet effects (186). In an insulin resistant state, this protective anti-platelet effect of insulin is lost. This is supported by studies demonstrating reduced platelet insulin receptor number and affinity in subjects with T2DM (186).

To summarise, enhanced platelet activation seen in patients with the metabolic syndrome is multifactorial. Platelet resistance to the inhibitory action of insulin, in association with the intrinsic platelet abnormalities induced by hyperglycaemia and oxidative stress, coupled with defective endothelial production of anti aggregants such as NO and prostacyclins are central in the pathogenesis of platelet dysfunction in subjects with the metabolic syndrome.

1.6.9 Haemostatic abnormalities and the metabolic syndrome

As far as the pro thrombotic state is concerned, patients with metabolic syndrome are characterised by elevated fibrinogen and plasminogen activator inhibitor 1 (47), which could be linked to enhanced clotting activation, However, it is not clear if this occurs in vivo and the underlying mechanism is unknown.

The adipocytes are an important source of plasminogen activator inhibitor-1 (PAI-1). Its levels correlate with the amount of visceral adipose tissue and are positively associated with features of the metabolic syndrome (47). There is increasing evidence that tissue plasminogen activator (t-PA) and PAI-1 are associated with insulin resistance in the general population. PAI-1 levels have also been shown to predict type 2 diabetes in the general population (47). PAI-1 is an inhibitor of fibrinolysis leading to a pro thrombotic state and a resultant increase in the risk of CVD. There is also increasing evidence of a link between elevated plasma levels of haemostatic factors (fibrinogen, factor VII, Von Willebrand factor) and the risk for CHD (48).

Inflammation is thus being increasingly recognised for its role in the pathogenesis of both IR and atherosclerosis. Various studies have now shown that atherosclerosis, from its origins to its ultimate complications, involves inflammatory cells (T cells, monocytes, macrophages), inflammatory proteins (cytokines, chemokines), and inflammatory responses from vascular cells (endothelial cell expression of adhesion molecules) (187).

1.7 Dysglycaemia, Receptor for advanced glycation end products (RAGE) and vascular disease in the metabolic syndrome

Obesity is one of the strongest predictors of type 2 diabetes. Bonora *et al* in a 10 year follow up of the Bruneck study showed that the relative risk for new- onset type 2 diabetes was 9.9 in individuals with a BMI of >30kg/m ² compared to individuals with a BMI of < 25kg/m² (59).

Pooled prospective studies demonstrated that subjects with the metabolic syndrome had 6-7% risk for all-cause mortality, 12-17% risk of cardiovascular disease and 30-53% risk of diabetes compared to those without the metabolic syndrome (61). Many studies confirmed the effectiveness of using a definition of the metabolic syndrome to predict future type 2 diabetes (62, 63, 64, and 65).

Obese non diabetic subjects who reduced weight have been shown to benefit from increases in insulin sensitivity and consequently reduced insulin hyper secretion (66, 67).

Data from the West of Scotland Coronary Prevention Study (WOSCOPS) study also showed that the odds of developing diabetes and CHD increase dramatically with the number of metabolic syndrome components (figure 1.13) (68).

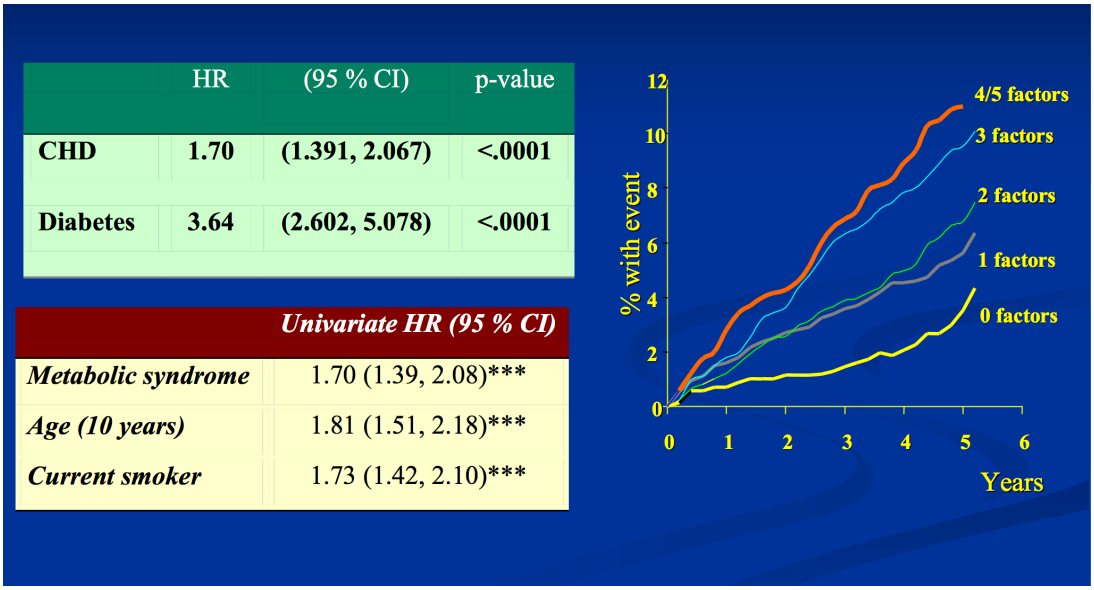

Figure 1.13 The risk of diabetes and cardiovascular disease in subjects with the metabolic syndrome Adapted from Sattar N *et al*. *Circulation* 2003; 108: 414-419

Type 2 diabetes on its own is an established risk factor for coronary heart disease, the presence of metabolic syndrome in this group of subjects accentuates the risk of coronary heart disease as shown by Alexander *et al*. (15). Patients with type 2 diabetes mellitus (T2DM) have a two to four fold increase in the risk of CHD and a patient with diabetes but without previous myocardial infarction (MI) carries the same level of risk for subsequent acute coronary events as a non-diabetic patient with previous MI. Diabetes is therefore considered to be a coronary equivalent (189-190).

A prospective study by Guzder and colleagues reported similar results. The risk of cardiovascular disease, including cerebrovascular disease and peripheral vascular disease, was also increased (60).

Hyperglycemia can cause vascular damage through at least three apparently unrelated pathways: advanced glycation end product (AGE) formation, activation of protein kinase C (PKC), and sorbitol accumulation by way of the polyol pathway (200). Diabetes amongst other conditions is associated with an accelerated and excess formation of advanced glycation end products (AGEs) which are believed to be primary instigators of the inflammatory process.

1.7.1 Formation of AGEs (Maillard Reaction)

The original Maillard hypothesis on the formation of AGEs proposed that chemical modification of proteins by reducing sugars (glycation of proteins) in diabetes alters the structure and function of tissue proteins such as type IV collagen, laminin and fibronectin resulting in capillary basement membrane thickening and hypertrophy of the extravascular matrix, and precipitating the development of diabetic complications **(191)** (Fig.1.14)

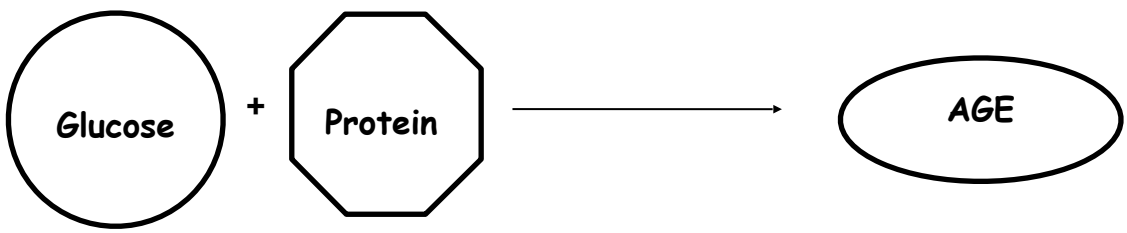

Figure1.14 Formation of AGE

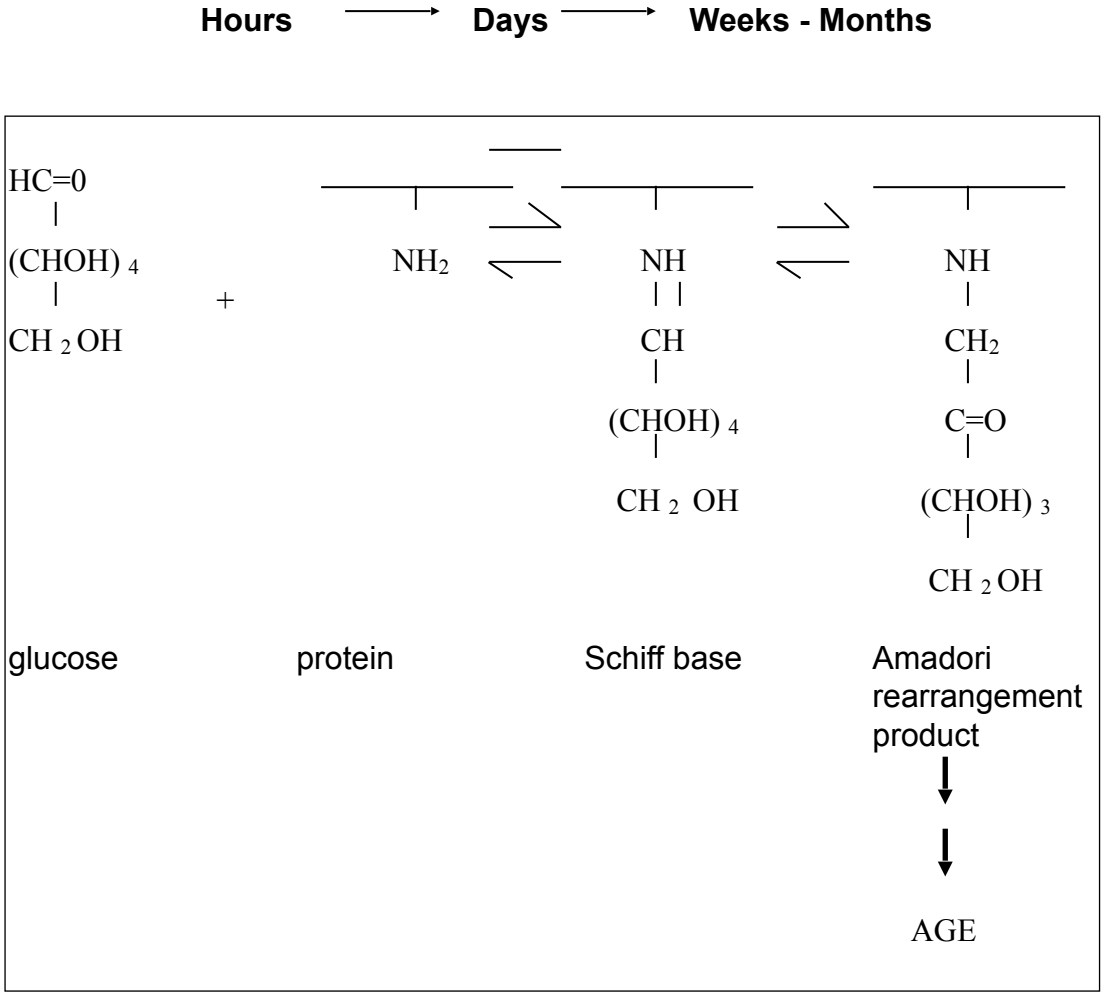

Figure 1.15 - Maillard Reaction

Glycation involves the formation of chemically reversible early glycosylation products with proteins, so called Schiff bases and Amadori adducts (e.g. glycated haemoglobin; HbA1C). With time, these early adducts undergo slow and complex rearrangements to form advanced glycation end-products (AGEs) (Figure 1.15)

Baynes and colleagues noted the importance of oxidizing conditions and reactive oxygen species in the formation of glycoxidation products, the major

class of AGEs that accumulate in tissues in diabetes (192-193). Besides the formation of carbohydrate intermediates, there is increasing evidence that maillard products are also formed via lipid - derived intermediates, resulting in advanced lipoxidation products (ALEs) (194). Dyslipidaemia is a common phenomenon in subjects with the metabolic syndrome and lipids are an important source of protein modifications. So, in these patients both AGEs and ALEs may be formed at the same time in atherosclerotic plaques.

Other pathways which may lead to AGE formation is through autoxidation of glucose by reactive oxygen species, and through carbonyl compounds (195,196). In particular methylglyoxal, a reactive dicarbonyl metabolite of glucose, has received considerable attention as the most reactive AGE precursor in endothelial cells. Decreased clearance of serum AGEs may further increase tissue AGE accumulation and de novo formation, and absorption of AGEs from food or smoking may aggravate AGE accumulation in renal failure (197-199).

1.7.2 Receptor for advanced glycation end products (RAGE)

Advanced glycation end products bind to cells via a receptor for advanced glycation end products (RAGE). RAGE is a multiligand cell-surface protein that was isolated from bovine lung in 1992 by the group of Schmidt and Stern (204). It is a member of the immunoglobulin super family of molecules possessing variable and constant domains and a short cytoplasmic tail which is essential for post RAGE signalling (211) (Figure 1.16).

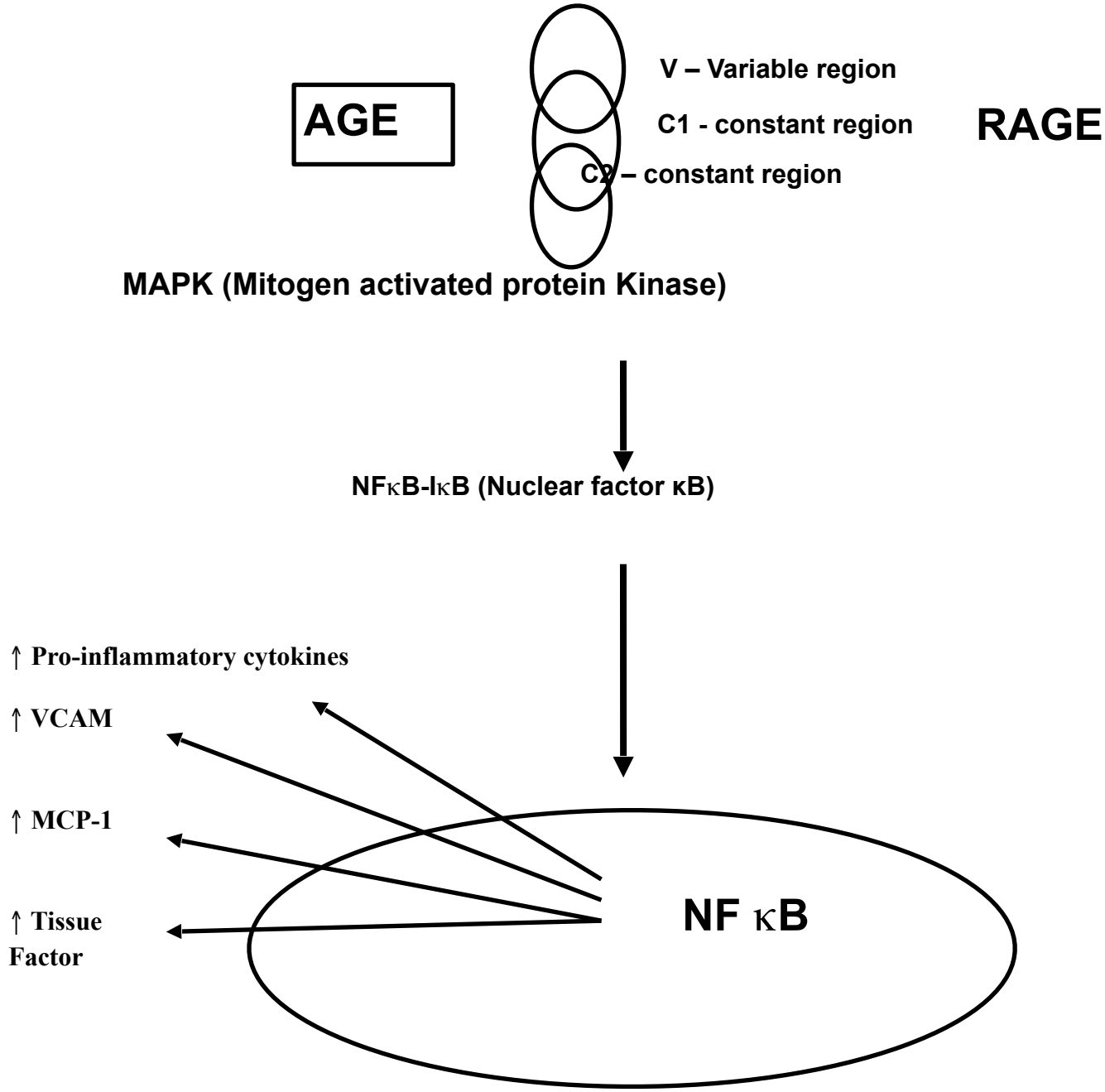

Figure 1.16 - AGE binds to its receptor RAGE

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Physiological Role of Receptor for advanced glycation end products (RAGE)

The exact function of RAGE has yet to be fully characterised. Many cells including T-Lymphocytes, endothelial cells, smooth muscle cells, mesangial cells and monocytes/macrophages express RAGE. In the majority of healthy adult tissues, RAGE is expressed at a low basal level. In contrast, the lung exhibits high basal level expression of RAGE suggesting a potentially important role for the receptor in maintaining lung homeostasis. RAGE is believed to control elastin expression in the lung which is critical for maintaining epithelial integrity. Therefore, disruption of RAGE levels has been implicated in the pathogenesis of a variety of pulmonary disorders including cancer and fibrosis (348-351). Apart from its role within the lung, RAGE is also believed to play an important role in embryonal neuronal growth, activation and differentiation of 'T' cells, mobilisation of dendritic cells and osteoclast maturation (352, 353). As mentioned previously, RAGE can bind several ligands apart from AGEs including amphoterin and b-amyloid substances.

Ligand - RAGE binding activates key cell signalling pathways including mitogen activated kinase and transcription factor NF-κβ both in endothelial (201) and vascular smooth muscle cells (202). This regulates the expression of genes encoding a number of pro inflammatory mediators commonly found in atheroma. Activation of the NF-κβ pathway may also cause a switch of

endothelial function toward a pro-thrombotic condition that, together with an altered platelet metabolism and changes in intra platelet signalling pathways, contributes to the pathogenesis of atherothrombotic complications of DM(203). As a result of the cross linking of AGE with the endothelium the synthesis of a number of new proteins occurs. This mechanism is relevant to the development of both macro (209,210) and micro vascular complications in the diabetic state (205-208).

Recent studies have identified novel gene splice variants of the human receptor RAGE (212-214). One of these encodes the full-length RAGE, which has full signalling and AGE binding potential. Another encodes an N-terminal truncated form, a membrane-bound isoform that contains no AGE-binding domain as it lacks the v-domain which is critical for binding of the ligand (215). A third splice variant encodes C-truncated soluble RAGE (sRAGE), which has AGE-binding properties in the absence of a signalling cascade because it lacks the sequences encoding the transmembrane and intra cytoplasmic domains (216). At least two potential mechanisms of sRAGE production have been identified: either by expression of a splice variant of sRAGE or as a result of cleavage from the cell surface through the activation of metalloproteinases (ADAM10) (217-218). Experimental studies have suggested that sRAGE can act as a decoy for RAGE ligands and thus have cytoprotective properties against AGE actions (217-219). Furthermore, studies investigating total circulating level of sRAGE have shown that sRAGE levels were reduced in non-diabetic subjects with coronary heart disease and in those with essential hypertension (220-221). Another

Japanese research group also found an inverse correlation between plasma sRAGE and carotid atherosclerosis in type 1 and type 2 diabetic subjects (222-223).Plasma sRAGE levels have been shown to be decreased in patients with the metabolic syndrome and are inversely correlated with several components of the metabolic syndrome including BMI, BP, insulin resistance index, triglycerides and low HDL levels (224).This remained significant even when the non diabetic subjects were extracted suggesting an important role for sRAGE even in non diabetic populations (225).

Increasingly RAGE has been proposed as a therapeutic target as up-regulation of RAGE has been shown to be associated with diverse pathological events ranging from atherosclerosis to Alzheimer's disease (346).Currently available therapeutic agents including PPAR-g agonists, statins and ACE inhibitors have been shown to be capable of modulating RAGE expression (226-229).

Taken altogether, understanding the molecular mechanisms underlying RAGE regulation will provide important insights into potential targets for prevention and treatment of cardiovascular disease.

1.8 Management of the metabolic syndrome

Abdominal obesity is the principal factor determining insulin resistance and the range of abnormalities associated with the metabolic syndrome. This is the reason the ATP III definition of the metabolic syndrome lists waist circumference as a diagnostic factor instead of BMI. Weight loss has been shown to improve many of the abnormalities associated with the metabolic syndrome including insulin resistance, dyslipidaemia, hypertension and arterial dysfunction (69, 70, and 71).

1.8.1 Effects of Lifestyle interventions in Diabetes, Hypertension and Dyslipidemia in subjects with the metabolic syndrome

The optimal diet for patients with the metabolic syndrome should focus on quantity of calories as well as the quality and composition of the diet. Reduction of the amount of saturated fat along with a reduction in portion size and consumption of foods rich in dietary fibre have been shown to have long lasting effects on the risk factor profile.

The United States Diabetes prevention program (DPP) (72), the Finnish Diabetes prevention study (DPS) (73) and the Chinese Da Qing Diabetes prevention programme (74) has provided convincing evidence for the benefits of lifestyle interventions in the prevention of type 2 diabetes.

The Chinese Da Qing study showed that 68% of the control group compared to 43% of those randomised to lifestyle interventions went on to develop type 2 diabetes during the 6 years of intervention. Similarly both the DPS and DPP studies reported that 58% fewer people progressed to type 2 diabetes in the intensive lifestyle intervention groups compared to the control group. Interestingly only modest weight loss (4-6kgs) was required in the above studies to achieve these impressive outcomes.

Weight loss in obese hypertensive subjects was associated with a decrease in BP and an improvement in insulin sensitivity (75). Cassano *et al* have shown that small amounts of weight loss in subjects with abdominal obesity could lead to dramatic lowering in BP.

The dyslipidaemia in the metabolic syndrome is characterised by elevated TG, increased small dense LDL and a decrease in HDL-cholesterol. The Framingham offspring study has demonstrated that weight reduction and increased physical activity are effective in improving the lipid profiles in these subjects.

1.8.2 Medical treatment of the metabolic syndrome

Management of patients with the metabolic syndrome should therefore focus on various strategies for weight reduction and a combination of diet and exercise remains the cornerstone for the treatment of the metabolic syndrome. However, despite lifestyle interventions many patients with the metabolic syndrome do not achieve targets and need pharmacological treatment. Medical treatment should be considered for all cardiovascular risk factors that are not normalised following lifestyle modifications and based on global risk estimates.

1.8.2.1 Medical treatment of obesity

Drug therapy can be considered in subjects with a BMI of >30kg/m² or lower in those with associated co-morbidities. However their widespread use is limited by their side effect profile.

Orlistat, an intestinal lipase inhibitor, remains the only medication that is currently licensed for the treatment of obesity in the UK. In the Xendos study, Orlistat has been shown to reduce the incidence of newly diagnosed diabetes in obese subjects with impaired glucose intolerance (77, 78, and 79).

Sibutramine, a centrally – acting serotonin-nor epinephrine reuptake inhibitor, has been shown to improve lipid profile and HbA1c in patients with

type 2 diabetes (80). However, in a recent large interventional trial in obese patients with the metabolic syndrome (SCOUT study), the risk of non-fatal MI and stroke was shown to be higher in the Sibutramine group compared to placebo (81). As a consequence, Sibutramine was withdrawn from the market.

Rimonabant, an endocannaboid (CB1) receptor antagonist has been removed from the market due to side effects, especially depression and suicide risk.

Bariatric surgery is a therapeutic alternative that has been shown to cause total and lasting resolution of all components of the metabolic syndrome. NICE recommends bariatric surgery as a treatment option for adults with obesity if they have a BMI of 40 kg/m² or more, or between 35 kg/m² and 40 kg/m² and other significant disease (for example, type 2 diabetes or high blood pressure) that could be improved if they lost weight. Bariatric surgery is also recommended as a first-line option (instead of life style interventions or drug treatment) for adults with a BMI of more than 50 kg/m² in whom surgical intervention is considered appropriate (82).The first major meta-analysis in this field showed a weight loss of 47.5% in patients who underwent gastric banding, 61.6% for gastric by pass, 68.2% for gastroplasty and 70.1% for biliary – pancreatic diversion (BPD) / duodenal switch (83). These findings were replicated by a Meta analysis in 2009 and also by the Bariatric Outcomes Longitudinal Database (BOLD) study (84, 85).

1.8.2.2 Treatment of Dyslipidaemia

Dietary modifications to replace saturated fats with mono and poly unsaturated fats should be encouraged along with an increase in physical activity. High intake of poly unsaturated fatty acids (PUFA, fish oils) is associated with a reduction in non essential fatty acids (NEFA), triglycerides (TG) and very low density lipoprotein (VLDL) levels along with a beneficial effect on insulin sensitivity and inflammation (reduced TNF α and NF-κβ levels) (86,87).

Fibrates are a group of drugs that are highly effective in reducing TG, LDL-C and increasing HDL-C by acting as ligands for the Peroxisome proliferator Activated Receptor alpha (PPAR-α) receptors thereby modulating the genes involved in lipid metabolism.

Nicotinic acid has been shown to have a beneficial effect on the lipid triad in patients with the metabolic syndrome. However its widespread use is often limited by its side effect profile including worsening of glucose tolerance.

Statins are the most effective LDL-C lowering drugs with modest effects on HDL-C and TG. Apart from being effective for treating the lipid abnormalities, drug treatment should not have any deleterious effects on the other components of the metabolic syndrome.

The therapeutic thresholds for treating dyslipidaemia are the same as the general population with lower target values for individuals with diabetes or established cardiovascular disease.

1.8.2.3 Treatment of Hypertension in the metabolic syndrome

Angiotensin converting enzyme (ACE) inhibitors / Angiotensin receptor blockers (ARB), β blockers, Calcium channel blockers and diuretics are used either as mono therapy or in combination for treatment of hypertension. Selection of drugs and combinations are determined in part by their effects on other components of the syndrome.

ACE inhibitors / ARBs have been shown to improve insulin sensitivity and reduce microalbuminuria unlike β blockers / diuretics which have been shown to have adverse effects on lipids and glucose tolerance (88).

Calcium channel blockers have no significant effects on the other components of the metabolic syndrome.

Therefore ACE inhibitors / ARBs should be the drugs of first choice in patients with the metabolic syndrome without co-existing CVD, followed by calcium channel blockers. However cardio selective β blockers should be considered in combination with ACE inhibitors / ARBs for patients with associated CVD.

1.8.2.4 Medical treatment of pre diabetes and type 2 diabetes in patients with the metabolic syndrome

Currently there are no data from controlled drug studies in patients with impaired fasting glucose and all studies have been performed in patients with impaired glucose tolerance.

In the STOP-NIDDM trial, Acarbose, an α-glucosidase inhibitor was shown to reduce progression to type 2 diabetes in patients with impaired glucose intolerance (IGT). Furthermore, acarbose reduced the incidence of newly diagnosed hypertension, lowered triglyceride levels and reduced major cardiovascular events compared to placebo (89).

The beneficial effects of Metformin on various parameters of the metabolic syndrome were amply demonstrated in both the DPP and UKPDS studies.

Insulin resistance / hyperinsulinaemia may have effects on the various components of the metabolic syndrome. For example, insulin resistance in the skeletal muscle leads to glucose intolerance, increased hepatic gluconeogenesis and increased production of triglyceride - rich VLDL, and raises blood pressure by various mechanisms. Weight loss with resultant re-distribution of body fat leads to an improvement in insulin sensitivity which is believed to be the ‘’common soil’’ of the metabolic syndrome. The clinical importance of insulin resistance lies in the fact that improving insulin resistance by various treatment methods may reduce the risk factors of subjects with the metabolic syndrome.

1.8.3 Peroxisome Proliferator activator receptors (PPARs) – Historical aspects

A group of drugs that evoked considerable interest in this context are the Peroxisome proliferator-activated receptors (PPARs). PPARs are ligand activated transcription factors that attracted enormous scientific and clinical interest because of their ability to regulate insulin sensitivity. Furthermore they are involved in the regulation of energy homeostasis, lipid metabolism, inflammation and atherosclerosis (90-98).

In 1990 Isseman and Green reported the cloning and initial characterisation of a novel murine nuclear receptor that is now referred to as PPAR α (99). Following this, Wahli *et al* reported the cloning of Xenopus Laevis ortholog of PPAR α in 1992, which like the mouse receptor was activated by micro molar concentrations of peroxisome proliferators. They also reported the cloning of two closely related orphan receptors encoded by distinct genes, which they named PPAR β and PPAR γ (100).

The PPARs are therefore comprised of three subtypes designated as PPARα, PPAR γ and PPAR β/δ. PPARs have a broad but isotype-specific tissue expression pattern, which account for the variety of cellular functions they regulate (Table 1.9).

Table 1.9 Tissue Distribution of PPAR isoforms

PPAR isoforms	Tissue Distribution
PPARα	Liver, heart, skeletal muscle, adipose tissue, Endothelial cells(EC), Vascular smooth muscle cells (VSMC), lymphocytes and macrophages
PPARγ 1	Cardiac muscle, skeletal muscle,kidney,intestine,VSMC and pancreatic β cell
PPARγ 2	Adipose tissue
PPARγ 3	Adipose tissue,colon,macrophages
PPARγ 4	Macrophages
PPAR β/δ	Widely expressed in many tissues and cell types

PPARs are activated by a variety of natural and synthetic ligands some of which have been used to treat type 2 diabetes (Thiazolidenediones) (TZDs) and dyslipidaemia (Fibrates). Upon activation by endogenous or synthetic ligands, PPARs hetero dimerise with the 9-cisretinoic acid receptor (RXR). The PPAR–RXR heterodimer undergoes conformational changes, binds to Peroxisome Proliferator responsive elements (PPRE) in the promoter region of the target genes and alters co activator / co repressor dynamics to modulate the transcription machinery, which in turn causes up regulation or down regulation of the target genes (101,102) (Figure 1.17).

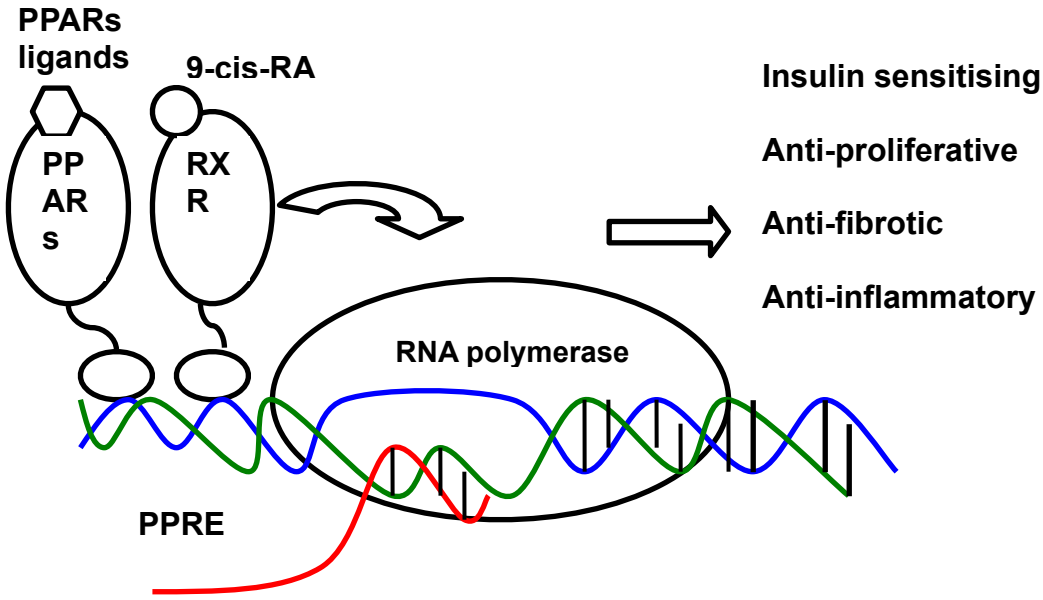


Figure 1.17 Mechanism of action of PPARs

1.8.3.1 PPAR α

PPAR α is expressed abundantly in tissues with a high rate of fatty acid oxidation such as the liver, heart and skeletal muscle where it acts as a major regulator of fatty acid homeostasis. PPAR-α also significantly expressed in the kidney, adipose tissue (especially brown adipose tissue) and most cell types present in the vasculature including endothelial cells, smooth muscle cells and macrophages.

PPAR α receptors are believed to act as general sensors of overall fatty acid load. An increase in the circulating levels of free fatty acids (FFA) transcriptionally activate PPARα, which, in turn, up regulates the expression

of enzymes involved in mitochondrial and peroxisomal β-oxidation with resultant enhanced hepatic fatty acid catabolism and reduced accumulation of lipids in liver (103,104,105). Consequently as a result of this they restrict the availability of fatty acids for VLDL with a resultant reduction in the circulating levels of TGs. In addition, activated PPARα reduces triglyceride levels by directly modulating the expression of certain apolipoproteins and the critical steps involved in VLDL-TG assembly and secretion (105,106,107).

PPAR α activation within the vasculature reduces inflammation by interfering with the recruitment of inflammatory cells and also reduces the expression of the adhesion molecules ICAM-1, VCAM-1and MCP-1 (108,109,110,111,112). In summary PPARα primarily regulates genes involved in lipid metabolism and is the molecular target for the fibrate group of drugs.

1.8.3.2 PPAR β/δ

PPAR β/δ is expressed ubiquitously with relatively high levels in the liver, skeletal and cardiac muscle, adipose tissue, brain and the vasculature (113,114).Unlike PPARα and PPARγ, its physiological function is much less studied and understood. However many studies, using recently available and potent synthetic ligands such as GW501516 and L165041, and animal models suggest that PPAR β/δ is involved in regulating lipid and glucose metabolism (115). Currently there are no marketed drugs that target PPAR β/δ.

1.8.3.3 PPAR γ

PPAR γ receptors are found in insulin - sensitive tissues such as adipose tissue, liver, skeletal muscle and macrophages, and primarily regulate adipocyte differentiation, insulin action, inflammation and lipid homeostasis (93,117,118)

PPAR γ receptors are expressed in 4 isomeric forms:

PPAR γ 1 - occurs in white and brown adipose tissue, skeletal muscle, liver and cells of the immune system and vasculature.

PPAR γ 2 - is restricted to white and brown adipose tissue.

PPAR γ 3&4 – is expressed in macrophages and adipose tissue.

They serve as receptors for thiazolidenediones (TZDs).

Thiazolidinediones are predominantly PPAR γ agonists that improve insulin sensitivity in the liver, skeletal muscle and adipose tissue. In addition, pioglitazone has been shown to improve the lipid profile in these patients through its effects on PPAR α. Moreover, TZDs have been shown to exert anti inflammatory and anti thrombotic effects, increase arterial compliance and decrease carotid intima - media thickness (IMT) (119,120).

Troglitazone, in the TRIPOD (troglitazone in prevention of diabetes) study (121), Rosiglitazone in the DREAM (Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication) study (122) and Actos (Pioglitazone) in the ACT NOW study in the prevention of type 2 diabetes (123) showed the potential of TZDs to reduce progression to type 2 diabetes in subjects with impaired glucose tolerance.

TZDs, such as Rosiglitazone and Pioglitazone, are associated with weight gain but cause a reduction of intra abdominal fat and an increase in subcutaneous fat with a resultant improvement in insulin sensitivity (124,125). The landmark study 'ProActive' showed that Pioglitazone significantly reduced the risk of strokes, non fatal MI and deaths in patients with type 2 diabetes at high risk of CVD (127).

Thus, thiazolidenediones act on two major pathogenic mechanisms of the metabolic syndrome i.e. insulin resistance and intra-abdominal obesity. They should thus be effective in preventing CVD (126).

However these two drugs, the antidiabetic TZDs and lipid lowering Fibrates affect only a single component of the metabolic syndrome which limits their use as monotherapy. Furthermore, their use is often associated with side effects especially TZDs which have been associated with an increased rate of cardiovascular events (128,129). Rosiglitazone has since been withdrawn as a result of such safety concerns.

In recent years extensive efforts have been underway to develop more effective and safer single PPAR agonists along with dual, Pan and partial agonists and selective peroxisome activator receptor modulators (SPARM) compounds that would improve the multiple metabolic abnormalities associated with the metabolic syndrome including obesity, insulin resistance with associated dysglycaemia, atherogenic dyslipidaemia and inflammation.

The aim has been to develop drugs with specificity for at least two PPAR isoforms (e.g. PPAR α / PPAR γ, PPAR α / PPAR β/δ and PPAR γ / PPAR β/δ), or PPARs that exhibit cell or tissue specificity which would be more efficacious and have relatively less undesirable side effects compared with currently used agonists with specificity towards single PPAR isoforms. Unfortunately efforts to develop these novel molecules have so far not met with much success and a number of trials have to be discontinued due to serious safety concerns (130,131,132,133,134).

However, PPARs are also activated by a variety of endogenous and natural ligands including n-3 and n-6 fatty acids and eicosanoids. PPAR γ is activated by PGJ_2 (a prostaglandin) whilst in contrast, PPAR α is activated by leukotriene B_4, a few endocannabinoids and phospholipids, as illustrated in the following table (table 1.10).

Table 1.10 List of endogenous PPAR ligands

PPAR α	**PPAR β/δ**	**PPAR γ**
Fatty acids		
Arachidonic acid	Arachidonic acid	Arachidonic acid
Linoleic acid		Linoleic acid
Saturated fatty acids	Saturated fatty acids	Saturated fatty acids
Eicosanoids		
Prostaglandins	Prostaglandins	Prostaglandins
Prostacyclin	Prostacyclin	
Leukotrienes		
Lipoproteins		

These natural ligands modulate PPAR activity and are therefore likely to be useful in treating a wide range of metabolic disorders including obesity, dyslipidemia, type 2 diabetes and atherosclerosis, hopefully without any associated serious side effects or safety concerns. In this context, conjugated linoleic acid (CLA), an important dietary polyunsaturated fatty acid with reported PPAR α and γ activity has attracted interest because of its effects on body composition. Specifically, it has been shown to cause a reduction in body fat mass and an increase in lean body mass (230,231). CLA has also been shown to have beneficial effects on adipogenesis, inflammation (232,233) and insulin sensitivity (234).

1.8.4 Conjugated linoleic acid (CLA)

CLA is a collective term for a class of conjugated dienoic isomers of linoleic acid which are naturally occurring polyunsaturated 18-carbon compounds. The isomers of CLA are distinguished by differences in the position and the orientation (cis or trans) of the double bonds along the fatty acid chain (figure 1.18).

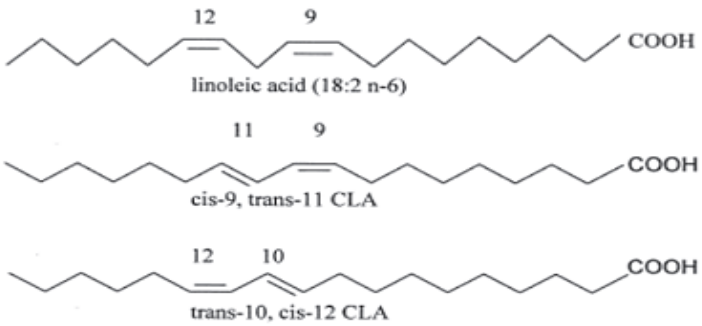


Figure 1.18 Biologically Active CLA isomers

It is possible that a number of these CLA isomers have biological activity. However, all of the known physiologic effects of CLA are induced by two isomers: c9, t11-CLA and t10, c12-CLA. There is also increasing interest in the fact that the biological effects of cis-9, trans–11 CLA and trans-10, cis-12 isomers may be different, with the former exhibiting PPAR α activity whilst the t10, c12-CLA has been shown to have effects predominantly on PPAR γ receptors (235,236).

1.8.4.1 CLA – Historical aspects

In 1978, Pariza et al (241, 242) reported that grilled ground beef contained both bacterial mutagens and a substance that inhibited mutagenesis. They went on to identify the new anti carcinogen as conjugated linoleic acid (CLA) (243). Interest in CLA was further kindled by this serendipitous observation some 30 Years ago. Since then, in various animal models, CLA has been shown to reduce or prevent adiposity, improve insulin resistance, inhibit adipogenesis, tumour development, and modulate inflammatory reactions (242). These observations have led to much speculation about the potential role of CLA in human health. Research on CLA has markedly increased over the last three decades and possible health benefits for humans continue to emerge.

1.8.4.2 Sources of CLA in humans

CLA is produced from linoleic acid in the rumen of cattle, goats, sheep and other ruminants. It cannot be made in any substantial quantities by humans and the main source of CLA in humans are food products derived from ruminants including meat, dairy products and partially hydrogenated vegetable oils, where more than 90% of CLA is in the c9-t11 configuration (237). The content of CLA in the diet varies considerably and is a direct reflection of the diet the animals are fed. Evidence suggests that pasture fed cows had 500% more CLA in their milk fat than cows on supplementary feeds. Similarly cow's milk produced from late Spring through early Autumn (seasons of rapidly growing green grass) will have almost twice the CLA content of milk produced in winter months (239,240).The average consumption of CLA in the diet has reduced from between 2-3g per day in the diet of 30 years ago to about 100-300 mg / day in the modern diet. This reduction of CLA in the diet may partly explain the global epidemic of obesity and diabetes (239,240). For humans, the recommended therapeutic daily intake of CLA ranges from 1.5 to 3.5 g/day (239,240).

The cis-9, trans-11 and trans-10, cis-12 (t10, c12) isomers, which are considered to be the most physiologically active isomers, can also be commercially synthesised from linoleic acid rich oils such as sunflower and safflower oil (238). These two isomers are usually represented in about equal amounts in synthesised CLA, with ten other minor CLA isomers representing the remaining 10-15 percent of these mixtures (238).

1.8.4.3 Beneficial effects of CLA in humans

Extensive research has been undertaken to determine the extent of CLA's health benefits in animals and humans as well as the mechanism of action through which CLA mediates these processes

1.8.4.3.1 Effects of CLA on Body fat and composition

Most research studies have supported a beneficial effect of supplemental CLA on inducing weight loss. Animal studies on mice, rats, and hamsters have shown an alteration in overall body composition following CLA supplementation (244,245,246,247). Not only did the studies in laboratory animals confirm a benefit of CLA on fat loss, they also showed that the anti adiposity characteristics of CLA were stronger in young animals during growth than in mature animals (248, 249).

One human study of middle aged men found a significant reduction in abdominal fat but no change in the body mass index (BMI) (252). In addition, a longer term study indicated a significant decrease in body fat mass after 12 months of CLA supplementation and a recent meta-analysis indicated that CLA is beneficial in reducing body fat mass (256,257).

A 2012 study found that among 80 overweight and obese Chinese subjects, two daily doses of CLA (1.7 grams each) for 12 weeks reduced body fat by 2 percent, though cholesterol levels worsened slightly (324).

The mechanisms underlying the effects of CLA are not yet established. However, there are clear indications that the anti adiposity effects are specifically mediated by the t10, c12 CLA isomer rather than the c9, t11 CLA isomer (255,256).

In vitro studies using murine adipocyte cell lines (253) as well as primary human adipocytes (254) show that t10, c12-CLA supplementation reduces tri acyl glycerol accumulation and adipocyte differentiation. These studies are supported by in vivo animal studies where t10,c12-CLA has been shown to reduce the transcription of several adipocyte-specific genes such as adiponectin, glucose transporter 4, leptin, and lipoprotein lipase (LPL) in white adipose tissue (254,256). The actions are believed to be mediated by the effects of t10, c12-CLA isomer on PPAR γ receptor, because several of the adipogenic genes are PPAR γ target genes.

1.8.4.3.2 Effects of CLA on Atherosclerosis

Atherosclerosis is a chronic inflammatory disease as previously described. It has been proposed that CLA can reduce inflammatory gene expression, thus altering the signalling pathways that lead to the formation of reactive oxygen species (ROS). Furthermore, CLA inhibits the synthesis of arachidonic acid and thus modulates the inflammatory response which is generally under the control of eicosanoids produced by arachidonic acid (258). To date, most animal and human studies on the effects of CLA on atherosclerosis have been inconclusive and contradictory (258).

Several studies have indicated a decrease in LDL, total cholesterol, triglyceride, and fat deposition levels with CLA supplementation (259,260), while other studies indicate an increase in HDL with an increase in fatty streak development (261) (McGuire and McGuire 2000).

1.8.4.3.3 Effects of CLA on Diabetes and Insulin sensitivity

Short-term clinical trials indicate that there is a strong isomer - dependent relationship, with the *t*-10, *c*-12 CLA having a detrimental effect on insulin sensitivity (262). However, the *c*-9, *t*-11 CLA isomer has been shown to have anti-diabetic effects (264). Furthermore, interventions that provided supplements that contained a 50:50 combination of both CLA isomers (trans-10, cis-12 and cis-9, trans-11 CLA) have, in general, produced mixed results on insulin sensitivity (263). Similarly, various animal studies have shown a wide range of effects related to blood glucose levels, insulin sensitivity, and insulin resistance (265).

1.8.4.3.4 Immune Response Modulation

The effects of CLA in the modulation of immune responses have several positive implications for human health. CLA has been shown to modulate the production of eicosanoids, prostaglandins, cytokines, and immunoglobulin's. Specifically, CLA reduces the concentration of immunoglobulin E (IgE) and thus reduces allergic reactions (266). Overall, CLA modulates allergic and infectious immune responses by different pathways and different mechanisms of action.

Table 1.11 Summary of key clinical trials using CLA in human subjects

STUDY	OBJECTIVES / DESIGN	KEY OUTCOMES
Treatment with dietary trans10 cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care. 2002 ; 25 (9): 1516-21	Randomized, double-blind controlled trial, abdominally obese men (n = 60) were treated with 3.4 g/day CLA (isomer mixture), purified t10c12 CLA, or placebo. Euglycemic – hyper insulinaemic clamp, serum hormones, lipids, and anthropometry were assessed before and after 12 weeks of treatment.	t10, c12 CLA increased insulin resistance (P < 0.01) and glycaemia (P<0.001) and reduced HDL cholesterol (P < 0.01) compared with placebo. The CLA mixture did not change glucose metabolism, body composition, or weight compared with placebo but lowered HDL cholesterol (P < 0.05).
Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: a potential link to fatty acid-induced insulin resistance. Circulation. 2002: 8; 106(15): 1925-9	60 men with metabolic syndrome were randomized to one of 3 groups receiving t10,c12CLA, a CLA mixture, or placebo for 12 weeks in a double-blind placebo-controlled trial. Insulin sensitivity (euglycaemic clamp), serum lipids, in vivo lipid peroxidation plasma vitamin E, plasma C-reactive protein, TNF-α, and IL-6 were assessed before and after treatment.	Supplementation with t10c12 CLA markedly increased 8-iso-PGF(2alpha) (578%) and C-reactive protein (110%) compared with placebo (P<0.0001 and P<0.01, respectively) and independent of changes in hyperglycemia or dyslipidemia
The CLA isomer, t10c12-CLA, is inversely associated with changes in body weight and serum leptin in subjects with type 2 diabetes mellitus. J Nutr. 2003,133:257S-60S	In this double-blind study, subjects with type 2 diabetes mellitus were randomized into one of two groups receiving either a supplement containing mixed CLA isomers (CLA-mix; 8.0 g daily, 76% pure CLA; *n* = 12) or a supplement containing safflower oil (placebo; 8.0 g daily safflower oil, *n* = 9) for 8 wk.	t10c12-CLA, but not c9t11-CLA, was inversely associated with body weights (*P* < 0.05) and serum leptin (*P* < 0.02). These findings strongly suggest that the t10c12-CLA isomer may be the bioactive isomer of CLA to influence the body weight changes observed in subjects with type 2 diabetes
Effects of cis-9,trans-11 CLA supplementation on insulin sensitivity, lipid peroxidation,and proinflammatory markers in obese men. Am J Clin Nutr. 2004; 80(2):279-83	25 abdominally obese men received 3 g c9,t11 CLA/d or placebo (olive oil) for 3 months in a randomised, double-blind,placebo-controlled study	CLA preparation containing the purified c9,t11 CLA isomer increased insulin resistance and lipid peroxidation compared with placebo in obese men.

Supplementation with trans10cis12-conjugated linoleic acid induces hyper pro insulinaemia in obese men: close association with impaired insulin-sensitivity. Diabetologia 2004, 47:1016-1019	57 non-diabetic abdominally obese men were randomised to receive 3.4g t10c12CLA, CLA-isomer mixture or control oil for 12 weeks. Insulin sensitivity, intact pro-insulin, insulin, the proinsulin: insulin ratio, C-peptide, glucose and adiponectin were assessed before and after supplementation.	Supplementation with t10c12CLA increased proinsulin ($p<0.01$), the proinsulin : insulin ratio ($p<0.05$) and C-peptide concentrations ($p<0.001$) in comparison with control subjects. Adiponectin, however, did not change significantly. The change in proinsulin, but not the proinsulin: insulin ratio, was related to impaired insulin sensitivity ($r= -0.58$, $p<0.0001$), independently of changes in insulin, C-peptide, glucose, adiponectin and BMI.
Effects of two conjugated linoleic Acid isomers on body fat mass in overweight humans. Obes Res 2004, 12:591-598	Volunteers were randomized over five groups receiving daily either 3 g of high oleic acid sunflower oil, 1.5 g of cis-9,trans-11 (c9t11) CLA, 3 g of c9t11 CLA, 1.5 g of trans-10,cis-12 (t10c12) CLA, or 3 g of t10c12 CLA administrated as triacyl glycerol in a drinkable dairy product for 18 weeks in this placebo-controlled, double-blind, randomised study	A daily consumption of a drinkable dairy product containing up to 3 g of CLA isomers for 18 weeks had no statistically significant effect on body composition in overweight, middle-aged men and women.
CLA supplementation for 1 year reduces body fat mass in healthy overweight humans. Am J Clin Nutr. 2004;79(6): 1118-25.		Long-term supplementation with CLA-FFA or CLA-triacylglycerol reduces Body Fat Mass in healthy overweight adults
Supplementation with conjugated linoleic acid for 24 months is well tolerated by and reduces body fat mass in healthy, overweight humans. J Nutr 2005, 135:778-784	After 12 mo in a randomized, double-blind, placebo-controlled trial of CLA supplementation, 2 groups received CLA as part of a triglyceride or as the free fatty acid, and 1 group received olive oil as placebo.	This study shows that CLA supplementation for 24 months in healthy, overweight adults was well tolerated. It also confirms that CLA decreases BFM in overweight humans, and may help maintain initial reductions in BFM and weight in the long term.
Lack of effect of dietary conjugated linoleic acids naturally incorporated into butter on the lipid profile and body composition of overweight and obese men. Am J Clin Nutr 2005, 82:309-319	In a crossover design study including an 8-wk washout period, 16 men were fed with modified butter naturally enriched with CLA	These results suggest that a 10-fold CLA enrichment of butter fat does not induce beneficial metabolic effects in overweight or obese men.
Effects of the individual isomers cis-9, trans-11 vs. trans-10, cis-12 of CLA on inflammation parameters in moderately overweight subjects with LDL-phenotype B.Lipids, 2005; 40 (9):909-18.	The objective of this study was to evaluate the immune-modulating effects of 3 g cis-9, trans-11 (c9, t11) vs. trans-10, cis-12 (t10,c12) CLA isomers in a population with a high risk of coronary heart disease in a 13 week study	Daily consumption of 3 g of c9, t11 or t10, c12 CLA isomer did not affect LPS-stimulated cytokine production by PBMC or whole blood and plasma CRP levels.

The effect of 6 months supplementation with conjugated linoleic acid on insulin resistance in overweight. J Obesity 2007, 31:1148-1154	Randomized, double-blind, placebo-controlled trial with change in body composition as primary end point comprising 118 subjects receiving supplementation with either placebo (olive oil) or CLA for 6 months.	CLA does not affect glucose metabolism or insulin sensitivity in a population of overweight or obese volunteers.
Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans Am J Clin Nutr 2007; 85:1203–11.		Given at a dose of 3.2 g/d, CLA produces a modest loss in body fat in humans.

1.8.5 Hypothesis:

There is thus substantial evidence from animal and experimental studies that CLA possesses anti-carcinogenic, anti-atherogenic, anti-diabetic effects and anti-inflammatory effects in addition to its beneficial effects on lipids and on body fat composition. However, evidence from direct studies on humans have been inconclusive (Table 1.11). A clear rationale also exists for the hypothesis that improving insulin sensitivity may influence many of the components of the metabolic syndrome. Treatment with PPAR ligands such as CLA may thus have the potential to optimise both metabolic control and cardiovascular risk.

I hypothesised that CLA may exert its benefits not only by improving insulin sensitivity and body composition, but also through indirect mechanisms involving changes in sRAGE mediated inflammation and changes in adipocytokine profile. To investigate this hypothesis and in order to clarify the effects of CLA supplementation in-vivo, a randomised double blind cross over study in subjects with the metabolic syndrome was undertaken over a 22 week period.

1.8.6 Our Study Objectives / Aims

1. To assess the effects of CLA supplementation on inflammation and sRAGE expression in subjects with the Metabolic Syndrome
2. To determine whether treatment with CLA in a group of patients with the Metabolic Syndrome has a beneficial effect on body composition, metabolic profiles and other markers of cardiovascular risk
3. To evaluate the differences between a CLA mixture and 9,11 isomer on the above parameters

This study used the CLA mixture (50:50 cis-9, trans-11 and trans-10, cis-12 isomers) as this is the one that is commercially available to population groups interested in losing weight. The c9, t11-CLA isomer has not been as well examined as the t10, c12-isomer in humans and as this is the isomer most abundant in natural foods and it is possible to increase the content of this isomer in bovine milk via feeding strategies, we found it relevant to include this isomer in our study. We did not use the t10, c12-isomer as this has been shown in some studies to worsen metabolic profiles (262,263,264).

CHAPTER 2

A randomised, double blind, crossover study of the effects of CLA isomers on body composition, metabolic profiles, inflammation and vascular function in patients with the Metabolic Syndrome – Study design

2.1 Overview

There is evidence from experimental and animal studies that CLA exerts beneficial effects on body weight, insulin sensitivity, adipogenesis, tumour development, inflammation and vascular function (243 -249). The data from these studies suggest that CLA could have a beneficial effect on body weight, metabolic profiles and vascular risk profile in overweight insulin resistant patients.

However, none of these results have been consistently reproduced in humans, possibly because of the differences in the dosages, duration and types of isomers provided in these studies.

In order to clarify the effects of CLA supplementation in vivo, we conducted a clinical trial to investigate direct measures of inflammation including RAGE expression, body composition, insulin sensitivity and metabolic profiles as well as various markers of cardiovascular risk in subjects with the metabolic Syndrome.

The clinical trial described in this chapter compares the effects of our investigational medicinal product (IMP), the CLA mixture (50:50 cis-9, trans-11 and trans-10, cis-12 isomers) with the cis-9, trans-11 isomer on various parameters in subjects with the metabolic syndrome using a randomised, double - blind, cross-over study design.

2.2 Materials and Methods

2.2.1 Study approval and funding

Prior to study commencement, approval to conduct this clinical trial was obtained from Cardiff & Vale NHS trust Research and Development department (reference number 04/CMC/3239E), the South East Wales Research Ethics Committee (reference number 05/MRE09/60) and the Medicines and Healthcare Products Regulatory Authority (EudraCT No: 2005-003197-17) who also, during the course of the study undertook an inspection of the trial as part of their routine statutory inspection of clinical trial activity at Cardiff and Vale NHS trust (December 2007).Funding was obtained form the Diabetes research fund (University hospital Llandough) and the medicinal product was provided by Stephan Lipid nutrition, Netherlands. The pre-initiation approval process began in 04/07/2005 and final approval was obtained in December 2006 following which patient recruitment commenced (First enrolment 12/01/2007). The delay in approval was mainly related to the clinical trials authorisation as part of the final approval process by the MHRA.

2.2.2 Overall study design

This was a 22 week randomised, double-blind, cross - over study of the effects of Conjugated linoleic Acid isomers on inflammation and RAGE expression, body composition, metabolic profiles and vascular function in patients with the metabolic syndrome.

Thirty patients were randomly assigned to consecutive 2-month treatment periods of either 2 grams of cis-9, trans-11 CLA isomer or a CLA mixture (50:50 cis-9, trans-11 and trans-10, cis-12 isomers). A washout interval of 6 weeks separated the two treatment phases. Randomisation was performed prospectively by an independent source (St Mary's Pharmaceuticals, Cardiff), with half receiving 9, 11 CLA isomer first and the other half receiving the CLA mixture. Patient allocation details were coded and kept confidential until the trial was completed.

Subjects underwent detailed metabolic evaluation including measurement of insulin sensitivity, glycaemic status and lipid profiles. Systolic, diastolic, and mean BP were measured along with markers of inflammation including RAGE expression, surrogate markers of endothelial function and measures of platelet function. Anthropometric measurements were carried out including BMI and waist measurements, and arterial compliance was assessed non-invasively by means of pulse wave analysis and pulse wave velocity measurements at baseline and after each treatment phase.

These measurements were made on entry into the treatment phase and again after 8 weeks of treatment with CLA mix followed by 9,11 CLA separated by a washout period of 6 weeks and vice versa. Sequential measurement of these parameters enabled an assessment of the potential mechanisms by which CLA may produce its effects.

2.2.3 Discussion of the design

Metabolic Syndrome is a common condition affecting 25% of the population based on accepted diagnostic criteria (6). We employed a crossover design for this study because a substantial within-subject correlation was anticipated, such that smaller sample sizes might be required to detect significant differences. The main advantage of the crossover study design is that each individual acts as his or her own 'control' thus reducing the influence of confounding variables. Removing patient variation in this way makes cross over trials more efficient than similar sized parallel group trials. The main draw back is the potential to carry–over a treatment effect from the 1st treatment phase to the 2nd. This can be overcome to a great extent by ensuring an adequate wash-out period and performing baseline measures prior to each treatment phase (267-271).

A dose of CLA of 1 gram twice daily was used in this study as the recommended therapeutic daily intake of CLA ranges between 1.5 to 3.5 g/ day (239,240). Furthermore, higher doses used in previous studies were shown to be associated with worsening insulin sensitivity (252). CLA has a short circulatory half life of a few hours and therefore a washout interval of 6 weeks was used to separate the two treatment phases to minimise the chance of 'carry over' into the next treatment phase. Similar dosages and washout intervals have been employed in previous studies (257,258,259,262-265).

2.2.4 Study participants - inclusion and exclusion criteria

Patients with the metabolic syndrome as defined by NCEP ATP III criteria with at least 3 of the following (6) were enrolled into the study

Fasting plasma glucose	> 5.6 mmol/l
Serum triglycerides	> 1.7 mmol/l
Serum HDL cholesterol	< 1.0 mmol/l
Blood Pressure	> 130 / 85 mm Hg
Abdominal Obesity	
Waist Girth	> 102 cms in males
	> 88cms in females

Patients were excluded from participation if they had pre existing diabetes. Exclusion criteria were age less than 18 years or greater than 70 years, pregnancy, breastfeeding and any intercurrent significant medical or psychiatric condition. All patients took their usual medications with dosage and timing kept unaltered before and during the study.

2.2.5 Recruitment

Potential participants were initially identified from the Diabetes Clinics at the University Hospital of Wales and Llandough, and eligible subjects were invited to participate through a letter from the clinician responsible for their care. I provided each potential participant with a patient information sheet (see appendix 1) and explained the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it might entail.

Each subject was informed that participation in the study was voluntary and they could withdraw from the study at any time and that withdrawal of consent would not affect her subsequent medical treatment or relationship with the treating physician. Patients were given adequate time to review the patient information sheet and ask questions about any aspects of the study. If subjects were still in agreement, informed consent was obtained using a participant-investigator signed consent form (appendix 2). All study visits took place in the temperature controlled ward area of the Clinical Research Facility at the University Hospital of Wales, Cardiff, with each visit lasting 3-4 hours.

2.2.6 Interruption or discontinuation of treatment

The term discontinuation refers to a patient's non completion of study. Information about discontinuation and an explanation why the patient was withdrawn from the study was recorded. Patients who discontinued prematurely due to side effects were followed until resolution of the event.

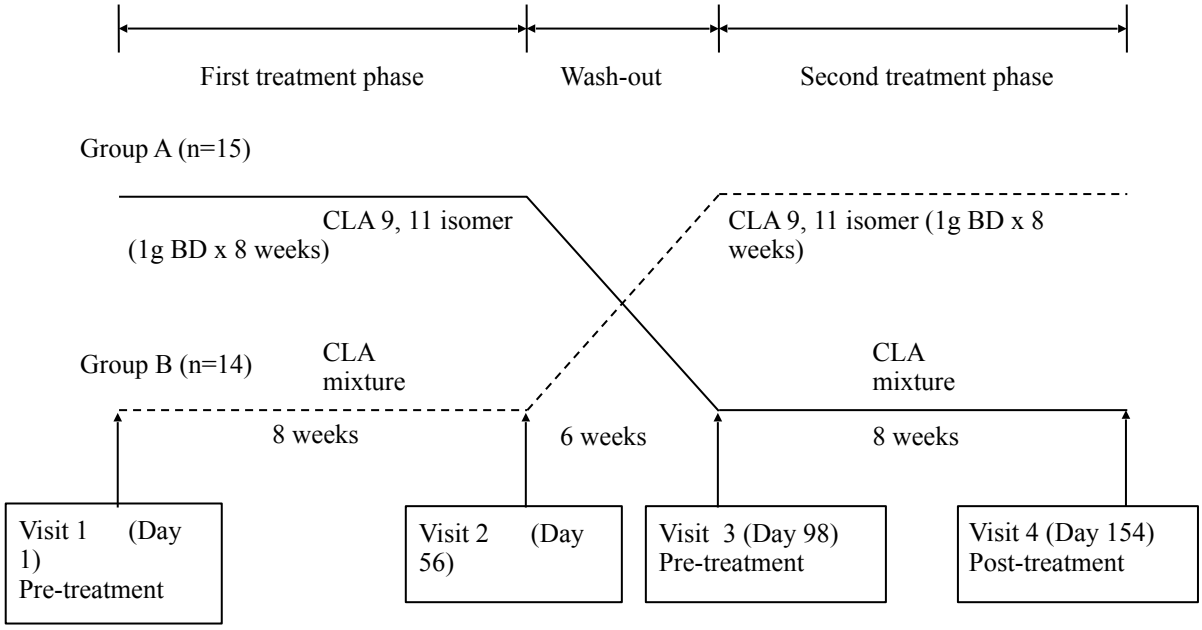
Reasons that a patient could discontinue participation were considered to constitute one of the following

1. Adverse event
2. Abnormal laboratory value
3. abnormal test procedure result
4. protocol violation
5. subject withdrew consent
6. subject lost to follow up
7. administrative problems
8. Death

2.2.7 Treatments and Interventions

Thirty participants were randomised to receive consecutive 8 week treatment periods of either 1g BD of cis-9, trans-11 CLA isomer or a CLA mixture (50:50 cis-9, trans-11 and trans-10, cis-12 isomers). A washout interval of 6 weeks separated the two treatment phases. CLA isomer capsules were both supplied by Lipid Nutrition and packaged in a licensed pharmaceutical laboratory (St Mary's Pharmaceutical Unit, Cardiff, UK).

Figure 2.1 Study plan / flow chart



2.2.8 Measurements

Participants were assessed in the Clinical Research Facility at the UHW at four points: baseline tests at visit 1 and at the end of the wash-out phase (visit 3) and after each treatment (9, 11 CLA isomer or CLA mixture) phase (visits 2 and 4).

Following an overnight fast, subjects were admitted to the Clinical Research Facility at 08:00 hours. Pre menopausal patients had a pregnancy test and would have been excluded if this was positive. Patients were not assessed if they had evidence of recent or ongoing infection as this would skew measurement of CRP.

2.2.8.1 Anthropometric Measurements

Baseline anthropometric data, consisting of blood pressure, height, weight, body mass index (BMI), waist circumference and body composition measured by bioelectrical impedance were collected. Body height (without shoes) was measured to the nearest 0.5 cms using a stadiometer and the body weight to the nearest 0.5 kg on a balance beam scale. BMI was calculated as weight (kg) divided by height (m^2). Standard bio impedance was performed using a commercial bio impedance meter (Tanita Body fat analyser TBF-305, Tanita Corporation, Japan).

2.2.8.2 Metabolic Biochemistry Measurements

On each occasion blood samples were collected after an overnight fast for lipids, glucose, markers of inflammation (RAGE, hs CRP), Adipokines (Adiponectin, Resistin and PAI-1) and surrogate markers of endothelial and platelet function (eSelectin, pSelectin and von Willebrand factor (vWF).

Serum total cholesterol, HDL-cholesterol and triglycerides were measured by an enzymatic-colorimetric method. LDL-cholesterol was estimated from quantitative measurements of total and HDL-cholesterol and plasma triglycerides (TG) using Friedewald's equation as follows, [LDL- Chol] = [Total Chol] - [HDL-Chol] - ([TG]/2.2 (272).

2.2.8.3 Quantifying glycaemic control, insulin resistance and metabolic parameters

The normal response to glucose load is suppression of hepatic glucose production and enhancement of glucose uptake in the liver and muscle. In subjects with IFG, the impairment of insulin sensitivity in the liver to control hepatic glucose output causes the elevated fasting glucose. Insulin sensitivity in subjects with isolated IFG showed a significantly increased HOMA-IR (Homeostatic Model Assessment) (273-276). HOMA-IR primarily reflects hepatic insulin resistance, which is the underlying mechanism of increased fasting glucose.

As opposed to IFG, peripheral insulin resistance i.e. muscle, appears to be the main abnormality in the IGT subjects which, along with a defect in 'early-phase' insulin secretion results in an inability to suppress hepatic glucose production, leading to an excessive rise in plasma glucose during the first 60 mins of oral glucose tolerance test (OGTT). (279-281).

The key question of which one is the primary contributor to the development and the subsequent maintenance of hyperglycaemia remains controversial. Insulin resistance is believed by some, to be the primary abnormality and the increased secretory demand on the ß-cells by insulin resistance leads in those susceptible to ß-cell dysfunction (277,279,282). Other groups have suggested that reduced ß-cell function with decreased insulin secretion is a prerequisite for the early and subsequent development of T2DM (290-292 (283-285). In early type 2 diabetes development, insulin resistance may appear to be the more prominent abnormality with a compensatory increase in insulin secretion.

2.2.8.3.1 Measures of insulin secretion and β- Cell mass

Glycaemic control was assessed by measuring fasting plasma glucose (glucose oxidase method).

Insulin resistance can be defined clinically by measurement of a fasting plasma insulin concentration, but lack of standardisation of insulin assays precludes defining a specific insulin concentration above which a person is defined as insulin resistant. The measurements of basal insulin or insulin levels after an oral glucose challenge alone does not give a sufficiently accurate representation of ß-cell function. The gold standard for

measurement of insulin sensitivity is considered to be the euglycaemic hyperinsulinaemic clamp technique (276). However, in our study we used the Homeostatic model assessment to estimate insulin sensitivity / β cell function (286).

2.2.8.3.2 Homeostatic Model Assessment (HOMA-B and HOMA-IR)

Homeostasis Model Assessments (HOMA) estimate steady state ß-cell function (HOMA-B) and insulin sensitivity/resistance (HOMA-IR). These measures correspond well to non-steady state estimates of ß-cell function and insulin sensitivity derived from stimulatory models, such as a hyperinsulinaemic clamp, hyperglycaemic clamp or an intravenous glucose tolerance test. HOMA-B was calculated using a HOMA calculator version 2.2 (Diabetes Trial Unit, Oxford University). This is a method assessing ß-cell function from basal glucose and insulin. HOMA of beta-cell function and insulin resistance was first described in 1985 (286). The insulin resistance (HOMA-IR) is calculated from basal plasma glucose and insulin. Both the equations are simplified to : HOMA-B% = (20 x FPI)/(FPG -3.5), HOMA-IR = (FPI x FPG) / 22.5 for ß-cell function and insulin resistance, respectively where FPI is fasting plasma insulin concentration (mU/l) and FPG is fasting plasma glucose (mmol/l)(286).

2.2.8.3.3 Plasma insulin

Insulin was measured by a highly specific and sensitive assay (MLT Research Ltd, Cardiff, UK) which measures ‘true’ insulin levels, as it does

not cross-react with intact and split proinsulin. It employs monoclonal antibodies which restrict the cross-reactivity to a minimum. The MLT insulin assay is an immuno chemiluminometric assay (ICMA) for the quantitative measurement of insulin in human samples. The duration of the assay is approximately 2.5 hours. All assays contain control samples to ensure the results are within an acceptable range. The MLT insulin assay is a two-site immunoassay, employing an insulin specific solid phase antibody immobilised on micro titre wells, and a soluble antibody labelled with a chemiluminescent acridinium ester which recognises insulin. Plasma is incubated in the micro titre well, along with the labelled antibody solution. The plate is then washed to remove unbound, labelled antibody and measured using a micro titre plate luminometer. The luminescent reaction is a flash type (>95% complete in 1 second) which allows the entire plate to be read in approximately 5 minutes. The normal range for fasting insulin is 6-100pmol/L and the CV for the insulin assay is <4.0% with sensitivity of 1.5 pmol/l and specificity of 100%.

2.2.8.3.4 Plasma total proinsulin

The MLT Total Proinsulin assay is an immuno-chemiluminometric assay for the quantitative measurement of the sum of concentrations of intact, des 3132 split proinsulin and 32-33 split proinsulin in human samples. The assay is able to handle approximately 80 samples in one run and the duration of the assay is approximately 3.5 hours. All assays contain control samples to ensure the results are within an acceptable range. The MLT Total Proinsulin assay is a two-site immunoassay, employing a solid phase antibody

immobilised on micro titre wells, and a soluble antibody labelled with a chemiluminescent acridinium ester, which recognises all circulating forms of proinsulin. Plasma is incubated in the micro titre well, along with a buffer and after a wash step, labelled antibody solution is added before a second incubation. The plate is then washed to remove unbound, labelled antibody and measured using a micro titre plate luminometer. The luminescent reaction is a flash type (>95% complete in 1 second), which allows the entire plate to be read in approximately 5 minutes A calibration curve is constructed permitting estimation of proinsulin concentrations in the samples by comparison with calibrators of known proinsulin concentration. The normal range for fasting proinsulin is < 20pmol/L. The CV for proinsulin assay is <5.0% with sensitivity of 0.35 pmol/l and specificity of 100%.

2.2.8.3.5 Pro insulin / Insulin ratio

Insulin production in normal subjects involves cleavage of insulin from proinsulin; 10 to 15 percent of secreted insulin is proinsulin and its conversion intermediates. In contrast, the proportion of immuno reactive insulin that is proinsulin in type 2 diabetes is increased considerably in the basal state (>40 percent). Proinsulin is an insulin precursor which is released in small amount under normal conditions. In the fasting state, the proportion of proinsulin-like molecules in T2DM is two to three-folds greater than in healthy subjects (287). Increased demand on the ß-cells also results in the release of less mature ß-cell granules at a time when conversion of proinsulin to insulin is incomplete, hence higher levels in the IGT subjects (288). Prospective studies have demonstrated that the proinsulin levels and

proinsulin to insulin ratio may be used to predict early deterioration in glucose tolerance (289,290). It has been seen that the proinsulin to insulin ratio is negatively correlated with acute insulin response suggesting that it can be used as a surrogate marker of ß-cell dysfunction (291-293). Because proinsulin concentration has been associated with beta-cell dysfunction, it was also measured in our study.

2.2.8.3.6 Plasma Non Esterified Fatty acids (NEFA)

Normally there is suppression of lipolysis and a reduction in plasma non esterified fatty acids (NEFA) following insulin secretion. In patients with insulin resistance there is an increase in fasting NEFA levels which is less suppressible to insulin and which can inhibit skeletal muscle glucose utilization (294).The assay for determination of NEFA is carried out using the Wako NEFA-C kit which relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA- synthetase (ACS). The acyl-CoA thus produced is oxidised by adding acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(b-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm. Results are entered into MultiCalc and calculated using a regression equation. The normal range for fasting NEFA is 0.1-0.6mmol/L. The CV for this assay is <3.0% with a sensitivity of 0.01 mmol/l.

Insulin, proinsulin, NEFA, hsCRP and PAI-1 assays were performed by the Diabetes Research Unit at Swansea.

2.2.8.4 Markers of inflammation, adipokines and endothelial activation

Serum was prepared by centrifugation of blood at 4000 rpm for 8 minutes and the samples were frozen at - 70^{o}C until the analyses. Measurement of circulating sE selectin, sP selectins, adiponectin, resistin, sCD40L, vWF and sRAGE secretion was quantified using standard ELISA kits purchased from R&D systems (Wantage, UK). Briefly, the sandwich ELISA involved the adherence of the capture antibody, monoclonal mouse anti-human e-Selectin, sp-Selectin, adiponectin, resistin, sCD40L, to a maxisorb 96 well plate (Nunc, Gibco, UK). 100µL of the capture antibody, in PBS at a concentration of 4µg/mL, was added to all wells of the plate, which was incubated overnight at room temperature. The plate was washed three times with PBS/0.05% Tween 20 (PBS/Tween) before the addition of blocking buffer (PBS/ 1% BSA) and incubation at room temperature for 1hr. After further washing (x3 in PBS/Tween) 100µL of samples and standards were added to the wells of the plate. A seven-point standard curve was constructed using recombinant human individual standards (R&D Systems) 0-1000pg/mL. Each standard and sample was applied to the plate in duplicate. The plate was covered and incubated at room temperature for 2 hrs before washing and the addition of 100µL of biotinylated detection antibody (200ng/mL in reagent diluent), the plate covered and incubated at room temperature for 2hrs. Following washing, 100µL of a 1/20,000 dilution of streptavidin-HRP conjugate (R&D systems) was added to each well and left at room temperature for 20 minutes, before washing and the addition of 100µL of TMB (tetramethylbenzidine, R&D systems) substrate to each well.

The plate was covered and incubated at room temperature for 30 minutes. To each well 50µL of 1M HCL was added to stop the colorimetric reaction and the absorbance of each well was determined at 450nm on a spectrophotometer. These assays were performed at the Cardiff Metropolitan University clinical laboratory. All assays performed as single batches and all intra batch CV were < 5%.The performance characteristics of each of these assays are summarised in Table 2.1.

Biochemical test characteristics

Test	**Reference range**	**Sensitivity**
Adiponectin	(5 –10.0)	0.989 ng/mL
Resistin	(4 – 8.0)	0.055 ng/mL
sP Selectin	(21-47)	0.5 ng/mL
sE Selectin	(25.4–40.9)	0.027 ng/mL
sCD40L	(20-700)	10.1 pg/mL
sRAGE (pg/mL)	(442-870)	16.14 pg/mL

2.2.8.5 Measurement of platelet function

Platelet activation was measured using a platelet chronolog aggregometer and through the measurement of surrogate markers including s CD40 ligand and vWF factor.

Figure 2.2: Chronolog platelet aggregometer used in our study to quantify platelet aggregation.

2.2.9 Platelet aggregation studies:

Platelet aggregation is a term used to denote the adherence of one platelet to another. This phenomenon can be induced by adding aggregating agents to whole blood or platelet rich plasma (PRP). In this study, Platelet aggregation was performed on a Chronolog 900 impedance platelet aggregometer (Chronolog, Coulter-Beckman UK) (Figure 2.2).

The chronolog aggregometer has separate channels for quantifying:

1. Whole blood platelet aggregation
2. Platelet aggregation in platelet rich plasma (PRP)

2.2.9.1 Whole blood platelet aggregation

I measured whole blood platelet aggregation in our study. This was quantified by the electrical impedance method (353). Two fine electrodes are immersed in whole blood. Initially, a monolayer of platelets coats the electrodes. Addition of an aggregating agent causes additional platelets to adhere to the monolayer, leading to increasing electrical resistance or impedance between the electrodes. This increase in electrical impedance is converted to a DC voltage, which is recorded on a linear strip chart recorder, thus allowing the quantification of platelet aggregation in ohms. Whole blood collected in citrated vacutainer tube (Beckton Dickinson, Oxford, U.K) was used for the study. Blood (0.5 ml) was diluted 1:1 with phosphate buffer saline in siliconized cuvettes and these were placed in the heater block incubator wells of the aggregometer. 10μl of ADP at 0.2μM concentration was used to induce platelet aggregation and the maximum impedance at 10 minutes was determined and each result is expressed as the mean of triplicates.

2.2.9.2 Reproducibility of platelet aggregation studies using the Chronolog platelet aggregometer

Measurement of platelet aggregation in whole blood by the impedance method requires a consistent technique in conducting the tests. I myself performed all the platelet aggregation studies after having familiarised with the technique. The following results were obtained on sequential samples of whole blood from a healthy volunteer, using 10μl of ADP at 0.2μM as an aggregating agent (Table 2.1). The mean platelet aggregation was 11.6 ohms with a variation of about 5%, which is acceptable.

Table 2.1: Demonstrating reproducibility of platelet aggregation studies

Run	Platelet aggregation in Ohms
1	12
2	11.5
3	11.2
4	11.5
5	12
6	11.8

2.3 Measurement of Arterial Stiffness

2.3.1 Non invasive Assessment of Arterial stiffness

There are numerous invasive and non invasive methods to assess arterial compliance in vivo. The invasive techniques are more accurate; however their widespread use is precluded in research and clinical practice. The non invasive methods focus on analysis of arterial waveforms, measurement of pulse wave velocity (PWV) and changes in the arterial diameter to distending pressure. We used the sphygmocor™ apparatus (AtCor Medical Ltd) which utilises analysis of specific components of the arterial pressure wave forms acquired using applanation tonometry to measure arterial stiffness. This involves placing a highly sensitive pressure transducer compressing the artery against a firm structure such as the bone.

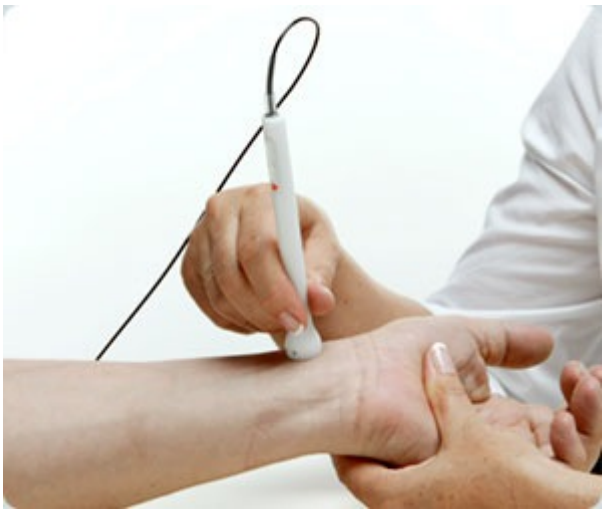


Figure 2.3: Showing the use of a hand held tonometer to obtain pulse waves

The sensor is then able to detect pressure and volume changes in the underlying vessel to generate the peripheral arterial waveform. This waveform is calibrated to the conventionally measured peripheral blood pressure. The system software applies a validated mathematical transfer function to generate the central arterial waveform and measure the central blood pressure (158).PWA can then be performed to calculate Augmentation index (Aix) and the time to the reflected wave (T_R).

2.3.1.1 Measurement of PWV

PWV refers to the speed with which the arterial wave travels across a specified distance and doppler ultrasound or applanation tonometry can be used to record the arterial waveform from a proximal site, e.g. the carotid and a distal site, e.g. the femoral artery. The arterial wave will need to traverse the abdominal aorta, a vessel that is prone to atherosclerosis to travel between these two locations. The time delay for the arrival of the arterial wave between the two sites can be calculated by gating to the peak of the ‘R, wave of the ECG or by simultaneous measurements at both sites. The distance between the two sites is measured and PWV is calculated as distance/time in metres per second.

2.3.1.2 Relating changes in Arterial Diameter to distending pressure

The change in the diameter of the artery can be related to the distending pressure and measured using ultrasound, less commonly MRI or applanation tonometry through the generation of diameter – pressure curves

of the common carotid artery with increasing distensibility indicating a higher degree of arterial stiffness. Measurement of AIx by PWA using applanation tonometry requires the least training and can be measured with a high degree of reproducibility (159), it has been shown to carry significant prognostic value. For these reasons, it was decided to measure PWV and AIx using PWA to assess arterial stiffness in the present clinical trial.

2.3.1.3 Measurement of Arterial stiffness

I performed all the measurements to avoid any intra-observer variability. Blood pressure was measured at the right brachial artery after 10 minutes rest in the seated position. Peripheral pressure waves were then captured from the radial artery at the wrist using a transducer connected to the sphygmocor apparatus. After 20 sequential waveforms had been acquired, with an operator quality index of over 75%, the central waveform was derived from an average peripheral waveform using a validated transfer factor (259).The AIx and central aortic pressure was derived from analysis of this waveform. The estimated aPWV was calculated as the time between the foot of the pressure wave and the first reflection point (T_R). Pulse wave velocity (PWV) was measured by the sphygmocor system by sequentially recording ECG-gated carotid and femoral wave forms (for aortic PWV) and carotid and radial waveforms (for brachial PWV) as previously described.

2.3.1.4 Reproducibility of AIx using Sphygmocor

This was determined before commencement of the main study. Each of the above measurements was performed twice and averaged prior to data entry.

Intra-observer variability on 20 healthy volunteers was determined prior to study commencement. The mean difference ± SD between repeated measurements of the AIx was 1.5 ± 1.1 %. An example of the study visit case report form can be found in the appendix.

2.4 Treatment assignment / randomisation / blinding

The treatment assigned to each participant was determined according to a computer generated randomisation list (Microsoft XL random number generator) produced by an independent external pharmacist (St Mary's Pharmaceutical Unit, Llanishen, Cardiff, UK). The participant packs/study drugs were labelled with a unique patient identification number. When a participant was found eligible for the study and completed all the baseline procedures, he/she was allocated a unique patient identification number in sequential, chronological order. The participant was then treated with the medication labelled with the same number. If a participant was withdrawn from the study, his or her identification number was not reallocated. All participants and investigators remained blind to treatment allocation for the duration of the study.

2.4.1 Emergency procedure for unbinding

The randomisation code for each patient was delivered to the Pharmacy department at the University Hospital of Wales in individual sealed envelopes for emergency use only. In the absence of any emergency, patient allocation details were kept coded and remained confidential until the trial

was complete. Only in the case of a serious adverse event was there a plan for the Investigator to open the relevant sealed envelope containing the patient's treatment allocation and only if this would be of help in the treatment of the patient.

2.5 Outcome Measures

The primary outcome measures were defined as changes in inflammation and RAGE expression. Secondary outcome measures were defined as changes in Body composition (Waist, BMI, body fat and waist circumference), insulin sensitivity and β cell function, endothelial markers (eSelectin, p-Selectin, and vWF), adipokines, platelet function and metabolic profiles.

2.6 Sample Size and power calculation

Sample size advice was obtained from Dr Keith Morris, Department of Applied sciences, Cardiff Metropolitan University. The main outcomes of this study were based on the known ability of CLA to regulate NF-κB activity and hence NFκB genes, in this case the main outcome was to investigate for the first time CLA modulation of RAGE expression. No previous data from human studies was available on CLA regulated RAGE expression, however, laboratory based studies at Cardiff Metropolitan University, suggested that incubation of human monocytes with Conjugated linoleic Acid at physiological concentrations, resulted in at least a 28% reduction in surface RAGE expression in these cells (295). Based on this estimate it was calculated (paired t-test, a required power of 95% and a significant value set

at 0.05) that this study required a sample of at least 7 subjects in each group. We however felt that this small sample would be better adjusted to at least 20 in each group, to take into account for subject loss during the study period. Precise estimates of the actual power are not readily available for cross-over studies such as this. Twenty nine subjects completed the treatment and one withdrew from the study.

2.7 Statistical methods

All statistical analyses were performed using Minitab version 16. All analyses were performed as per pre-established protocol and in line with previous guidance on analysis of crossover trials (267 -271) and described in detail in chapter 3.

2.8 Procedures and Instructions

2.8.1 Adverse Event Reporting

Information about all non-serious and serious adverse events (SAEs), irrespective of causality, whether volunteered by the subject, discovered by investigator questioning, or detected through physical examination, laboratory test or other means, was collected and recorded. An adverse event was defined as any undesirable sign, symptom or medical condition occurring after starting the study treatment. Study treatment included the study drug under evaluation (9, 11 CLA isomer or CLA mixture) given during any phase of the trial. Medical conditions/diseases present before starting the study were only considered adverse events if they worsened after starting the study drug.

Abnormal laboratory values or test results constituted adverse events only if they induced clinical signs or symptoms or required therapy, or were considered clinically significant for any reason, in which case they were recorded. Where possible, each adverse event was also described by:

1. Its duration
2. The severity grade (mild, moderate, severe)
3. Its relationship to study drug (suspected, not suspected)
4. The action(s) taken

2.8.2 Safety Results

Overall the study medication was well tolerated. Four adverse events were reported during the course of the study (nausea, flare up of gout, thrombophlebitis & uveitis), which were mild and self-limiting. One subject decided to withdraw from the study due to work commitments and a standard protocol was adhered to. Periodic safety reports were provided to all regulatory authorities.

2.8.3 End of study Unblinding

After completion of the final subject's final visit, a complete electronic database was created which contained all the data collected on vascular, anthropometric and metabolic parameters. A copy of the database was supplied on a CD-ROM to a research governance officer from Cardiff and vale NHS trust who undertook a final study closure inspection and confirmed source verification of the data. The pharmacy department was then given

permission to release the randomisation codes so data analysis could be performed.

2.8.4 Project Management and Administrative Procedures

I was responsible for the day-to-day conduct of the study and co-ordinated recruitment, obtained consent, performed the blood sampling and measured pulse wave velocity and augmentation index in the study participants. A formal meeting took place on a monthly basis with Dr Marc Evans, the principal investigator, to discuss all aspects of the study and to plan the work for the forthcoming week. Informal discussions regarding any difficulties that arose with the project also took place as required. Source data verification was undertaken by Dr Marc Evans at regular intervals who cross-checked all data entries for the final dataset before study closedown; the data were stored securely on the Trust shared drive with daily back up. A copy of this was taken on CD by the Sponsor as evidence of the final data before permission was granted for release of the randomisation codes.

2.8.5 Auditing Procedure

As part of Good Clinical Practice, the study protocol and all documentation were closely monitored. The study was conducted as outlined in the study protocol and in accordance with all applicable government regulations. All study documentation was made available for inspection at any time by appropriate regulatory authorities.

CHAPTER 3

A randomised, double blind, crossover study of the effects of CLA isomers on body composition, metabolic profiles, inflammation and vascular function in patients with the Metabolic Syndrome – Results

3.1 Overview

There is a reasonable body of work that suggests that CLA and its isomers may have beneficial effects on adipose tissue, inflammation, vascular function and metabolic profiles (230-235). IR plays an important role in the pathophysiology and therefore subjects with the metabolic syndrome provide a particularly good model in which to examine the impact of CLA replacement.

To date, a majority of the studies that investigated the effects of CLA have utilised animal and tissue culture systems and models. Inconsistencies and contradictions have occurred when attempting to reproduce beneficial results in human trials. The doses used in animal studies are much higher compared to those used in clinical trials and these studies fail to account for any in vivo co-activating or co-suppressing interactions. It is therefore not always possible to compare and extrapolate results from in vitro and in vivo studies. For these reasons it becomes necessary to confirm or refute any in vitro observations in vivo.

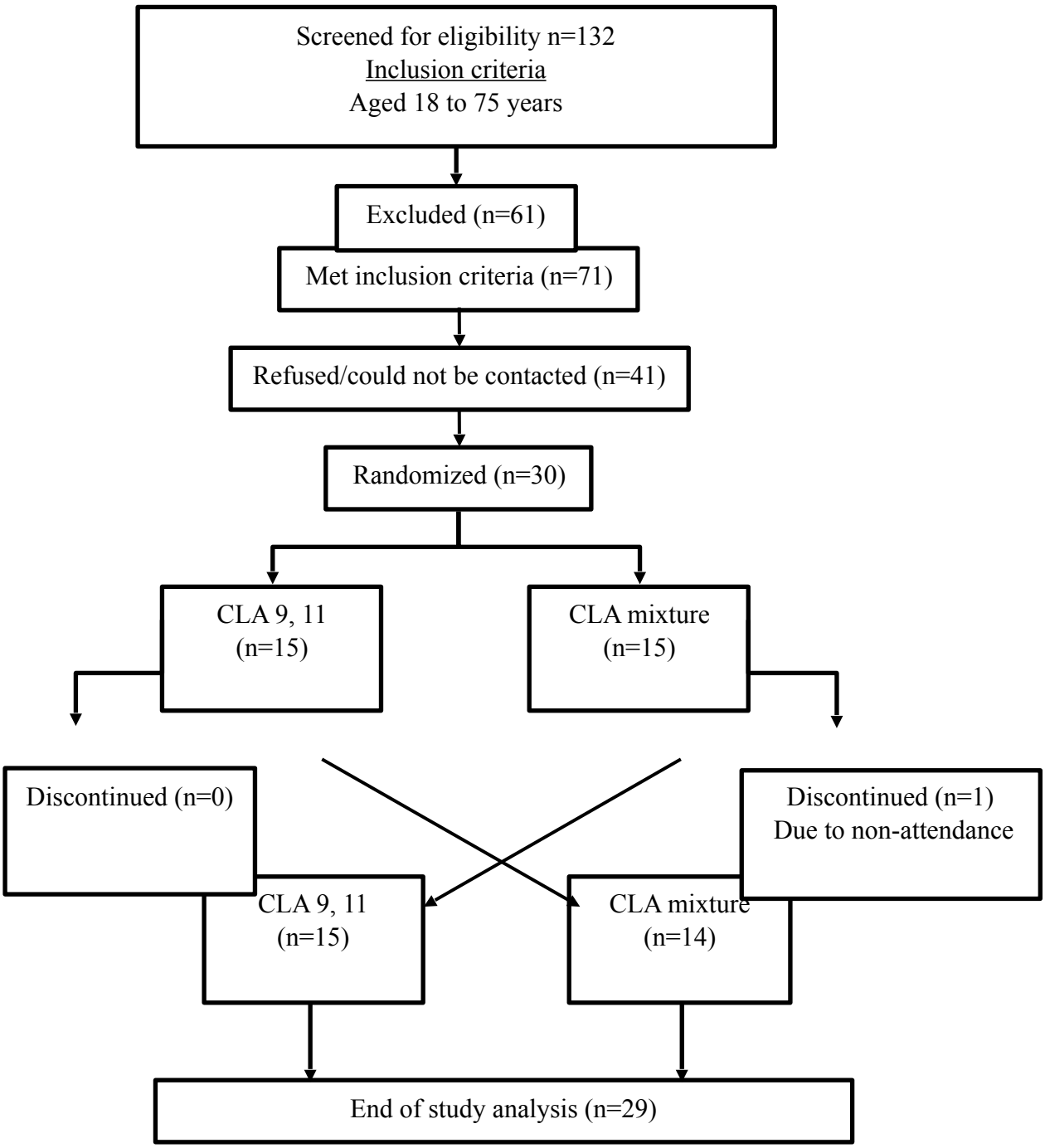
There have been a number of human studies that examined the effects of CLA, however most of these studies were limited by their numbers and duration of treatment and in the variables studied. Furthermore, the inconsistencies observed in subject based clinical trials may be a feature of the sheer variety of CLA doses used in these studies and most studies investigating CLA have used a mixture of predominantly 9:11 CLA and 10:12 CLA which is not typical of ruminant derived dietary CLA, primarily because

the individual CLA isomers are costly and difficult to purify. To our knowledge, this is the first study to prospectively test the effect of these isomers on sRAGE expression in a randomised, double-blind, crossover design. We chose a crossover design because a substantial within-subject correlation was anticipated, such that smaller sample sizes would be required to detect significant differences, and used a washout period of six weeks to minimise the potential for a carry over effect, which was not evident in this study. This chapter aims to describe the results obtained from a clinical trial analysing the effects of CLA and its isomers on inflammation, vascular function, body composition and metabolic profile in subjects with the metabolic syndrome. The primary outcome measure for this study was changes in measures of RAGE expression. The secondary outcome measures were to evaluate the effects of CLA therapy on anthropometric measures of body composition (Weight, BMI, and fat percentage and waist circumference) and adipocytokines, changes in markers of arterial stiffness (AIx, BP, bPWV and aPWV), endothelial and platelet function and metabolic profiles (lipids, homeostasis model of assessment for insulin resistance).

One hundred and thirty two patients from the diabetes clinics at the University Hospital of Wales and University Hospital Llandough were screened for trial eligibility of whom seventy one patients fulfilled the inclusion criteria. Of these, thirty patients consented to trial participation and were eventually randomised. One subject failed to attend the final study visit due to work commitments. Twenty nine patients completed the study and data on these patients are presented.

3.2 Patient flow

Figure 3.1 Flow of participants through each phase of the study



3. Safety, tolerability and compliance

Overall the study medication was well tolerated. Four adverse events in different patients were reported during the course of the study (nausea, flare up of gout, thrombophlebitis & uveitis), which were mild and self-limiting and probably unrelated to CLA treatment.

A summary of the adverse events is provided in table 3.1

AE number	Patient ID	Description	Start date	Finish Date	Outcome	Intensity	Expectedness	Causality	Seriousness
1	7	Nausea and headache	29/01/07	29/01/07	Resolved	Mild	Unexpected	Not related	Not Serious
2	11	Thrombophlebitis at venflon site	28/04/07	30/04/07	Resolved	Mild	Expected	Not related	Not serious
3	35	Uveitis	18/02/08	20/02/08	Resolved	Mild	Un expected	Not related	Not serious
4	37	Flare up of Gout	09/01/08	10/01/08	Resolved	Mild	Unexpected	Not related	Not serious

Table 3.1 Adverse events recorded during the study

4. Statistical Rationale

All Statistical analyses were performed using Minitab version 16 (Havertown, Pa). All analyses were performed in line with previous guidance on analyses of cross over trials as recommended by Altman and others (270-273). Each patient received a treatment at a specific time point (**period one**), and then

crossed over to receive another treatment at another time point (**period two**). The subjects in this study were randomly allocated into two groups so that half (n=15) received the treatments in the order (9, 11 CLA) (**period one**) and then the CLA mixture (**period two**), while the other half (n=14) received treatments in the reverse order, that is, CLA mix followed by 9, 11 CLA with an intervening wash out period. The effects of the treatments were then compared in the same person during the different treatment periods. The benefit of using this design is primarily through eliminating the variability between the patients, since each person acts as their own control. In addition, such a within-subjects comparison of treatments is regarded as being more sensitive than between-subject comparisons and consequently, the sample size needed for the study is smaller (270-273).

However in a crossover trial, there may be some systematic differences between the two periods regardless of the treatment which is termed as a period effect. Furthermore, there may be a carry-over of treatment effect from one period to the next and in the presence of significant period effects or a treatment – period interaction, the data from the second period may have to be discarded. If there is no period effect and no treatment-period interaction the analysis of a crossover trial is simple.

“Pre statistical” analyses were used to determine whether there were any period effects or treatment-period interaction as a result of the crossover design. This analysis was simplified by calculating the absolute change for each marker tested (mean value at baseline subtracted from the mean value

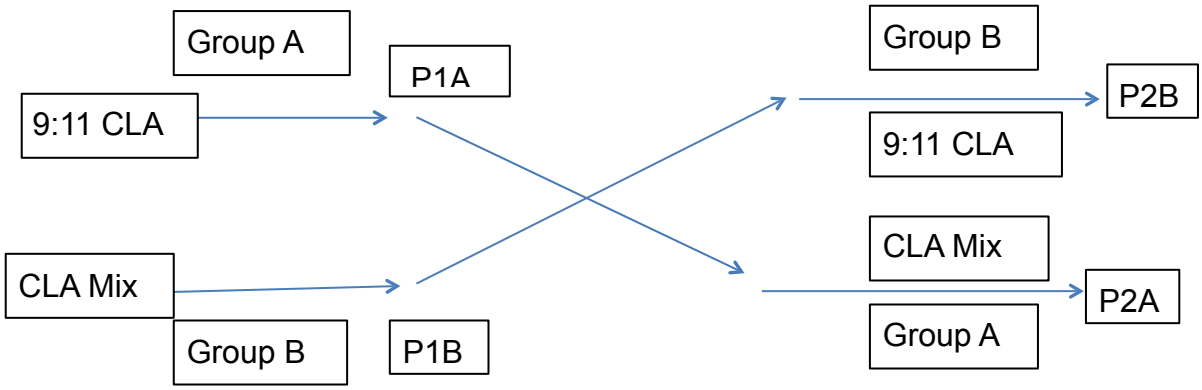
at the highest dose of each isomer for each subject) and testing differences between periods with use of two sample *t* tests or Mann-Whitney test comparing the medians. Analyses of the results in this crossover study did not reveal any significant period or interaction effects in relation to any of the parameters measured. In addition, a two sample *'t'* test or Mann-Whitney test compared the means or medians of within subject differences to measure the treatment effects.

Patients were thus randomised into two groups:

(Group A) - Participants treated with 9, 11 CLA (period 1) initially followed by CLA mix (period 2).

(Group B) - Participants treated with CLA mix (period 1) followed by 9, 11 CLA (period 2).

Figure 3.2 Analysis utilised the following model



Period I A (P 1A) – 9, 11 isomer, Period 2A – CLA mix, Period 1B – CLA mix, Period 2B – 9, 11 isomer.

3.5 Baseline Demographic and Anthropometric Characteristics

Twenty nine of the 30 subjects completed the study. Compliance was 97 % (capsule count) with no differences between the groups. The treatment was well tolerated with no major side effects. During the trial, the subjects were advised to continue with their usual medications and I did not record other variables including smoking status and dietary habits which could have an impact on the various parameters measured. However the crossover nature of the study ensures minimal variability, as each participant served as his own control.

Table 3.2 Comparison of Baseline demographic and anthropometric data

Values are expressed as mean (SD) and when indicated as median and range.

Characteristic	**Group A(n=15)** Mean (±SD)	**Group B(n=14)** Mean (±SD)	**'p' Value**
Age(Yr)	51.5(8.5)	53.7(8.4)	0.48
Wt(kg)	87.2(3.6)	88.9(4.7)	0.78
BMI(kg/m	31.1(3.6)	29.1(3.8)	0.42
Body fat (%)	35.9(8.3)	33.4(5.5)	0.35
Waist Circumference(cm)	105.3(3.8)	103.4(5.1)	0.64

The baseline characteristics indicate that the study cohort was representative of the general population with the ms, with a mean BMI in the overweight range and associated with an increased mean waist circumference. The mean age of the subjects were 51 years in group A and 53 years in group B. There were no significant baseline differences in body composition between the two treatment groups (Table 3.2).

3.6 Baseline Vascular Parameters of Study participants

Table 3.3 summarises the baseline vascular parameters for the study population. The blood pressure for our study population was in the hypertensive range as would be expected with these subjects.

Table 3.3 Baseline vascular markers for the study population

Characteristic	**Group A (n=15)** Mean (±SD)	**Group B (n=14)** Mean (±SD)	**'p' Value**
Peripheral DBP(mm Hg)	92.3(9.4)	93.9(9.5)	0.65
Peripheral SBP(mm Hg)	130.9(14.2)	139.8(11.5)	0.09
Central DBP(mm Hg)	92.3(9.4)	96(11.7)	0.35
Central SBP(mm Hg)	124.4(12.3)	128.2(11.4)	0.39
AIx (%)	20.2(6.8)	21.2(10.9)	0.78
bPWV(m/sec)	8.5(1.2)	8.6(2.2)	0.85
aPWV(m/sec)	9.0(1.3)	9.0(1.5)	0.88
T_R(m/sec)	142.2(12.2)	145.0(10.9)	0.51
sE- selectin(ng/ml)	71.37(30.63)	70.1(35.33)	0.90
sP- selectin(ng/ml)	7.59(2.51)	7.66(2.07)	0.93
vWF(ng/ml)	133.2(23.9)	136.9(29.2)	0.65
sCD40L(ng/ml)	1277(875-1674) *	915(859-971)*	0.64
Platelet aggregation(ohms)	16.9(4.1)	17.1(3.9)	0.88
hsCRP(mg/litre)	1.48(1.3)	2.49(1.6)	0.37
sRAGE(pg/ml)	699.1(220.5)	788.4(223.1)	0.21

*Indicates Median and Inter Quartile Range and comparison using Mann-Whitney test.

The baseline Augmentation index (AIx) in our study population at baseline ranged between 20 % and 21 % compared with a value of 18 % for a similar mean aged population. The reference values for Aix using the sphygmocor apparatus have been provided by the manufacturer and available at http:// atcormedical.com. There were no statistically significant differences in vascular parameters at baseline between both the groups.

3.7 Baseline Adipokines and Metabolic Parameters (Table 3.4)

Characteristic	**Group A (n=15)** Mean (SD)	**Group B (n=14)** Mean (SD)	**'p' Value**
Insulin(pmol/litre)	101 (30.4-141.7)*	71.3 (6.7-119.0)*	0.42
Proinsulin(pmol/litre)	5.6(3.7)	3.7(3.0)	0.35
NEFA(mmol/L)	0.19(0.2)	0.5(0.5)	0.64
Fasting glucose (mmol/L)	6.05 (0.7)	5.6(0.8)	0.17
HOMA-IR	32.9(25.5)	25.8(21.2)	0.11
HOMA-B	806 (425-1134)*	413 (215-1326)*	0.36
TC(mmol/litre)	5.4(1.18)	5.4(0.96)	0.85
HDL(mmol/litre)	1.09(0.22)	1.07(0.27)	0.87
LDL(mmol/litre)	3.2(0.96)	3.2(1.15)	0.86
Triglycerides(mmol/litre)	2.7(1.55)	2.4(1.28)	0.58
Adiponectin(ng/ml)	6.2(2.66)	4.4(2.78)	0.19
Resistin(ng/ml)	3.2(1.32)	3.7(2.02)	0.45
PAI-1(ng/ml)	2.7(1.02)	3.6(1.47)	0.15

* Indicates Median and Inter Quartile Range and comparison using Mann Whitney test.

The data in Table 3.4 shows there were no statistically significant differences between the groups at baseline as shown by the ‘p’ values.

8. Summary of Results

3.8.1 A crossover design to investigate the differential effects of the 9, 11 CLA isomer and CLA mixture

A crossover design has the ability to identify differences between the two treatments. The first part of the analysis was therefore to compare the effects of the 9, 11 CLA isomer and the CLA mixture on both primary and the secondary end points.

Analyses of the results in this crossover study did not reveal any significant period or interaction effects in relation to any of the parameters measured. As previously stated, in the absence of an interaction, a patient’s average response to the two treatments is considered to be the same regardless of the order in which they were received (270-273). Consequently, the analysis of the data was undertaken to identify any treatment effects i.e. differences in overall response associated with one treatment in comparison to the other.

A negative mean difference in outcomes was suggestive of a 9:11 isomer mediated effect and a positive mean difference indicates that the effects are primarily associated with the CLA mixture (270-273).

3.8.1.1 Effects on primary end points – (Inflammation and sRAGE expression, endothelial markers, platelet function)

A summary of the effects on Primary outcomes for the study is given in Table 3.5.

Table 3.5 Changes in sRAGE, Inflammation, Endothelial & Platelet function (Comparing 9, 11 isomer Vs CLA mix)

Variable	**Mean Difference** (period1 – period 2)	**95% CI for the difference**	**'p'-value**
sRAGE (pg/ml)	-22	-308, 264	0.87
hsCRP(mg/litre)	2.1	-3.2, 4.4	0.37
sE-Selectin(ng/ml)	5.6	-11.5, 22.7	0.56
sP-Selectin(ng/ml)	0.1	-1.7, 1.9	0.90
vWF(ng/ml)	1.6	-22.3, 25.4	0.89
sCD40L(ng/ml)	95	-716, 606	0.81
Platelets Aggs(ohms)	2.8	0.6, 5.0	0.01**†**

† - denotes a significant effect

A negative mean difference = Effect mediated by 9,11 CLA

A positive mean difference = Effect mediated by CLA mixture

From these results it is apparent that the two treatments did not significantly differ with regards to circulating sRAGE levels and markers of endothelial function. However, the results for platelet aggregation were significant ('p'=0.01) and these are shown to be mediated by the CLA mix in comparison to the 9, 11 isomer. This was somewhat surprising as it was not associated with any changes in surrogate markers of platelet function such as sCD40L or sP-selectin levels or measures of endothelial function (sE-selectin, vWF).

3.8.1.2 Effects on Secondary outcome measures

3.8.1.2.1 Changes in Vascular Outcomes of the study cohort

Table 3.6 summarises the vascular outcomes for the crossover cohort. The results demonstrate a significantly enhanced treatment effect for peripheral SBP ('p'=0.04) and aortic PWV ('p'=0.01) and the negative value of the mean difference would suggest that these effects are mediated by the 9:11 CLA isomer. There was however no changes observed with peripheral or central diastolic BP, central systolic BP, AIx or T_R suggesting that wave reflection is not significantly affected.

Table 3.6 Effect of 9, 11 isomer Vs CLA mix on Vascular Outcomes in subjects with the metabolic syndrome

Variables	**Mean Difference** (period1 – period 2)	**95% CI for the difference**	**p-value**
P-SBP (mm Hg)	-8.50	-16.6, - 35	**0.04†**
S-DBP (mm Hg)	-1.25	-7.61, 5.12	0.68
C-DBP (mm Hg)	-2.35	-7.85, 3.14	0.38
C-SBP (mm Hg)	-5.33	-12.51, 4.84	0.13
AIx (%)	-1.05	-6.88, 4.78	0.70
aPWV(m/s)	-0.81	-1.47, -0.14	**0.01†**
bPWV(m/s)	-0.03	-0.47, 0.55	0.88
T_R	-1.64	-6.89, 3.61	0.52

† - denotes a significant effect

A negative mean difference = Effect mediated by 9,11 CLA

A positive mean difference = Effect mediated by CLA mixture

3.8.1.2.2 Changes in Body Composition, Lipids and Adipokines for the crossover cohort

The results summarised in table 3.7, demonstrates a significant beneficial effect on HDL levels ('p'=0.03) which are shown to be mediated by the 9:11 isomer as suggested by the negative value of the mean difference. Furthermore, treatment with the CLA mixture significantly increased adiponectin concentrations ('p'=0.01). These changes were however not associated with significant changes in body composition or other lipid parameters or adipokines.

Table 3.7 Changes in Body composition, Lipid profiles and Adipokines (9, 11 isomer Vs CLA mix)

Variables	**Mean Difference** (period1 – period 2)	**95% CI for the difference**	**p-value**
BMI(kg/m	-0.11	-0.83,0.61	0.75
WC(cm)	-0.66	-3.88,2.57	0.67
TC(mmol/litre)	0.001	-0.59,0.59	0.99
HDL(mmol/litre)	-0.12	-0.26,0.00	**0.03†**
LDL(mmol/litre)	-0.59	-0.05,1.23	0.06
Triglycerides(mmol/litre)	-0.63	-1.99,0.72	0.33
Adiponectin(ng/ml)	1.43	0.253,2.62	**0.01†**
Resistin(ng/ml)	-0.20	-0.62,-0.20	0.31
PAI-1(ng/ml)	0.69	-0.84,2.23	0.35

† - denotes a significant effect

A negative mean difference = Effect mediated by 9, 11 CLA

A positive mean difference = Effect mediated by CLA mixture

3.8.1.2.3 Changes in Insulin Sensitivity and β cell function (9, 11 isomer Vs CLA mix)

This study also measured fasting glucose, insulin, proinsulin and NEFA levels at baseline and following the treatment periods during this crossover study in a sub-section of our patient cohort. The proinsulin / insulin ratio's, NEFA and calculated HOMA– B values were used as surrogate measures of β cell function and HOMA– IR was used as a marker of insulin sensitivity.

Table 3.8 Effects of CLA on Insulin sensitivity and β-cell function (9, 11 isomer Vs CLA mix)

Variables	Mean Difference (period 1- period 2)	95% CI for the difference	p-value
Insulin(pmol/L) Grp A (n=10) Grp B (n= 9)	-122	-221,76.8	0.20
Proinsulin(pmol/L) Grp A (n=10) Grp B (n= 9)	-7.03	-19.31,5.24	0.23
NEFA(mmol/L) Grp A (n=10) Grp B (n= 9)	-0.09	-0.97,0.78	0.82
FPG(mmol/L) Grp A(n=15) Grp B (n=14)	0.15	-0.770,1.08	0.72
HOMA-IR Grp A (n=10) Grp B (n= 9)	919	-1263,3102	0.36
HOMA-B Grp A (n=10) Grp B (n= 9)	2728	1008,4448	**0.004†**
Pro ins / Ins Ratio Grp A (n=10) Grp B (n= 9)	-0.13	-0.781,0.521	0.65

† - denotes a significant effect

A negative mean difference = Effect mediated by 9,11 CLA

A positive mean difference = Effect mediated by CLA mixture

Insulin sensitivity and β-cell function data are presented in Table 3.8. A solitary significant CLA mixture mediated effect was observed for HOMA-B. However, the changes seen in HOMA-B levels should be interpreted with caution given the wide confidence intervals evident and the relatively small sample size.

3.8.2 Combined Effects of 9, 11 CLA / CLA mix treatment on primary and secondary outcomes (Comparison between Baseline values and End of treatment)

This study utilised a cross-over design with no placebo component to the study. Furthermore, no significant differences at baseline, period or carry over effects between the two groups were observed and a relatively small number of significant outcomes reported (peripheral SBP, aPWV, HDL-cholesterol, adiponectin, platelet aggregation and HOMA B).These significant results were found to be mediated by either the 9,11 isomer or with the CLA mixture that contained 50% of 9,11 CLA.

Essentially this part of the study did not suggest any clear overall benefits to the use of the expensive and difficult to obtain 9,11 isomer over the CLA mixture. However, it was also thought expedient to investigate if combining the data from both groups (for which no baseline differences were reported) and comparing the outcomes at baseline and at the end of the 16 weeks of treatment. Therefore, further analysis of the samples were undertaken to

investigate the overall effects of 9, 11 CLA / CLA mix using a paired‘t’ test. This was undertaken specifically because of:-

1: The similar baseline characteristics of the sample.

2: The similarity of the treatments and their likely mode of action and with no period or carry over effects observed during the cross-over phase.

3:To investigate the combined effect of the treatment over the 16 week period on the larger sample of 29 subjects.

Comparing the baseline parameters with the end of the treatment phase has a number of analytical advantages as it involves increased numbers of subjects and entails paired analysis with its enhanced ability to detect *overall* significant differences between the baseline and the end of the intervention period.

The combined results for the entire cohort are presented in the following tables.

3.8.2.1 Effects of 9, 11 CLA / CLA mix on inflammation and Vascular outcomes – Paired test comparing baseline values with end of treatment (visit 4) (Table 3.9)

Treatment with 9, 11 CLA / CLA mix significantly improved aPWV. This was accompanied by a lowering of blood pressure along with significant changes in circulating sRAGE, markers of endothelial (sE selectin, vWF) and platelet function (sP-Selectin, sCD40l).

Table 3.9 - Results of Vascular Outcomes for the entire study cohort

Variables	Mean Difference (visit 4 – visit 1)	95% CI for the difference	'P'-value
P-SBP(mm Hg)	0.14	-4.16, 4.44	0.94
P-DBP(mm Hg)	-3.14	-6.38, -0.10	0.05
C-SBP(mm Hg)	-3.60	-7.32, 0.12	0.05
C-DBP(mm Hg)	-4.22	-8.24, 0.20	**0.04†**
AIx (%)	-0.79	-3.38, 1.80,	0.53
aPWV(m/s)	-0.63	-1.02, 0.22,	**0.003†**
bPWV(m/s**)**	-0.27	-0.60, 0.05	0.10
T_R	-2.71	-5.91,-0.49,	0.09
sE- selectin(ng/ml)	-13.3	-23.8, 2.78	**0.01†**
sP- selectin(ng/ml)	-1.43	-2.2, 0.58	**0.002†**
vWF(ng/ml)	-16.1	-29.2, 3.33,	**0.01†**
sCD40L(ng/ml)	-362	-704, 21,	**0.03†**
Platelet aggs(ohms)	-1.52	-3.05, 0.00	0.05
hsCRP(N=16)	-1.76	-4.94, 1.43	0.23
sRAGE(pg/ml)	320	511, 129	**0.002†**

† - denotes a significant effect

Figure 3.3 Effect of 9,11CLA / CLA mix treatment on aPWV and bPWV (Visit 1 – Baseline, Visit 4 - Post treatment)

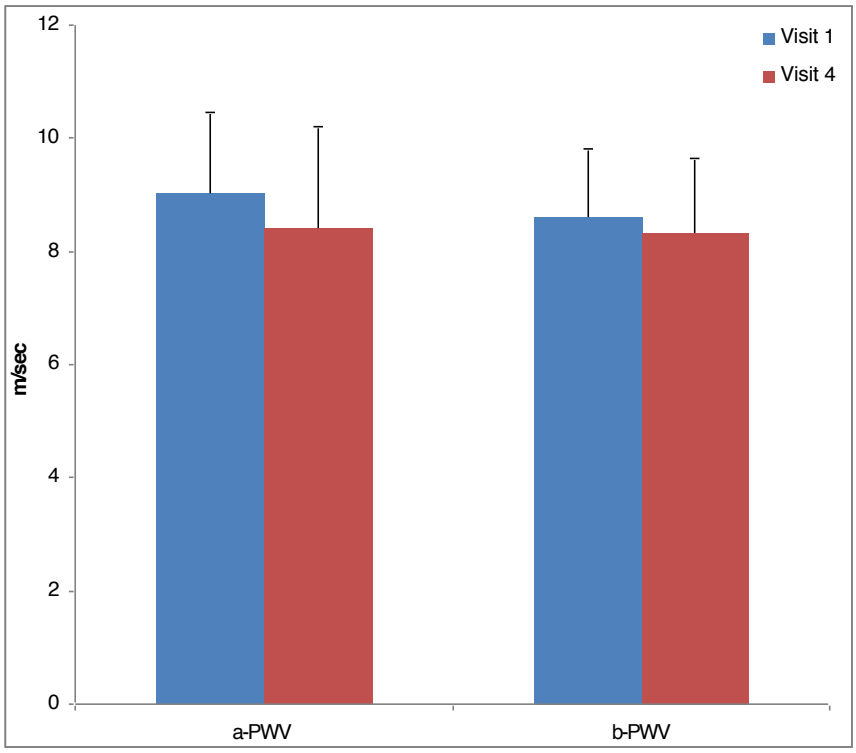
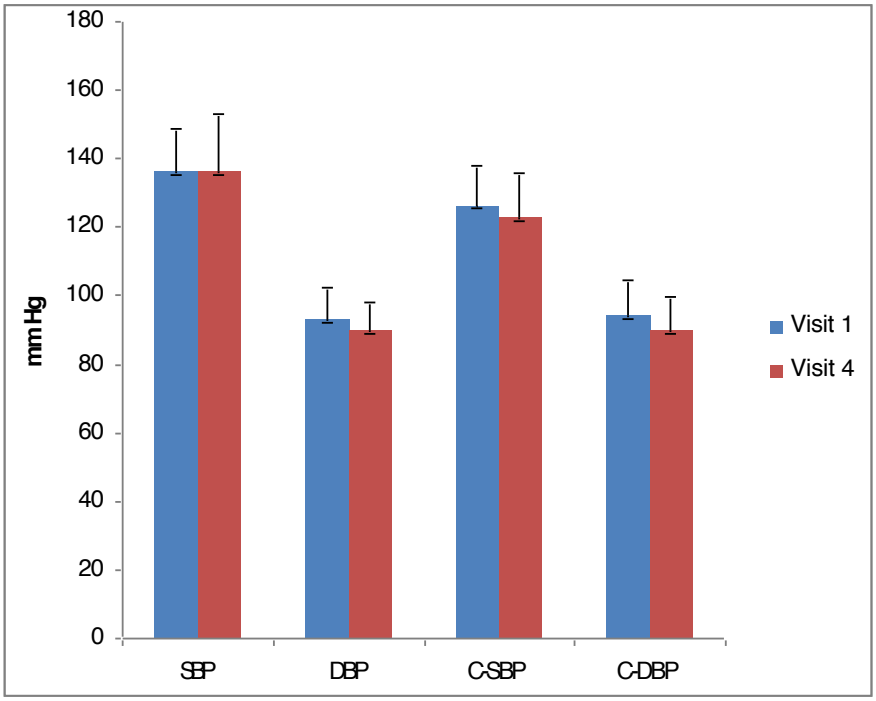


Figure 3.4 Changes in BP following treatment with 9, 11 CLA / CLA mix in the metabolic syndrome



3.8.2.2 Changes in Body Composition, Lipids and Adipokines for the entire cohort (comparison between baseline data with end of visit 4)

(Table 3.10) - Effect of 9, 11 CLA / CLA mix therapy on Body Composition, Lipids and Adipokines in subjects with the metabolic syndrome.

Variables	**Mean Difference (visit 4 – visit 1)**	**95% CI for the difference**	**p-value**
BMI	-0.68	-1.03,0.34,	**0.005†**
Body fat (%)	-1.28	-2.86,0.28,	0.10
WC(cm)	-1.31	-3.32,0.70,	0.19
TC(mmol/litre)	-0.05	-0.37, 0.26,	0.73
HDL(mmol/litre)	0.04	-0.01, 0.09	0.15
LDL(mmol/litre)	-0.25	-0.63, 0.12	0.17
Triglycerides(mmol/ litre)	0.21	-0.14, 0.57	0.22
Adiponectin(ng/ml)	0.90	-0.34,1.46,	**0.003†**
Resistin(ng/ml)	-1.07	-1.61, 0.52	**<0.0005†**
PAI-1(ng/ml)	1.20	-0.31, 2.71,	0.10

†- denotes a significant effect

Figure 3.5 Effect of 9, 11 CLA / CLA mix therapy on lipid profiles in subjects with the metabolic syndrome (Visit 1 – Baseline, Visit 4 - Post treatment)

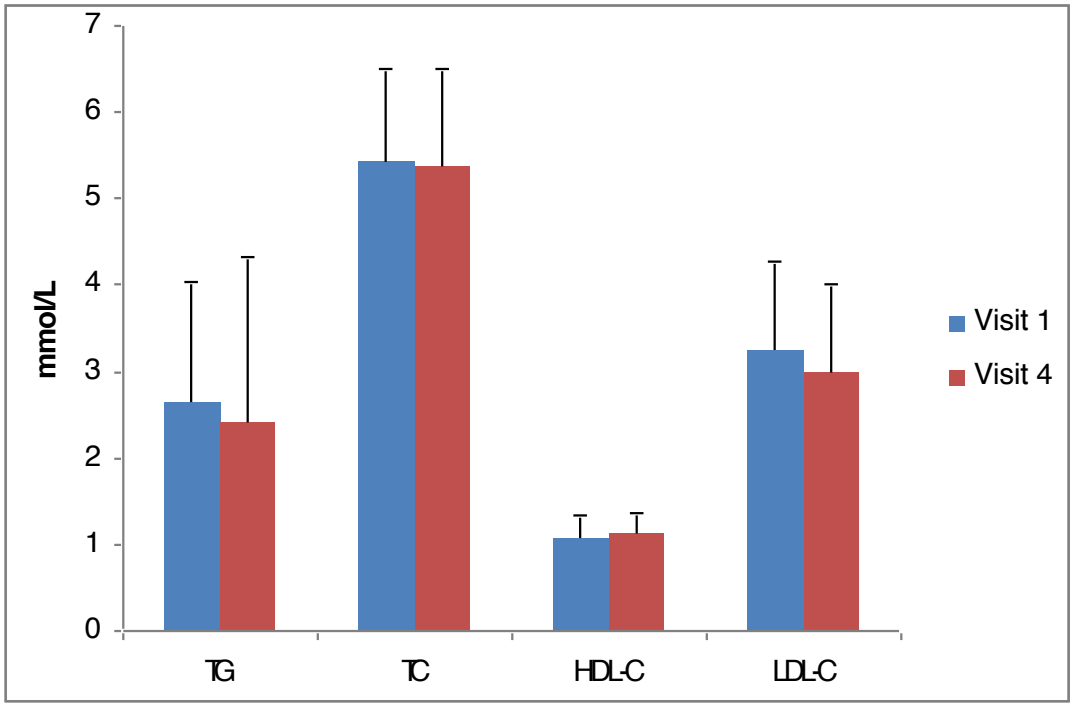


Figure 3.6 Effect of 9, 11 CLA / CLA mix on Adipokines (Visit 1 – Baseline, Visit 4 - Post treatment)

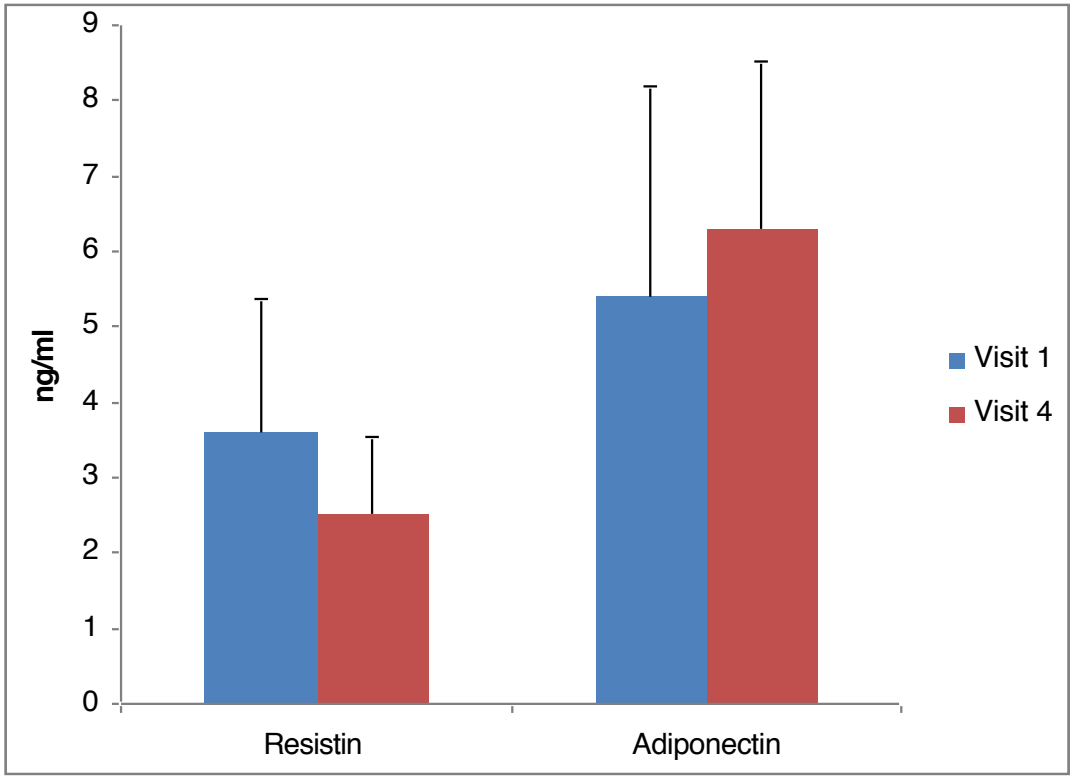


Table 3.11 Effects of 9, 11 CLA / CLA mix on insulin sensitivity and β-cell function (Comparing baseline data with end of treatment values)

Variables	Mean Difference (visit 4 – visit 1)	95% CI for the difference	p-value
Insulin(pmol/L)	18.2	- 59.5, 95.8	0.63
Pro insulin(pmol/L)	-5.07	- 0.88, 11.0	0.09
NEFA(mmol/L)	-0.21	- 0.66, 0.24	0.34
FPG(mmol/L)	-0.02	- 0.47, 0.43	0.92
HOMA-IR	-13.3	- 42.3, 15.7	0.35
HOMA-β	-434	- 1404, 535	0.36
Pro ins / Ins ratio	-0.09	- 0.39, 0.21	0.53

There were no significant changes observed in measures of either insulin sensitivity or β cell function following treatment with CLA 9, 11 CLA / CLA mix.

3.9 Correlation Analysis for the Combined Data

Table 3.12 Pearson's Correlation coefficients for the change in sRAGE versus selected vascular and metabolic parameters investigated, the correlation coefficient is given with the significance in brackets.

variables	**diff s RAGE**
diff aPWV	-0.045, 0.812
diff e-Selectin	0.247 0.196
diff vWF	-0.090 0.641
diff sCD40L	0.058 0.767
diff WC	-0.209 0.275
diff HOMA-IR	-0.230 0.315
diff HOMA-B	-0.420 0.065
diff Hdl	-0.097 0.616
diff LDL	0.200 0.297
diff adiponectin	-0.035 0.862

Analysis of the data shows that sRAGE did not significantly correlate with any of the parameters analysed. However the numbers of subjects are small and must be taken into account when interpreting the correlation data.

Further statistical extrapolation of the inter-dependence of these variables was not undertaken in view of the small numbers. However, the lack of significant correlations demonstrated herein, would support further analysis through multi regression methods and this is presented in 3.10 below.

3.10 Regression Analysis for the Combined Data

One of the reasons for undertaking the statistical analysis of such as that gathered in this study is to describe relationships between the variables for which data has been collected. To investigate which variables investigated as part of this study could act as predictors of the significant change in aPWV regarded as the dependant variable and sRAGE observed after 9:11 isomer / CLA treatment, a stepwise regression analysis was undertaken. This method involves selecting predictor or predictive variables using an automatic procedure that takes the form of a sequence of F-tests or alternatively t-tests. Since the significant predictors of the difference in aPWV between baseline and end of study were being investigated, likewise the predictor variables used were also the differences between visit 1 and visit 4.

The stepwise regression analysis undertaken here demonstrated that the only two predictors, namely the difference in Sp-Selectin and difference vWF identified as being significant in predicting the improvement in aPWV due to 9:11 isomer / CLA mix treatment in this metabolic group during the study period analysis. The regression equation describes the relationship between

the two dependant variables and the explanatory variables selected by the stepwise regression. The regression equation is aPWV = 1.14 - 0.141 Sp-Selectin - 0.00931 vWF. Analysis of variance ANOVA confirmed that this relationship was significant (p=0.037)

Table 3.13 Regression analyses of aPWV vs difference Sp-Selectin and vWF.

aPWV = 1.14 - 0.141 Sp-Selectin - 0.00931 vWF

Predictor	Coef	SE Coef	T	P
Constant	1.1407	0.2531	4.51	0.000
Diff Sp Selectin	-0.14073	0.07861	-1.79	0.041
DIFF vwf_1	-0.009313	0.005936	-1.57	0.086

R-Sq = 17.9% R-Sq(adj) = 13.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	4.8613	2.4307	2.84	0.037
Residual Error	26	22.2723	0.8566		
Total	28	27.1336			

For the increase in sRAGE observed after treatment with CLA, the sole best predictor selected by stepwise, was the difference in HOMA-IR observed during the study period and the regression equation is:- diff sRAGE = - 472 - 0.117 diff HOMA IR. Analysis of variance ANOVA confirmed that this relationship was significant (p=0.041) and the regression analysis is presented in Table 3.14

Table 3.14 Regression analysis of sRAGE vs difference in HOMA-IR

diff sRAGE = - 472 - 0.117 diff HOMA IR

Predictor	Coef	SE Coef	T	P
Constant	-471.9	122.9	-3.84	0.001
diff HOMA IR	-0.11676	0.05953	-1.96	0.045

S = 537.482 R-Sq = 17.6% R-Sq(adj) = 13.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1111538	1111538	3.85	0.045
Residual Error	18	5199972	288887		
Total	19	6311511			

3.11 Discussion

Although a number of in vitro and in vivo studies have examined the anti obesity and metabolic effects of conjugated linoleic acid, to my knowledge this is the first study to prospectively study the effects of CLA isomers on sRAGE expression in subjects with the metabolic syndrome.

A crossover design was chosen because a substantial within-subject correlation was anticipated such that small sample sizes would be required to detect significant changes and this study used a washout period of 6 weeks to minimise any carry over effects.

Furthermore, differences in the characteristics of the two major isoforms of CLA (9:11 and 10:12) have also been reported with some evidence of non-beneficial effects observed with 10:12 CLA supplementation (262). Hence, this study initially investigated the 9:11 isomer in comparison with a 50:50 mixture of 9:11 and 10:12 isomers in a cross-over design.

Detailed analysis of the cross-over study in relation to SBP, aPWV and HDL and suggested that differences observed with regards to these parameters were associated with the 9:11 isomer. These lipid and vascular effects associated with the 9, 11 isomer are supported by previous published studies (91, 95,103,104,105) and it has been proposed that they are features of the ability of 9:11 isomer to act as a ligand of the PPARα receptor.

On the other hand the CLA mix was shown to increase adiponectin and HOMA-B levels. This could partly be mediated by the effects of the 10, 12 isomer which constitutes 50% of the CLA mixture. The 10, 12 CLA isomer has been shown in previous studies to be responsible for weight loss and consequently changes in adipokines which are believed to be mediated through their effects on PPAR γ receptors (93, 94, and 98).

The results from the crossover study however showed no significant differences between the effects of either the 9, 11 isomer or the CLA mix with regards to the other outcomes. This could partly be explained by the small sample size, relatively short duration of treatment and that the sample size was chosen to primarily observe changes in sRAGE expression. Both the treatments were however well tolerated and did not demonstrate any adverse outcomes.

The study initially investigated the differences in the outcomes of the 9,11 and CLA mixture using the cross-over design. However, there was a lack of substantial differences in the two treatments and also an inconsistency in specifying these differences to a specific isomer. There is also a similarity in the actions of the treatments and therefore a further sub-analysis of the data was undertaken. This sub-analysis involved combining the two groups and analysed the differences in baseline data and the results at the end of the treatment period. This sub-analysis was specifically undertaken to determine if there was an overall effect on the two groups of the treatment with the

lipids after the sixteen weeks of treatment. It could be argued legitimately that the presence of the wash-out period required that the analysis should have two baselines and hence four analytical stages. However, it could also be argued that the subjects received an overall treatment of 16 weeks and hence two analytical points were selected, namely, baseline and the final end of treatment for the combined groups. The results from the combined data revealed a number of beneficial treatment effects as discussed below.

Low grade inflammation has been demonstrated to be associated with IR and CVD and is proposed to be a useful clinical marker of CVD risk (296, 297). Inflammation is believed to be responsible for the development of the many complications of the metabolic syndrome including atherosclerosis (298). The increased vascular risk associated with the metabolic syndrome may be partly initiated by glycated protein binding to their receptor RAGE, which through a series of complex reactions induces secretion of inflammatory cytokines which activates key cell signalling pathways both in endothelial (201) and vascular smooth muscle cells (202). Activation of the NF-κB together with an altered platelet metabolism and changes in intra platelet signalling pathways, contributes to the pathogenesis of atherothrombotic complications in these subjects (203). Recently it has been reported that human vascular cells express several RAGE variant proteins, including three novel human RAGE transcripts (308), all encoding truncated soluble forms of RAGE (sRAGE), consisting of only the extracellular ligand binding domain and lacking the cytosolic and transmembrane domains. Soluble RAGE (sRAGE) lacks the ability to activate the key signalling

pathways and therefore sRAGE has recently emerged as a biomarker in several RAGE-mediated vascular disorders, including coronary artery disease, diabetes and hypertension (309). Falcone and colleagues also showed that endogenously lower levels of sRAGE were associated with enhanced risk of coronary artery disease, as detected by angiography and suggested that individuals with the very lowest levels of sRAGE displayed the greatest overall risk for disease (310). Therefore measurement of sRAGE might be a powerful complement to assessment of high sensitivity C-reactive protein in subjects predisposed to atherosclerosis.

To our knowledge this is the first clinical study to look at the effects of CLA on sRAGE expression in subjects with the metabolic syndrome which was the primary objective of this study.

There were no significant differences between the 9, 11 isomer or CLA mix on sRAGE expression in our study cohort. However, the combined results show a significant increase in sRAGE levels following 9:11 isomer / CLA mix supplementation. The increase in sRAGE levels can therefore act as a “decoy” receptor and attenuates the inflammatory response mediated by RAGE activation as proposed by previous studies (308).

The dietary lipid CLA and its specific isomers are ligands of the nuclear transcription factors PPAR and have been shown to suppress NF-κB and one of its target genes RAGE in animal models (307). It is possible that activation of PPARs with associated changes in BP and lipid profile is in all

likelihood responsible for the changes in sRAGE expression in these subjects (217,218,219)

Analysis of the combined data (Table 3.12) showed that sRAGE did not significantly correlate with any of the parameters measured. However this lack of correlation with the other parameters should be interpreted with some caution on the basis of a relatively small sample size and as the statistical power was based primarily on changes in sRAGE expression which is a limitation of this study. The results however support our primary hypothesis of the ability of these isomers to regulate circulating sRAGE and suggest a role for CLA in reducing vascular risk through attenuating inflammation. This seems to be predominantly associated with changes in endothelial and platelets function and independent of the changes in the metabolic profile.
As treatment with CLA has the potential to favourably modulate these risk factors, such therapy is likely to confer additional vascular benefits beyond improved glycaemic control. This however will need to be confirmed and replicated in larger studies.

Arterial stiffness is independently associated with cardiovascular mortality in a number of disease states including type 2 diabetes, renal failure and hypertension (311-313) and interventions to reduce stiffness such as lowering of BP is associated with improved survival (314). Normal blood pressure is dependent on the net balance between vasoconstrictor and vasodilator forces. In the insulin resistant state as in subjects with the metabolic syndrome, there appears to be a selective resistance to the

effects of insulin on endothelial NO production mediated by the PI3 kinase signalling pathway, whilst leaving the insulin action on ET-1 production mediated by the MAP kinase signalling pathway unchecked tilting the balance towards a vasoconstrictor influence. Hence, improving insulin sensitivity has a potential to reduce blood pressure in these subjects by promoting insulin mediated vasodilatation.

Therefore, this study also looked at the effect of CLA supplementation on BP (central and peripheral), markers of arterial stiffness (BP, aPWV, bPWV and AIx) along with surrogate markers of endothelial (sE-selectin, vWF) and platelet function (sCD40L, sP-selectin), all of which are considered to be traditional risk factors for atherosclerosis.

Treatment with 9, 11 CLA / CLA mix was associated with a significant improvement in aortic PWV('p'=0.003) which is considered in epidemiological studies to be an important surrogate marker of atherosclerotic risk and has been shown to be independently predictive of cardiovascular mortality (311). A slowing of aortic PWV was not associated with significant changes in AIx or T_R. The reduction in aortic pulse wave could be explained by changes in BMI and lipid parameters and partly by the increase in adiponectin levels which may have contributed to changes in vascular function because adiponectin enhances nitric oxide production by endothelial cells (324). The change in aortic PWV may be a consequence of the reductions in central and peripheral blood pressure and also by significant changes in surrogate markers of endothelial (sE-selectin, vWF)

and platelet function (sP-Selectin, sCD40l). The BP lowering effects of CLA are shown in this study to be independent of improvement in glycaemic control and changes in insulin sensitivity.

Conjugated linoleic acid has received considerable attention because of its anti diabetic and anti obesity actions. The results from the clinical studies have been variable and somewhat conflicting. Riserus et al showed that the t10, c12 CLA caused marked insulin resistance, oxidative stress and dyslipidaemia in obese men (262). However these effects were not replicated in other studies (263,264). We did not therefore use the 10, 12 isomer in our study. In the present study, we hypothesised that 9,11 CLA could improve insulin sensitivity and lipid profile on the basis of recent data (263,264) and compared this with the CLA mix containing equal proportions of both the isomers which is found in weight loss products sold over the counter.

Insulin, proinsulin, NEFA, HOMA-B and HOMA-IR values remained unchanged after CLA supplementation in our study in contrast to other studies which reported worsening insulin sensitivity (262,263,264). The insulin sensitising effects of CLA are suggested to be mediated via activation of PPARγ and subsequent stimulation of adipocyte differentiation. There was however no evidence in our study that either isomer adversely or positively influenced insulin sensitivity.

This study utilised HOMA-IR and HOMA-B for measuring insulin sensitivity and β-cell function rather than using a more robust method (hyperinsulinaemic euglycaemic clamp) and this along with the sample size, relatively short duration of treatment and the consequent wide confidence intervals with insulin levels may be responsible for the lack of effects on insulin sensitivity.

The body fat lowering effects of CLA in experimental animals has led to its use as a tool in body weight management in human subjects. The effects of CLA on body composition have been divergent (315,316). In contrast to findings from other studies (263,264), no significant changes in body composition were observed in our study apart from a reduction in BMI when the results for both groups were combined. The lack of effect of CLA supplementation on body composition may be due to the relatively short duration of treatment and the doses of CLA used.

Treatment with CLA has been shown to affect serum lipoprotein concentrations both in vitro and clinical studies (262,264,317-319). Within the 9, 11 group in this crossover study, there was a significant increase in HDL concentrations. These beneficial effects could be mediated by altering the rate of de novo lipogenesis and / or the rate of lipolysis or increasing carnitine palmitoyl transferase activity all of which are recognised to be the consequence of PPARα activation (320,321). However these effects were not replicated when data was combined and were not associated with significant changes in body composition.

Adiponectin levels were noted to be significantly elevated following CLA supplementation and were associated with a significant reduction in resistin levels. These changes however did not correlate with changes in body composition or glycemic control. This is probably not surprising given the short time frame of treatment and the doses used in this study.

PAI-1 has been found to be elevated in obesity and subjects with the metabolic syndrome. PAI- 1 activity has been observed to decrease with reducing body fat or body weight. However no such effect was seen in our study.

In conclusion, the data presented in this chapter may support a role for CLA supplementation in reducing vascular outcomes and the risk of atherosclerosis. The BP, endothelial and platelet effects seem to be independent of the improvements in glycaemic control and insulin sensitivity and possibly mediated through attenuation of inflammation and up regulation of circulating sRAGE. These findings will need to be replicated in larger studies before its routine use in subjects with an increased vascular risk.

CHAPTER 4 GENERAL DISCUSSION

Chapter 4 General Discussion

A sedentary lifestyle, excess calorie intake and obesity have contributed to a huge increase in the prevalence and incidence of the metabolic syndrome, type 2 diabetes and their associated cardiovascular disease. Insulin resistance, intra-abdominal obesity, and low grade inflammation are accepted to be the 'common soil' of the metabolic syndrome. This common soil regards diabetes and atherosclerosis as being a complication of the metabolic syndrome, both conditions have common genetic and environmental antecedents.

There is mounting evidence that centrally located adipose tissue secretes a variety of adipocytokines with the capacity to influence appetite, insulin resistance, inflammation, blood flow and thrombosis. Adiponectin is believed to be an important link between obesity, type 2 diabetes and CVD. AMP expression has been shown to improve insulin signalling though the activation of AMP kinase signalling and effects on fatty acid oxidation (324). AMP kinase has also been purported to have anti inflammatory and anti atherosclerotic effects and is known to reduce the expression of adhesion molecules and to also increase NO production in endothelial cells (324).

Insulin resistance is associated with a wide array of defects including dyslipidaemia, hypertension, glucose intolerance, inflammation and associated haemostatic abnormalities that predate the onset of type 2 diabetes and cardiovascular disease by many years.

Inflammation as a causal factor in the atherogenic process is currently a major topic of interest in the cardiovascular arena. There is abundant evidence that inflammation directly mediates activation of the coagulation cascade and modulates fibrinolysis. Increased Inflammation leads to the release of chemokines, cytokines and the expression of cell adhesion molecules. The increased cardiovascular risk associated with the metabolic syndrome can therefore be partially explained by enhanced inflammation and subsequent thrombosis (296,297).

Thus the metabolic syndrome represents a heterogeneous cluster of diseases which need a well tailored but integrated approach.

While weight loss especially reducing central adiposity is crucial to reduce the impact of insulin resistance and associated consequences in subjects with the metabolic syndrome, maintenance of this weight loss is probably the most difficult aspect of any intervention program. However in the majority of cases pharmacotherapy will be needed and the drugs should be selected with respect to their ability to modulate the ‘common soil’ of the metabolic syndrome.

There has been a long search for a single drug which could deal with all aspects of the syndrome. It was felt that the TZDs and dual PPARs might fulfil this role, as they act on two major pathogenetic factors of the metabolic syndrome, i.e. IR and central adiposity. PPARs improve insulin sensitivity in the liver, muscle and adipose tissue, promote favourable effects on lipid

profiles, exert anti inflammatory and anti thrombotic effects by reducing CRP, PAI-1, vWF and NFκB activity and increase adiponectin levels. Unfortunately these drugs were associated with serious side effects and the dual PPARs had to be withdrawn from the market (132,133,134).

The relationship between fatty acids, atherosclerosis and other inflammatory diseases has been well known and increased intake of saturated fatty acids has been shown to be positively associated with development of atherosclerosis and inflammation (299). In contrast, omega-3 (ω-3) fatty acids have shown to be protective against CVD (300).

In the context of the need for alternatives to the prevention and treatment of metabolic syndrome and diabetes, the polyunsaturated fatty acid CLA has received considerable attention. This is a result of animal experiments that report modulation of body composition, anti diabetic and anti atherogenic effects associated with its dietary supplementation. These beneficial effects of CLA have in part been thought to be mediated through their ability to behave as ligands of various isoforms of the PPAR. However, of particular interest has been the ability of CLA to act as an activator of both PPARγ and PPARα and thereby modulate the transcription of a large number of genes with a role in inflammation and immune function.

Several studies of CLA supplementation in human subjects have now been published, but in contrast to animal experiments (301,302), there have been marked variation between reports on health related outcomes (303,304,305

and 306). There have also been reports of some interesting isomer specific effects on body weight, lipids and insulin sensitivity and these isomer specific effects need further investigation (235,236).

Thesis Aims

The aims of this thesis was to investigate the role of CLA, a 50:50 mixture of the 9, 11 CLA and 10, 12 isomers versus 9, 11 CLA alone in regulating events crucial in the pathogenesis of atherosclerosis through the ability of the lipid and its isomers to modulate both circulating sRAGE levels and induced cytokine secretion. The study also studied the effects of CLA supplementation on markers of arterial stiffness / endothelial function, body composition, adipokines and insulin sensitivity.

I hypothesised that CLA may exert its benefits not only by improving insulin sensitivity and body composition, but also through indirect mechanisms involving changes in sRAGE mediated inflammation and changes in adipocytokine profile.

Role of sRAGE

CLA and its isomers may thus have the ability to regulate and modulate atherosclerosis-related events in monocytes, macrophage and endothelial cells. However, the mechanisms through which CLA and its isomers exert their effects on the vasculature are not fully understood.

The results from this study show a significant up regulation of sRAGE levels following treatment with 9,11CLA / CLA mix. The results from the combined data also showed improvement in markers of endothelial (sE-selectins, vWF) and platelet function (sP-selectin). The benefits in vascular function could well be driven by changes in adipocytokines and sRAGE expression. These changes interestingly appeared to be independent of changes in insulin sensitivity.

Inflammation is therefore pivotal in the metabolic syndrome and hence a dietary component that has the potential to reduce inflammation in cells such as monocytes, macrophages and endothelial cells that are all crucially involved in the development of atherosclerosis is of considerable clinical relevance. The use of such a dietary component may open new perspectives in the development of effective strategies to reduce the CVD burden in the metabolic syndrome.

In contrast to findings from other studies (231,232), no changes in body composition were observed in our study. An interesting view of this study is that the participants were weight stable and judging from the effect on body composition in the present study, it could be hypothesised that CLA may be useful in inhibiting weight gain rather than in weight reduction.

In contrast to our hypothesis, there were no significant differences between the 9, 11 isomer compared with the CLA mix on various parameters except with regards to aortic PWV, lipid profiles, HOMA B and platelet function.

This could be the result of a possible antagonistic effect of the 10, 12 isomer within the CLA mixture used here. The effects of a single isomer might vary from those observed when the two isomers are given in combination. It was not possible to use the 10, 12 isomer in this study due to its associated detrimental effects as discussed previously (250,252,253).

A limitation of this study could be the small sample size. The power calculation suggested that the present sample size was large enough to detect significant differences in sRAGE expression. However, before any firm conclusions can be drawn about the effects of CLA on body composition and metabolic variables, the present results should be confirmed by conducting larger studies. These studies should target either the CLA mixture, that is readily available or the 9, 11 isomer alone as compared to a placebo. This would allow specific effects associated to the isomer or the CLA mixture to be more readily determined.

A further limitation of this study was that we did not assess dietary intake or physical activity during the study and thus the possibility that change in dietary habits could affect the results although all subjects were instructed to maintain their usual lifestyle habits. This study did not specifically confirm changes to cellular membranes associated with increased 9, 11 CLA / CLA dietary intake. It has been shown that CLA supplementation, changes the fatty acid composition of cell membranes (322). To confirm that the subjects involved in this trial, through CLA supplementation were modulating fatty acid membrane uptake would have been beneficial. This change in fatty acid

composition and hence the ability to regulate PPARs would have been a potential mechanism by which at least some of the significant events observed here could have been mediated. However, the ability to measure this fatty acid uptake through advanced chromatography was not available and was not undertaken. It is also important to acknowledge that some of these significant effects with CLA may simply reflect changed patient behaviour simply as a motivation in taking part in a clinical trial or a placebo effect.

Another study limitation is that I did not prove that CLA directly activated both PPARα and γ receptors. It is possible to confirm this through demonstration of PPARα and γ dependent genes being activated. PPARα dependent genes include fatty acid oxidation such as LCFA β-oxidative enzymes (323), and for PPARγ the scavenger receptor CD36. Alternatively, increased PPAR activity could have been confirmed using luciferase based gene reporter assays. These methods could not be applied during this study, but could be considered for future studies involving supplementation with PPAR ligands such as those used here. Another important observation from this study is that the results do not confirm any hazardous effects of consuming CLA, if anything, it seems as if this can protect against developing vascular disease. The results from this study therefore could have important implications for human nutrition and the food industry.

Future studies

1) Further trials will be needed to corroborate these findings in individuals at high risk of cardiovascular disease such as subjects with type 2 diabetes or in individuals with previous MI using hard end points e.g. FMD for endothelial function / Meal tolerance tests (MTT) for assessment of insulin secretion / insulin sensitivity.

2) Studies should also examine the ability of CLA in inducing solubilisation / clarify the role of various splice variants of RAGE gene in subjects with type 2 diabetes.

3) Continuing research on gene expression, fatty acid metabolism, and immune and inflammatory responses will ultimately help determine the efficacy of CLA as a prevention and treatment method for atherosclerosis

Conclusion

Dietary supplementation with CLA seems to produce a number of beneficial vascular effects which are noted to be independent of its metabolic effects and possibly caused by attenuation of inflammatory diathesis. This opens up another putative mechanism for the role of CLA in regulating RAGE expression. It is possible that CLA could be inducing the solubilisation of RAGE, which can occur either by the formation of splice products at the gene level or by metalloproteinase induced shedding of the RAGE receptor from the cells surface with resultant suppression of RAGE mediated inflammation providing yet another potential future target for treatment of the metabolic syndrome.

The following table 4.0 presents the key outcomes from the most recent studies.

Table 4.0 Update on Recent Clinical Trials with CLA in Human subjects

STUDY	OBJECTIVES / DESIGN	KEY OUTCOMES
An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 CLA in postmenopausal women. JNutr; 2008, 138:1445-1451	75 healthy postmenopausal women were given a daily supplement of 5.5 g of oil rich in either CLA mixture, an oil rich in the naturally occurring c9 , t11 CLA (CLA milk), respectively, or olive oil for 16 wk in a double blind, randomised, parallel intervention study.	In conclusion, oil containing trans-10, cis-12 CLA has several adverse effects on classical and novel markers of coronary vascular disease, whereas the c9, t11 CLA isomer is more neutral, except for a small but significant increase in lipid peroxidation compared with olive oil.
Effect of a conjugated linoleic acid and omega-3 fatty acid mixture on body composition and adiponectin. Obesity, 2008, 16:1019-1024	In this double-blind placebo-controlled, randomised, cross-over study. Subjects received either 6 g/day control fat or 3 g/day CLA (50:50 cis-9, trans-11:trans-10, cis-12) and 3 g/day n-3 LC-PUFA for 12 weeks with a 12-week wash-out period between crossovers	Supplementation with CLA plus *n*-3 LC-PUFA prevents increased abdominal fat mass and raises fat-free mass and adiponectin levels in younger obese individuals without deleteriously affecting insulin sensitivity, whereas these parameters in young and older lean and older obese individuals were unaffected, apart from increased fasting glucose in older obese men.
Conjugated linoleic acids as functional food: an insight into their health benefits.Nutrition & Metabolism ,2009, **6**:36		As far as human consumption is concerned, a definite conclusion for CLA safety has not been reached yet on anti-obesitic, anti-carcinogenic, anti-atherogenic, anti-diabetagenic, immunomodulatory, apoptotic and osteosynthetic effects.
Comparison of dietary conjugated linoleic acid with safflower oil on body composition in obese postmenopausal women with type 2 diabetes mellitus. Am J Clin Nutr 2009; 90:468–76.	This was a 36-wk randomised, double-masked, crossover study. Fifty-five obese postmenopausal women with type 2 diabetes received SAF or CLA (8 g oil/d) during two 16-wk diet periods separated by a 4-wk washout period.	Thirty-five women completed the 36-wk intervention. Supplementation with CLA reduced body mass index (BMI) (P =0.0022) and total adipose mass (P = 0.0187) without altering lean mass. The effect of CLA in lowering BMI was detected during the last 8 wk of each 16-wk diet period. In contrast, SAF had no effect on BMI or total adipose mass but reduced trunk adipose mass (P =0.0422) and increased lean mass (P = 0.0432). SAF also significantly lowered fasting glucose (P = 0.0343) and increased adiponectin (P = 0.0051).

Effects of a dairy product (pecorino cheese) naturally rich in *cis* 9, *trans*-11 conjugated linoleic acid on lipid, inflammatory and haemorheological variables: A dietary intervention study. Nutrition; 2010 (20): 2; 117-124.	Ten subjects (6 F; 4 M) with a median age of 51.5 followed for 10 weeks a diet containing 200 g/week of cheese naturally rich in CLA (*intervention period*) and for the same period a diet containing a commercially available cheese of the same quantity (*placebo period*).	Consumption of the dairy product naturally rich in *cis*-9, *trans*-11 CLA determined a significant ($p < 0.05$) reduction in inflammatory parameters such as IL-6(pre: 8.08 ± 1.57vs.post:4.58 ± 0.94 pg/mL),IL-8 (pre: 45.02 ± 5.82 vs. post: 28.59 ± 2.64 pg/mL), and TNF-α (pre: 53.58 ± 25.67 vs. post: 32.09 ± 17.42 pg/mL) whereas no significant differences in the placebo period were observed. Dietary short - term intake of the tested dairy product naturally rich in cis - 9, *trans*-11 CLA appeared to cause favourable biochemical changes of atherosclerotic markers.
Dietary supplementation with cis-9, trans-11 conjugated linoleic acid and aortic stiffness in overweight and obese adults. Am J Clin Nutr, 2010; 91:175–83.	In this double blind randomised , placebo controlled parallel group study, 400 subjects were randomised to receive 4g of CLA / placebo for 6 months	During the intervention, mean PWV did not change in the c9, t11 CLA group compared with the placebo group. There was no effect of c9, t11 CLA supplementation on BP, body composition, insulin resistance, or concentrations of lipid, glucose, and C-reactive protein. This study does not support an anti atherosclerotic effect or an effect on CV risk factors of c9, t11 CLA.
Effect of conjugated linoleic acid on body fat accretion in overweight or obese children. Am J Clin Nutr 2010; 91:1157– 64.	This was a 7 -month randomised, double-blind, placebo-controlled trial of CLA in 62 pre-pubertal children aged 6–10 y who were overweight or obese but otherwise healthy. The subjects were randomly assigned to receive 3 g/d of 80% CLA mixture or placebo in Chocolate milk.	CLA supplementation for 7 months ↓ body fatness in 6–10-y-old children who were overweight or obese but did not improve plasma lipids or glucose and ↓ HDL more than in the placebo group
Effect of a High Intake of Conjugated Linoleic Acid on Lipoprotein Levels in Healthy Human Subjects PLoS ONE February 2010 | Volume 5 | Issue 2 | e9000	Sixty-one healthy women and men were sequentially fed each of three diets for three weeks, in random order, for a total of nine weeks.Diets were identical except for 7% ofenergy(approximately 20 g /day), which was provided either by oleic acid, by industrial trans fatty acids, or by a mixture of 80% cis-9, trans-11 and 20% trans-10, cis-12 CLA.	High intakes of an 80:20 mixture of cis-9, trans-11 and trans-10, cis-12 CLA raise the total to HDL cholesterol ratio in healthy volunteers

Conjugated Linoleic Acid Supplementation for 8 Weeks Does Not Affect Body Composition, Lipid Profile, or Safety Biomarkers in Overweight, Hyperlipidemic Men The Journal of Nutrition; 2011; 141:1286-1291	A double-blinded, 3-phase cross-over trial. During three 8-wk phases, each separated by a 4-wk washout period, 27 participants consumed under supervision in random order 3.5 g/d of safflower oil (control), a CLA mixture, and c9, t11 isomer. At baseline and endpoint of each phase, body weight, body fat mass, and lean body mass were measured by DXA. Blood lipid profiles, insulin sensitivity, adiponectin, and inflammatory (hs-crp, TNFa, and IL-6) and oxidized-LDL, were measured.	Compared with the control treatment, the CLA treatments did not affect changes in body weight, body composition, or blood lipids. In addition, CLA did not affect the b-oxidation rate of fatty acids or induce significant alterations in the safety markers tested. In conclusion, although no detrimental effects were caused by supplementation, these results do not confirm a role for CLA in either body weight or blood lipid regulation in humans..
CLA Does Not Impair Endothelial Function and Decreases Body Weight as Compared with Safflower Oil in Overweight and Obese Male Subjects. J Am Coll Nutr. 2011 Feb;30(1):19-28	Eighty-five overweight men (aged 45–68 years, body mass index 25–35 kg/m2) were randomized to receive 4.5 g/d of the CLA isomeric mixture, safflower oil, heated safflower oil, or olive oil in a 4-week double blind study. Endothelial function was assessed by peripheral arterial tonometry (PAT) index determination in the fasting and postprandial state	CLA did not impair endothelial function. Other parameters associated with metabolic syndrome and oxidative stress were not changed or were slightly improved
The efficacy of long-term CLA supplementation on body composition in overweight and obese individuals: a systematic review and meta-analysis of randomised clinical trials. European Journal of Nutrition, March 2012, Volume 51, Issue 2, pp 127-134		The evidence from RCT's does not convincingly show that CLA intake generates any clinically relevant effects on body composition in the long term.

Appendix 1 - Patients Information Sheet - Version 2 (20/08/2005)

Title of the study

A study to assess the metabolic, vascular & inflammatory effects of Conjugated linoleic acid in patients with the metabolic syndrome.

Introduction

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the study is being carried out and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish. Ask us if there is anything not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you do not wish to take part, you may refuse without explanation and this will no way affect your current or future medical treatment.

Consumers for ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy of this leaflet is available on request.

What is the purpose of the study?

Conjugated Linoleic acids (CLA) are a group of isomers of conjugated octa - dienoic acid (fatty acids) that occur naturally in food, mostly in diary products. CLA has attracted much interest since the discovery that it has body fat-lowering, immune enhancing & anti-cancer effects. It is increasingly being used as a dietary supplement in patients with the metabolic syndrome. (Overweight subjects with high blood pressure, high blood glucose & cholesterol related abnormalities) who have an increased risk of developing heart disease. An earlier study found that a group of people who took CLA had reduction in body fat, along with a reduction in cardiovascular risk factors.

This study will look at the effects of dietary supplementation of CLA on body fat, blood glucose, blood vessel function. The main aim being to answer the question whether CLA would improve blood vessel function, thus reducing the risk of heart disease.

Why have I been chosen?

You have been diagnosed to have the metabolic syndrome. This study will determine whether CLA taken as dietary supplements is effective in improving blood vessel function, thereby reducing cardiovascular risk.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you decide not to take part or to withdraw at any time, you may do so without giving a reason and without that decision affecting current or future medical care in any way.

What will happen to me if I take part?

Before you enter the study your doctor will give you a full explanation of what the study will involve and you will be able to ask any questions that you may have. You will then be asked to sign the consent form, before any other procedures are performed. Please read this carefully before signing. If you agree to take part you will be asked about your medical history and medicines you are taking. Your doctor will carry out a medical examination and a variety of tests described in greater detail below will be carried out. A total volume of 40ml (8 teaspoonfuls) of blood will be taken. You should not eat or drink anything (except water) for approximately 9 hours before attending the clinic (from midnight before the visit). You will then be given the study medication.

You will be asked to return to the clinic after 8 weeks for blood tests. After this, for the next 6 weeks you will not receive any medications. You will be asked to return to the clinic at the end of this period and will be given the study medication for another 8 weeks. You will have tests before commencing the medication and on completion of the study. During this period we will be in regular contact with you by telephone to ensure that you have no problems. If you have any problems or any questions, you may contact us on the telephone numbers given below. Each visit will take no longer than 2 hours to complete.

The table below shows which of the tests and procedures that will be done at each visit. The tests are explained below:

Study Procedures	Visit 1	Visit 2	Visit 3	Visit 4
Physical Examination	×	×	×	×
Blood Pressure and Heart Rate	×	×	×	×
Blood Test	×	×	×	×
Pulse wave analysis	×	×	×	×
ECG	×	×	×	×
OGTT	×	×	×	×

- Physical examination: includes measurements of heart rate, blood pressure, height, weight and waist measurement.
- Blood test: up to 40 mls (equal to 8 teaspoons) of blood will be removed from a vein in your arm at visits 1, 2, 3 & 4 during the study period proper.

- An ECG (Electrocardiogram) will be performed at the first and final visits to measure the electrical activity of your heart.
- During these visits you will undergo pulse wave analysis . These investigations are painless and involve an ultrasound device being placed over one of the major blood vessels in the arm. These tests will provide information on how stiff the arteries are and whether the study has any effect on this.

What type of study is this?

This study is a randomised, double blind, crossover study; sometimes because we do not know which way of treating patients is best, we need to make comparisons. People will be put into groups and then compared. The groups are selected by a computer, which has no information about the individual, i.e. by chance. Patients in each group then have a different treatment and these are compared. Neither you nor the researchers will know which of the treatments you are taking until the end of the study when you may be told if you wish. Only in the event of an emergency will the medication you are taking be identified before the study is completed.

Treatment Group 1: Conjugated Linoleic acid (C9, t11 isomer)
Treatment Group 2: Conjugated Linoleic acid mixture (t10, c12 isomer and C9, t11 isomers in a 1:1 ratio)

You have a 50% chance of receiving either isomer. Neither you nor your doctor will know which treatment group you are in (although if your doctor needs to find out he/she can do so). This is to limit the potential for bias throughout the study.

It is very important that you take the medications as directed by the study doctor. Only the person for whom it was prescribed must take study medication. It must be kept out of the reach of children or persons of limited capacity to read or understand.

What are the possible benefits of part?

We hope that this research will help you by improving blood vessel function. However, this cannot be guaranteed. The information we get from this study may help us in the future treatment of people with metabolic syndrome.

What are the possible risks of taking part?

The drug used in the study may involve other risks that are not known at the present time. CLA has been used in previous studies and so far no major adverse events have been reported.

What if something goes wrong?

If you become ill as a result of the medical treatment provided and if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism should be available to you.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study.

Will my taking part in this study be kept confidential?

Your participation in the study will be treated as confidential, that is, any personally identifiable information (e.g. initials and date of birth) will be held and processed under secure conditions with access limited to appropriate staff involved in the study. You will not be referred to by any name in any report of the study. Your identity will not be disclosed to any person, except

for the purposes described above and in the event of a medical emergency or if required by law.

You may be entitled under law to access your personal data and to have any justifiable corrections made. If you chose to take part in this study, Your GP or any other doctors involved in your medical care will be informed of your participation, with your permission.

What will happen to the results of the research study?

A report will be written after the study has completed after which your doctor may tell you the type of treatment you received. You will not be identified in any report or publications.

Who has reviewed the study?

An independent ethics review board reviewed the protocol.

Who are the people doing this research?

The team who are conducting this project includes Dr Marc Evans who is Consultant Diabetologist at the University Hospital of Wales and Llandough Hospitals and Dr Hemanth Bolusani -Clinical Research Fellow Llandough Hospital. Full contact addresses are given below:

Dr Marc Evans

Consultant Diabetologist
Llandough Hospital
Cardiff

Dr Hemanth Bolusani

Clinical Research Fellow
Llandough Hospital
Cardiff

If you decide to take part in this research study, you will be given a copy of this information to keep. If at any time you have any further questions or require any further information, please contact a member of the research team whose details are given below.

Name: Dr Marc Evans (Principal research doctor)
Tel No: 02920716478(Office hours); 07866 528821(24 hours)

Name: Dr Hemanth Bolusani (Clinical Research Fellow)
Tel No: 07876704538(24 hours)

Thank you for taking time to read this information leaflet. You are entitled to receive a copy of this form.

Appendix 2

CONSENT FORM-Version 2.0(20/8/2005)

A study to assess the metabolic, vascular & inflammatory effects of Conjugated Linoleic acid in patients demonstrating insulin resistance.

Name of Researcher: Dr L M Evans

Please initial box

1. I confirm that I have read and understood the full information sheet Dated 20/08/2005 for the above study to assess the effects of dietary supplementation of conjugated linoleic acid (fatty acids) on body fat,blood glucose and blood vessel function and have had the opportunity to ask the questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals involved in the study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (If different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature
_____	_____	_____
. _____	_____	_____

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3

CLA STUDY

Discuss and sign consent form
All patients must be fasted

Date……ID No……D.O.B……

Name……

Address……

……

Ethnic group: Caucasian…..Indo-Asian….Afro-Carribean…

Inclusion Criteria

1) Plasma Glucose > 5.6 Y/N
2)Serum Triglycerides >1.7 Y/N
3)Serum HDL-C
Males <1.03 Y/N
Females <1.30 Y/N

4) Blood pressure >130/85mm
5) Abdominal obesity
Males >102Cms
Females >88Cms

Exclusion Criteria

1)Diabetes Y/N
2)Aspirin /NSAID Y/N
3)Acute illness Y/N
4) Renal failure Y/N
5) Liver failure Y/N
6)Pregnancy/LactationY/N

MEDICAL HISTORY.

1.High B.P Y/N…….Duration……
2.High Cholesterol Y/N
3.Diabetes Y/N
4.Stroke Y/N CVA……. TIA……
5.CHD Y/N Angina……MI…..
6.PVD Y/N

……

……

……

Life Style

1.Smoker Y/N .Duration ……. Amount …… Ex… Pack Years ……

2.Alcohol Y/N Units/week ……..

Family History

……

……

……

Current Drug Therapy

Drugs

……. …….....

……. …….....

……. ……..

……. ……..

…… ……..

Exercise Y/N . Duration/type…….

ANTHROPOMETRIC MEASUREMENTS

Height(cms)……. Wt(Kg)…….BMI……..

Body Composition. % Fat……..Body Fat…….

Waist circumference(cms)…….....

CARDIOVASCULAR MEASUREMENTS:

Seated BP a) …….. b)…….. Avg ……

Seated AIx a) …….. b)…….. Avg ……

Central Aortic Pressure
a) …….. b)……...Avg ……

Tr a) …….. b)……...Avg ……

Pulse Wave Velocity SSN to Carotid ……..mm

SSN to Radial ……..mm

SSN to Umbilicus……mm

SSN to Femoral ……mm

CAROTID RADIAL a) ……. b)…….. Avg ……. m/s

CAROTID FEMORAL a) …….. b)……. Avg ……..m/s

Bloods and Oral glucose tolerance test

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