The Influence of Age and Nutrients

on Insulin Sensitivity

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Abstract

The studies presented in this thesis aimed to investigate the effects of nutritional modulation and age-associated changes on insulin sensitivity. Four separate studies were performed; three of these had insulin sensitivity as the primary outcome. Existing studies show that ageing is associated with insulin resistance, but data may be confounded by several factors that also occur with increasing age, such as increased adiposity, skeletal muscle lipid accumulation and reduced physical activity. To elucidate this further the first study compared body composition, skeletal muscle lipid content, fat metabolism during light-intensity exercise and whole-body and skeletal muscle insulin sensitivity between 7 healthy young and 14 older males. Ageing and insulin resistance are also associated with impaired skeletal muscle protein synthesis, however the effects of insulin resistance per se on amino acid metabolism and associated insulin signalling pathways are not really known. The second study involved 8 young healthy males and aimed to explore the effect of insulin resistance on the protein synthetic response to amino acid ingestion and muscle protein signalling pathway in humans. Dietary intake has been shown to affect insulin sensitivity; however it is unclear if diet composition affects liver fat content independent of energy balance. Therefore the third study aimed to investigate the effects of hyperenergetic diets high in fat or carbohydrate on liver fat and insulin sensitivity. The study involved 23 healthy but overweight and obese males who initially consumed an isoenergetic diet for one week, and then were randomised to 2 weeks of either hyperenergetic (+25% excess) high fat or high carbohydrate diets. Liver fat content, abdominal visceral fat, skeletal muscle fat content, hepatic lipid metabolism and insulin sensitivity were assessed before and after the 2 week intervention period. Whilst dietary excess can exacerbate insulin resistance, certain

micronutrients may improve insulin sensitivity. Carnitine has shown encouraging outcomes in relation to promoting fatty acid oxidation, metabolism and modulating body composition in healthy young volunteers. However the effects on older people have never been explored. This formed the basis of the fourth study that investigated the effects of 6 months of oral carnitine supplementation or placebo in 14 older ($\geq$65 years of age) healthy males in relation to fatty acid metabolism, skeletal muscle lipid and insulin sensitivity.

The main findings are summarised. Irrespective of age, adiposity and physical activity are associated with impaired fatty acid oxidation, greater skeletal muscle lipid accumulation and reduced insulin sensitivity. However ageing per se appears to increase the sympathetic response to exercise and enhance systemic fatty acid delivery and adipose tissue lipolysis. Insulin resistance induced by acute elevation of lipid was found to affect the skeletal muscle protein synthetic response to amino acid ingestion, and this impairment appeared to occur downstream from the Akt insulin signalling pathway. Energy excess per se increases liver fat content and affects liver metabolism but there were no differential effects of carbohydrate or fat on hepatic insulin sensitivity and liver fat content. Finally, oral carnitine ingestion for 6 months successfully increased skeletal muscle total carnitine content of older healthy people and resulted in increased fatty acid oxidation and intramyocellular lipid (IMCL) utilisation during light-intensity exercise, but no effect on skeletal muscle insulin sensitivity was seen.

These studies have increased mechanistic insight into the associations between ageing, nutrients and insulin sensitivity, paving the way to further research.

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help provide answers to conditions that are huge public concerns today. Their friendly camaderie, banter and motivation will not be forgotten.

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Declaration

Apart from specific contributions attributed to individuals in the acknowledgement section of this thesis, I declare that this dissertation is of my own work based on studies undertaken by me at the David Greenfield Human Physiology Unit/ MRC/ARUK Centre for Musculoskeletal Ageing Research laboratory, School of Life Sciences, University of Nottingham from 06/02/2012 to 05/12/2014.

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Poster Presentations arising from this thesis

1) Intramyocellular fat oxidation is reduced during low-intensity exercise in older men and can be partially restored by increasing muscle total carnitine content. British Society for Research in Ageing. Liverpool, UK June 2014

2) Increasing muscle total carnitine content can increase fat oxidation during low-intensity exercise in older men. American College of Sports Medicine. Miami, USA Sept 2014

3) Subsarcolemmal lipid accumulation in ageing skeletal muscle. The Physiological Society. Edinburgh UK 2015

4) Differential effects of carbohydrate vs fat overfeeding on liver fat content and lipid metabolism in healthy overweight males, European Association in theStudy of Diabetes. Stockholm, Sweden Sept 2015

5) The efects of hyperenergetic carbohydrate vs. Fat diets on liver fat content and insulin sensitivity in overweight healthy males. Diabetes UK, Glasgow UK March 2016.

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1) Stephens FB, Chee C, Wall BT, Murton AJ, Shannon CE, van Loon LJ, Tsintzas K. Lipid-induced insulin resistance is associated with an impaired skeletal muscle protein synthetic response to amino acid ingestion in healthy young men. Diabetes. 2015; 64(5):1615-20.

2) Carolyn Chee, Chris E. Shannon, Aisling Burns, Anna L. Selby, Daniel Wilkinson, Kenneth Smith, Paul L. Greenhaff, Francis B. Stephens. The relative contribution of intramyocellular lipid to whole body fat oxidation is reduced with age, but subsarcolemmal lipid accumulation and insulin resistance are only associated with overweight individuals.Diabetes. 2016; 65: 840-50.

Abbreviations

° C	degree Celcius
2DG	2-deoxy-D-glucose
2DG6P	2-deoxy-D-glucose 6- phosphate
4E-BP1	Eif4e binding protein 1
β-OHB	Beta- hydroxybutyrate
Acetyl-coA	Acetyl-coenzyme A
Akt2	Protein kinase B
ALT	Alanine transaminase
AMPK	AMP activated kinase
APE	Atom per excess
AST	Aspartate transaminase
BCAA	Branched-chain amino acids
BIA	Body impedance analysis
BMR	Basal metabolic rate
BSTFA	Bis (trimethylsilyl) trifluoroacetamide
CaMK	Calmodulin-dependent protein kinase
CPT	Carnitine palmitoyl transferase
CPT-	Carnitine palmitoyl transferase-1
CRP	C reactive protein
CS	Citrate synthase
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DEXA	Dual-energy x-ray absorptiometry
DNL	*de novo* lipogenesis
ECW	Extracellular water
EDTA	Ethylenediaminetetraacetic acid

EGP	Endogenous glucose production
EGTA	Ethylene glycol tetraacetic acid
FDG	Fluorodeoxyglucose
FFM	Free fat mass
FOX-O	Forkhead protein box O
G6PDH	Glucose-6 phosphate dehydrogenase
GCMS	Gas chromatography mass spectrometry
GDR	Glucose disposal rate
GEM	Gas Exchange Machine
GLUT-2	Glucose transporter 2
HDL-C	High density lipoprotein cholesterol
^{1}H MRS	Proton magnetic resonance spectroscopy
HOMA-IR	Homeostatic model of analysis of Insulin Resistance
HPLC	High performance liquid chromatography
IGT	Impaired glucose tolerance
IHCL	Intrahepatic cellular lipid
IκbK	Inhibitor of kappa b kinase
IL	Interleukin
IMCL	Intramyocellular lipid
IMF	Intramyofibrillar
IMTG	Intramuscular triglyceride
IPAQ	International physical assessment questionnaire
IRMS	Isotope ratio mass spectrometry
IRS	Insulin receptor substrate
ISWT	Incremental shuttle walk test
JNK	c-Jun N-terminal kinase
LDL-C	Low density lipoprotein cholesterol

LPS	Lipopolysaccharide
MetS	Metabolic syndrome
MPS	Muscle protein synthesis
MRS	Magnetic resonance spectroscopy
mTORc2	Mammalian target of rapamycin complex 2
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acid
NMR	Nuclear magnetic resonance
PAL	Physical activity level
Pcr	Phosphocreatine
PDC	Puruvate dehydrogenase complex
PI3-k	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PPAR	Peroxisome-proliferator activated-receptor
PUFA	Polyunsaturated fatty acid
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RMR	Resting metabolic rate
ROS	Reactive oxygen species
SAE	Severe adverse event
SBP	Systolic blood pressure
SFA	Saturated fatty acid
SSL	Subsarcolemmal lipid
TAG	Triacylglyceride
TBDMS	Tert-butyldimethylsilyl

TBW	Total body water
THL	Tetrahydrolipstatin
TLR-4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-alpha
VLDL	Very low-density lipoprotein

CHAPTER 1: INTRODUCTION

'Der Mensch ist, was er iBt'

'Man is what he eats'

Ludwig Andreas Feuerbach 1863

Concerning Spiritualism and Materialism

Coined over 150 years ago, the expression that man is what he eats and that the food one eats has bearing of ones' state of health and mind still holds true to today.

What has become very apparent in modern times is the increasing prevalence of Type 2 Diabetes (T2DM), which is strongly associated with diet and lifestyle. This has been demonstrated in large-scale population studies (Tuomilehto *et al.* 2001, Knowler *et al.* 2002, Li *et al.* 2008). A plethora of studies have attempted to shed light on the fundamental question of how diet and nutrition drive the development of insulin resistance. However what remains uncertain and has given rise to conflicting study outcomes is what aspect of diet exerts the greater influence; increased energy intake or change in the proportion of the macronutrient. Further, can introducing certain micronutrients improve insulin sensitivity? How does insulin resistance as a result of acute and chronic manipulation of the diet affect human metabolism?

In line with diet and insulin resistance, the obesity epidemic is also of concern, afflicting people of all ages. In particular, the 21st century and beyond will see an increased prevalence of ageing and development of diabetes, with a growing number of studies examining underlying mechanisms and strategies to impede or even 'reverse'

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the development of insulin resistance associated with ageing. However overweight and obesity and other changes in metabolism occur with ageing and not necessarily due to ageing per se, so it is not entirely clear what directly influences insulin sensitivity as people age.

An analysis of evidence in the literature reveals inconsistent results on the effects of dietary modulation, obesity and ageing per se on insulin sensitivity, but cautious consideration to interpreting these results has to be taken due to confounding variables and difficulty in conducting nutritional and age-related studies in controlled settings. This thesis presents new and unique evidence to the discussion and topical issues surrounding the association of diet, ageing, obesity, insulin sensitivity and metabolism, and attempts to reconcile differing concepts in insulin resistance development.

1.1 Historical perspective
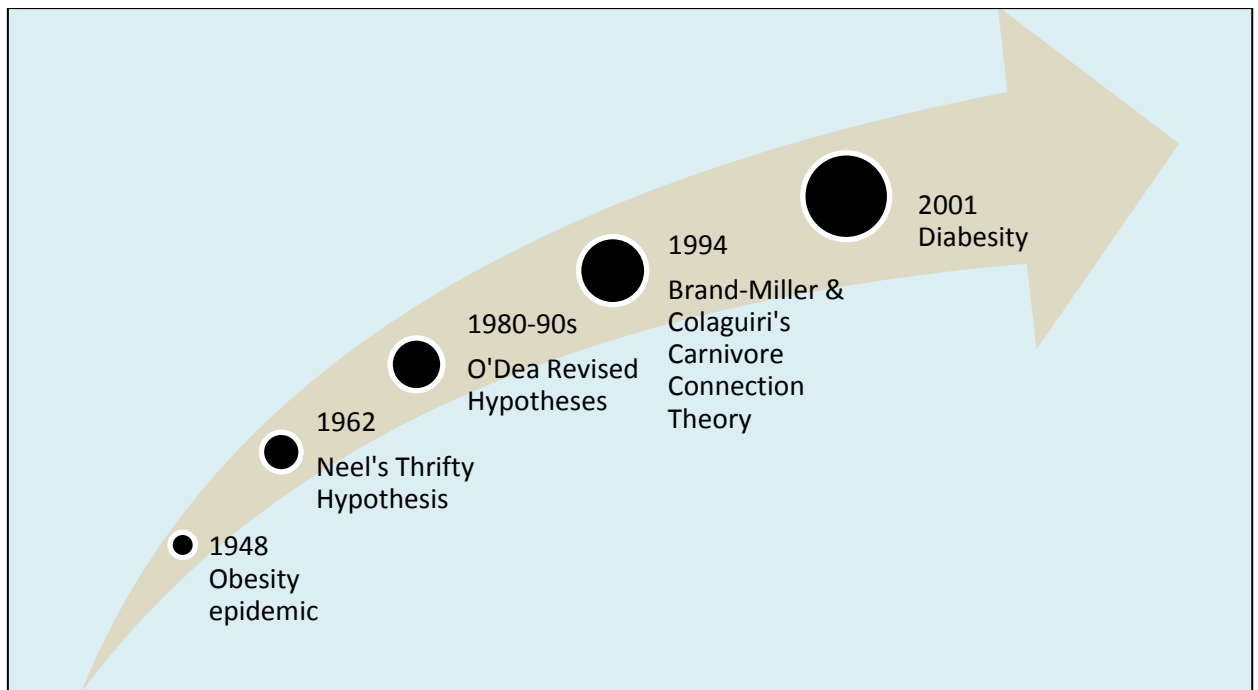
Figure 1.1: Concepts of diet and development of insulin resistance over the last 60 years.

1.1.1 Concepts and hypotheses on nutrients and insulin sensitivity

The association between diet and insulin sensitivity has been a matter of much interest, debate and controversy for more than 60 years. The first major and well-known prospective study investigating risk factors for heart disease (Framingham, Massachusetts) began in 1948 and probably led to the term 'lifestyle disease' whereby over-nutrition and low physical activity led to positive energy balance and obesity (Dawber, Kannel *et al.* 1959).

Until the end of the 19th century, at least a proportion of the population in developed countries were struggling with famine and malnutrition, considered a major cause of industrial unproductivity (Fogel, Proceedings of the World Food Programme/United Nations University Seminar; 1997). In the early 20th century studies of poor children indicated that adding carbohydrate and fat to their diets improved growth and this was beneficial in improving malnutrition and industrial productivity (Boyd-Orr 1937). The

US Food and Agriculture Organisation was an important protagonist; one of their major aims was to increase low-cost calorie foods, namely fats and sugars. Following this, by 2002 global food production reached 2600 kilocalories (kcal) per day per capita, and is projected to reach 3000 kcal by 2030 (FAO. Rome, Italy: Food and Agriculture Organization of the United Nations; 2002. World agriculture: towards 2015/ 2030).

Understanding the significance of the growing prevalence of obesity arose in the early 20th century, when life insurance companies used body weight data to determine premiums, having identified an association between excess weight and premature death (Caballero 2007). Breslow (1952) proposed a direct link between the increasing prevalence of obesity and increasing rates of cardiovascular disease in the US population which then became a recurrent theme with emphasis in US government reports in the 1960s and 1970s (US Department of Health. Washington, DC: US Public Health Service; 1966. Obesity and health (Report no.1485). Data over the past 30 years continue to show an alarming rise in obesity prevalence (Harlan, Landis *et al.* 1988) and by the year 2000, 65 percent of the adult population in the United States had a body mass index (BMI) above 25, and 30 percent had a BMI above 30 (Hedley, Ogden *et al.* 2004). The projected global number of people who will be overweight or obese is estimated at 1.4 billion and 570 million respectively by 2030 (Kelly, Yang *et al.* 2008).

1.1.2 Concepts and hypotheses linking diet, nutrition and insulin resistance

Several hypotheses in the development of the insulin resistance syndrome have been postulated ever since an association with nutrition, overweight and insulin resistance was recognised (Figure 1.1). Indeed it is difficult not to mention obesity in the same context as insulin resistance or diabetes.

In 1962, Neel hypothesised that a genotype in diabetes existed (Neel 1962), a condition that suited conditions of 'feast' and 'famine', and this simply evolved over time due to changes in diet and lifestyle. The 'thrifty gene hypothesis' puts forth the notion that some people possess a 'thrifty tendency and flexibility' to minimise energy loss and encourage storage of energy as fat during the 'feast' period, and produce energy to aid survival during periods of 'famine'. When the hypothesis was first proposed, it was thought that only small numbers of populations were highly susceptible to diabetes particularly in the West, but more recently it has become evident that obesity and insulin resistance are 'consistent sequelae of lifestyle transition' (Rowley, Best *et al.* 1997). The increased availability of food and overconsumption, so characteristic of urbanisation and modern lifestyle have led to increased fat storage, obesity and associated insulin resistance.

Over the years the thrifty gene hypothesis underwent refinements through studies carried out in populations with a high prevalence of T2DM. For example whilst investigating indigenous people and other populations in Australia, O'Dea recognised that besides food stored during the 'feast' period, other food were readily available (termed subsistence diet) (O'Dea 1991). Therefore a transition from a traditional to a Westernised lifestyle occurred, characterised by a change to diets high in refined carbohydrates and fat that were readily available, coupled by reduced physical activity levels. However, O'Dea also demonstrated in population studies that development of insulin resistance could be reversed. When the traditional lifestyle consisting of ingesting high- protein, low- carbohydrate diet and increased physical activities was simulated over 2 weeks, glucose tolerance improved significantly and hyperinsulinemia was reduced. A subsequent study by the same group showed that when subjects with diabetes followed a low-fat diet consisting of lean meat for a period of 7 weeks and

increased physical activity, mean fasting blood glucose levels fell from 11.6 to 6.6 mmol/L, with a mean weight loss of 8 kilograms (kg) (O'Dea *et al.* 1992).

 Another one of the most intensely studied population group in the aetiology of T2DM is that of the Pima Indians of the Gila River Indian Community in Arizona. This community has participated in longitudinal studies since the 1960s (Knowler 1979) and their clinical characteristics have defined T2DM across most populations (Knowler 1990). In fact T2DM diagnostic criteria adopted by the World Health Organisation (WHO) were initially established in this tribe. Whilst their traditional diet consisted of high carbohydrate but low fat nutrients (24% saturated fat of total energy) (Hesse F 1959, Boyce *et al.* 1993), by the mid-20th century they had begun conforming to modern lifestyle changes, consuming diets of fat that contributed up to 40% of total energy and became less physically active. This tribe is now one of the most obese in the world, with 50% or more of the population developing diabetes. Despite little genetic disparity between Pima Indians in Mexico and Arizona and non-Pima Mexicans, Mexican Pima Indians have a much lower prevalence of T2DM and obesity providing evidence that lifestyle changes play an important role in the development of diabetes (Schulz *et al.* 2006).

Brand-Miller and Colagiuri in 1994 proposed the 'carnivore connection' theory, where insulin resistance evolved as a consequence of scarcity of carbohydrates and high intake of animal protein. This provided an advantage to populations that adapted to high-protein but low carbohydrate diets, but proved deleterious in a high-carbohydrate environment. Perhaps the switch to consuming high-carbohydrate and fat diets in modern times has facilitated an increase in insulin resistance rates. In this context it is of interest to note that populations that have changed dietary high-protein 'hunter gatherer' intakes to modern high carbohydrate diets relatively later than their European

counterparts have shown a proportionally greater increase in the incidence of developing T2DM, compared to European descent populations who switched to high carbohydrates thousands of years ago. A prime example is China. The prevalence of diabetes in the 1980s was 1%, but by 2008, around 10% of the population developed diabetes (Yang, Lu *et al.* 2010). However, the relationship between overweight, obesity and diabetes varies between different populations. In Asia diabetes appears to develop at a lower BMI and younger age, a modest increase in weight appears to lead to rapid development of insulin resistance (Yoon, Lee *et al.* 2006). It could be that the greater tendency for populations in Asia to develop increased abdominal adiposity but less muscle mass increases risk of insulin resistance. Indeed tomography has shown greater visceral fat in Asians compared to White Europeans despite similar waist circumferences (Lear, Humphries *et al.* 2007).

Nevertheless overweight and obesity appear to be the prime drivers of insulin resistance. The association between obesity and diabetes is so strong that the term 'diabesity' emerged around the turn of the century (Zimmet P 2012).

In summary, the association between diet and insulin sensitivity has been established for at least the past half century, interspersed with various concepts and hypotheses that attempt to describe the emerging global prevalence of diabetes. 'Diabesity' has become a great concern of the 21st century with growing diabetes and obesity rates. Insulin resistance appears to be associated with changes in dietary intake that predisposes to adiposity, particularly in these times of increased availability and easy access to food. The following sub-chapters attempt to define insulin resistance, examine the mechanisms involved in impaired insulin sensitivity and how certain nutrients might contribute to the development of insulin resistance. How diets and exercise improve insulin sensitivity will also be discussed.

1.2 Mechanisms of Insulin Signalling and action

Understanding the mechanisms underlying normal insulin action is paramount to appreciating how insulin resistance develops and what influences insulin homeostasis. One would have predicted that when Banting and Best first purified insulin and treated a patient with diabetes in 1922, a mechanism that explained insulin action would soon follow. Almost a century later the mechanism has proven complex, still not completely understood and is continuously being unraveled.
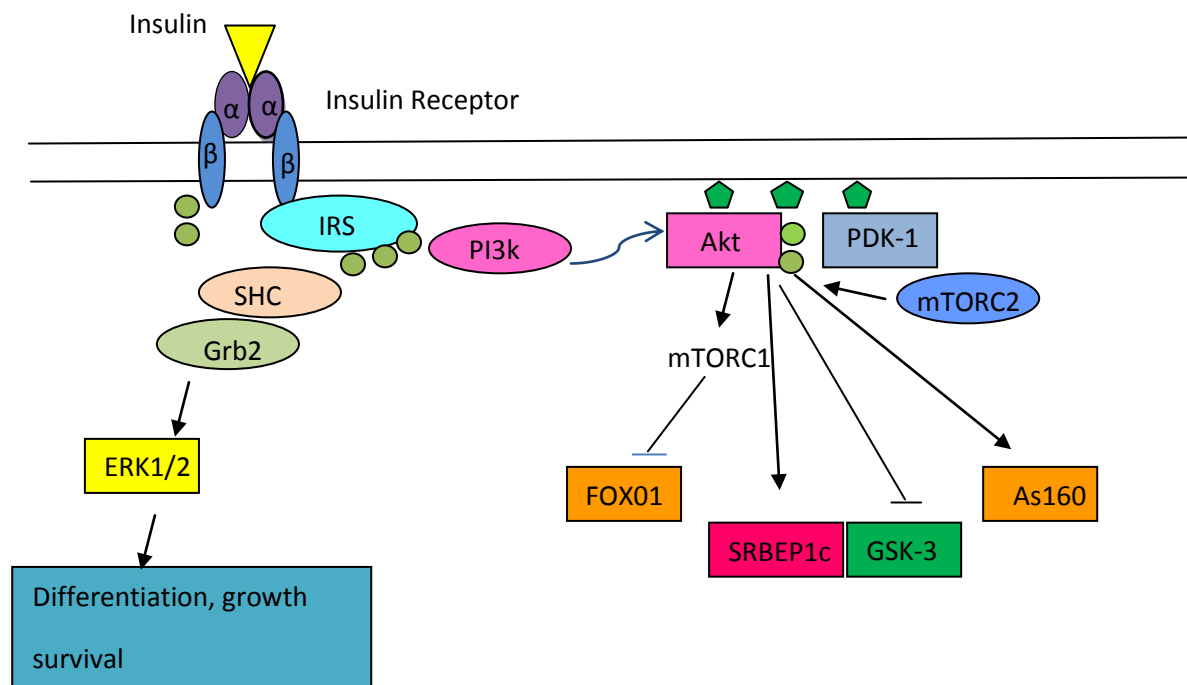
Figure 1.2: Mechanism of insulin signalling pathway: Early work by Saltiel and Kahn (2001) describes the binding of insulin to the insulin receptors, phosphorylating serine-threonine protein systems of insulin receptor substrates (IRS-1-4). These then become coupled to protein kinase signal systems of phosphatidylinositol 3 kinase (PI3k) and formation of PI (3,4,5) Phosphate 3 (PIP3). PIP3 then binds to the surface membrane and associates with PDK-1 leading to phosphorylation and activation of protein kinase b/Akt). Akt is phosphorylated by phosphoinositide-dependent protein kinase -1 and mammalian target of rapamycin complex 2 (mTORc2) (Shepherd P 2005).

What is well established is that insulin action is initiated by the binding of the hormone insulin to its membrane receptor consisting two α and two β subunits (Freychet P 1971, Kahn CR, 1985). The insulin receptor is present in varying quantities in almost all mammalian tissues, with the greatest concentration seen on adipose tissue and liver cells. Insulin binds to the α-subunits, stimulating tyrosine phosphorylation of the intracellular portion of the β-subunit such as tyrosine, serine and threonine – IRS 1 to 4. At least until the end of the 1980s, it was uncertain how the receptor kinase ultimately transmitted its signal. Models proposed and largely accepted as playing a central role include receptor autophosphorylation (Herrera R *et al.* 1985) or dephosphorylation of cellular enzymes and conformational change of beta-subunit receptor (White MF, Kahn CR 1986) leading to interaction of secondary effector systems such as PI3k activity and phospholipase C (Sale *et al.* 1986, Machicao and Wieland 1984). So it became clear that activation of PI3-K associates with phosphoinositol dependent kinase 1 (PDK1) leading to phosphorylation of protein-kinase B (Akt).

The relatively recent discovery of novel insulin receptor products indicates that there are more undiscovered receptors and mechanisms involved in insulin signalling. Activation of inositol hexakisphosphate (IP6) kinase (IP6K1) yields 5-diphospho-inositolpentakisphosphate (5-PP-IP5) or IP7 that binds to Akt, inactivating it and preventing its function as a substrate for mTORC2 and PDK1 phosphorylation (Chakraborty *et al.* 2010, Manning B *et al.* 2010). mTOR is a nuclear serine/threonine protein kinase found in two complexes (mTORC1 and mTORC2) in most of the body's tissues. These appear to be essential in the regulation of metabolism, including organization of the insulin and growth factor signals.

Insulin resistant states such as T2DM are characterised by reduced receptor numbers due to down-regulation, altered kinase activity and reduction in insulin-receptor autophosphorylation. These appear to be regulatory in nature (Bar *et al.* 1979).

1.3 Role of insulin in nutrient metabolism

1.3.1 Role of insulin in carbohydrate metabolism

Insulin is the hormone central to glucose homeostasis on ingestion of nutrients particularly dietary carbohydrates (Bessessen 2001). Post-prandial, glucose is taken up by hepatocytes via GLUT-2 transporters (Guyton 1996) and phosphorylated to glucose-6-phosphate by the enzyme glucokinase. This process is not regulated by insulin but is reliant on the glucose concentration outside the hepatocyte (Frayn 1999). Insulin stimulates glycogen synthase which polymerises glucose to glycogen (Petersen *et al.* 1998) and inhibits glycogen phosphorylase. When glucose metabolism in liver exceeds hepatocyte glycogen-storing capacity, insulin and glucose promote conversion of excess glucose into fatty acids via the transcription factors sterol regulatory element-binding protein-1c and carbohydrate response element-binding protein-1c respectively, a process known as *de novo*-lipogenesis (DNL) (Postic C 2007). DNL is limited in humans under normal conditions (<5% of triacylglycerol pool); (Diraison 2003) compared to people with non-alcoholic fatty liver disease (NAFLD) with 25% of the triacylglycerol pool (Donelly 2005). Hepatic glucose production originates from glycogenolysis and gluconeogenesis, the breakdown of which depends on glycogen phosphorylase and glucose-6-phosphate. Insulin inhibits glucagon secretion, lipolysis and proteolysis in muscle and lipolysis in adipose tissue.

1.3.2 Role of insulin in lipid metabolism

Insulin's role on lipid metabolism is evident by the immediate reduction in lipidaemia and acetonuria following insulin administration in an individual with diabetes (Fonseca 1924, Killian 1923).

Insulin controls fatty acid and triacylglycerol synthesis through activation of pyruvate dehydrogenase and acetyl-coenzyme A (acetyl-coA) carboxylase by promoting its polymerisation, and appears to regulate esterification of fatty acids to triacylglycerol. Fatty acids taken up by the liver also go through beta-oxidation (Frayn K 1999). Through the process of beta-oxidation, non-esterified fatty acids (NEFA) are split into acetyl-coA which enters the tricarboxylic cycle for energy production or alternatively condensed into ketone bodies. Insulin promotes storage of NEFA in the form of triglycerides through reesterification. It is well-documented that insulin possesses a potent inhibiting effect on adipose tissue lipolysis via activation of insulin receptors (IR) expressed on these cells (Fain, J *et al.* 1966; Lavis, V and Williams, R 1973).

1.3.3 Effect of insulin on Protein metabolism

A classical action of insulin is stimulation of protein synthesis. This is exemplified in untreated Type 1 Diabetes (T1DM) where lack of insulin leads to weight loss, muscle loss and growth cessation (Kimball and Farrell *et al.* 2002; Proud and Denton 1997). Insulin is widely described as a potent stimulus for muscle protein, indeed hyperinsulinaemia can increase muscle protein synthesis particularly when muscle amino acid availability is also increased (Bennet, Connacher *et al.* 1990). However insulin has been regarded to have a permissive rather than a modulatory role in muscle protein synthesis (MPS), and it is the availability of amino acids rather than insulin that appears to regulate rate of protein synthesis (Cuthbertson *et al.* 2005). Insulin has been shown to stimulate growth of cell cultures in vitro due in part to insulin binding to

receptors for insulin growth factor (IGF)-1 and IGF-2, demonstrating its role in tissue protein synthesis. Its effects are complex involving changes in synthesis and degradation; its mechanisms are beyond the remit of this thesis. Briefly the effects of insulin on protein take place over several timeframes. The rapid actions of insulin on protein metabolism involve increases in messenger ribonucleic acid (mRNA) translation. Insulin controls several translation factors. In particular, the relatively newly-discovered protein-signalling system based on the Ser/Thr protein kinase, mTOR has been found to have a major role in the control of mRNA translation. Indeed similar signalling elements involved in protein metabolism have been found to be utilised in carbohydrate metabolism. Specifically, mTOR has been shown to be controlled by the PI3-k pathway, an important mediator of the metabolic actions of insulin stimulates biosynthesis of certain proteins such as albumin, acetyl coA-carboxylase, fatty acid synthase, glucose-6 phosphate dehydrogenase, and pyruvate dehydrogenase, amino acid uptake, translation factors, and alterations in phosphorylation state of proteins involved in protein metabolism. Insulin activates protein synthesis by activating translational factors and increasing cellular ribosomes mediated primarily through phosphoinositide 3-kinase and activation of protein kinase B.This allows for overall activation of protein synthesis through its regulation of mammalian target of rapamycin (mTOR) and 4E-BP1, both of which are mainly involved in synthesis of protein (Proud 2006).

Other signalling pathways in skeletal muscle cells involved in MPS include in particular the mTORc1 that targets and activates kinases such as S6K1 that ultimately results in ribosomal biogenesis and translation.
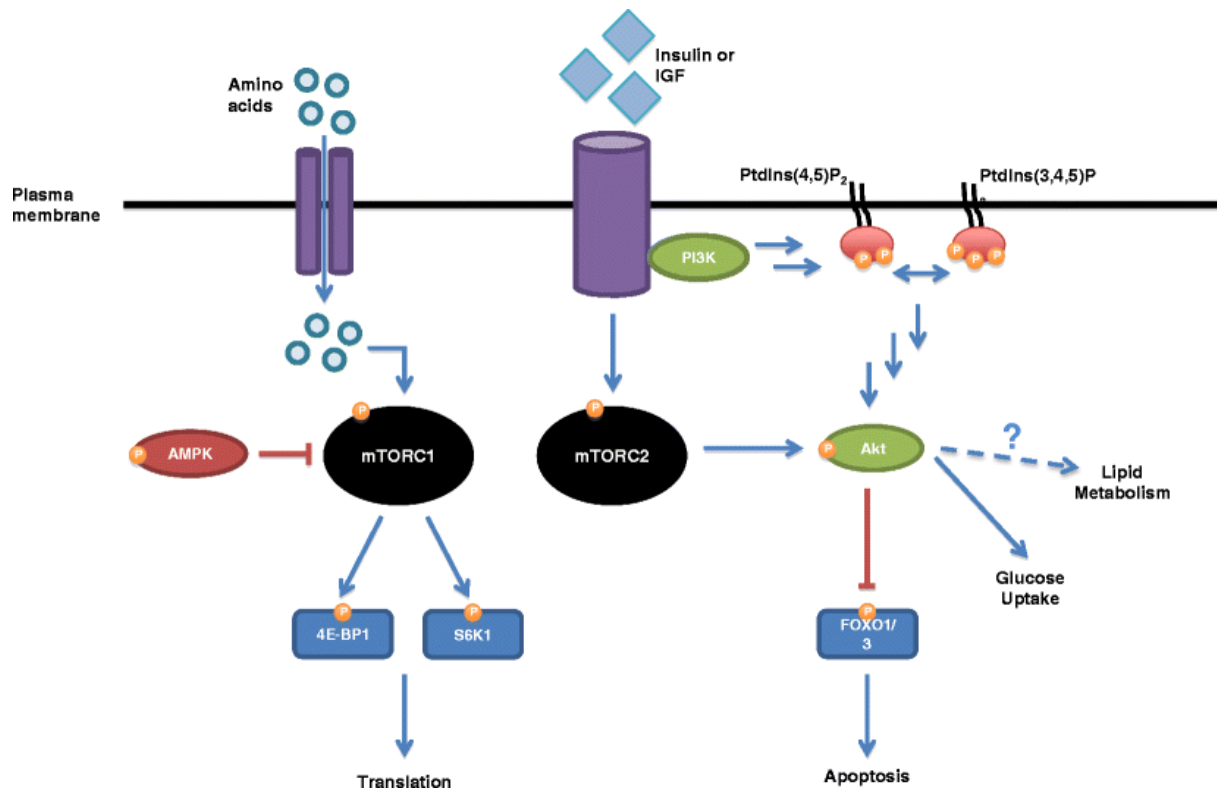
Figure 1.3 from Haran, Rivas *et al.* 2012.

Anabolic stimuli (amino acids, growth factors (insulin, IGF-1), and exercise (not

shown) act through the mTOR and Akt signalling pathways. mTORC1 is involved in

the phosphorylation and activation of S6K1 and phosphorylation and inactivation of 4E-

BP1, resulting in ribosome biogenesis, increase in translational efficiency, and

heightened MPS. The energy sensor AMPK inhibits this pathway, and is active when

the AMP/ATP ratio is high. Growth factor stimulates activation of the PI3K pathway as

well as mTORc2; both eventually activate Akt, which can inhibit stress signalling and

apoptosis through inactivation of FOXO1/O3 transcription factors. Akt also plays a role

in carbohydrate metabolism. P- phosphorylation, activation or inactivation; PI3K-

phosphoinositide 3 kinase; Akt- protein kinase B; FOXO- forkhead protein box O;

AMPK -AMP-activated kinase; 4E-BP1- eIF4E-binding protein 1; S6K1- S6 kinase 1

1.4 Defining Insulin Resistance

Harold Percival Himsworth, who as author of the pivitol paper 'Diabetes mellitus: its differentiation into insulin-sensitive and insulin-insensitive types' was perhaps the first person to introduce the concept of insulin resistance. (Himsworth, 1936). Through insulin administration to different subjects with diabetes and comparing the rate and extent of fall in blood glucose levels, Himsworth was able to classify diabetes into two types: "those in whom insulin produced an immediate suppression of hyperglycaemia following glucose intake, that is subjects who are insulin-sensitive but appeared to be insulin deficient; and those in whom insulin had little or no effect in suppressing hyperglycaemia, described as insulin-insensitive." He proposed that the observed insulin insensitivity was through restriction of a then unknown sensitising factor which in normal healthy humans produces a response to insulin in the presence of carbohydrate. It is now recognised that several possible mechanisms exist to explain this phenomena, as discussed in the subsections before. Of interest and significance is that Himsworth preferred the term insulin insensitivity to resistance, as he found no convincing evidence of a factor that 'resists' the action of insulin per se. The term insulin resistance had also been used to refer to preceding observations of an individual requiring more insulin than expected to produce hypoglycaemic symptoms or when vast amounts of insulin were insufficient to prevent the development of diabetic coma (Joslin 1935, Root 1929) and patients on insulin requiring increasing large amounts of insulin to control hyperglycaemia (Kahn CR, Rosenthal AS 1979). The term is now used interchangeably to describe the precursor or preceding state of diabetes.

Following on from what is now regarded 'a seminal contribution'(Reaven 2011), further experimental works from Himsworth published in the late 1930s to 60s did not have as much impact, and it was not until the 1970s when work by Reaven, whose experimental

methods in quantifying insulin-mediated glucose rekindled interest in insulin resistance. He found insulin resistance to be characteristic of patients with glucose intolerance (Shen S 1970) and later to the association of insulin resistance and different human diseases (Reaven 1988) and the development of clinical syndromes such as the metabolic syndrome (Reaven 2005).

At its simplest level insulin resistance can be defined as decreased sensitivity or responsiveness to the metabolic actions of endogenous or exogenous insulin to increase whole body glucose uptake and utilisation (Lebovitz 2001) characterized by higher fasting and post-glucose loading insulin levels (Kelley 2000). No universally-agreed definition of insulin resistance exists but various descriptions have been proposed according to methods of assessments in vivo. Using fasting plasma insulin to measure insulin action on a population, the definition of insulin resistance used to identify at risk individuals in a study by Salazar *et al.* 2011 was based on results of a study where 25% of an apparently healthy population with highest insulin concentrations developed glucose intolerance, hypertension and coronary vascular disease to a greater degree than the remaining 75% of the population.

The term insulin resistance has now been widely used and closely associated with a cluster of metabolic and cardiovascular abnormalities that define the metabolic syndrome (DeFronzo RA, 1991). This close association is acknowledged by the fact that the Adult Treatment Panel III (ATP III) have in their diagnostic guidelines used the terms 'Metabolic Syndrome', 'Syndrome X' and 'insulin resistance syndrome' synonymously. Although there appears to be a close affiliation, the metabolic syndrome is by no means a defining term for insulin resistance. In fact using criteria diagnostic of the metabolic syndrome (MetS) based on the harmonized version of the Adult Treatment Panel III (ATP III) and International Diabetes Federation (IDF) (see Figure

1.4), a number of studies (Cheal *et al.* 2004, Liao *et al.* 2004, Siera-Johnson *et al.* 2006, Salazar *et al.* 2011) concluded that MetS criteria did not provide an effective way of identifying individuals who are insulin resistant and that care should be taken when referring to a state of insulin resistance based on these factors.

- **Waist Circumference : ≥ 94 cm in men and ≥ 80 cm in women**
- **HDL-C: <1.0 mmol/L (40 mg/dl) in men and <1.3 mmol/L (50 mg/dl) in women**

- **Triglycerides: ≥1.7 mmol/L (150 mg/dl)**
- **Systolic BP ≥130 mmHg or Diastolic BP ≥ 85 mmHg**
- **Glucose: ≥6.1 mmol/L (110 mg/dl)**

Figure 1.4: Harmonised version of ATP III and IDF diagnostic criteria of MetS. 3 out of 5 criteria are required to diagnose MetS.

1.4.1 Insulin resistance and its association with cardiovascular disease.

Perhaps the concept of an association between clusters of cardiovascular risk factors and diabetes was best described by Reaven in 1988, who identified a clear relationship between hyperinsulianemia, glucose intolerance and hypertension, and also led to the descriptive term 'Syndrome X'. However this probably had its origins much earlier in 1923 when Kylin described the clustering of hyperglycaemia, hypertension and hyperuricaemia (Kylin E 1923). This was followed by others who associated obesity (Vague 1947) and hyperlipidaemia (Avogaro and Crepaldi 1965) with diabetes. Hypertension is more common in obesity as these individuals tend to be hyperinsulinaemic, and those who underwent exercise training experienced a decline in blood pressure limited exclusively to those who also had reducing insulin levels. The possible pathogenesis underlying this are that increases in insulin concentration promotes an increase in catecholamine levels and therefore sympathetic activity. Insulin

has also been shown to act on the kidney at the level of the renal tubules in promoting

sodium resorption and proximal tubule to promote volume resorption (Reaven GM

1988). Other metabolic associations with insulin resistance are listed in Figure 1.4.

Epidemiological studies suggest that hyperinsulinaemia is a risk factor for Coronary

Artery Disease (CAD). However the mechanisms are not entirely clear.

Hypertriglyceridaemia appears to be secondary to insulin resistance. An inverse

relationship between high-density lipoprotein cholesterol concentration and glucose

intolerance and direct relationship between triglycerides and hyperinsulinaemia are

observed. These clusters of risk factors of CAD may be important in the development

of CAD and in the early days have been termed Syndrome X. This included resistance

to insulin-stimulated glucose uptake or insulin resistance, glucose intolerance,

hyperinsulinaemia, increased VLDL triglyceride and decreased high-density lipoprotein

cholesterol and hypertension. The common feature is insulin resistance, which has often

led to some groups referring to insulin resistance as Syndrome X or metabolic syndrome

(Reaven 1988).

1.4.2 Metabolic syndrome

As alluded to earlier, the terms metabolic syndrome, Syndrome X (Reaven 1988),

insulin resistance syndrome (Haffner 1992); and less so the plurimetabolic syndrome

and the deadly quartet (Kaplan 1989) have been used interchangeably to describe the

risk factors associated with insulin resistance and cardiovascular disease. As a

consequence, the prevalence and indeed the identification of the syndrome vary among

different studies given the lack of unifying and accepted criteria as a definition. The

insulin resistance syndrome appears to be widely accepted as insulin resistance is a

common denominator seen in descriptions of the syndrome. However the term has also

raised differing and sometimes controversial views on what constitutes an accepted

definition. In 1998 the World Health Organisation (WHO) agreed on the term metabolic syndrome, supported by the third report of the National Cholesterol Education Programs Adult Treatment Panel (ATP III) but with different definitions. The ATP definition or factors used to define the metabolic syndrome is the most widely used in literature (Figure 1.4), however some argue that criteria to define the syndrome remain ambiguous. Doubt also remains over whether all patients that meet criteria of the metabolic syndrome are indeed insulin resistant. Unsurprisingly it became difficult to directly compare data from research studies using different definitions of the syndrome. The International Diabetes Federation (IDF) 2005 current definition attempts to address both the clinical and research needs establishing a 'platinum standard' of diagnostic criteria. However not all groups or associations were on board with the new definition, with the ADA/EASD questioning as to whether the syndrome was indeed a 'syndrome', if it actually served a purpose and whether the term was 'medicalising' people unnecessarily, driven by industry to create markets for pharmacological intervention (Kahn *et al.* 2005). Despite the differences in opinion the IDF'S criteria is strongly felt to identify individuals at high risk of developing T2DM and cardiovascular disease, advocating lifestyle changes over drug treatment (IDF 2005, Alberti, Zimmet, Shaw 2005). Further research in this area will clearly identify more accurate predictive indices for the future.

1.4.3 Role of insulin resistance in the development of Type 2 Diabetes

Two major conclusions were reached from a series of experiments utilising insulin clamps conducted by Himsworth *et al*. (1939). First, insulin-stimulated uptake of glucose is significantly lower in people with impaired glucose tolerance and T2DM compared to normal healthy subjects. The degree to which this is reduced was comparable between those with IGT and T2DM, indicating no relationship between

fasting plasma glucose concentration and insulin resistance in these groups. This observation was in line with studies carried out on Pima Indians. Secondly, it was showed that there was almost a three-fold variance in insulin-stimulated uptake in glucose in individuals with normal glucose tolerance. Around a quarter of the normal population would exhibit insulin resistance to a similar degree of severity seen in individuals of impaired glucose toleranc (IGT) and T2DM, regardless of degrees of hyperglycaemia. These apparent inconsistencies are explained by the extent of insulin that can be secreted to compensate for insulin resistance. The individuals' rate and sustainability of beta-cell insulin secretion determines their ability to stimulate glucose uptake and maintain glucose levels. It is not clear why the amount of insulin secreted differs from one person to another, but what is clear is that the beta cells play a crucial role in determining the degree of glucose tolerance and maintaining glucose homeostasis and that the inability to maintain hyperinsulinaemia leads to development of hyperglycaemia seen in IFG and T2DM. Elevated FFA concentrations can also inhibit insulin-stimulated glucose uptake (Ferrannini 1983). There is a direct relationship between plasma FFA concentrations and hepatic glucose production and plasma glucose concentration (Golay *et al.* 1987). An increase in FFA flux to the liver augments hepatic glucose production in humans and rat liver and causes hyperglycaemia by stimulating gluconeogenesis. This relationship is further supported by evidence showing agents that interfere with hepatic fatty acid oxidation can lower plasma glucose concentration (Ratheiser K *et al.* 1991, Hubinfer A *et al.* 1997). Administration of a potent inhibitor of the hepatic carnitine palmitoyl transferase (CPT) system lowered plasma glucose concentrations in rats with experimental diabetes (Conti R *et al*. 2001). It is also interesting to note that with the etoximir, FFA concentrations increased whilst insulin concentrations remained the same, indicating that the fall in

glucose was not entirely due to enhanced beta-cell function but inhibition of hepatic FFA oxidation.

1.4.4 Liver fat and development of insulin resistance

Excess energy is stored as glycogen and in particular as fat in liver, adipose tissue and muscle. Excessive accumulation of fat in liver has been linked to hepatic insulin resistance and indeed whole-body insulin resistance. The presence of liver fat independent of other cardiovascular risk factors has been shown to predict the onset of T2DM, and observed to be present before the onset of T2DM (Yki-Jarvinen 2005). This is because fatty liver overproduces very low density lipoprotein (VLDL), glucose, C-reactive protein (CRP), fibrinogen and coagulation factors, well-known cardiovascular risk factors. In contrast, reduction in liver fat content is associated with an improvement in insulin suppression of glucose production and fasting plasma glucose (Petersen *et al.* 2005). Carbohydrate (sucrose) overfeeding for 3 weeks has been shown to increase liver fat content and liver enzymes of overweight individuals that were reversible following reverting to a hypoenergetic diet (Sevastianova *et al.* 2012). Higher plasma insulin levels are associated with and to an extent influences the rate of hepatic DNL (Petersen *et al.* 2012, Schwarz *et al.* 2003). This is further substantiated by studies showing hypoenergetic diet (Nobili *et al.* 2007), physical activity (Perseghin *et al.* 2007) and thiazolinedione (Ravikumar *et al.* 2005) use reduces insulin secretion and decreases liver fat content.

DNL following carbohydrate intake causes transport of free fatty acids into mitochondria. But because malonyl-CoA (produced during DNL) inhibits oxidation, newly produced triacylglycerol is preferentially directed towards storage or export, contributing to increased liver fat and VLDL (Taylor 2013). Accumulation of liver fat

also occurs when oxidation of fatty acids is perturbed or reduced. Hepatic insulin resistance develops when fatty acids are esterified to diacylglycerol (DAG, an intermediate of triacylglycerol and phospholipid metabolism), which activates protein kinase C epsilon type (PKCε) that in turn inhibits the signalling pathway from the insulin receptor to IRS-1 (Samuel *et al.* 2010). This effect on the first post- receptor step is underscored by disruption to the insulin signalling pathway, an important part of the mechanism involved in insulin action as described in Subchapter 1.2. In obesity intrahepatic DAG correlates with hepatic insulin sensitivity (Magkos *et al.* 2012). Ceramides (a derivative of sphingomyelin) cause sequestration of Akt2 and activation of gluconeogenesis, but no relationship to hepatic insulin resistance in humans could be demonstrated (Kumashiro *et al.* 2011). Despite a strong relationship between increased liver fat and insulin resistance, high levels of liver fat do not inevitably correlate with hepatic insulin resistance. This observation is analogous to normal insulin sensitivity seen in trained athletes despite raised intramuscular triacylglycerol (van Loon and Goodpaster 2006) and in muscle of mice overexpressing the enzyme DGAT-1(Liu *et al.* 2009).
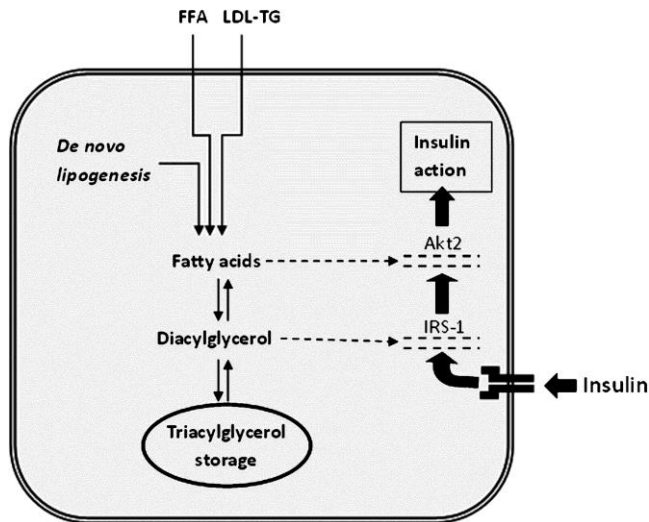
Figure 1.5: Mechanism of interaction between excess amounts of fatty acids, diacylglycerol and ceramide and insulin action within the hepatocyte. DAG activates PKCε and inhibits activation of IRS-1 by the insulin receptor. Ceramides cause sequestration of Akt2 by PKCζ and inhibit insulin control of gluconeogenesis (Taylor, *Diabetes Care* 2013).
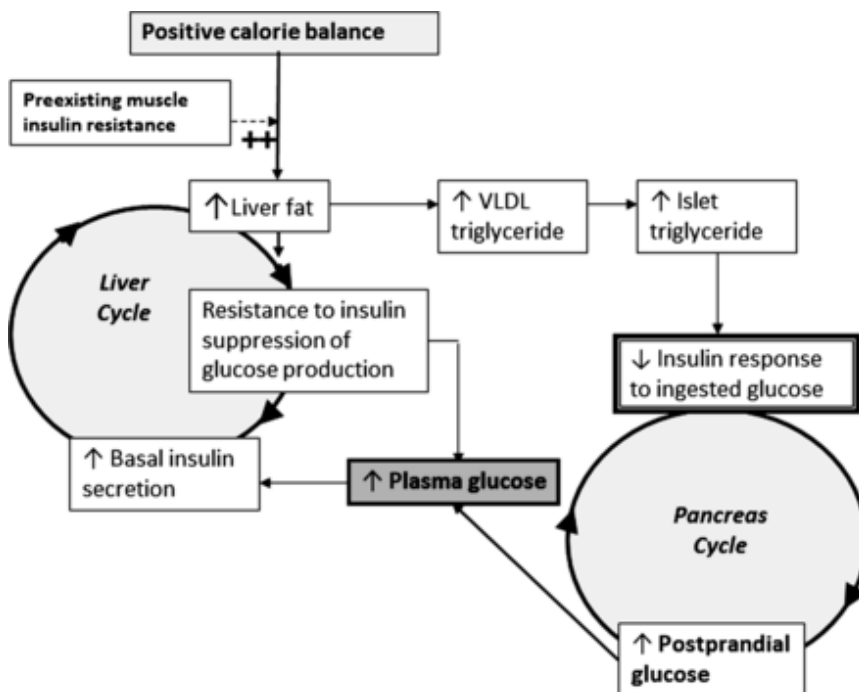
Figure 1.6: Twin cycle hypothesis in the development of T2DM (Taylor, *Diabetologia* 2008).

So now the increased liver fat not only causes resistance to insulin suppression of hepatic glucose production, but increases fasting plasma glucose over a period of time and thus basal insulin. Chronic hyperinsulinaemia promotes lipogenesis and increases conversion of excess energy to fatty acids, and so begins the cycle of hyperinsulinaemia and blunted suppression of hepatic glucose production. VLDL triglycerol increases delivery of fatty acids to tissues, including the islet cells of pancreas. Excess fatty acid availability in the pancreatic islet impairs the acute insulin secretion in response to nutrients, and at a certain level of fatty acid exposure, postprandial hyperglycemia ensues. Hyperglycaemia further increases insulin secretion rates, enhances hepatic lipogenesis, and further drives the liver and pancreatic cycle. Eventually, the fatty acid and glucose inhibitory effects on the islets reach a trigger level that leads to a relatively sudden decompensation into clinical diabetes (Taylor 2008).

1.4.5 Skeletal muscle insulin resistance

Although Taylor touched upon the contribution of muscle insulin resistance albeit a chronic pre-existing contribution to the twin cycle hypothesis and recognised that muscle insulin resistance reflects whole body insulin resistance as the earliest predictor of onset of T2DM (Petersen *et al.* 2007), it would appear that he did not regard muscle to exert as large of an influence on development of T2DM than the liver or pancreas. Taylor argues that resumption of normal or near-normal blood glucose control leads to improvement of liver insulin sensitivity and not muscle, and this is supported by early animal studies. Mice with absent skeletal muscle insulin receptors do not develop diabetes (Bruning *et al.* 1998). Moreover humans with the PPP1R3A genetic variant of muscle glycogen synthase who cannot store glycogen in muscle after meals are not necessarily hyperglycemic (Savage *et al.* 2008). Normoglycemic individuals have been shown to maintain normoglycaemia with a degree of muscle insulin resistance identical

to those with T2DM (Taylor 2008). He also maintained that mitochondrial defects in muscle of insulin resistant individuals/people with diabetes (Petersen *et al.* 2004) were irrelevant to the aetiology of T2DM. It is not difficult to comprehend why Taylor was opposed to muscle insulin resistance as a major contributor to development of T2DM as he argues it is not muscle insulin resistance per se that causes blood glucose to rise but chronic hyperinsulinaemia that expedites accumulation of fatty acids, causing rise to chronic hyperglycaemia and ultimately insulin resistance.

This brings us to discussing how muscle insulin resistance develops, giving rise to whole body insulin resistance, perhaps adding to part of Taylor's theory that it is liver fat and the pancreas that acutely trigger onset of overt diabetes.

 De Fronzo and Tripathy (2009) considered skeletal muscle insulin resistance as the initiating factor for development of T2DM long before beta cell failure of the pancreas (Lillioja *et al.* 1998, Warram *et al.* 1990) with impaired muscle glycogen synthesis as the primary defect in the development of insulin resistance. Under hyperinsulinaemic clamp conditions, around 75-80% of insulin-mediated glucose uptake occurs in skeletal muscle (Thiebaud *et al.* 1982, DeFronzo *et al.* 1981) whilst the remainder 20-25% of insulin-stimulated muscle glucose disposal is oxidised to CO_2 and H_2O (De Fronzo 1997). The earliest metabolic manifestation in the pathogenesis of T2DM appears to be moderate to severe insulin resistant muscle, demonstrated by examining lean, normal glucose tolerant, first degree relatives of people with T2DM (Warram *et al.* 1990) and long-term follow-up of normal glucose tolerant individuals as they progress from impaired glucose tolerance to overt diabetes (McCance *et al.* 1994). This approach provides a robust examination of development of diabetes as it excludes confounding factors such as obesity and hyperglycaemia. The earliest detectable metabolic abnormality seen in humans in T2DM is impairment of glycogen synthesis secondary to

reduced glycogen synthase activity (Bogardus 1984, DeFronzo 1997). In non-glucose tolerant offspring of parents with T2DM, using the two-step euglycaemic insulin clamp, impairment of glucose uptake was entirely accounted for by non-oxidative glucose metabolism (which represents glycogen synthesis). No defect was noted in the suppression of hepatic glucose production by insulin (Gulli *et al.* 1992). Insulin secretion was significantly increased excluding a primary defect in beta cell function. Perseghin *et al.* (2007) directly quantified the defect in muscle glycogen synthesis over time in (non-glucose tolerant) NGT offspring of parents with diabetes using nuclear magnetic resonance (NMR) spectroscopy and demonstrated that reduced glycogen synthesis accounted for almost all of the decrease in insulin-stimulated muscle glucose disposal. Using the triple isotope method of ^{12}C mannitol, ^{13}CO methylglucose and ^{3}H glucose and euglycaemic clamp, defects in muscle glucose transport and phosphorylation were seen in NGT offspring of T2DM parents, similar to that seen in T2DM individuals. Using ^{14}C NMR, a similar defect along the glucose transport/phosphorylation pathway in particular the glucose-6-phosphate levels were seen by Rothman *et al.* (1995). A follow-up prospective study on Pima Indians (Weyer *et al.* 1999) found that at-risk individuals were resistant to insulin but at the stage of NGT, beta cells were able to secrete sufficient insulin to offset the insulin resistance. Over time progressors (at-risk individuals who progressed to T2DM) and non-progressors (at-risk individuals who remained as NGT) experienced further reduction in insulin sensitivity (11-14%). However an interesting observation was that progressors were able to off-set muscle insulin resistance by augmenting insulin secretion by 30% whilst non-progressors were associated with almost an 80% decline in the acute insulin response to the intravenous glucose challenge. As the majority of glucose disposal after intravenous glucose administration occurs in muscle, this provides compelling evidence

that insulin resistance in muscle is the earliest demonstrable defect in the development of T2DM. However for overt T2DM to occur, beta cells must be at a stage where they are unable to compensate for the defect in insulin action (Gastaldelli 2004, Abdul-Ghani *et al.* 2006).

The molecular mechanisms involved in the aetiology of skeletal muscle insulin resistance in genetically-predisposed individuals have been identified and appear to manifest at an early stage in NGT insulin resistant offspring of parents with T2DM. Using the euglycaemic insulin clamp and skeletal muscle biopsies, defects in IRS-1 tyrosine phosphorylation and PI-3 kinase and Akt activation were consistently demonstrated in T2DM subjects (Krook *et al.* 2000, Cusi *et al.* 2000, Bouzakri 2003). Basal and insulin-stimulated IRS-1 tyrosine phosphorylation (a requisite for glucose transport and glycogen synthesis) and PI-3 kinase activity are significantly reduced in NGT subjects with a strong family history of T2DM (Morino *et al.* 2005). A similar significant reduction in PI-3 kinase activity has been observed during conditions of elevated FFA levels induced in lean healthy subjects during insulin-mediated glucose disposal (Belfort, Mandarino et al. 2005)

NGT offspring of parents with T2DM were shown to possess muscle insulin resistance but normal sensitivity to the suppressive effects of insulin on hepatic glucose production (Gulli *et al.*). Elevated fasting plasma FFA concentration during conditions of fasting hyperinsulinaemia and impaired suppression of plasma FFA during the euglycaemic insulin clamp indicate the presence of adipocyte resistance to the anti-lipolytic effects of insulin. Impaired insulin –mediated suppression of whole-body lipid oxidation was also present. The accumulation of lipid in muscle (IMCL) has been demonstrated in offspring of T2DM (Petersen *et al.* 2005), and as elaborated below is one of the primary drivers to the development of muscle insulin resistance as a result of accumulation of

lipid metabolites (ceramides, DAG and long-chain fatty acyl-coAs) that interfere with the insulin-signalling pathway (Hotamisligil *et al.* 1996, Schmitz-Peiffer 2000). In relation to this, accumulation of lipid in muscle is associated with skeletal muscle insulin resistance (Phillips, Caddy *et al.* 1996). Ceramides and DAG have been shown to be elevated in obese insulin resistant rat muscle with increased intra-myocellular lipid (IMCL) content (Turinsky, O'Sullivan *et al.* 1990) . DAG and ceramide act as second messenger involved in intracellular signalling with roles in cPKC and nPKC –mediated activation and reduction in Akt and GLUT4 translocation respectively.

However improvements in human skeletal muscle insulin sensitivity have also been seen with little or no change (Bruce, Thrush *et al.* 2006) and even an increase in IMCL concentrations (Phillips, Green *et al.* 1996). For example, master athletes have been shown to have high levels of IMCL, yet they are insulin sensitive (Goodpaster, He *et al.* 2001) , indicating it is not the quantity of IMCL per se but the complex interplay and balance of lipid availability, uptake and oxidation. The significance of cellular uptake and oxidation despite differing FFA availability will be discussed in chapter 3.

Lower fat oxidation has been demonstrated to occur in people with T2DM and in obese-insulin resistant but non-diabetes individuals (Kelly DE 2005), suggesting impairment of mitochondrial oxidative capacity. NGT offspring of parents with diabetes had reduced expression of key mitochondrial genes involved in regulation of oxidative metabolism in skeletal muscle such as energy generation, glycolytic, tricarboxylic acid cycle and oxidative phosphorylation. Shulman and others demonstrated impaired mitochondrial activity via ^{1}HNMR and postulated that mitochondrial dysfunction was a primary defect leading to accumulation of lipid metabolites as a result of reduced fat oxidation and subsequent insulin resistance. However, small increases in palmitoyl-carnitine have been shown to impair ATP synthesis in mitochondria of human muscle,

therefore it is not clear which is the cause, and which is the effect (Abdul Ghani *et al.* 2006).

Plasma lipid concentrations determine the rate of muscle FFA uptake, during conditions of hyperinsulinaemia (Brechtel, Dahl *et al.* 2001). Obese and insulin resistant states are often accompanied by high levels of FFAs and in addition to reduced lipid oxidation seen in obese muscle is likely to lead to excess IMCL deposition(Kim, Hickner *et al.* 2000). Excess muscle FFAs are either converted to lipid droplets for energy use or signalling molecules (ceramides, DAG) which may play a central role in lipid-mediated insulin desensitisation. Fatty acids are converted into fuel for muscle use and for this to occur FFAs are converted to long-chain fatty acyl co-As (LCFA co-As). These are transported into mitochondria by carnitine acyltransferase for beta-oxidation. The importance and beneficial effects of carnitine and fatty acid metabolism will be discussed at length in Chapter 6.

To summarise, although there is no universally-accepted definition for insulin resistance, it can simply be described as a state of reduced sensitivity to the metabolic effects of endogenous or exogenous insulin of glucose uptake and use. There are differing opinions on what should constitute the metabolic syndrome, but factors that appear to be mutually agreed upon include factors that increase cardiovascular risk such as impaired glucose tolerance/diabetes, overweight/obesity, hypertriglyceridaemia and hypertension. Mechanisms underlying hepatic and skeletal muscle insulin resistance contribute to development of T2DM.

1.5. Skeletal muscle, lipid accumulation and insulin resistance

Elevated levels of FFA concentrations are associated with obesity and skeletal muscle insulin resistance, and may even predict development of T2DM (Jenssen *et al.* 1989, Poulisso *et al.* 2005, Samuel and Shulman 2012). Indeed, Randle *et al.*, besides proposing their theory on the glucose-fatty acid cycle, were one of the first to demonstrate that elevated plasma FFA concentrations were responsible for the reduced insulin sensitivity in diabetes mellitus (Randle *et al.* 1967). Randle's proposal of the glucose-fatty acid cycle described FFA's inhibitory effect on glucose metabolism in rat's diaphragm and isolated heart, whereby under aerobic conditions increased FFA availability increases fatty acid oxidation. This results in mitochondrial citrate and acetyl-coenzyme A (CoA) build-up and a rise in the cytoplasmic NADH/NAD ratio. The increase in acetyl-coA thus inhibits pyruvate dehydrogenase activity and subsequent inhibition of phosphofructokinase. This would cause a rise in glucose-6-phosphate, inhibition of hexokinase II activity, increase in intracellular glucose and subsequent reduction in glucose uptake (Randle 1998, Dresner *et al.* 1999). In muscle and adipose tissue, glucose promotes reesterification of circulating FFA, whereas FFA inhibits muscle glycolysis, thus restricting glucose utilisation.

However in contrast to Randle's hypothesis not all subsequent studies have yielded similar findings (Schonfeld and Kipnis 1968). Using lipid infusion, insulin clamp technique and nuclear magnetic resonance (Roden *et al.* 1996, 1999) demonstrated that in contrast to Randle's theory, FFAs induced insulin resistance at the level of glucose transport/phosphorylation, which then leads to a reduction in muscle glycogen synthesis and carbohydrate oxidation. In concert with these findings, others (Griffin *et al.* 1999, Rothman *et al.* 1992) showed that lipid infusion decreases intracellular glucose and glucose-6-phosphate content secondary to inhibition of skeletal muscle glucose uptake.

Plasma lipid concentrations determine the rate of muscle FFA uptake, during conditions of hyperinsulinaemia (Brechtel, Dahl *et al.* 2001). Obese and insulin resistant states are often accompanied by high levels of FFAs and in addition to reduced lipid oxidation seen in obese muscle is likely to lead to excess IMCL (Kim, Hickner *et al.* 2000). On a mechanistic level, lipid infusion during hyperinsulinaemic euglycaemic clamp has been demonstrated to affect the intracellular signalling pathways in skeletal muscle by reducing IRS- 1 tyrosine phosphorylation, IRS-1 and 2 associated PI3-kinase activity and Akt phosphorylation and activity (Kim *et al.* 2000, Yu *et al.* 2002). Mechanisms implicated include activation of kinases such as PKCs, IKKβ, and JNK and p38 MAP kinase, thought to reduce phosphorylation of tyrosine residues of IRS-1 by insulin, blocking its downstream signal transduction (Tirosh *et al.* 1999, Bloch-Damti 2005; Evans *et al.* 2005).

Another putative mechanism underlying lipid-induced insulin resistance include accumulation of DAG that can activate novel protein kinase C (PKC) isoforms that cause serine phosphorylation of insulin receptor substrate-1 (Samuel and Schulman 2012) and ceramides as a consequence of binding of fatty acids to Toll-like receptor 4 (TLR-4), which impairs Akt activation (Bikman and Summers, 2012). High-fat diets and lipid and heparin infusion induced accumulation of intracellular DAG in muscle (Schmitz-Peiffer *et al.* 1997) and activation of PKC, caused by reduction in tyrosine phosphorylation of IRS-1. Ceramides on the other hand may affect insulin signalling by activation of activation of Akt dephosphorylation at threonine 308 and inhibition of its translocation to the plasma membrane (Summers 2010).

There also appears to be a causal role for the inflammatory pathways and development of insulin resistance. Fatty acids, like the bacteria-derived lipopolysaccharide (LPS), may bind to TLR-4 on macrophages and adipocytes and activate the proinflammatory

pathways (Medzhitov 2007) promoting c-Jun NH(2)-terminal kinase (JNK) and Iκb kinase (IKK) complex activation. These kinases cause phosphorylation of serine residues on insulin IRS-1 and inhibition of the stimulatory phosphorylation of tyrosine residues (Hotamisligil *et al.* 1993, 2008). This results in inhibition of insulin action Ingestion of high-fat meals and infusion of low-dose LPS can raise circulating cytokines such as tumour necrosis-α (TNF-α), IL-1β and IL6 (Krogh-Madsen *et al.* 2008) which in turn could cause elevation of lipid metabolites (Bikman and Summers 2011).

Reactive oxygen species (ROS) are involved in important physiological processes, produced in response to stimuli such as nutrient metabolism and signalling through plasma membrane receptors. High levels of ROS are associated with chronic elevation in plasma lipid levels and intramyocellular fatty acids (Carvalho-Filho *et al.* 2005) and can negatively affect various biological signalling pathways. Impairment of the insulin signalling pathway is not completely understood, but ROS has been demonstrated to induce IRS serine/threonine phosphorylation, decrease GLUT-4 gene transcription and decreasing mitochondrial activity (Bloch Damti 2005, Morino *et al.* 2005). Animals fed a high-fat diet were found to have increased ROS production and were insulin resistant. Conversely when treated with antioxidants this improved oxidative stress and insulin sensitivity (Ogihara *et al.* 2004; Blouet *et al.* 2007).

Several studies have shown mitochondrial dysfunction to play a pivitol role in the development of insulin resistance, whilst others have not (Dela, Helge 2013). Mitochondrial content, function and oxidative capacity in muscle were found to be reduced in insulin resistant obese and in T2DM (Holloway 2007, Schrauwen-Henderling *et al.* 2007). These findings are corroborated by studies involving high fat diet or lipid infusion to healthy humans and rodents, resulting in impaired oxidative phosphorylation and ATP synthesis (Chanseaume *et al.* 2006, Szendroedi *et al.* 2009).

With regards to the interaction between FFA and the liver, early studies have shown that FFA stimulates gluconeogenesis from lactate (Struck *et al.* 1966), alanine (Herrera *et al.* 1966) or pyruvate (Friedman *et al.* 1967) in perfused rat liver. A relatively new hypothesis proposed is the association between skeletal muscle insulin resistance and excessive fat oxidation rates and mitochondrial overload. According to this hypothesis, when beta-oxidation exceeds the mitochondrial's capacity to utilise acetyl-coA in the TCA cycle, incompletely oxidised, short-chain fatty acid products such as acylcarnitines may impair skeletal muscle insulin sensitivity (Muoio *et al.* 2008). This proposal is supported by increased levels of partially oxidised acylcarnitines seen in skeletal muscle of insulin-resistant high fat diet (HFD)-fed mice and diabetic fatty rats. The mechanism however has not been largely explored and investigated in humans.

1.6 Lipid infusion

Several of the studies described above utilised intravenous lipid infusion and heparin. In at least the last five decades intravenous lipid infusion has been used as a means of examining the effects of an acute increase of FFA levels on glucose uptake and underlying mechanisms of insulin resistance development in healthy subjects. Felber and Vannotti (1964) were one of the earliest to observe that lipid infusion caused glucose intolerance in normal, healthy individuals. Several studies substantiating this finding show a reduction in rate of plasma glucose disappearance following lipid infusions (Schalch and Kipnis (1965), Balasse and Neef (1975)).

Ferrannini *et al.* explored the effects of physiological elevations of FFA concentrations on glucose production and utilisation under controlled hyperinsulinaemic conditions and findings were shown to be compatible with Randle's glucose fatty acid hypothesis

(Ferrannini *et al.* 1983). In this study, heparin was infused concurrently to raise FFAs three to four fold, by intravascular lipolysis of infused lipids which stimulates activity of lipoprotein lipase in blood. When FFA concentrations were raised following IV lipid infusion under hyperinsulinaemic euglycaemic conditions (where endogenous glucose production was entirely suppressed), total glucose uptake was significantly lower compared to control (lower M/I ratio; where M is glucose uptake and I is steady state insulin concentration). This clearly demonstrated that by acutely elevating FFA levels, insulin-stimulated glucose uptake is inhibited even in the presence of hyperglycaemia. When insulin levels were low, lipid infusion had no effect on glucose utilisation, thought to be due to uptake of glucose by non-insulin dependent tissues. It was also speculated that inhibition by FFAs occurred at a peripheral level, most likely muscle. In this regard, inhibition of glucose metabolism by FFA is more likely to occur in conditions where there is insulin present at particularly high levels, ie insulin resistant states or T2DM.

1.7 High fat overfeeding

The issue concerning high-fat diet and its association with insulin resistance is of topical interest and continues to be a matter of intense investigation, discussion and debate. But there is without doubt that high fat diets play a pivotal role in the subject of insulin sensitivity and is an independent risk factor for overweight and obesity (Astrup, 2001). The role of dietary fat in weight gain relies on the individual's genetic background, environment and ability to oxidise dietary and endogenous fat (Giacco *et al.* 2004).

Data on interventional human studies investigating the effects of high-fat diets on metabolism and insulin sensitivity are plentiful but largely inconclusive given the short period of intervention and relatively small number of subjects.

Whereas excessive intake of fat (>37% of daily energy intake) appears to impair insulin sensitivity irrespective of the composition of fatty acids in the diet (Thomas and Pfeiffer 2011), substantial evidence from animal studies suggest that certain fats promote accumulation of intramuscular triglygeride (IMTG). In line with this the first way in which fatty acids can affect insulin sensitivity is the type or quality of fat. There appears to be 'good' and bad' types of fatty acid that improve or reduce insulin sensitivity respectively (Marshall, Bessesen *et al.* 1997). Specifically a selective increase in saturated fat intake may impair insulin action (Maron *et al.* 1991, Parker *et al.* 1993; Mayer *et al.*, Mayer-Davis *et al.* 1997) whereas increasing unsaturated fat (when total fat intake is low) has been linked to improving insulin sensitivity (Vessby *et al.* 1994, Pan *et al.*1995). One way by which fatty acids can influence insulin sensitivity is the fatty acid composition of cellular membrane which may influence cell insulin signalling (Inokuchi 2006). Fatty acid types in human diet; saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), trans-unsaturated FAs differ by spatial configuration and chemical property. The efficacy of molecular signalling and transduction is highly dependent on the complex positioning of various proteins within the fatty acid cellular membrane, and so intake of various lipid molecules that can affect membrane fluidity and rigidity could potentially alter the mechanism's sensitivity to configurative changes. Animal studies (Storlien, Pan *et al.* 1996) show that saturated fat–laden membranes promote insulin resistance, whereas more unsaturated membranes protect against it, a finding also noted in humans.

The monounsaturated fatty acids (MUFAs) may improve insulin sensitivity by exerting an effect on cell membrane FA composition, membrane fluidity, insulin receptor binding/affinity and up-regulation of glucose transporters (Riserus, Willett *et al.* 2009). It may also be that MUFA are preferentially oxidised over saturated fatty acids (SFA) that gradually accumulate in muscle (DeLany, Windhauser *et al.* 2000).

PUFA may improve insulin sensitivity by its anti-inflammatory effects mediated by toll-like receptors in particular TLR 2 and 4 (Thomas and Pfeiffer 2011). Other postulated effects of PUFA on insulin sensitivity include beneficial changes to membrane fluidity, increased binding affinity of the insulin receptor and increased glucose transport into cells and effects on triglycerides.

The MUFAs may improve insulin sensitivity by exerting an effect on cell membrane FA composition, membrane fluidity, insulin receptor binding/affinity and up-regulation of glucose transporters (Riserus, Willett *et al.* 2009). MUFA diets have been found to prevent central fat redistribution and prevent insulin resistance from ingesting a carbohydrate-rich diet (Paniagua, Gallego de la Sacristana *et al.* 2007).

In contrast SFAs have been shown to have a direct relationship with the incidence of insulin resistance and T2DM. Under conditions of hyperglycaemia SFA influence enzyme activities and transcription factors and serine kinases interfering with insulin signalling pathways and inflammatory pathways associated with impaired insulin sensitivity (Rioux and Legrand 2007; Poitout and Robertson 2008). Furthermore, high intakes of TFA may lead to insulin resistance and increased risk of cardiovascular events. (Axen, Dikeakos *et al.* 2003; Mozaffarian 2006).

With respect to ingestion of these different fatty acid types on muscle, it would appear that accumulated lipid metabolites may be influenced by the type of fat ingested, that may be detrimental to mechanisms involved in insulin signalling. Saturated fatty acids such as palmitate, stearate or arachidate induce ceramide and DAG synthesis, inhibit Akt activation (Storz, Doppler *et al.* 1999; Chavez, Knotts *et al.* 2003), reduce glucose uptake by desensitisation of insulin stimulation (DAG) (Montell, Turini *et al.* 2001) and inhibit IR or IRS-1 phosphorylation (palmitate)(Storz, Doppler *et al.* 1999). A mediterranean- style diet consisting 25-35% of fat, containing mostly monounsaturated fats, has been linked to preventing risk factors of metabolic disease. (Kastorini, Milionis *et al.* 2011).

N-3 PUFAs found in fish oil have been shown to be beneficial against development of insulin resistance, although the reversal of insulin resistance by increasing intake is not definitive. Rats fed a high-fat diet enriched with n-3 PUFA maintained IR, IRS-1, PI3-k activity and GLUT-4 content in skeletal muscle. N-3 PUFA are also preferentially oxidised over saturated fatty acids, and can up-regulate genes involved in lipid oxidation such as PPARs. In contrast n-6 PUFA appear to exhibit effects opposite of that seen with n-3 PUFA.

1.7.1 Fat overfeeding studies, liver fat and insulin resistance

One of the more pertinent studies involved overfeeding rats a high-fat diet for 3 days such that a model of NAFLD was simulated to determine the effect of hepatic fat on hepatic insulin responsiveness without the possible confounding effect of peripheral insulin resistance (Shulman 2004). After 3 days of overfeeding fat (59% fat, 26% carbohydrate, 15% protein), hepatic triglycerides and fatty acyl-coA content nearly tripled but remained unchanged in muscle. Therefore high fat overfeeding resulted in marked hepatic insulin resistance whilst peripheral (muscle and adipose tissue) insulin

sensitivity was unaltered. This was attributed in part to decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 which may result from activation of PKC-ε or JNK, limiting the ability of insulin to activate glycogen synthase.

In humans ingestion of a 2 week isocaloric high-fat diet increased liver fat, but body weight and subcutaneous fat mass were unchanged implying possible uptake of excess fat by the liver (Westerbacka *et al.* 2005). This was in accordance with a 3 week study incorporating isocaloric high-fat diet in overweight men (van Herpen *et al.* 2011). Although these studies demonstrate accumulation of liver fat, it would appear that this did not translate to hepatic insulin/peripheral insulin resistance or that to see a significant effect on insulin sensitivity a hyperenergetic diet and longer duration of overfeeding is required. In contrary, Brons et al showed that a hyperenergetic high fat diet for 5 days led to a significant increase in hepatic glucose production.

1.8 High Carbohydrate feeding

Fructose and glucose overconsumption have long been associated with fatty liver but there is still controversy as to whether it is the macronutrient or energy excess from increased consumption that contributes primarily to liver fat accumulation and insulin resistance. Fructose in particular has been related to fatty liver and severity of histological changes in people with NALFD (Kang *et al.* 2006) independent of obesity and total caloric intake. However studies on the effect of excessive carbohydrate on liver fat content are relatively few. One of the first few studies examining this possible relationship showed no effect on liver fat after 4 weeks of fructose overfeeding in lean healthy men (Kang *et al.* 2006, Silbernagel 2011). Insulin sensitivity in the latter study also remained unchanged. In contrast, others shown that a high fructose diet does have a positive effect on increasing liver fat (Le at al. 2009, Ngo *et al.* 2010, Sobrecases H *et*

al. 2010). The reason for the discordance in results is not entirely known, however a possible explanation could be that some studies were conducted in lean healthy men with little or absence of liver fat at baseline. The use of isoenergetic vs. overfeeding diets in different studies should also be taken into consideration. When carbohydrate overfeeding (fructose/sucrose/glucose) was examined in overweight or offspring of people with T2DM, an increase in intrahepatic lipid content was observed, further suggesting that overweight (Sevastianova *et al.* 2012) and offspring of T2DM subjects tended to have a predisposition towards increased TAG/liver fat content. The I148M variant in the palatin-like phospholipase domain containing protein 3 (PNPLA3) gene is associated with liver fat (Sookoian and Pirola 2011) and mutant PNPLA3 is unable to hydrolyse intrahepatic triglycerides (He *et al.* 2010). Therefore possession of this genotype may exert an influence an individuals' response to carbohydrate feeding. A 3 week hyperenergetic high carbohydrate diet (1000kcal/day with 98% energy from carbohydrate) in overweight healthy men resulted in weight gain and a 27% increase in liver fat content from baseline (Sevastianova *et al*. 2012). This study did not primarily study the effects of high carbohydrate diet on insulin sensitivity, but no difference in fasting plasma glucose was seen post-overfeeding period although a trend in increased fasting serum insulin concentrations. Johnston *et al.* 2013 showed that overweight men on isocaloric high-fructose or high-glucose diets did not develop significant changes in hepatic concentration of TAGs; however under hyperenergetic conditions these diets produced significant increases in liver fat indicating an energy, rather than a specific macronutrient-mediated effect. No effect on hepatic insulin sensitivity was seen.

Fructose is a unique monosaccharide in that most of its metabolism and extraction (50-70% of fructose delivery) takes place in the liver. Therefore increased availability of fructose places greater demands on hepatocytes and increase abnormal glucose flux.

There are data to suggest that high concentrations of fructose produce adaptations in the liver that include metabolic intermediates, gene expression and insulin action. Increased fructose supply has been shown to induce a hepatic stress response activation of stress-activated protein kinases such as c-Jun N-terminal kinases (JNK) and subsequent hepatic insulin signalling. Hepatic DNL (fatty acid and triglyceride synthesis) contributes to fatty liver and NAFLD (Donnelly *et al.* 2005) and substrates involved include primarily glucose, fructose and amino acids.

Table 1.1: Studies of iso-or hyperenergetic high carbohydrate or fat diets

Authors	Study description	Results
Horton *et al.* 1995	14 days of high energy (+50%) fat or carbohydrate in 9 lean and 7 obese males	Carbohydrate overfeeding increased energy expenditure and carbohydrate oxidation and less fat oxidation. Fat was more likely to be stored as excess energy. No differences in energy or nutrient balance were seen in both obese and lean subjects in response to fat overfeeding. Obese subjects had greater RQ and oxidised more carbohydrate. Insulin concentrations and FFA were higher and lower respectively in the carbohydrate-fed group.
Lammert *et al.* 2000	Effects of 3 weeks of isoenergetic high carbohydrate and fat overfeeding on body composition, sleep calorimetry and DNL in 20 healthy non-obese males	Both groups had similar weight and fat mass gain. Carbohydrate overfeeding caused a greater increase in fractional hepatic DNL compared to fat overfeeding.

Bisschop *et al.* 2001	6 healthy men on 3 different fat content diets for 11 days. 0% and 85% [low-fat, high-carbohydrate (LFHC) diet], 41% and 44% [intermediate-fat, intermediate-carbohydrate (IFIC) diet], and 83% and 2% [high-fat, low-carbohydrate (HFLC) diet]	A high-fat, low-carbohydrate intake reduces the ability of insulin to suppress endogenous glucose production and alters the relation between oxidative and non-oxidative glucose disposal in a way that favours storage of glucose.
Westerbacka *et al.* 2005	2 weeks of isoenergetic high fat diet (56%) in 10 overweight/obese healthy females	Liver fat at baseline averaged 10 +/- 7%. It increased by 35 +/- 21% during the high-fat diet. Fasting serum insulin increased during the high-fat diet. Serum lipids, free fatty acids, and intraabdominal and subcutaneous fat mass were unchanged.
Le *et al.* 2006	4 weeks of overfeeding in lean healthy male subjects with 18% excess energy per day of carbohydrate (fructose)	Increased TAG, VLDL-TAG and fasting glycaemia. Unchanged hepatic, adipose and whole-body insulin sensitivity

Kechagias *et al.* 2008	4 weeks of high energy overfeeding of carbohydrate, fat and protein in the form of convenience food in young healthy individuals	Increased weight, serum ALT and hepatic triglyceride content. Maximal ALT/baseline ratio correlated with carbohydrate intake during the second week. HDL cholesterol increased
Le *et al.* 2009	7 days of overfeeding (+35% energy excess) of carbohydrate (fructose) in healthy controls and offspring of T2DM	Increased VLDL-TAGs, IHCLs, IMCLs in healthy controls and offspring of T2DM, IHCL higher in offspring of T2DM
Ngo Sock *et al.* 2010	7 days of overfeeding carbohydrate (fructose and sucrose) in healthy young males	High fructose and sucrose diets increased VLDL-TAGS and IHCL. IMCL increased on the high glucose diet
Sobrecases *et al.* 2010	7 days of high fructose (+35%), high fat (+30%) or high fructose high carbohydrate diets on liver fat	Hyperenergetic diets increased IHCL, highest with combination high fat and carbohydrate followed by high fat and high carb. VLDL concentration increased post overfeeding with fructose, decreased with fat and remained unchanged with the combination of high fructose and fat.

Silbernagel *et al.* 2011	4 weeks of overfeeding with 150g excess carbohydrate (fructose vs. glucose) in lean healthy male and female subjects	Increased TAG in fructose group; but liver, visceral, subcutaneous abdominal fat and IMCL unchanged
Van Herpen *et al.* 2011	3 weeks high fat isoenergetic diet (15%protein, 30% CHO and 55% fat) for 3 weeks in overweight men.	IHL increased by 17%, IMCL and peripheral insulin sensitivity unaffected, reduced metabolic inflexibility. Plasma parameters insulin, free fatty acids, high-sensitivity C-reactive protein, and liver enzymes and body weight were unaffected by diet.
Brons *et al.* 2011	5 days high energy (+60% energy)high-fat (+50%) in healthy men	Hepatic glucose production and fasting glucose levels increased significantly in response to overfeeding. Peripheral insulin action, muscle mitochondrial function, and general and specific oxidative phosphorylation gene expression were unaffected.
Sevastianova *et al.* 2012	3 week high energy carbohydrate feeding (1000kcal/day, 98% energy) in healthy overweight subjects	Liver fat increased by 27% above baseline of 9.2% with significant increase in DNL.

Yu *et al*. 2013	10 wk overfeeding of sucrose or high fructose corn syrup equivalent to 25th, 50th and 90th percentile in 138 subjects	Weight and total energy intake increased. There was no difference in waist circumference, fat mass, liver or muscle fat. There was an increase in triglycerides but not cholesterol levels.
Johnston *et al*. 2013	Effects of 2 weeks of isoenergetic vs. hyperenergetic fructose vs. glucose on liver TAG, insulin sensitivity and liver biochemistry	No difference between high-fructose and high-glucose diets on liver triacylglycerol or biochemistry in healthy overweight men.

1.9 Carbohydrate and fat overfeeding diets

Studies comparing carbohydrate vs. fat overfeeding are scarce. When healthy men with normal body mass index were fed either a hyperenergetic (30-35% excess energy from fructose or fat) or 65% excess energy from fructose and fat) for a period of 4-7 days, intrahepatocellular lipid (using ^{1}H MRS imaging) increased with all diets, but more so with fat overfeeding (+86% compared to fructose, +16%). Interestingly subjects were only overfed fat for 4 days compared to a week in the fructose group. Fasting glycaemia remained unchanged whilst serum insulin tended to increase in all groups. Hepatic glucose production remained unchanged (Sobrecases *et al.* 2010).

Another pertinent study examined the effect of subjects overfed isoenergetic amounts (+50% above energy requirements) of fat or carbohydrate on energy expenditure for 2 weeks. The study showed that carbohydrate overfeeding produced progressive increases in carbohydrate oxidation and total energy expenditure resulting in 75-85% excess of energy being stored, whilst fat overfeeding had minimal effects on fat oxidation and total energy expenditure resulting in greater fat storage of 90-95% excess energy (Horton *et al.* 1995). This suggests that fat overfeeding results in greater fat accumulation than carbohydrate excess. This is in concert with other studies showing a similar result (Flatt *et al*. 1985, Acheson *et al.* 1988, Schutz *et al.* 1989, Bennett *et al.* 1992).

In summary, a number of mechanisms have been implicated in association with development of insulin resistance, from well-established Randle's fatty-acid glucose cycle to relatively recent hypotheses concerning accumulation of lipid metabolites and partially oxidised lipid by-products that may interfere with insulin signalling pathway. Intravenous lipid infusion has been shown to induce insulin resistance, providing an ideal condition for investigating insulin-resistant states without the influence of confounding co-existent factors. High fat

diets appear to modulate insulin sensitivity depending on the type of fatty acids and composition ingested that may be as important as lowering total fat consumed. It would appear that certain fatty acids are more likely to undergo oxidation in skeletal muscle and lipid metabolites such as ceramide and DAG can exert a negative effect on insulin sensitivity. High carbohydrate diets seem to increase hepatic triglycerides particularly in overweight and obese humans but do not appear to have an effect on insulin resistance, probably influenced by the timescale and small numbers investigated in these trials.

Another significant association with development of insulin resistance is that of ageing and shall be discussed in the next section.

1.10 Ageing and insulin resistance

Ageing- related insulin resistance deserves particular attention given the rising ageing population and its association with diabetes and the metabolic syndrome.
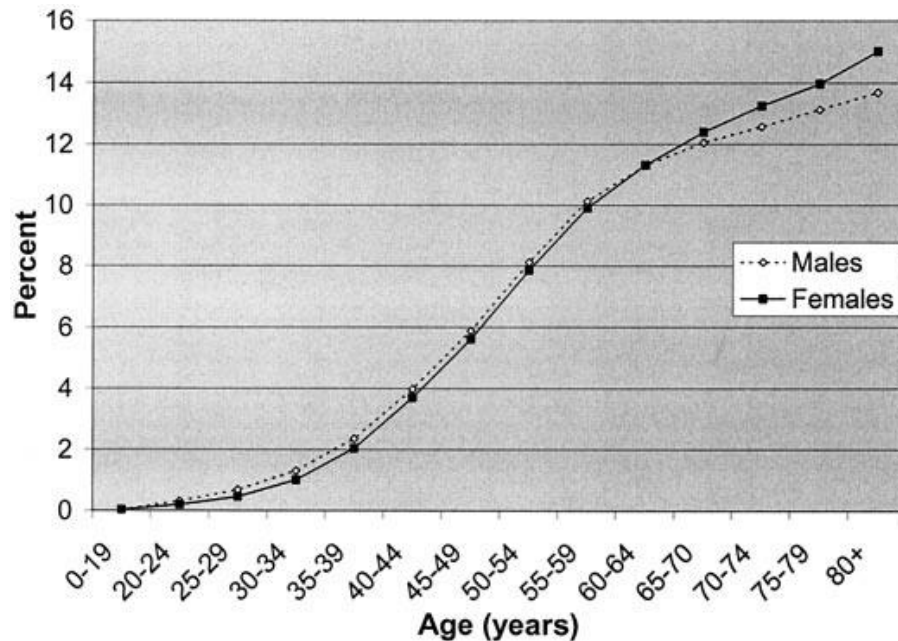
Figure 1.7: Global diabetes prevalence by age and sex for year 2000. Figure taken from Wild, S *et al.* Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27(5): 1047-1053.

A report published in 2004 estimated diabetes prevalence for all age-groups to rise by 1.6% and the number of people with diabetes projected to more than double in 2030. A striking observation from this study was that the increase in the number of people > 65 years has become the major contributor to global demographic change in diabetes prevalence (Wild, Roglic *et al.* 2004).

An inevitable consequence of ageing and undeniably the most significant contributor to development of T2DM, cardiovascular disease and metabolic syndrome seems to be an increasing risk of the development of insulin resistance. Impaired glucose tolerance and

development of T2DM is more common in older persons, moreover the Third National Health and Nutrition Examination Survey (NHANES III) found that the prevalence of impaired glucose tolerance, impaired fasting glucose and diabetes mellitus increases with advancing age; T2DM was evident in >20% while impaired glucose tolerance was found in another 20% of those between 60 and 74 years of age (Harris, Flegal *et al.* 1998). It is well established that when methods of assessing insulin sensitivity was compared in old and young, there appears to be a clear negative correlation of insulin sensitivity and increasing age. Table 1.2 presents studies supporting these observations. A comprehensive review by Davidson in the 1970s reported at least 60 papers using the oral glucose tolerance method and demonstrated a progressive deterioration in oral glucose tolerance as the population aged (Davidson 1979).

Table 1.2: Studies supporting an association between age and decline in insulin sensitivity utilising various methods of assessing glucose uptake.

Authors	Study	Method	Results
DeFronzo R 1979	19 young subjects (26±1 year) vs 17 older subjects (64±2 yrs)	Hyperinsulinaemic euglycaemic clamp	Amount of glucose infused (M) to maintain euglycaemia was significantly higher in young than older subjects. M (amount of glucose)/I (insulin concentration) (x 100) ratio was higher in young vs older subjects.

Refaie *et al.* 2006	40 healthy non-obese older men (mean age 65 +/-4.8 years vs 30 young and 40 T2DM subjects	IV GTT Insulin sensitivity index	Lower fasting insulin, insulin sensitivity index,insulin resistance index,HOMA-IR, QUICKI, second phase insulin response,fractional insulin clearance in old vs young
Amati *et al.* 2009	7 endurance trained young athletes vs 12 older athletes vs 11 young normal weight vs 10 normal weight older vs 15 young obese vs 15 older obese	Hyperinsulinaemic euglycaemic clamp	Higher glucose disposal rates regardless of age in athletes followed by normal weight and obese individuals
Karakelides *et al.* 2010	12 young lean, 12 young obese, 12 elderly lean vs 12 elderly obese	Hyperinsulinaemic euglycaemic clamp	Lower insulin sensitivity in older people thought to be secondary to age-related increase in obesity

The effect of age on insulin secretion remains a matter of debate, likely due to differing methods of assessing glucose disposal in the elderly and generally few studies that focus on insulin sensitivity in this age group. There's the pertinent question of whether it is 'age' per se or other factors associated with ageing that ultimately affects insulin sensitivity. It is well established that ageing is associated with glucose intolerance and increasing insulin resistance (Davidson 1979, DeFronzo 1981, Morley 2008). Both fasting and post-prandial glucose concentrations are generally higher in the elderly leading to compensatory hyperinsulinaemia. The mechanisms underlying age and reduced insulin sensitivity are not completely understood, although factors associated with ageing may likely contribute to impairing glucose tolerance in the older population. Insulin resistance may not be a direct consequence of aging per se but due to obesity (Kelley, Goodpaster et al. 1999) and physical inactivity. (Amati, Dube et al. 2009) Early studies seem to point to a post-receptor defect in target insulin function (Rowe, Minaker et al. 1983). In recent years several causes that have been implicated include changes in body composition such as increasing fat mass (Schutz, Kyle et al. 2002),(Amati, Dube et al. 2009), together with accelerated decline in skeletal mass and strength (Baumgartner, Wayne et al. 2004), and accumulation of lipid in muscle (IMCL) (Goodpaster, He et al. 2001). In addition an increasing sedentary lifestyle seen in the elderly attenuates muscle use, leading to atrophy and promotes weight gain (Stenholm, Harris et al. 2008), in line with sarcopenia (Zamboni, Mazzali et al. 2008). Other metabolic perturbations in ageing include abdominal obesity, unrestrained hepatic gluconeogenesis, adipose lipogenesis, defective glycogen synthesis and glucose uptake in skeletal muscle (Morley 2008). An increase in pro-inflammatory cytokines derived from age-associated accrual of visceral fat and increasing senescent cells are also known to interfere with insulin action (Sepe, Tchkonia et al. 2011). As part of the normal biology of ageing, lipid accumulates in muscle. Compared to younger individuals IMCL content is increased by up to

40% in older people, as seen on ^{1}H NMR (Petersen, Befroy *et al.* 2003). Mitochondrial activity and oxidative phosphorylation was reduced in elderly insulin resistant individuals. In particular intermediate lipid and metabolites associated with IMCL such as ceramides, DAG and acyl-coA cause insulin resistance by activating key proteins that antagonise insulin-responsive and signalling pathways (Savage, Petersen *et al.* 2007), therefore accumulation of lipid in ageing is correlated with skeletal muscle insulin resistance (Dube, Amati *et al.* 2008) Lipid accumulation also arise from a reduction in mitochondrial quantity and function (Hoeks and Schrauwen 2012) resulting in decreased fatty acid oxidation rate (Petersen *et al.* 2003). The association between impaired mitochondrial function and how it might affect insulin-signalling is complex and is thought to involve incomplete beta-oxidation of fatty acid substrates in liver and muscle.
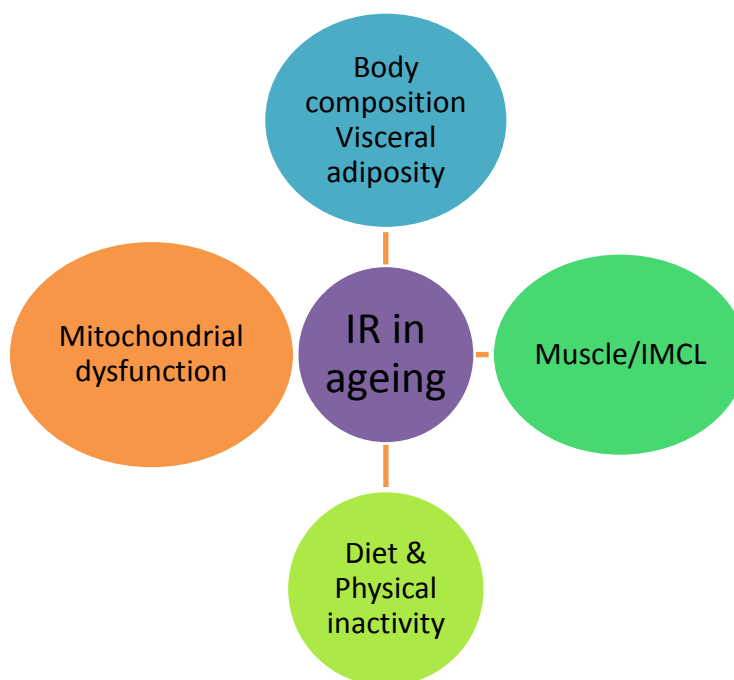
Figure 1.8: Factors contributing to insulin resistance in ageing. IR=Insulin resistance, IMCL=intramyocellular lipid

The factors contributing to insulin resistance in ageing are most probably multifactorial and changes seen to accompany ageing have to be considered as playing an influential role.

1.10.1 Visceral adiposity

A common feature of ageing is increased visceral fat (sum of fat depots within visceral cavity) (Folsom *et al.* 1993), a risk factor for insulin resistance and correlates strongly with insulin sensitivity in human and animal models (Atzmon *et al.* 2002). Putative mechanisms underlying visceral fat and its association with insulin resistance include increased portal release of FFAs (Muzumdar *et al.* 2008) and/or abnormal expression and secretion of fat-derived peptides such as leptin, ACRP30, and inflammatory cytokines eg TNF-α. Surgical removal of visceral fat in a young rat model improved hepatic insulin action, and was reproduced in older rat models (Muzumdar *et al.* 2008). In animal models at least, selective intra-abdominal depots (perinephric and epididymal) appear to modulate insulin action and glucose tolerance.

1.10.2 Sarcopenic obesity

An inevitable consequence of advancing age is the decline in muscle mass and strength (Morley *et al.* 2001) and this appears to accelerate from around the fourth decade at a rate of just under 1% per year (Zamboni *et al.* 2008, Stenholm 2008). Muscle strength appears to decline three times faster than mass, which suggests changes within muscle associated with age or physical inactivity: accumulation of fat, structural proteins and mitochondrial dysfunction (reduction in mitochondrial numbers, morphology change, oxidative damage, reduced oxidative capacity and increased DNA mutations and oxidative stress and damage (Jensen *et al.* 2001).

1.10.3 Mitochondrial content

The reduction in mitochondrial content and activity with ageing (Hoeks and Schrauwen 2012) may contribute to the development of insulin resistance through the observation that muscle of older healthy people contained increased muscle fat and a reduction in mitochondrial oxidative phosphorylation activity. Non-invasive magnetic spectroscopy demonstrate a 40% reduction in resting muscle mitochondrial tricarboxylic acid (TCA) cycle flux and ATP synthesis rate in lean insulin-resistant elderly subjects (Petersen *et al.* 2003). However there is emerging evidence to suggest that although mitochondrial dysfunction and insulin resistance occurs concurrently, this association may occur coincidentally or through a common factor such as physical inactivity (Lanza *et al*. 2009).

1.10.4 Intramyocellular lipid

A strong association has been demonstrated between ageing, IMCL accumulation in muscle and skeletal muscle insulin resistance. Indeed whilst the physiological role of IMCL was once a topic of interest solely associated with exercise physiology, it has become increasingly important since the finding of an association with insulin resistance was found. This association and the mechanisms underpinning IMCL and insulin resistance have been touched upon earlier. In relation to ageing, IMCL is increased by up to 40% in the elderly as seen on ^{1}H NMR spectroscopy (Petersen *et al.* 2003). They tended to have larger IMCL droplets, fewer mitochondria and lower proportion of IMCL in contact with mitochondria. These are likely to contribute to reduction in mitochondrial function and lipid metabolism, and thus reduced fatty acid oxidation rates (Crane *et al.* 2010). The underlying cause for the accumulation of IMCL in ageing is not completely understood. It may be because use of muscle (as discussed above) declines with age, however muscle loss and strength are also seen in highly-trained older athletes (Faulkner *et al.* 2007). It could be because there is an

imbalance between lipid supply and proportional fatty oxidation. Despite the accumulation of lipid in muscle of highly–trained endurance athletes, insulin sensitivity was not affected, raising the possibility that capacity of lipid oxidation metabolism is one of the major mediators in the development of insulin resistance.

1.10.5 Physical inactivity

Ageing is often associated with a decline in habitual physical activity and adopting a more sedentary behaviour. In early old age (65-75 years of age), a modest increase in activity may be observed in an attempt to improve health and fill the time resulting from retirement. By middle old age (75-85 years) most would develop some physical disability and by late old age (85 years and above), many would be become physically dependent. However inter-individual variances in functional capacity occur at any given chronological age (Shephard *et al.* 1998). Limiting factors include maximal oxygen consumption, fatigability, muscle loss and strength, metabolic and hormonal responses, risks of exercise and motivation. Maximal oxygen uptake declines by about 5ml per kg per min per decade from 25 to 65 years, with possible acceleration thereafter. It is uncertain whether this is an inevitable consequence of the ageing process per se or reduced physical activity that occurs with advancing age. Age-related decline in aerobic capacity may be caused by decrease in maximal heart rate, stroke volume and arterio-venous difference. Increase accumulation of fat mass, IMCL, reduction in physical activity therefore increases the risk of development of insulin resistance.

1.11 Mechanisms of insulin resistance in ageing

The exact underlying mechanisms of insulin resistance in ageing are mostly unknown. Early studies described receptor and post-receptor perturbations in insulin action (Rowe *et al.* 1983, Lonnroth and Smith 1986). Despite the maximally-stimulatory effect of insulin concentration, glucose disposal was reduced in the elderly, confirmed with hyperinsulinaemic euglycaemic

clamps (1200 mU/m^2/min) causing a rightward shift in the insulin-action dose-response curve (Fink and Kolterman 1983).

Molecular mechanisms underlying insulin resistance in ageing was studied at the level of the insulin signalling cascade in rats in response to in-vivo insulin infusion (Ropelle *et al.* 2013). White adipose tissue of aged rats showed altered subcellular distribution of insulin receptors and IRS-1 and a reduction in insulin-stimulated IR tyrosine phosphorylation. Activation of Akt and GLUT-4 translocation to the plasma membrane was impaired. Muscle from rats also exhibited a defect in GLUT-4 trafficking; however insulin signalling at IR and Akt was increased. No difference was seen in liver, indicating that IR in adipose tissue precedes development of insulin resistance in liver and skeletal muscle.

Of the molecular mechanisms implicated in the development of insulin resistance in the elderly, one of the more robust mechanistic evidence associated with negative regulation of insulin action appears to be stimuli that activate inflammatory serine/threonine kinases eg c-Jun NH$_2$-terminal kinase and inhibitor of κb kinase. These stimuli include over-nutrition (particularly lipids), oxidative and endoplasmic reticulum stress (Samuel and Schulman 2012, Evans *et al.* 2012, Hotamisligil 2003, 2010) and are linked to reduced mitochondrial content and/or function predisposing to accumulation of IMCL. IMCL appears to be increased in ageing (see below) that in turn promotes production of toxic lipids as discussed earlier.

Ageing has also been associated with increased nitric oxide production as a result of inducible nitric oxide synthase (iNOS). iNOS is increased in macrophages and inflammatory cells, stimulated by proinflammatory cytokines and has been implicated in insulin resistance in the context of obesity (Kaneki *et al.* 2007). Recent research (Ropelle *et al.* 2013) report that ageing mice exhibited increased iNOS expression and S-nitrosation of the insulin

receptor IRS-1 and Akt/PKB leading to insulin resistance. More studies should be conducted to substantiate similar findings in humans.

1.12   Improving insulin sensitivity

Diet and lifestyle is one of several factors that can entirely reduce or even prevent the development of diabetes. Several randomised controlled population studies can attest to this, including the Da Qing Study, Finnish Diabetes Prevention Study and U.S. Diabetes Prevention Program.

The Da Qing Study was conducted in a province of China involving 6 years of active intervention, where diabetes risk was reduced by 31, 46, and 42% in the diet-only, exercise-only, and diet-plus-exercise groups, respectively, compared with the control group (Pan, Li et al. 1997). The intervention proved effective in the long-run assuming the intervention was adhered to as in a subsequent 14-year follow-up study, the intervention groups had a 51% lower risk of diabetes during the active intervention period compared to control, and a 43% lower risk over a 20-year follow-up. (Li, Zhang et al. 2008)

In both the Finnish Diabetes Prevention Study (Lindstrom et al. 2003) and the U.S. Diabetes Prevention Program (DPP), lifestyle intervention significantly reduced diabetes incidence by 58% (Knowler, Barrett-Connor et al. 2002).

1.11.1 Diet

As weight gain and adiposity are strongly associated with insulin resistance (McAuley and Mann 2006) most dietary plans proposed in improving insulin sensitivity focus on promoting weight loss and remain one of the main principles in managing people with T2DM.

The American Diabetes Association (ADA, 2011) published nutritional recommendations for treatment of individuals with T2DM and subjects at high risk of developing diabetes that include:

1) Weight loss of at least 7% in overweight/obese individuals

2) Restriction of intake of saturated fats to < 7% of energy intake

3) Cholesterol intake of <200mg/day including reduction of trans-fat intake

4) High-fibre intake of > 14g/1000kcal

5) Protein intake is no longer restricted, providing 15-20% of energy as long as renal function is normal.

Various dietary concepts on modification of macronutrients have been used and are discussed below. It has to be borne in mind that some of these studies are short-term and warrant a longer period of study under controlled settings to appreciate meaningful outcomes and adherence to diets. The latter is key to maintaining weight loss, but often difficult to achieve and depends on the practicality, sustainability and palatability of the diets proposed and motivation of the individual.

1.11.1.1 Low-energy diets

Energy-restricted dietary intakes have been proven to reverse pancreatic cell failure and hepatic insulin resistance associated with T2DM (Lim *et al*. 2011). Short term very low calorie diet (VLCD) has also been shown to cause a significant reduction in IMCL and an increase in insulin sensitivity in the absence of substantial changes in total body fat in people with and without T2DM (Lara-Castro *et al.* 2008).

1.11.1.2 Low-fat diets

Short-term low-fat intervention diets appear to lead to weight loss in overweight individuals (Astrup, Grunwald *et al.* 2000); however these diets appear to have no more an advantage than other energy-restricted diets in maintaining weight loss in the long-term. Low-carbohydrate non-energy restricted diets appear to be at least effective as low-fat non-energy restricted diets in promoting weight loss for up to a year. Furthermore adherence to a low-fat diet particularly in insulin-resistant individuals is difficult and particularly challenging (McClain, Otten *et al.* 2013).

1.11.1.3 High fibre diets

The beneficial effects of diets high in fibre is well-known and is regarded an important recommendation in nutritional guidelines. Fibre slows gastric emptying and absorption of dietary fat and carbohydrate contents attributed to the viscous water soluble types of dietary fibre (Weickert and Pfeiffer 2008). However data on fibre's effects are inconclusive, with some only showing moderate effects on weight loss (Howarth, Saltzman *et al.* 2001).

Of the different fibres guar gum has received the most attention but its effect on insulin resistance is equivocal, varying amongst different populations. No effect was seen in obese individuals but a reduction in insulin levels was seen in T2DM patients.

1.11.1.4 Low glycaemic index (GI) diets

Glycaemic index (GI) is a measure of the ability of carbohydrate in a particular food to raise glucose levels defined by the incremental area under the glycaemic response curve (AUC) (Wolever, Mehling et al. 2008). Low-GI and/or low-glucose load diets may reduce the risk of metabolic syndrome, T2DM, cardiovascular disease and chronic inflammation. In contrast, carbohydrates high in GI lead to increases of postprandial glucose and insulin concentrations

that may compromise metabolic flexibility (Brand-Miller, McMillan-Price et al. 2009; Isken, Klaus et al. 2010). Overweight or obese subjects on short duration low glycaemic index (GI) diets lost more weight and have better improvement in lipid profiles than controls receiving other diets (Thomas, Elliott *et al.* 2007). However other findings have been inconclusive, most likely due to small study samples and short duration of these studies. In one of the largest intervention studies published to date, weight regain at 1 year was only marginally lower with a reduction of the GI (Larsen, Dalskov *et al.* 2010). In a meta-analysis, 2 servings per day of whole-grain intake were associated with a 21% decrease in insulin resistance risk. It is likely that diet only will not entirely prevent development of diabetes, but could work in conjunction with other factors such as exercise, as will be discussed in more detail in the next subsection.

1.11.1.5 Micronutrients

Evidence has shown that micronutrients such as magnesium (Hua *et al.* 1995, Lima *et al.* 1998), zinc (Chen *et al.* 1991, Singh *et al.* 1998), chromium (Thomas and Groper 1996, Anderson *et al.* 1997), L-carnitine (Heller *et al.* 1986, Mingrone *et al.* 1999) and fish oils (Bathena *et al.* 1991) can positively influence insulin sensitivity, however results are often equivocal and require larger, well-designed human studies. The amino acid L-carnitine will be discussed in greater detail as a possible contributor to insulin sensitivity (Chapter 5).

1.11.2 Exercise

It has become well established that physical activity and exercise training are effective means of increasing insulin action in muscle of insulin resistant individuals and have been proven to reduce the risk of development of diabetes. As previously described, the Da Qing IGT and Diabetes Study demonstrated that exercise intervention (20 min of mild or moderate, 10 min of strenuous, or 5 min of very strenuous exercise one to two times a day) reduced the

incidence of diabetes by 46% in people classified as having impaired glucose tolerance (IGT) more than diet and exercise (42%) and diet alone (31%) (Pan, Li *et al.* 1997).

Briefly, in the early stages of exercise and with increasing exercise intensity, fuel for muscles is provided for predominantly by glycogen. When this becomes depleted blood glucose and free fatty acids from adipose tissue become the primary source for fuel utilisation (Bergman, Butterfield et al. 1999). Intramuscular lipid stores are mostly used during longer duration activities (Borghouts, Wagenmakers et al. 2002).

Exercise training induces improvements in insulin action by:

(1) up-regulation of GLUT4 expression and facilitation of insulin signal transduction

(2) chronic activation of AMPK

(3) promoting mitochondrial biogenesis and increasing lipid oxidation and turnover thereby preventing the accumulation of deleterious lipid species.

1.11.2.1 Chronic exercise

Although a single bout of exercise has been shown to improve insulin sensitivity in previously sedentary adults (Devlin, Hirshman *et al.* 1987) and increase glucose uptake by up to 40% (Perseghin, Price *et al.* 1996), these effects only last for 48-72 hours post-exercise (King, Baldus *et al.* 1995). Regular long-term exercise has been shown to exert chronic effects on insulin sensitivity. Aerobic exercise at all intensities over a period of weeks has been shown to improve insulin sensitivity, enhance the responsiveness of skeletal muscle to insulin with increased expression and/or activity of proteins involved in glucose metabolism, insulin signalling and fat oxidation capacity. Endurance exercise induced an adaptive increase in the GLUT-4 protein concentrations, activities of both glycogen synthase and hexokinase, the enzyme that phosphorylates glucose of the glucose transporter in skeletal muscle

(Holloszy 2005). Despite the presence of increased intramuscular triglycerides (IMTG) in highly-trained athletes, the observation of a paradoxical increase in insulin responsiveness may be reconciled by the muscles ability to increase lipid turnover (increased uptake, transport, utilisation and oxidation). Following 12 weeks of endurance training, ageing human skeletal muscle that contained higher IMCL content showed improvements in mitochondrial biogenesis and electron transport chain activity (Menshikova, Ritov *et al.* 2006). The same improvements were seen in Bruce *et al.*'s study but involved obese subjects and IMTG content remained relatively unchanged (Bruce, Thrush *et al.* 2006). This would imply that improved fatty acid oxidation and improvement in insulin sensitivity may be driven by greater FFA delivery and uptake and reductions in deleterious lipid metabolites from a greater lipid flux. Bruce *et al.* also showed that following endurance training in obese individuals fatty acid metabolites were also reduced.

Another type of exercise, resistance training, enhances whole-body glucose disposal capacity by increasing muscle mass and strength and even a single resistance exercise training session can improve insulin sensitivity for up to 24 hours after cessation of exercise (Koopman *et al.* 2005). These benefits are possibly partly attributed to reductions in IMTG stores. In randomised controlled trials (RCTs), resistance or aerobic training for 10 - 16 weeks in men with newly diagnosed or established T2DM improved insulin action, blood glucose control and HbA1c values and resulted in significant losses in visceral fat and improved fat oxidation (Ibanez *et al.* 2005; Bweir S *et al.* 2009).

Exercise training may improve insulin sensitivity indirectly through its effect on lipids. Goodpaster *et al.* 2003 postulated that the strongest predictor of insulin sensitivity in obesity following endurance training is enhanced whole-body fat oxidation. Increased oxidative capacity following exercise training is associated with increased CPT-1 activity and decreased ceramides and DAG in muscle of obese individuals (Bruce *et al.* 2006).

Collectively this suggests that exercise training may improve insulin sensitivity by increased lipid oxidation, therefore preventing accumulation of lipids that may interfere with insulin signalling pathways. Four weeks of exercise training was shown to attenuate effects of a high-fat diet on muscle lipid storage and was associated with increased palmitate oxidation and elevated PGC-1 expression (Lessard *et al.* 2007). Despite the 'metabolic inflexibility' and reduced fatty acid oxidation at rest commonly seen in insulin-resistant individuals, they have been shown to readily utilise lipids during exercise. Obese sedentary individuals with abdominal adiposity were shown to have increased fatty acid oxidation rates during submaximal exercise compared to lean sedentary exercised controls (Goodpaster *et al.* 1999). Thus it would appear that the molecular/insulin signalling events that accompany contraction during exercise can override metabolic flexibility and predispose to increased fat oxidation.

CHAPTER 2: METHODS

2.1 Common Methods

Methods common to all studies undertaken are described as follows.

2.1.1 Ethical approval

Ethical approval for studies carried out and described in this thesis was obtained from the University of Nottingham Medical School Research Ethics Committee.

Study 1 Comparing skeletal muscle lipid, fat metabolism and insulin sensitivity in older vs. young healthy men- medical ethics approval number E13102011BMS

Study 2 Investigating the effects of lipid on amino acid and insulin sensitivity- medical ethics approval number F131020122BMS

Study 3 Investigating the effects of hyperenergetic high fat vs high carbohydrate diet on liver fat and insulin sensitivity- medical ethics approval number B14022013BMS

Study 4 Investigating the effects of carnitine on improving skeletal muscle insulin sensitivity in healthy ageing - medical ethics approval number E13102011BMS

All volunteers who participated in these studies were informed of risks associated with experimental procedures before obtaining informed written consent. All studies were performed in accordance with the Declaration of Helsinki of the World Medical Association (World Medical 2013).

2.1.2 Pre-screening procedure

Volunteers were medically screened and completed a health questionnaire, blood screen of full blood count, urea and electrolytes, liver function tests (including Hepatitis B and C viral screen and ferritin for Study 3), coagulation, lipid profile, thyroid function test and

electrocardiograph (ECG). Subjects were excluded if they had a history of diabetes, cardiovascular, metabolic or respiratory disease. Those who did not meet inclusion criteria were excluded from further participation.

2.1.3 Randomisation of volunteers

Randomisation of volunteers in these studies was done using randomisation codes generated electronically (www.randomisation.org). Studies 1, 2 and 3 were partially blinded randomised trials whilst study 4 was a double-blinded randomised trial. The intervention or placebo groups for study 4 were revealed once data collection was complete. Magnetic resonance imaging (MRI) operators involved with scanning the volunteer's liver and investigators analysing liver fat in study 3 were blinded to the groups receiving either high carbohydrate or fat diets.

2.1.4 Blood sample collections

Blood samples at screening were taken via venepuncture into the appropriate Vacutainer blood collection tubes. In all studies arterialised venous blood were obtained by inserting a cannula (Venflon, Ohmeda, Sweden) retrograde into a vein of the dorsum of the hand, warmed in a device with static air heated at 55^0C. The heated hand technique has been proven under experimental conditions to provide a safe and practical approach to an otherwise difficult and less feasible way of sampling blood from arteries (Gallen, Macdonald 1990). In previous studies blood chemistry values of pH, pCO_2, pO_2 and O_2 saturations of blood sampled from a heated hand vein were consistently similar to that in the arterial range (Liu *et al.* 1992). For purposes of sampling in metabolic studies, compared to true arterial blood, 'arterialised' or heated venous blood sampling provided no significant differences in concentrations of glucose, non-esterified fatty acids, insulin, glucagon and several amino acids (Morris, Ueda *et al.* 1997). With pertinence to the sampling of blood during the

hyperinsulinaemic euglycaemic clamp mean glucose concentrations in 'arterialised' venous blood and arterial blood have been shown to produce a difference of only 0.1 mmol/L under basal and hyperinsulinaemic conditions (Liu *et al.* 1992).

A critique of using the hand-heated device in studies presented in this thesis is that the temperature of the environment in the device was not routinely measured during the studies and it can only be assumed that the temperature remained constant (50-55°C) from study to study to avoid inter-visit variability. Other perturbations that contribute to this include the volunteers having their hands out of the device during toilet breaks. Hand-warming has been shown to effectively arterialise venous blood and gives significantly higher insulin sensitivity values but induces systemic haemodynamic effects that may affect measurements of insulin sensitivity (Morris, Ueda *et al.* 1997).

Figure 2.1: A subject having his left hand warmed in the hand heated device during the hyperinsulinaemic euglycaemic clamp.

2.1.5. Blood samples analyses

Blood obtained for plasma analyses was spun at 3.3G at 4°C for 10 minutes. Plasma was divided into aliquots and frozen at -80°C until analysis. Serum samples were left to clot for at

least 30 minutes before being spun and frozen also at -80°C. The following assays were performed by technicians at the High Performance Liquid Chromatography (HPLC) laboratory of the Metabolic Physiology Group, School of Life Sciences, University of Nottingham: serum insulin, non-esterified fatty acid (NEFA), lactate, adrenaline and noradrenaline (studies 2 and 3) and glucagon, C-peptide, β- hydroxybutyrate, leptin and cytokines (Study 2).

Measurements of plasma to determine enrichment and isotope ratios of tracers $[6,6-^2H_2]$ glucose (Study 3), 2-Deoxy-D-Glucose (2DG) and $[U-^{13}C]$ Palmitate (Studies 1 and 4) were analysed by researchers and technicians at the Human Physiology Labs at Derby Medical School, University of Nottingham.

In all studies utilising the hyperinsulinemic euglycaemic clamp, arterialised blood glucose concentrations were measured using the Yellow Springs Instrument 2300 Stat Analyser (YSI; YSI Inc, Yellow Springs, Ohio, USA) every 5 minutes. The YSI uses steady-state measurement methodology whereby the membrane-based immobilised glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (Chua and Tan 1976). The difference between the sample generated plateau current and the initial baseline current is proportional to the glucose concentration (YSI STAT 2300 Laboratory manual 5-4).The YSI 2300 STAT uses an aqueous rather than a serum or plasma –based standard. On injection of a test sample into the YSI, the following reactions take place:

1) D-Glucose + oxygen   (glucose oxidase) $\longrightarrow$ gluconolactone + H_2O_2 oxidase
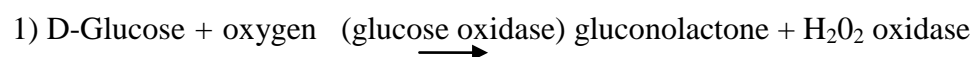
2) The H_2O_2 sensitive electrode oxidizes a constant portion of the H202 at the platinum anode: $H_2O_2 \longrightarrow 2H^+ + 2e^- + O_2$.
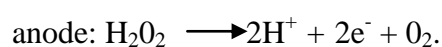
3) The circuit is completed by a silver cathode at which oxygen is reduced to water: $4H + O_2 \longrightarrow 2H_2O + 4e^-$

The YSI was found to have good intra and interbatch precision with a coefficient variation of 1.2% or less for glucose concentrations of 0.94 to 3.98g/litre (5.2 – 22.1mmol/L) and 5.8% or less for glucose concentrations of 0.29 to 2.91 g/litre (1.6-16.2 mmol/L) respectively. It is simple to use, analyses are quick and requires only small samples (Chua and Tan 1978). Determination of glucose using the YSI correlates well with Beckman's Glucose Analyser (r=0.997) and the Haemacue device (r= 0.979).

2.1.6 Radioimmunoassay

The radioimmunoassay method as described by Yalow and Berson 1968 was used to determine serum insulin and glucagon. This technique involves making a known quantity of antigen radioactive by labelling it with it with gamma-radioactive isotopes of iodine, such as ^{125}-I, attached to tyrosine.

2.1.7 Serum Insulin assays

Serum insulin aliquots were frozen at a minimum of $-20^{\circ}C$ and measured using a solid-phase ^{125}I radioimmunoassay using standard insulin kits.

2.1.8 Non-esterified fatty acid assays

FFA was measured using an automated immunoassay analyser and commercially available kit. Tubes used to collect blood were pre-filled with 7.5ul per ml plasma of ethylene glycol tetraacetic acid (EGTA)-Gluthathione, a preservative to prevent degradation of FFAs during storage. 10uL of Tetrahydrolipstatin (THL) was added to vials to inhibit *in vitro* lipolysis and prevent falsely high plasma FFA readings, thus increasing accuracy of determining plasma FFA concentrations (Krebs M *et al.* 2000).

2.1.9 Glucagon assays

Blood samples for glucagon were collected into tubes containing aprotinin, a protein bovine pancreatic trypsin inhibitor used for rapidly-degraded proteins and stored in glass tubes at -80°C until analysis. Total glucagon concentrations were determined using solid-phase ^{125}I radioimmunoassays as described above.

2.1.10 β-Hydroxybutyrate (β-OHB) assays

Serum βOHB and lactate were measured using an automated immunoassay analyser and commercially available kits.

2.1.11 Hyperinsulinaemic euglycaemic clamp

The glucose clamp technique as described by DeFronzo *et al.* (DeFronzo, Tobin *et al.* 1979) remains the gold standard method of directly determining metabolic insulin sensitivity in vivo, particularly when the assessment of insulin sensitivity is of primary interest and feasibility is not an issue. Under steady conditions it has a coefficient variation/reproducibility of 0.1 and a discriminant ratio of 6.4 (measure of reproducibility and ability to distinguish individual results) (Mather *et al.* 2001). To assess an individual's glucose disposal, a hyperinsulinaemic state is induced via intravenous infusion of insulin based on body surface area is achieved resulting in increased skeletal muscle and adipose tissue glucose disposal and suppressed hepatic glucose production. 20% glucose is infused at variable rates aiming to achieve euglycaemia (an arbitrary predetermined level of glycaemia within normal range of glycaemia, 4.5mmol/L was chosen for studies presented in this thesis). The glucose infusion rate (GIR) is thus equal to glucose disposal rate (M) under the assumption that hyperinsulinaemia is sufficient to completely suppress hepatic glucose production and there is no net-change in glucose concentrations under steady state conditions.

The method has been shown to be reproducible in subjects at high (insulin infusion rate 40mU/m^2/min; concentration 100µunits/ml) (De Fronzo, Tobin *et al.* 1979), mid-(60-70µunits/ml) (Soop *et al.*, 2000) and low (insulin infusion rate 20 mU/m^2/min; concentration 38µunits/ml) (Kingston, Livingston *et al.* 1986) physiological ranges of hyperinsulinaemia. M is typically normalised to body weight or fat free-mass. Lean body mass was determined from Dual energy x-ray absorpmetry (DEXA) (apart from Study 2 where fat-free mass was determined via skin calipers and Bioelectric Impedance Analysis (BIA) were used in estimating glucose disposal rates of volunteers in these studies. The validity of insulin sensitivity utilising the glucose clamp technique depends on steady state conditions being achieved. A more reliable approach of determining accurate values for glucose disposal rates is to take a period of at least 30 minutes, more than 1 hour post insulin start during which the coefficient of variation for blood glucose, plasma insulin and GIR is < 5% (KatzA *et al.* 2000).

Under ideal settings insulin infusion rates should be matched to the population studied, thus multiple infusion rates are adjusted according to an individual's insulin resistant or insulin sensitive state in a stepwise fashion. Although desirable, this approach is time-consuming and not feasible for both the operator and subjects (Muniyappa *et al.* 2008). Another significant advantage of the clamp is the ability to simultaneously measure and distinguish hepatic glucose production and peripheral (mostly skeletal muscle) insulin resistance/sensitivity, lipolysis and protein metabolism using radiolabelled tracers. The use of glucose tracers also proves useful at lower insulin infusion rates or in studies in insulin-resistant individuals so appropriate corrections can be made to glucose disposal.

Radiolabelled glucose tracers can also be used simultaneously during the clamp to quantify hepatic glucose production and whole body disposal, 2DG in Studies 1 and 3, deuterated

glucose in Study 2. Other isotope tracers such as phenylalanine may also be used to assess metabolism of glycerol or protein metabolism during the clamp.

A small but important limitation of this method is that it is laborious and time consuming, so may not be feasible for large population or epidemiological studies. It also requires trained personnel who are able to deal with complications related to the clamp. These are generally infrequent, indeed the commonest issue encountered during studies in this thesis was the cannula ceased working temporarily thus several blood glucose samplings were missed.

The clamp should also only be considered if the primary objective is to assess insulin sensitivity, as it is costly. Consideration should also be given to the assumption that levels achieved during steady state are supraphysiological and this may reverse the normal peripheral to portal gradient, so it may not accurately reflect insulin action and glucose dynamics under normal physiological conditions (Muniyappa *et al.* 2008).

The studies presented in this thesis utilised the hyperinsulinaemic euglycaemic clamp for a number of reasons:

1)   Assessment of insulin sensitivity was the primary or one of the primary objectives of each study.

2)   Gold standard technique of assessing insulin sensitivity

3)   Could be used simultaneously with phenylalanine, 2DG and $[6,6^2H_2]$ glucose tracers.

4)   Relatively small group of volunteers undergoing glucose clamp and availability of trained personnel to run the clamp.

Compared to other methods of quantifying insulin sensitivity such as the insulin suppression test, oral glucose tolerance test and Homeostatic Model of Assessment of Insulin Resistance

(HOMA-IR), where only fasting plasma insulin and glucose are required, running an insulin clamp is labour intensive, costly and requires an experienced operator to manage technical difficulties.

2.1.12 Other direct and indirect measures of assessing insulin sensitivity

Table 2.1 Summary of the advantages and disadvantages of other methods of assessing insulin sensitivity.

Test	Method	Advantages	Disadvantages
Insulin suppression test	Somatostatin/ octeotride is infused to suppress endogenous secretion of insulin and glucagon. Insulin and glucose are infused simultaneously for 180mins	Highly reproducible test Applied to larger populations Not as labour intensive or costly as the clamp	Used to determine skeletal muscle insulin sensitivity but does not specifically reflect hepatic insulin sensitivity
Oral Glucose Tolerance Test	75g glucose load Blood glucose and insulin every 30 mins for 120 mins	Mimics glucose and insulin dynamics of physiological conditions, (more so than the hyperinsulinaemic euglycaemic clamp)	Does not assess insulin sensitivity per se

Minimal model Analysis of frequently sampled intravenous glucose tolerance test	5 min infusion of insulin (4mU/kg/min) 20 mins after the IV bolus of glucose. Glucose and insulin blood samples taken at 1, 2, 10 mins then 20 min intervals until 180 mins. Data entered into a minimal model of analysis programme generates an index of insulin sensitivity (SI).	Advantages over the clamp: less labour intensive, steady-state and continuous infusion of insulin and glucose not required. Coefficient of variation of SI as good as clamp	Oversimplifies physiological glucose homeostasis. Estimates of SI are less reliable in individuals with impaired insulin secretion or significant insulin resistance
Simple indices of insulin resistance	1/Fasting insulin Glucose/Insulin ratio HOMA-IR, QUICKI	Simple, inexpensive and minimally invasive, can be used in large epidemiological studies. It is appropriate to be considered where insulin sensitivity is not the primary outcome, direct	Poor correlations with clamp, less predictive of insulin sensitivity in glucose intolerance/diabetes

		measurement of insulin sensitivity is not required or unfeasible to obtain.	

2.1.13 Indirect calorimetry

Figure 2.2: Indirect calorimetry during the hyperinsulinaemic euglycaemic clamp. A plastic hood is placed over the subject's head and connected to the GEM machine that measures VO_2 and VCO_2 which is then used to measure energy expenditure.

One of two ways (the other being direct calorimetry) and by far the most practical of measuring energy expenditure in humans is indirect calorimetry. This approach allows measurement of whole-body consumption of oxygen and production of carbon dioxide using a calorimeter with built-in on-stream analysers. A plastic hood is placed over the subject so air drawn through this canopy by a pump is collected and analysed. For resting studies presented in this thesis, indirect calorimetry was used using the Gas Exchange Machine) GEM (GEMNutrition Ltd, Cheshire, UK).

Indirect calorimetry quantifies resting energy expenditure (REE) or Basal Metabolic Rate (BMR), thus total energy expenditure (TEE) can be calculated using the following equation (Levine JA 2005):

TEE = (REE + diet induced thermogenesis)* activity factor

The REE or BMR is energy expended at complete rest in a post-absorptive state accounting for approximately 60% of TEE in sedentary adults.

Thermic effect of food describes energy derived from digestion, absorption, and storage of food and nutrients; accounting for around 10% of TEE.

The activity factor can account for up to 100% of TEE depending on the degree of activities of daily living and fidgeting.

Indirect calorimetry by gas exchange measurement can accurately predict energy expenditure and poses minimal burden on subjects and costs.

When VCO_2 and VO_2 values are obtained via breathing under the canopy or mask of the gas exchange machine, substrate oxidation rates are measured. Frayn's equation (Frayn 1983) for calculating substrate utilisation includes calculations for urinary nitrogen excretion, and as opposed to the equations below assumes the accumulation and excretion of metabolic intermediaries or end products through the form of ketogenesis, lipogenesis, gluconeogenesis or lactate. For studies utilising indirect calorimetry in this thesis, the non-protein equations of Peronnet and Massicotte (Peronnet and Massicotte 1991) were used, as urine was not routinely collected for measurement of nitrogen excretion.

Carbohydrate oxidation: $4.585 * VCO_2 – 3.226 * VO_2$

Fat oxidation: $1.695 * VO_2 – 1.701 * VCO_2$

Where VO_2 and VCO_2 are expressed in litres per min (L/min) oxidation rate in grams per minute (g/min).

There are few requirements to be observed during use of the indirect calorimetry method. These include an air-tight canopy to provide constant flow of air to provide oxygen and carbon dioxide within a reasonable range, sensitive, stable O_2 and CO_2 analysers for continuous sampling of expired air, a calibration routine using standard gas mixtures and a system to trap or condense out the moisture of the expired air line feeding into sensors. During the course of each study the indirect calorimeter was calibrated several times to correct for any drift in analyser sensitivity. This method can be used concomitantly with other research methods such as hyperinsulinaemic euglycaemic clamping and tracer methodology. Indeed indirect calorimetry was carried out during infusion of the tracers 2- deoxy-D-glucose, $[6,6-^2H_2]$ glucose and $^{13}C_6$ palmitate. It is important to take into account that indirect calorimetry estimates whole-body rates of substrate oxidation whilst most tracer techniques calculate blood or plasma turnover rates.

However achieving perfect conditions during indirect calorimetry measurements can be challenging, and certain conditions can contribute to errors in data acquisition. Clearly operator expertise and volunteer compliance help to minimise errors. Restlessness, hyper or hypoventilation and other perturbations may impinge on the assumed VO_2 and VCO_2 data derived from metabolic events.

2.1.14 Muscle Biopsies

Bergstrom's percutaneous muscle biopsy technique provides a simple and reproducible method of obtaining muscle samples (Bergstrom 1975). When handled correctly and appropriately, this method of sampling is quick, relatively atraumatic and well-tolerated. It also avoids the use of general anaesthesia, reduces scarring and the need for repeated biopsies (Goldberger, Henry *et al.* 1978; Edwards, Young *et al.* 1980; Edwards, Round *et al.* 1983), With suction applied to the biopsy needle whilst doing biopsies, the reliability of obtaining adequate amounts and size of tissue is increased. (Greig, Askanazi *et al.* 1985; Tarnopolsky, Pearce *et al.* 2011). Muscle tissue is obtained 10-15cm above the patella on the vastus lateralis or quadriceps which is the preferred site of biopsy (other than gastrocnemius (calf muscle), deltoid and biceps (arms).

Briefly once the volunteer has consented to the procedure, the location where muscle is to be obtained is identified and marked. The vastus lateralis muscle is located by measuring a spot 16cm above the patella bone, then 4-5 cm lateral. The volunteer is asked to tense the thigh to confirm the site. After the area is cleaned with betadine solution, 1 ml of 1% Lidocaine is injected just under the skin. A further 5-10mls of Lidocaine is injected intramuscularly, carefully avoiding blood vessels. After 1minute a scalpel is introduced into the anaesthetised area making a 2 cm incision after which firm pressure using gauze is applied immediately to the incision site. After about 2-3 minutes, a size 5 or 6 Bergstrom needle (size corresponds to diameter size of the window at the tip of the needle) is inserted into the incision, a further firm pressure is applied with the needle until a 'give' is felt. This corresponds to cutting through the tough fascia layer of muscle. The introducer needle is released and pushed into the needle holder a few times to 'snip' muscle, whilst suction connected to the top of the needle is applied to improve yield of the sample. The needle is then taken out completely and

pressure is applied once more to the site to stop further bleeding. The site is secured with steristrips and bandage.

Figure 2.3: Bergstrom muscle biopsy kit and incision site over vastus lateralis.

Of the over 350 muscle biopsies performed for the studies presented here, three subjects experienced haematomas/bruising, 3 temporary loss of sensation over biopsy sites, and 3 older volunteers experienced vasovagal syncope secondary to biopsy-induced pain. It could be contended that ultrasound-guided muscle biopsies should be used to avoid or minimise the risks of complications, however this is not used routinely and may not be practical particularly when biopsies are required immediately post-exercise. The use of ultrasound helps avoid major vessels but cannot avoid the possibility of biopsy induced pain.

Other methods of obtaining muscle samples include the open and bard needle biopsy methods (Dubowitz 2013). An advantage of the open biopsy method is that clamping the specimen prevents contraction of the biopsy fibres, but requires a larger incision and is slightly more invasive. Increased amounts of local anaesthesia may be required for obese and particularly anxious subjects and the wound is usually sutured closed. The bard biopsy technique uses the same preparation method as the Bergstrom biopsy technique but instead of the Bergstrom needle the bard needle is inserted into the incision site and repeated cuts are made using the retracting needle. An advantage of this technique is that repeated biopsies can be made that is relatively less invasive and traumatic. However only a small piece of muscle

is obtained each time and precise measurements of depth for needle insertion may be under or overestimated. At the David Greenfield MRC/ARUK research unit of the medical school University of Nottingham, the use of ultrasound prior to bard biopsies has minimised the risks of hitting a blood vessel and has proven invaluable in estimating depth of muscle, especially in overweight subjects.

A limitation of the percutaneous muscle biopsy technique is the potential delay in freezing muscle tissue specimens that can lead to inaccuracy of muscle metabolite analyses and variation in assays caused by non-muscle contaminants. Nevertheless this delay is not thought to largely influence most analytical methods. (Bergstrom 1975).

The muscle sample taken is frozen immediately to prevent degradation as this may affect biochemical studies. A problem of direct immersion of material into liquid nitrogen is that it allows gaseous nitrogen to coat the specimen, slowing the cooling process. To avoid this, liquid isopentane or propane is used to freeze the material which is subsequently immersed in liquid nitrogen to -160°C (Dubowitz 2013). Isopentane was used to freeze all muscle biopsy specimens in studies apart from visit 1 in Study 3 where fresh muscle specimens were required to prepare mitochondrial extracts for subsequent processing. Liquid nitrogen is preferred for long term storage of the specimen until ready for sectioning (Dubowitz 2013).

If IMCL content is the only determinant required from muscle then a less invasive method of analysing muscle is through using [1]H Nuclear Magnetic Resonance (^{1}HNMR) Spectroscopy (Petersen *et al.* 2003).

2.1.15 Stable Isotopes

2.1.15.1 Steele's Equation

Endogenous Glucose Production

F

Pool a

Vab

Glucose

Pool b

Vba

Glucose Disappearance
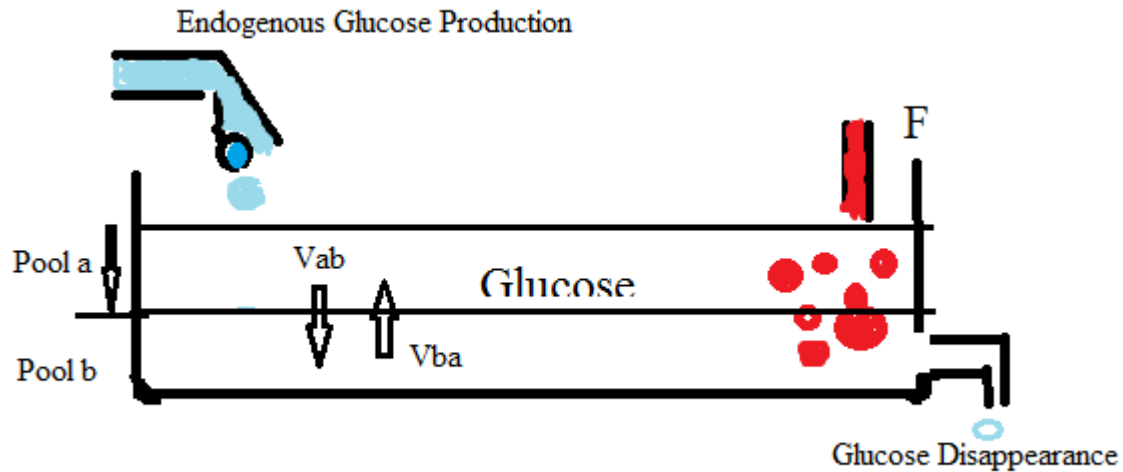
Figure 2.4: Steele's single compartment model

Explained in its simplest terms, Steele's single compartment model is analogous to a bathtub where water enters through a tap (endogenous glucose production, EGP) and leaves via the drain (glucose disappearance) Rd. The water level in the bathtub (glucose level in the single compartment) rises when water from the tap exceeds the water being drained (EGP>Rd), falls when Rd>EGP or remains in steady state when EGP=Rd. Steady state occurs during fasting or during the clamp when peripheral glucose concentrations remain constant. The tracer dilution technique is commonly used to measure endogenous glucose production where unlabelled glucose dilutes the tracer. Referring again to the bathtub analogy, when a dye (tracer) is added to the water in the bathtub it becomes uniformly distributed, such that when the water drains (Rd), it will not change the resultant dye concentration in the bathtub. However water coming from the faucet which has no dye will dilute the concentration over time (Vella and Rizza 2007).

Assuming the single compartment model can be applied to humans, where tracer and glucose are uniformly distributed this model can be used to measure endogenous glucose production.

Assuming the tracer is infused at a constant rate, equilibrium is achieved when tracer and trace are cleared at the same rate. At this point, the concentration of the tracer and tracee reflects the rate of infusion and disappearance respectively (Steele *et al.* 1956, 1959).

$$Ra = \frac{F}{Tracer} \times Tracee$$

$$Enrichment = \frac{Tracee}{Tracer} + tracee$$

$$APE \text{ (Atom per excess)} = \frac{tracer}{tracer + tracee} \times 100$$

Under most experimental conditions, where there is a sudden perturbation to steady state of the pool such as an insulin infusion, the tracer is diluted further by 'cold' glucose. To account for changes to tracer and tracee concentrations, the equation can be modified to take into consideration changes over time and multiple pools, rapidly and slower- equilibrating pools (Wall *et al.* 1957). These 2 pools can be further simplified to a single compartment separated into two by a pool fraction of the total extracellular glucose pool. The non-steady state Steel equation is therefore modified to two different time points:

$$\left(\frac{p\text{v X }[(\text{glucose}_1 + \text{glucose}_2)/2]}{1} \quad \text{x} \quad \frac{SA_2 - SA_1}{T_2 - T_1} \right) \quad \text{X} \quad \left(\frac{1}{[(SA_1 + SA_2)/2]} \right)$$

p = pool fraction, V = total extracellular pool, SA= concentration of tracer enrichments at times T1 and T2.

Applying the concept above, the rate of appearance (Ra) and disappearance (Rd) of palmitate (Study 3) was calculated using the single pool non-steady state Steele equations (Steele 1959) that were adapted for stable isotope methodology(Wolfe and Jahoor 1990).

$$Ra = \frac{F - V[(C_2 + C_1)/2][(E_2 - E_1)/(t_2 - t_1)]}{(E_2 + E_1)/2}$$

$$Rd = Ra - V (C_2 - C_1 / t_2 - t_1)$$

Where F is the infusion rate (μmol kg^{-1} min^{-1}), V is the distribution volume for palmitate or glucose (40 and 160ml kg^{-1}) respectively, C_1 and C_2 are the palmitate and glucose concentrations (mmol^{-1}) at times 1(t_1) and 2 (t_2), respectively, and E_1 and E_2 are the plasma palmitate or glucose enrichments (tracer to tracee ratio TTR) respectively at t_1 and t_2 respectively.

2.1.15.2 Determination of hepatic insulin sensitivity

The labelled euglycemic–hyperinsulinemic clamp is the most frequently used method for measuring hepatic insulin sensitivity in response to glucose and insulin infusions.

Among the stable-isotope glucose tracers, [6,6-^{2}H$_2$]glucose appears to be the most suitable because apart from being safe and non-recycling, it is also considered to give the best estimate of true endogenous glucose production.
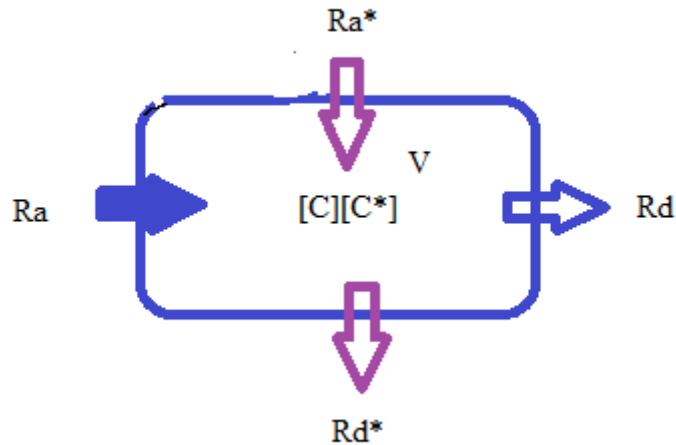
Figure 2.5: Monocompartment model (based on Steele's monocompartmental model and modified from Choukem and Gautier 2008). V: volume of compartment; Ra: hepatic glucose production rate; Ra*: tracer infusion rate; C: plasma glucose concentration; C*: plasma tracer concentration; Rd: rate of glucose disappearance; Rd*: rate of tracer disappearance

Basal hepatic glucose production rate corresponds to the hepatic response to physiological plasma insulin, under clamp conditions HGP measured is an estimate of the hepatic response to supraphysiological insulin concentrations (residual HGP). Under non-steady state conditions created by insulin and glucose concentrations, a one-compartment model with constant volume (V) is used (Fig. 3) to facilitate calculation of residual HGP. Steel's equation is the most widely used, although a more complex (Radziuk *et al.* 1978) and more recent model (Bluck and Clapperton 2005) that accounts for the error in Steele's equation has also been proposed.

Assuming basal HGP rate is equal to the liver's response to plasma insulin, under steady-state conditions, the glucose rate of appearance (Ra) equals its rate of disappearance (Rd); and the ratio of plasma tracer/tracee (C*/C), which corresponds to tracer enrichment (ε), is equal to the ratio of tracer infusion rate/glucose rate of appearance (Ra*/Ra). Thus, ε =Ra*/Ra (Ra=Ra*/ε). As Ra corresponds to the HGP rate in steady-state,

$$HGP = Ra*/\varepsilon$$

Although the isotope dilution method remains the best in determining hepatic insulin sensitivity *in vivo* and by far the safest and most practical compared to the arteriovenous-difference method and use of radioactive-isotope tracers, a number of limitations make it less than perfect. The liver is not the only glucose-producing organ during fasting conditions. The kidney's relative contribution to endogenous glucose production in the post- absorptive state is estimated to range from 5% to 28% (Cherrington *et al.* 1998, Gerich *et al.* 2001, Diraison *et al.* 2003). The monocompartmental model and the assumptions that constitute the basic principles of tracer methodology may also be subject to error. Another inconsistency is the computation of HGP rate using Steele's equation, which often generates negative values of HGP during the euglycemic clamp; this is perhaps due to an error in the equation itself. These negative values have been seen in studies (Choukem *et al.* 2008) and are assumed to correspond to zero, that is, complete suppression of HGP.

2.1.15.3 Palmitate tracer as a FFA tracer

FFAs are avidly bound to albumin and are insoluble in aqueous media. The stable isotope method described by Woolfe in the 1970s utilises palmitate labelled with ^{13}C or ^{2}H and provides a precise and sensitive method of FFA tracer kinetics. For Study 3, 63.6mg of ^{13}U palmitate was made up in 5mls of water. Because palmitate in its diluted form precipitates at room temperature, the palmitate was made up in warm water at 55°C and the syringe

containing the tracer was warmed again on a hot plate just before mixing with human

albumin solution. 100mls of 4.5% human albumin solution was heated in a bath at 55°C.

Once the palmitate became dilute it was injected sterilely into the bottle of albumin (as

palmitate will bind to albumin and this mimics biological processing) and allowed to cool to

room temperature before infusing.

Figure 2.6a and b: Palmitate solution is injected into a 100mls bottle of Human Albumin

Solution 4.5% warmed to 55°C, then left to cool at room temperature before infusing into the

human subject**.**

The sample tracer is extracted from plasma after addition of a stable isotope internal standard

to allow quantification of concentration, purified by thin layer chromatography and

derivatised to their methyl esters. Isotopic enrichment of palmitate is then determined by gas

chromatography mass spectrometry (GCMS). This method is superior to older methods of

tracing FFAs, such as calorimetry and enzymatic assays. It provides an accurate way of

identifying low individual and total FFA concentrations observed during an insulin infusion

and is specific in determining tracer enrichment. However relatively large amounts of tracer

and albumin were required to allow detection. Infusion rates can now be reduced with the

introduction of combustion isotope ratio mass spectrometry where $^{13}CO_2$ is measured. Of the

FFA tracers, palmitate, oleate and linoleate provide estimates of total FFA flux that are within 15% of actual values. Continuous palmitate infusion allows determination of regional kinetics in tissues. FFA oxidation can be estimated using FFA carbon tracers ^{13}C and ^{14}C. A correction for CO_2 fixation is often done to prevent inaccuracy (Sidossis *et al.* 1995).

2.1.15.4 Sodium bicarbonate

0.006375mg/kg of sodium bicarbonate was dissolved in 10mls of 0.9% saline and injected intravenously to prime the bicarbonate pool, to allow isotopic equilibrium of the palmitate. The main advantage of a primer is to achieve plateau in a shorter period of time, making infusions for volunteers feasible and cuts isotope costs and time of running studies. However some argue that the normal physiology of the tracer may be altered by priming due to rapid intake of tracer and that priming produces an artificial plateau (Wolfe and Chinkes 2005).

2.1.15.5 Breath samples enriched with $^{13}CO_2$

For Study 3 breath samples were collected into a bag and then transferred into a vacutainer. Briefly the subject is asked to form a tight seal with their mouth over the rubber tubing connected to the bag, blowing comfortably into the bag. Before removing the tube from their mouths the tube is clamped, and then connected to a 3 way tap. One end is connected to the vacutainer (BD) where expired air is injected into evacuated tubes for determination of $^{13}CO_2/^{12}CO_2$ ratio. As described by Siddossis *et al.* expired air is passed through a water trap followed by condensation in a liquid nitrogen trap to allow isolation of CO_2 and evacuation of other gases. $^{13}CO_2/^{12}CO_2$ is then determined by isotope ratio mass spectrometry (IRMS).

The ratio is expressed in units of tracer to tracee ratio (TTR), defined as:

$$\textbf{TTR} = (^{13}C/^{12}C)_{sa} - (^{13}C/^{12}C)_{ref} - [(^{13}C/^{12}C)_{bk} - (^{13}C/^{12}C)_{ref}]$$

Where sa = sample, ref = reference gas and bk = baseline sample

Figure 2.7: Breath bag and 3 way-tap to collect breath samples for $^{13}CO_2$ analysis

2.1.15.7 [6, 6-$^2H_2$] glucose quantification

50 µl of plasma was mixed with 10µl of internal standard (methyl glucopyranose 50µg/ml). Addition of the internal standard results in a characteristic spectrum where the peak of interest is divided by the internal standard peak to account for losses. 300µl of cold absolute ethanol was then added to the mixture to deproteinise it. This mixture was spun down and the supernatant removed. A tenth of the supernatant was evaporated under N_2 at 90^oC to form a dried solid residue. 50µl of pyridine and hydroxylamine (25mg/ml) was added to this solid residue to form an oxime group. This was then incubated at 70^oC for 45 minutes and allowed to cool slightly. 50µl of Trimethylsilyl-N-Trimethylsilyl Trifluoroacetimidate (BSTFA, 1% TMCS) was added and it was incubated at 70^oC again for 45 minutes.

The resultant mixture was then injected in a gas chromatography mass spectrometer at 240^oC for up to 240 minutes. An enrichment curve was generated by making up 0.5%-5% deuterated glucose standards. By this method, internal standard peaks, unlabelled glucose peaks and deuterated glucose peaks are generated.

2.1.15. 8 2-Deoxy-D-glucose (2DG) plasma concentrations

100uL of plasma was aliquoted and mixed with 10ul of fluorodeoxyglucose (FDG) standard. 1 ml of 100% ice cold ethanol is added to each tube and placed in ice/fridge for 20mins then spun for 2 mins at 10000rpm. The supernatant is dried completely in the techne® block at 90°C for 10mins. 100uL of oxime (20mg hydroxylamine HCL per 1ml pyridine) is added to each tube, vortex-mixed and incubated at 75°C for 30 mins in the oven. After cooling at room temperature, 70uL of BSTFA is mixed and incubated for 30 mins at 70°C in an oven in fume hood. Around 90uL is transferred into autosampler vials and capped.

These were run on GCMS, and the first major doublet peak area (2DG) and second major doublet peak area of FDG standard (elutes closely after second 2DG peak) are recorded.
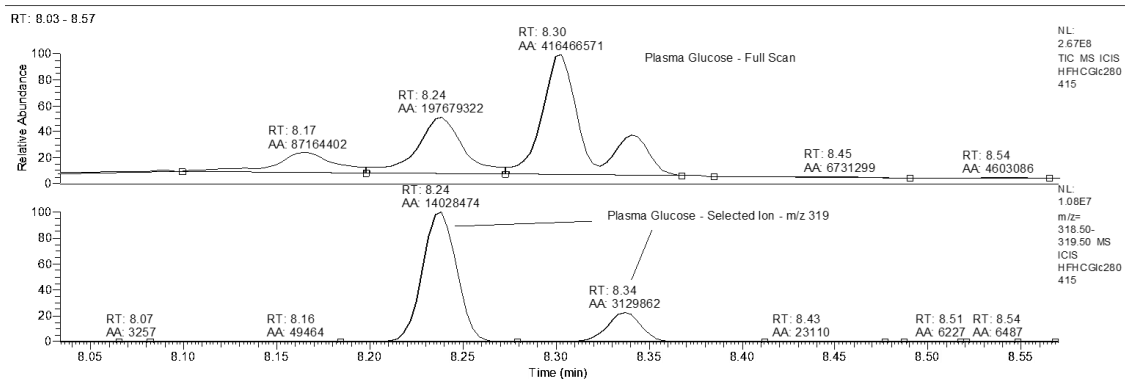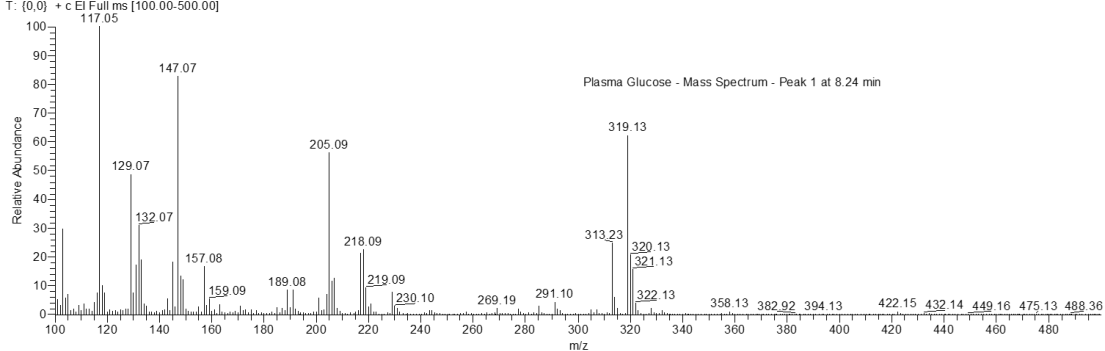
Figure 2.8: The trace shows 4 peaks, the lower shows 2, which are both glucose derivatives.

The mass spectrum shows the major fragments in the glucose-oxime-TMS derivative.
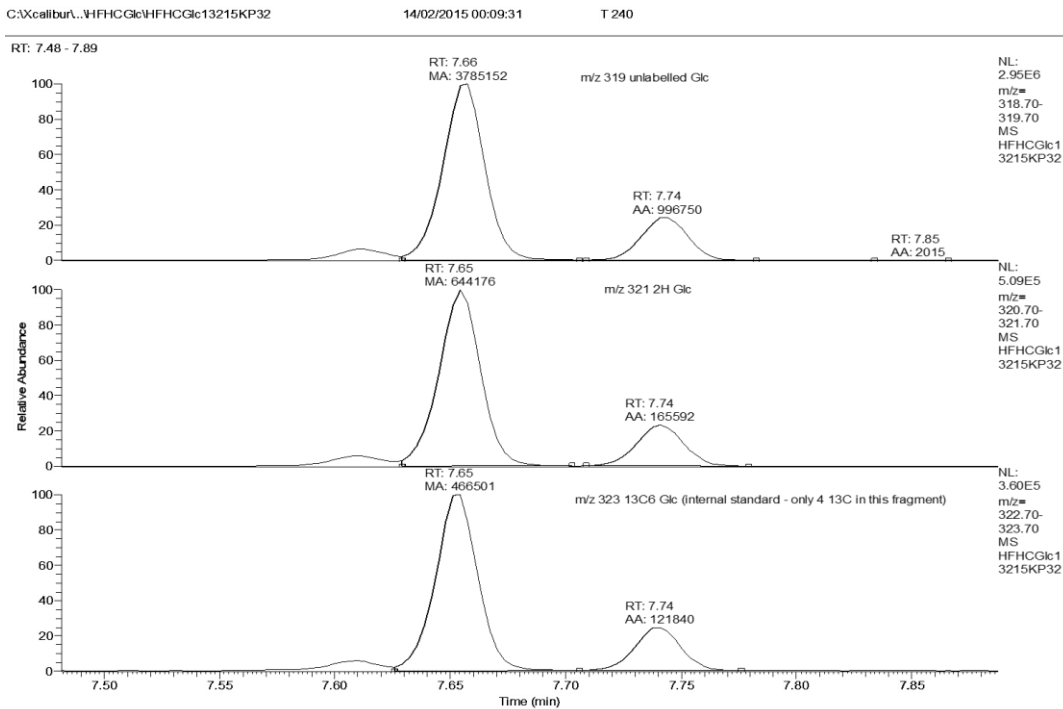
Figure 2.9: The chromatograms show the 3 masses monitored. The 319 mass is used for quantitation and is the major fragment where there are to labelled atoms (no deuterium). The tracer $^2H_2$, 2 deuteriums are replaced in the glucose molecule, so mass 319 + 2 =321 is looked at and quantified. The 323 mass represents the internal standard, added to quantify glucose. When added to plasma the ratio between glucose is fixed, so a standard glucose curve (of known concentrations & containing the same amount of internal standard) is generated. Diagram courtesy of Dr K Smith, School of Medicine, University of Nottingham, UK.

2.1.15.9 Determining skeletal muscle protein synthesis rate

The tracer L-[ring-$^2H_5$]-phenylalanine was utilised in Study 2 to determine the fractional rate of mixed muscle protein synthesis (FSR). An amino acid bolus (which excluded phenylalanine and tyrosine) was fed via nasogastric tube in the same study due to palability issues. Arterialised blood samples were obtained into evacuated-heparinised tubes (Vacutainer) and chilled on ice.

After deproteinisation on ice with dry 5-sulfosalicylic acid and derivatisation with tert-butyl dimethylsilyl (TBDMS) as described (Gorissen, Burd *et al.* 2014), plasma was analysed for phenylalanine and leucine concentrations and enrichment by GCMS.

Muscle protein synthesis rate was calculated by dividing the increment in enrichment in L-[ring-^{2}H$_5$]-phenylalanine by the enrichment of the precursor. Plasma and muscle free L-[ring-^{2}H$_5$]-phenylalanine enrichments were used to provide an estimate of the lower and higher boundaries of true FSR respectively. Thus the formula used was:

FSR (% hr $^{-1}$) = [ΔEp/Eprecursor x t] x 100

Where ΔEp is the delta increment of protein bound L-[ring-^{2}H$_5$]-phenylalanine during incorporation periods, Eprecursor is the enrichment of the precursor used during the time period for amino acid incorporation determination, and t denotes the time duration (hour) between biopsies. The equation is multiplied by 100 to express FSR as percentage per hour.

As the administered amino acid drink in Study 2 did not contain phenylalanine, whole body protein breakdown during the basal and fed periods could be determined from the rate of appearance of phenylalanine using the single-pool equation Ra=F/Ess where F is the infusion rate (μmol/kg min) and Ess is the steady state plasma phenylalanine enrichments (TTR) during the final hour of the basal and fed period.

2.2 Methods specific to studies

The following methods are specific to the studies presented in this thesis

2.2.1 Energy Predictions

Whilst indirect calorimetry is one of the most accurate in estimating basal metabolic rate (BMR), where this is unavailable or not feasible, BMR could be obtained by using equations based on anthropometric measurements. The FAO/WHO/UNU 1985 recommended the use of age and gender-specific equations based on weight and height or weight alone to predict BMR in all populations. These have been modified and widely adopted (Schofield *et al.* 1985), until recent times when it was observed that Schofield equations were inaccurate in predicting BMR in certain populations (Piers and Shetty 1993; Henry and Rees 1998). Specifically the inclusion of data from a population where the majority were Italian male military cadets and miners tended to result in overestimation of BMR in other populations. A challenging issue that persists is which predictive equation is most suitable for use in overweight and obesity. The issue lies with the lower metabolic rate of adipose tissue compared to lean tissue. Resting energy expenditure (REE) thus increases in a non-linear fashion with weight, and tends to over-estimate energy requirements in overweight and obese (Horgan and Stubbs 2003). Thus the Schofield equation is inappropriate for use in this group (Frankenfield 2005).

Based on comparisons made between the most commonly used predictive equations in clinical practice of Harris and Benedict (1919), FAO/WHO/UNU weight or weight and height equations (1985), Mifflin –St Jeor (1990) and Owen (1986, 1987), the Mifflin-St Jeor was found to be the most reliable, predicting resting metabolic rate (RMR) within 10% of measured in more nonobese and obese individuals than any other equation, and also had the

narrowest error range (Frankenfield 2005). However errors existed when applied to individuals and generalized to certain age and ethnic groups.

The current modified Schofield or Henry's/Oxford equation excludes Italian data and is currently accepted for use in most populations (Henry and Hayter 1999, Henry 2005). The new predictive formula includes height in addition to weight, and has been proven to be more accurate than the Schofield equation in an overweight or obese setting (Ramirez-Zea 2005; Weijs 2008) and showing a smaller deviation compared with REE determined with indirect calorimetry in adults with BMI within the normal range (Razalee *et al.* 2008).

Henry/Oxford prediction equations for BMR using height and weight:

18 - 30 years: **REE = 14.4 x W + 313 x H + 113**

30 - 60 years: **REE = 11.4 x W + 541 x H – 137**

Henry/Oxford prediction equations for BMR using weight alone:

18-30 years: **REE=16.0 X W + 545**

30-60 years: **REE=14.2 X W - 593**

Where W is weight in kilograms, H is height in meters, and REE is kcal/day

Although both equations incorporating weight and height and just weight alone could be used, the equation used for estimating energy requirements in study 2 used body weight as the only independent variable. Body weight, an easily and accurately acquired variable has been considered the best single predictor of BMR (Schofield 1985, Henry 1991). Regression equations with body weight and combination of body weight and height yielded similar R^2 values (Razalee 2010). Based on regression equations from the 1985 FAO/WHO/UNU Expert Consultation on Energy and Protein Requirements, including height as a second

predictor after weight, did not contribute significantly further to the equations for both genders, apart from those under three and above sixty years of age (Schofield 1985).

Once the predicted BMR has been estimated, total energy expenditure (TEE) is determined by factoring in the physical activity of the subject. Subjects' physical activity was assessed using the self-completed short version of the International Physical Activity Questionnaire (IPAQ) (Hagstromer, Oja *et al.* 2006) at screening. The IPAQ was developed in the late 1990s by a multi-national group, supported by WHO and has been shown to correlate with objective evidence of physical exercise (Mader, Martin *et al.* 2006). It assesses physical activity levels during the preceding seven days and generates a low (1.5), moderate (1.6) or high (1.8) activity score.

2.2.2 Determination of energy and macronutrient intake

For study 3 subjects completed 3 day food records of two weekdays and one weekend day before embarking on their study diet. 'Microdiet' software (Downlee Systems Ltd., Salford, UK) is a nutrient analysis software package that incorporates UK based nutrient data with the ability to edit and add additional foodstuffs not within the database. Nutrient content of foodstuffs could be added based on the nutritional information from food labels and published values. The Microdiet software was also used to design the food plans (isoenergetic with 50-55% CHO, 30-35% Fat and 13-15% Protein) for one week and 25% excess energy of either CHO or Fat for the following two weeks. Subjects were provided with and delivered commercially prepared food.

2.2.3 Methods of determining exercise capacity and performance

2.2.3.1 Incremental shuttle walk test

Baseline incremental shuttle walk test (ISWT) outcomes were compared between older lean and overweight volunteers in Study 1. Older volunteers performed the ISWT at the start and after the 6 month intervention for Study 4. Briefly this test involves shuttling between 2 cones 10 metres apart in time increments paced to a series of bleep signals until they are no longer able to keep up or exhibited symptoms limiting their performance. It provokes a symptom limited maximal performance that allows direct comparison of a patient's performance originally developed to evaluate functional capacity in patients with chronic progressive obstructive disease.(Singh *et al.* 1992) and later validated for use in other chronic diseases (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories). It is proven to be a feasible measure of functional capacity in older people and correlates well with other markers of disability (Dyer *et al.* 2002).

2.2.3.2 VO$_2$ max testing

VO$_2$ max testing was determined using an electronic-braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) during an incremental exhaustive exercise test to determine rate of VO$_2$ max measured using an online gas analysis system. This allowed further determination of the corresponding workload at 50% VO$_2$ max at which older subjects cycled to. Most data describing maximal oxygen uptake and requirements for achieving this have been developed using young healthy subjects. There is no single standardised or protocol of determining the maximum aerobic function in elderly people. Cycle ergometer and treadmill tests are considered the commonest method of exercise stress tests. Of the two, the treadmill has more diagnostic sensitivity and presents a more familiar and functional exercise modality ie walking (Lear, Brozic *et al.* 1999), but this may present a problem for

some elderly patients with balance problems or arthritis. VO_2 max values achieved with cycle ergometer are generally 11% (range 8-15%) lower than those with the treadmill due primarily to smaller volume of exercising muscle mass (Hermansen and Saltin 1969). VO_2 max tends to decline with age, at around 1% each year after the 3rd decade of life (Astrand 1960). This may be associated with the decline in cardiorespiratory function and muscular function with advancing age. The difficulty of directly measuring VO_2 max in the elderly stems from the physical limitations or chronic medical conditions in older people but also other factors such as muscle fatigue, perceived exhaustion, subject motivation and the clinicians willingness to continue exercising until exhaustion, particularly in elderly subjects who are at higher risk of coronary artery disease and arrhythmias.(Astrand 1976) Current literature indicates variability in protocols used to achieve VO_2 max and was mostly developed for use in young subjects. The requirements seen in literature include:-

1) Respiratory Exchange Ratio (RER)>1.0 (Cress, Thomas *et al.* 1991, Hollenberg, Ngo *et al.* 1998) or >1.1 (Shephard, Allen *et al.* 1968)

2) Peak exercise heart rate of at least 85% of age-predicted maximum (Borg 1982)or heart rate within 10 beats per minute of age-predicted maximum (Howley, Bassett *et al.* 1995)

3) Borg's perceived exertion scale >15

No single protocol for exercising elderly subjects exists. Table 2.2 current protocols used in various studies on VO_2 max. A protocol for VO_2 max tests for elderly subjects (65-75 years) in this study was proposed based on current protocols used in exercising young healthy subjects but modified to take into account the physical limitations, early fatigability, increased risk of arrhythmias and coronary heart disease. Of relevance is the study by Thomas *et al.* (Thomas, Cunningham 1987) who compared three different VO_2max testing protocols across the same group of elderly subjects and suggested that a continuous ramp-like

protocol with small increases in both speed and grade was the most appropriate for healthy older men. The protocol however had limited generalisation to the greater population of older adults due to the exclusion of women. A longitudinal study performed on 375 healthy women (48.6±16 yrs) and 435 men (51.9±16 yrs) from the Baltimore Longitudinal Study of Aging (BLSA) using treadmill testing considered achievement of maximal exercise to have been achieved when volunteers heart rate reached > 85% of predicted and appeared fatigue, and could not have carried on for at least another minute (Fleg and Lakatta 1988).

Therefore the requirements for ensuring VO_2 max has been achieved for this study were similar to that used for testing younger subjects and based on protocols seen in other studies involving elderly subjects ie RER>1.1 or HR> 85% of predicted maximal heart rate (HR) for age or Borg scale > 15. Modifications from that used in younger volunteers included a short familiarisation period (10 mins) of cycling at 40W. Power output then increased by 20 W every 3 mins, until 12 mins where it was increased every 20 W/min per minute until exhaustion. Subjects were attached to the heart rate (3 lead) monitor and the test was stopped if the volunteer exhibited chest pain, increasing dizziness, deceleration of heart rate, more than 2 ventricular ectopics on the ECG screen ie > 2 ectopics per 10 seconds.

It has been suggested that VO_2 peak data may provide sufficient information in determining maximum aerobic capacity in elderly people or perhaps redefining the maximum HR to be achieved during the max test.

Table 2.2: Protocols to determine VO$_2$ max in older subjects seen in literature and of that used in study 4.

Authors	Subjects	Protocol
Jones *et al.*, 1985	• 100 healthy subjects (50 male and 50 female), selected to provide an even distribution of age (15–71 y) and height	• Initial power setting 16.3 W • 10 men and 10 women between 55 and 71 y • Power increased by 16.3 W/min until a symptom-limited power output reached
Poehlman & Danforth, 1991	• 19 older adults (13 men, 6 women) (64 ± 1.6 y)	• Initial workload for women: 25 W at 50 rpm • Initial workload for men: 50 W at 50 rpm • Initial workload sustained for 3 min • Increased by 25 W every 2 min until exhaustion or until subjects unable to maintain 50 rpm
Fairbarn *et al.*, 1994	• 231 men and women equally divided within decades between 20 and 80 y (no breakdown of specific ages within decades provided)	• Initial power ouput at 16 or 32 W • Power increased by 16 W/min until subject reached symptom-limited maximal power output
Cress & Meyer, 2003	• Men and women ($N = 192$; 76 ± 7 y) from single-family community dwellings or retirement communities with multiple levels of care	• Familiarization period • Ramped test • Power output increased at a rate of 8–16 W/min

Pruchnic *et al.* 2004	13 healthy (67.3 ± 0.7 yr old) volunteers (8 women and 5 men)	Initial workload of 0-25W for first 2 mins then increment of 10-25W every 2 mins until volutional exhaustion and until one of established criteria reached based on the American College of Sports Nutrition
Study 4 of present thesis	23 healthy men (mean 69 years)	Initial workload 40W,increased by 20 W every 3 mins for first 12 mins,then 20w every min until subjects unable to maintain 60 rpm and until one of RER>1.2 or HR>85%predicted or Perceived Borg Score> 15

2.2.4 Methods of determining body composition

2.2.4.1 Anthropometric assessments

Skinfold measurements and bioelectric impedance analysis (BIA) were used to assess body composition in study 3.

Skinfold measurement is used to assess subcutaneous fat thickness at various regions of the body. It is traditionally used to assess the degree of fatness and specifically the size of subcutaneous fat depots The primary limitation is that most callipers have an upper measurement limit of 45 -55 mm, so this would not be ideal in very overweight or obese subjects (Duren, Sherwood *et al.* 2008). The mean of skinfold thickness measurements are calculated, per cent body fat is then calculated from the means of each four skin fold thickness measurements (biceps, triceps, subscapular and suprailiac) following the method of Durnin and Womersley (Durnin and Womersley 1974). Body fat percentage is estimated from the Siri formula (Siri1961), by dividing 495 by the body density (ε) calculated from the mean skinfold thickness measurements, minus 450 and multiplying by 100:

Body fat = (495/ε skinfold measurement -450) x 100.

2.2.4.2 Bioelectric Impedance Analysis

This method produces estimates of total body water (TBW), fat-free mass (FFM), and fat mass by measuring resistance of the body as a conductor to a very small alternating electrical current (Lukaski, Johnson *et al.* 1985; Chumlea and Guo 1994). The impedance index [stature squared divided by resistance (S^2/R) at a frequency] is proportional to the volume of total water and is an independent variable in regression equations to predict body composition (Baumgartner RN *et al.* 1990). BIAs use these equations to describe statistical associations for a specific population therefore these are useful only for subjects that closely

match the reference population in body size and shape. BIA's ability to predict the degree of fat in obese subjects can be difficult to interpret because these individuals have a greater proportion of body mass and body water accounted for by the trunk, the hydration of FFM is lower in the obese, and the ratio of extracellular to intracellular water is increased in the obese.(Gray, Bray *et al.* 1989). BIA's advantages are its portability, ease of use, relatively low cost and safety. The validity of BIA is influenced by gender, age, disease state, race and ethnicity (Rush, Chandu *et al.* 2006) and level of fatness (where TBW and relative ECW are greater in obesity compared with normal-weight individuals). Single-frequency BIA has better agreement than multifrequency BIA compared to DEXA as a criterion measure for fat mass and FFM estimates in overweight and obese men.(Pateyjohns, Brinkworth *et al.* 2006)

2.2.4.3 Dual energy x-ray absorptiometry (DEXA)

Dual energy x-ray absorptiometry (DEXA) scan (Lunar Prodigy, GE Healthcare) was used to measure body composition of volunteers in studies 1, 2 and 4. Study 3 utilised the methods of Bioelectric Impedance Analysis (BIA) and skinfold anthropometric measurements. Originally developed by Mazess *et al.* (Mazess, Peppler *et al.* 1981) to precisely measure total bone mineral content, DEXA is today widely and commonly used to measure fat and lean body mass. Commercially available DEXA scanners manufactured by Lunar, Norland and Hologic generate two low energy x-rays levels to distinguish total body adipose and soft tissue, bone mineral content and density. Calculations are based on the assumptions on levels of hydration, potassium and tissue density and vary between manufacturers. The method is based on the energy dependence of the attenuation coefficients for photon absorption of bone mineral (containing high atomic number of calcium) and soft tissue (contain low atomic number elements of carbon, hydrogen and oxygen) (Mazess, Barden *et al.* 1990) An x-ray tube behind a filter converts the polychromatic x ray beam into one with two main energy peaks. The attenuation of non-bone tissue was previously assumed to be constant but is now

measured therefore limiting errors in estimation of composition (Roubenoff, Kehayias *et al.* 1993).

The ratio of soft tissue attenuation (RST) at the two energy levels is measured. The subject's ratio of lean mass and fat (RL and RF) are known from theoretical calculations and human experiments. Thus the proportion of fat (alpha) and lean (beta) mass in each pixel can be determined:

RST (40Kv) = α (Rf) + β (RL)   (1)

RST (70Kv) = α (Rf) + β (RL)   (2)

In its' primary role of assessing bone mineral density, DEXA has a confidence value (CV) of ~ 1 %.(Mazess, Barden *et al.*1990)

Physical limitations include body weight, length, thickness and width of the subject. Indeed others have found that DEXA is unreliable in assessing body composition in obesity. Nevertheless DEXA is considered quick, convenient, non-invasive and exposes volunteers to a low radiation of <5mrem. This method can be applied to populations requiring minimal cooperation. It can also be performed by operators with minimal training.

Wang *et al.* (1992) described changes in body composition with growth, ageing and illness. When assessing composition, it has to be assumed that the hydration of lean body mass is uniform and fixed at 0.73ml/g (Roubenoff and Kehayias 1991). This may not hold true in elderly or hospitalised ill patients (Heymsfield and Waki 1991) and will clearly also affect analyses using the bioelectric impedance and underwater weighing method in these groups. Abnormal hydration affects the subjects RL (ratio of lean mass) leading to deviation from the RL in the equations 1 and 2, leading to an error in the amount of lean tissue contributing to total body tissue. DEXA measurements of bone are sensitive to the anteroposterior thickness

of the body so there may be a difference in thin and obese subjects. Bias can thus emerge from following body composition during weight-reduction studies or growth in adolescents. (Roubenoff *et al.*1993).

Particular regions such as the thorax and arms (where bone forms the greatest proportion of total mass) could lead to errors in soft tissue measurements. Pixels can sometimes miss small areas of bone and be included as lean tissue as the average absorption of coefficient for x-rays is closest to lean tissue than is bone, leading to possible overestimation of lean mass. It has also been shown that DEXA instruments from different manufacturers produce different measurements, and lack cross-validation using a standard phantom (Roubenoff *et al.* 1993).

For studies presented here, at least three trained technicians have carried out DEXA scans using the same DEXA scanner (Lunar Prodigy). Ideally the same operator should carry out both scans (at the beginning and end of the study) to minimise inter-operator variability. To attempt to minimise this inconsistency, the regions scanned were re-measured by one single operator at the end of the study.

Despite its limitations, considering the advantages, relative precision and practicality of DEXA compared to MRI/CT scanning and other indirect methods such as BIA and skinfold measurement, DEXA scanning appears to be the most useful in determining body composition.

Figure 2.10a and b: DEXA scanner and MRI scanner used to determine body composition in Studies 1, 2, 4 and hepatic and muscle triglyceride in study 3 respectively.

2.4.4.4 Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectrometry (MRS)

For study 3 magnetic resonance imaging and spectroscopy was employed at the beginning and end of the 2 weeks of overfeeding either fat or carbohydrate to quantify the effect of both diets on liver fat.

The basic principles of MR scanning depend on a nucleus with non-zero spin such as ^{1}H, ^{13}C and ^{19}F. As ^{1}H is the most naturally abundant element it produces the most intense MR signals and is thus the most frequently analysed element for MR and spectroscopy assessment (van der Graaf 2010).

The MRI process consists of five major elements (van der Graff 2010):

1) Magnet

2) Gradient systems

3) Radiofrequency (RF) coil system

4) Receiver

5) Computer

The magnet aligns the nuclei in low (parallel) and high (anti-parallel) energy states. A strong magnetic field is required and should be uniform over the area of interest; a shim system maintains this uniformity. The field uniformity can be altered using a magnetic field gradient (GZr) which varies linearly with position r to spatially encode the NMR signal. Gradients are generated by passing currents through arranged coils of wire placed surrounding the subject imaged.

To generate an NMR signal, an RF field flips the magnetisation away from its equilibrium state using an RF transmitter that is responsible for pulse shape, duration, power and timing. Each spin produces a sinusoidal signal at a frequency dependent on the local magnetic field. An RF coil detects the signal from the spins by coupling the nuclei to an external circuitry. Either a volume or surface coil can be used, with the former being the most efficient.

The RF signal is 'received' by a receiver system, where it is first amplified with a low-noise amplifier which is then transmitted to form an image on a remote computer. The computer system then retrieves images using a form of Fourier transform (FT). The fast FT can be used for two to three dimensional images.

As smaller metabolite signals other than water or fat are often required in MRS, a magnetic field of sufficient strength is required, generally 1.5 Tesla (T) or higher. Clinical MR systems with field strengths of 3T have improved signal-to-noise ratio (SNR) in MR spectra, enabling acquisition of spectra from smaller volumes (van der Graff 2010).

Liver fat content was quantified using H –MRS with a 3.0 Tesla (T) scanner (Philips Medical Systems) in Study 2. At 3T the chemical shift between water and the dominant fat peak corresponding to a frequency difference of -434 Hz. This oscillation period is half that of

1.5T and the corresponding IP and OP echo times at 3T are halved. A spectroscopy localisation technique with time of repetition of 3000ms, echo time of 30ms, and 16 acquisitions was used to obtain liver spectra. Measurements of abdominal subcutaneous and intraabdominal adipose tissue volume were obtained from 16T1-weighed axial magnetic resonance imagesby using selective fat excitation ranging from 8cm below and above the L4/L5 lumbar intervetebral disc. Adipose tissue volume and liver fat content were analysed by 2 different investigators using MRI software.

MRS is considered the most accurate non-invasive means of measuring liver fat content showing good correlations with histological samples (Longo *et al.* 1993, Thomsen *et al.* 1994, Szczepaniak *et al.* 1999). This method separates the liver signal into its water and fat components and calculates a signal fat-fraction. MRS is particularly recommended for assessment of small lipid fractions in liver as sensitivity to low signal intensities from fat is higher, and signals from water and fat are better distinguished. Resonance frequencies as depicted by MR correspond to protons in water and dominant protein signals in fat. When signal intensities at frequencies corresponding to water and fat are quantified the signal-fat fraction is calculated (Mitchell *et al.* 1991, Fishbein *et al.* 1997, Hussain *et al.* 2005).

Fat-signal fraction η is calculated as the signal from fat protons divided by combined signal from fat and water protons in the liver

$$\eta = F / W+F$$

F = fat signal

W =Water signal

It is assumed that the fat signal fraction reflects fat concentrations if the signals from W and F are corrected for confounding factors.

In particular ^{1}H MRS directly measures the protons in the fatty acids of the triglycerides, providing a quantitative assessment of hepatic triglyceride.

MRS carried out in this study used the Dixon method (Mitchell *et al.* 1991), the in phase and out of phase (IOP) imaging acquired in-phase images when signal from water and fat add, and out of phase images when signal from water and fat subtract.

Sagittal, coronal and axial slices through the right lobe of the liver were acquired and a single voxel measuring 3x3x3cm^3 was manually placed in liver parenchyma using multiplanar localising images. For reliable spectra from liver parenchyma to be recorded, voxels are carefully placed to avoid artificial signal contributions from intrahepatic blood vessels, large bile ducts, lateral margin of the liver and surrounding adipose tissue. Shimming (placing a box within area of the liver to be scanned) is performed to achieve a homogenous magnetic field across the voxel and to avoid non-uniformity of fat suppression (See figure 2.11). A spectroscopic sequence is then performed.
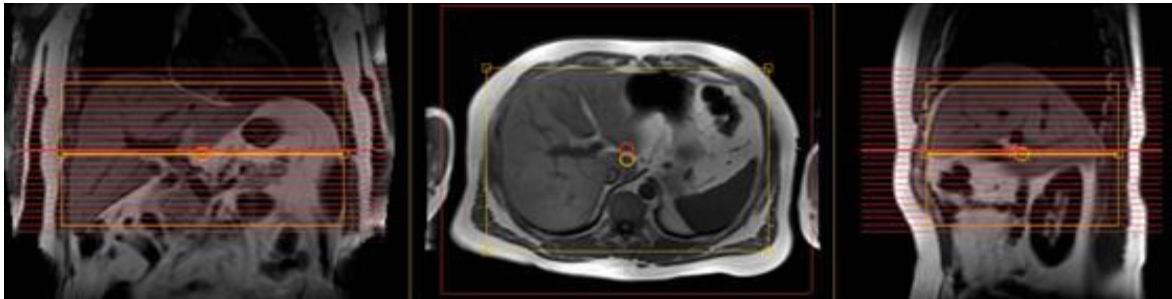
Figure 2.11: Placement of volume shim for optimum fat suppression in liver imaging: Increased phase shifts between fat and water occur creating image artifacts so complete fat suppression is required to eliminate this. This can be eliminated using a frequency selective fat suppression pulse (SPIR/SPAIR) with volume shimming. Air or soft tissue interfaces can create non-uniformity of fat suppression; therefore air within the vowel can be minimised by proper placement of volume shim box as above.
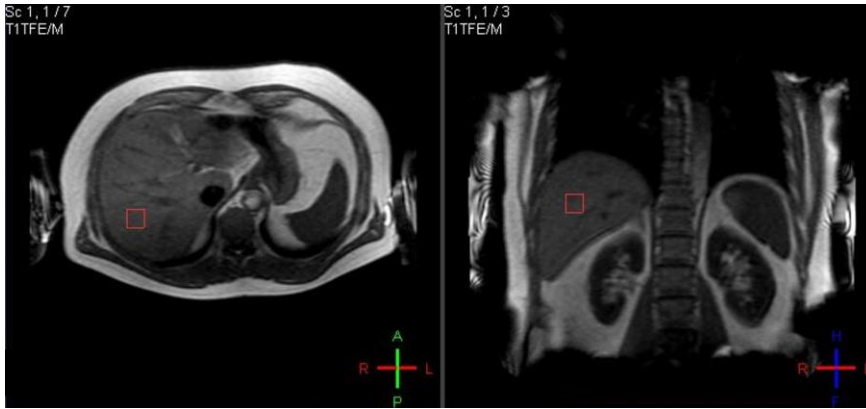
Figure 2.12. MRI scout images in the transverse (left) and sagittal (right) plane used for voxel placement of STEAM localized MRS (red box).

The two main methods used for single-voxel spectroscopy are point resolved spectroscopy (PRESS) (Ordridge *et al.* 1985) or stimulated-echo acquisition mode (STEAM) (Frahm *et al.* 1989). PRESS uses a 90-180-180 pulse sequence with long echo time, allowing for better visualisation of metabolites with long T1 relaxation times. The STEAM method on the other hand consists of a shorter echo time and lower signal yield compared to PRESS.

The magnitude-based approach is perhaps the most commonly used MR approach for liver fat assessment currently. Two gradient echos are acquired, one at an echo time (TE) in which the water peak and dominant fat peak are out of phase and one at a TE in which two peaks are 'in' phase. The TEs corresponding to in phase and out of phase (IOP) depend on the field strength. The MRI signal strength used in this study was 3 Tesla (3T).

MRS fat quantification techniques are shown to be safe, non-invasive and have high intra-individual reproducibility in repeated measurements. However it relies on overall volume fraction of lipids in liver parenchyma whereas quantification from liver biopsies is taken from percentage of hepatocytes that show distinct fat droplets. There may thus be a difference in reported percentage values of steatosis between ^{1}H MRS and histological analysis.

There is not as yet a clinically-defined cut-off value for normal vs abnormal levels of hepatic fat. A large MR spectroscopy study involving over 2000 participants of the Dallas Heart Study defined a 95th percentile cut-off of 5.56% fat-fraction as abnormal based on a subset of 345 patients with no identifiable risk factors for hepatic steatosis (Szczepaniak, Nurenberg *et al.* 2005). Indeed cut-off values may be modified slightly in the future when fat fractions are related to histological samples or clinical outcomes. Nevertheless based on current fat fraction cut-off values derived from the study above (~5%) the accuracy (bias) and precision (standard deviation) of a quantitative fat content biomarker must be smaller than 5-6% to provide a reliable diagnosis (Reeder, Cruite *et al.* 2011).

The MRI scanner may also not be suitable for people suffering claustrophobia, having orbital metallic implants, pacemakers or metallic heart valves. Although metallic implants generally do not pose safety risks, spinal fusion metallic plants or knee implants for example may distort images and affect quality of liver fat quantification.
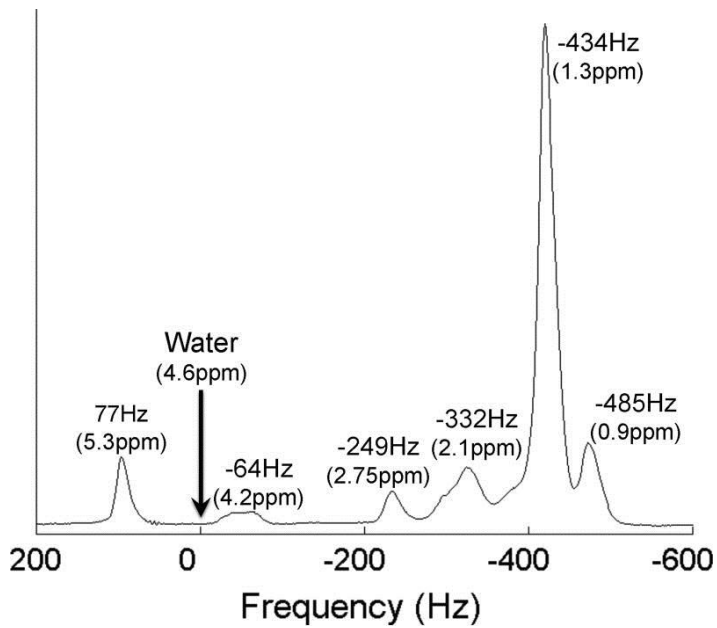
Figure 2.13: Single voxel MR spectrum of vegetable oil at 3.0T demonstrates the spectral complexity of fat. Triglycerides such as those in human liver fat have at least 6 identifiable peaks at clinical field strengths, similar to those shown in this figure. Methods such as IOP imaging and chemical shift based water-fat separation methods that model fat as a single NMR peak at -434Hz (-217Hz at 1.5T) will inaccurately estimate the concentration of fat within tissue if all peaks are not included in signal measurements. The frequencies that are shown present the chemical shifts of the different peaks at 3.0T relative to water (Reeder *et al.* 2011).
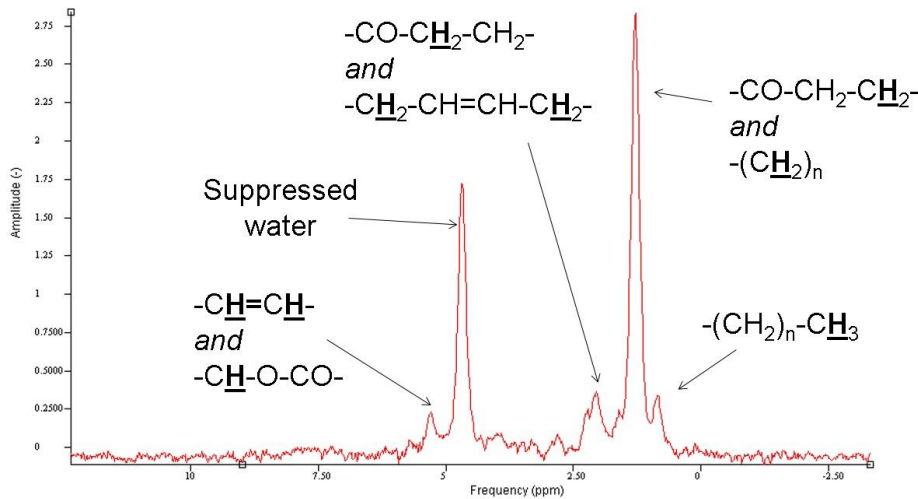
Figure 2.14. STEAM localized ^{1}H MRS with water suppression (TE=20ms) at 3T acquired from a 20x20x20mm voxel within the right lobe of the liver of one subject (9 averages in one breath hold). The spectrum shows high resolution of multiple fat peaks allowing easy peak fitting quantification. Diagram courtesy of Dr Stephen Bawding, School of Physics, University of Nottingham.

2.2.4.5 Other methods for quantifying liver fat

Liver biopsy and direct histological analysis of tissue samples are currently the gold standard for quantifying intrahepatic lipid content. However this method is invasive, relatively expensive compared to imaging methods and presents risks in otherwise healthy subjects (Bravo *et al.* 2001) so may be inappropriate for use in research or longitudinal studies. Biopsy is also subject to sampling variability as it evaluates a small portion ($0.05cm^3$) of the liver ($800\text{-}1000cm^3$) (Ratziu, Charlotte *et al.* 2005) and the distribution of fat throughout liver may be non-uniform. In this respect MRI/MRS provide a non-invasive means of measuring liver fat content. Compared with ultrasound and CT, it is capable of detecting small amounts of intrahepatic lipid content. Longo *et al.* 1993 described how H-MRS of liver parenchyma was well-correlated with data from CT studies and liver biopsies.

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Ultrasound is one of the most common modalities of imaging to evaluate hepatic steatosis; it is non-invasive, costs less, and easily available. Liver fat content is evaluated indirectly based on qualitative sonographic features such as liver echogenicity, echotexture, vessel visibility and beam attenuation. The use of ultrasound is operator and machine-dependent and thus has limited repeatability and reproducibility. It would be an inappropriate tool to use in the HFHC study (Chapter 4), as the use of ultrasound in overweight or obese subjects is challenging due to impaired beam penetration and limited liver visualisation. The positive predictive value for detection of hepatic steatosis is only 62-77% (Graif, Yanuka *et al.* 2000; Saadeh, Younossi *et al.* 2002).

Unlike ultrasound, CT evaluates hepatic steatosis indirectly based on hepatic X-ray attenuation that can be measured objectively and with high precision (Saadeh *et al.* 2002, (Kodama, Ng *et al.* 2007). Other factors such as iron, copper, glycogen and fibrosis and oedema can affect CT attenuation values resulting in errors in fat quantification and low sensitivity for mild to moderate steatosis. CT also relies on ionising radiation that will be appropriate for use in children or repeated monitoring of liver fat in adults (Fazel, Krumholz *et al.* 2009). The inherent variability in attenuation values across CT scanners manufactured by different vendors also exists (Birnbaum, Hindman *et al.* 2007).

2.2.4.6 Proton magnetic resonance spectroscopy (^{1}H MRS) of muscle

Besides liver, MRS of muscle was also determined. Within muscle, triglyceride is stored either intra (IMCL) or extra-myocellular (EMCL). The main proton signal from muscle is from water. Following water suppression the main peak seen on sprectroscopy is lipid, and EMCL and IMCL are closely opposed but can be separately identified. Unlike the liver, movement due to respiration is not an issue, so subjects are not required to carry out breath-holds during scanning. The reproducibility using this method for identifying IMCL is

acceptable with a coefficient of variation of 13% using 1.5 T scanner (Torriani, Thomas *et al.* 2005).

2.2.5 Skeletal muscle biopsy samples

The following methods were used for studies 1, 3 and 4.

2.2.5.1 Determining total muscle carnitine content

Muscle samples obtained by biopsy were immediately frozen in isopentane then stored in liquid nitrogen. Muscle was then dissected free of visible blood and connective tissue, pulverised and used for the determination of free-, acetyl- and long-chain acyl-carnitine content (Cederblad *et al.* 1990). Total muscle carnitine was calculated as the sum of these carnitine moieties. Long-chain acyl-CoA content was determined from the same extract as long-chain acylcarnitine using a modified version of the radioenzymatic method of Cederblad *et al.* (1990).

Freeze-dried powdered muscle was extracted with 0.5mM perchloric acid in 1mM Na2 EDTA and neutralised with 2.1M $KHCO_3$.

2.2.5.2 Acetyl-coA assay

Using a standard assay kit (Sigma-Aldrich), free CoA was quenched then Acetyl CoA is converted to CoA. The CoA was reacted to form NADH which interacts with PicoProbe to generate fluorescence (Ex=535/Em=587 nm). Briefly, tissue samples for measurement were deproteinised using perchloric acid/KOH protocol. The homogenate was spun at 10000g and neutralised with KHO_3 and put on ice for 5 minutes. Acetyl CoA standard curves were generated by diluting Acetyl CoA Standards whilst background free CoASH and succ-CoA in samples were corrected for by adding 10µl of CoASH Quencher to each standard, sample and background samples to quench free CoA. 2µl of Quench remover (ie NEM) was then added

and incubated for 5 mins at room temperature. CoA conversion was carried out by making up

50µl of reaction mix for all the wells, and incubated at 37°C for 10 minutes. Fluorescence

using Ex/Em =535/589nm with a plate reader was carried out. Background values and correct

Acetyl-CoA values for each sample were determined and a standard curve was plotted where

Acetyl-CoA amounts in the sample wells were obtained.

Acetyl CoA concentrations in the test samples:

C = Ay/Sv (pmol/µl; or nmol/ml; or µM)

Where: Ay is the amount of Acetyl CoA (pmol) in the sample from the standard curve.

Sv is the sample volume (µl) added to the sample well.

2.2.5.3 Determination of 2DG in muscle

 Frozen muscle (30–40 mg) was powdered under liquid nitrogen, and transferred to 70%

ethanol. The sample was vortex mixed and then centrifuged at 5,000g for 10 min. The

resultant supernatant was used for 2-deoxyglucose-6-phosphate (2DG6P) analysis using a

commercial kit (Cosmo Bio Ltd, Tokyo). 2DG accumulates in muscle as 2DG6P and is

oxidised by the introduction of glucose-6 phosphate dehydrogenase (G6PDH), resulting in

NADPH where it was quantified at 420 nm using a recycling amplification enzymatic-

photometric system (Saito K and Minokoshi Y *et al.* 2011).

2.2.5.4 Electron Microscopy of muscle

Muscle samples were first fixed in 1% osmium tetroxide, then dehydrated in ethanol and

embedded in Spurr's resin (25ml Araldite CY212 resin, 15ml Agar 100 resin, 55ml DDSA,

2ml Dibutylphthalate and 1.5ml DMP 30). Thin sections were cut using an ultramicrotome,

placed on Cu/Pd grids and stained for 5 min in uranyl acetate followed by 2 min in lead

acetate. Longitudinal images were visualized using a transmission electron microscope at a magnification of x4200. Approximately 40 micrographs from at least four different muscle fibres were taken at random and sequentially by an operator blinded to subject age, with around half of the images containing a SSL region. Images were analysed in a blinded manner described by Crane *et al.* 2009 using ImageJ (NIH) to determine percentage IMCL per fibre area, lipid droplet size, total number of lipid droplets per square micrometer of tissue and percentage IMCL area density.

2.2.5.5 Lipid metabolites

Quantification of intramuscular DAGs and ceramides was performed using high-performance liquid chromatography (HPLC) tandem mass spectroscopy. Tissue homogenates were fortified with internal standards and extracted into a one-phase neutral organic solvent system, evaporated and reconstituted in methanol. Quantitative analysis was performed in a positive multiple-reaction-monitoring mode, based on calibration curves generated by adding to an artificial matrix known amounts of target analytes, synthetic standards and an equal amount of internal standard. DAG and ceramide levels were normalized to total protein levels. For skeletal muscle DAG and ceramide analyses, 50 ng internal standard (1,3[d5]-15:0 DAG) was added to 5 mg freeze-dried muscle powder, total muscle lipids were extracted in $CHCl_3$:MeOH:H_2O (26) and the most abundant DAG (diC16:0, C16:0/C18:1, diC18:1) and ceramide (C16:0, C18:0, C18:1, C20:0, C24:1, C24:0) species were quantified using LCMS/MS. (Blachnio-Zabielska A *et al.* 2012, Blachnio-Zabielska A *et al.* 2013). Briefly, chromatographic separation was performed on a C8 MOS-1 Hypersil column (2.1 x 100 mm; 3.0 µm, Thermo Scientific) at a flow rate of 0.2 ml/min, using a binary gradient (90-99% solvent B; 0-20 mins) with 1.5 mM ammonium formate, 0.1% formic acid in water as solvent A and 2 mM ammonium formate, 0.15% formic acid in methanol as solvent B. DAG and ceramides were monitored in positive electrospray ionisation mode (Quattro Ultima triple

quad) as their $[M+NH_4]^+$ and $[M-H_2O]^+$ adducts, respectively. Peak areas (MassLynx 4.0, Micromass Ltd, UK) normalised to the internal standard were converted to absolute concentrations using standard curves constructed for each species undergoing the full extraction procedure.

2.2.5.6 Skeletal muscle oxidative capacity

Freeze-dried muscle was dissected free of visible blood and connective tissue, pulverised and used for the determination of muscle free carnitine, acetylcarnitine and long-chain acylcarnitine using the radioenzymatic method described previously by Cederblad (1990). Approximately 20 mg of wet muscle tissue was used to determine maximal CPT1 activity using the forward radioisotope assay (McGarry JDM *et al.* 1983). Briefly, muscle was homogenised in 50 mm Tris/HCl buffer (pH 7.5) and immediately used to determine malonyl-CoA (10 μm)-sensitive [14C]palmitoylcarnitine production from 100 μm palmitoyl-CoA, 1 mm L-carnitine and 0.05 μCi l-[14C]carnitine, which was normalised to total protein content using the Bradford assay.

Muscle phosphocreatine (PCr), lactate and glycogen were determined on freeze-dried muscle using the spectrophotometric method of Harris (1974). Muscle total creatine content was calculated as the sum of free creatine and PCr. In addition, maximal citrate synthase activity was determined spectrophotometrically on whole muscle homogenates based on the methods of Opie & Newsholme (Opie *et al.* 1967) and Zammit & Newsholme (1976) and expressed as mmol/min/ (kg wet muscle).

CHAPTER 3: STUDY 1

Comparing skeletal muscle lipid content, fat metabolism and insulin sensitivity in older vs. young healthy men

This chapter presents data comparing skeletal muscle lipid and metabolism at rest and during light-intensity exercise in healthy older and young men. The same older individuals participated in a further study to examine the effects of carnitine supplementation on skeletal muscle metabolism and insulin sensitivity, the results of which are presented in Chapter 6.

3.1 Introduction

It is established that insulin resistance increases with age but the proposed factors implicated in the decline of insulin sensitivity in ageing remains equivocal. Whilst it has been proposed that age influences insulin sensitivity independent of changes in body composition and physical activity (Shimokata *et al.* 1991), there is a growing body of evidence to suggest that increasing insulin resistance is closely related to increased adiposity (visceral fat) and sarcopenia (reduced muscle mass, strength and function), both closely associated with ageing (Evans and Campbell. 1993, Ferrannini *et al.* 1996, Imbeault *et al.* 2003). Age-related decline in glucose tolerance in population studies were also shown to be closely correlated with leisure-time activity (Wang *et al.* 1989). When age-related variables such as adiposity, dietary habits and physical activity were controlled for, the degree of correlation were reduced (Zavaroni *et al.* 1986, Zamboni *et al.* 1997), suggesting that elevation of glucose and insulin levels in ageing may be due to environmental factors. Moreover it has been shown that insulin resistance is not an obligatory finding in ageing as healthy centenarians have been demonstrated to have preserved insulin action compared to aged subjects (Barbieri *et al.*

2011). Thus ageing per se does not always appear to cause insulin resistance (Kohrt *et al.* 1993, Amati *et al.* 2009, Karakelides *et al.* 2010).

Elevated triglyceride levels in muscle consistently correlate with models of insulin resistance. Increased IMCL has been found in patients with T2DM (Falholt *et al.* 1988) and in cross-sectional studies of healthy humans, insulin resistance correlates most closely with IMCL compared to percentage body fat, BMI or age (Stein *et al.* 1997). Moreover, lipid metabolites associated with IMCL such as acyl-coA, diacylglycerol (DAG) and ceramide could cause insulin resistance in skeletal muscle by activating key proteins that antagonise insulin-responsive metabolic and signalling pathways (Savage *et al.* 2007). Compared to young, IMCL content is increased by up to 40% in older people as seen on [1]H Nuclear Magnetic Resonance (^{1}HNMR) spectroscopy (Petersen *et al.* 2007). As alluded to in the introduction chapter of this thesis, the accumulation of IMCL may be caused by the mismatch between lipid supply and reduction in proportional fat oxidation in ageing (Nair 2005) and not necessarily associated with insulin resistance. Indeed, studies on the causal relationship between skeletal muscle lipid accumulation and insulin resistance are inconclusive. Goodpaster's proposal of the 'athlete's paradox' described highly-trained endurance athletes who possessed high muscle oxidative capacity and increased insulin sensitivity despite increased muscle lipid content (Goodpaster *et al.* 2001). He speculated that it was not the accumulation of lipid in muscle per se but an individual's muscle's capacity for lipid oxidation that influenced development of insulin resistance. Indeed inhibition of mitochondrial carnitine-palmitoyl-transferase-1 (CPT-1) fatty acid oxidation by R –isomer of ethyl-2- [6-(4-chlorophenoxy) hexyl]-oxirane-2-carboxylate (etoximir) in rats elicited a reduction in whole-body insulin-mediated glucose disposal and endogenous glucose production (Dobbins *et al.* 2001), indicating the importance of fatty acid oxidation (or lack thereof) in the development of insulin resistance. Factors implicated in diminished fat

capacity in the elderly include increased fat mass (Nagy *et al.* 1996, Toth *et al.* 1996, Levadoux *et al.* 2011), reduction in skeletal muscle oxidative enzyme activity (Houmard *et al.* 1985, Petersen *et al.* 2007) and reduction in physical activity levels (Sial *et al.* 1996). Fat oxidation rates during sleep and over a 24- hour period of free activity were lower in older compared to young individuals regardless of activity levels (Levadoux *et al.* 2011). Moderate-intensity exercise in older people has been shown to elicit a 35% lower fat oxidation rate compared to younger subjects exercising at the same absolute and relative intensities, presumably caused by altered skeletal muscle metabolism in ageing as whole body lipolysis and plasma FFA availability were not-rate limiting (Sial *et al.* 1996).

With regards to exercise metabolism, other than impaired fatty acid metabolism the few studies investigating the effects of exercise in older individuals showed modified lactate and glycogen kinetics following exercise, but these studies were carried out in highly trained or master athletes and were investigated at maximal oxygen uptake (VO_2 max) (Masse-Biron *et al.* 1992). The changes seen in the ability of ageing skeletal muscle to respond to exercise may be due to loss of muscle mass, decreased muscle oxidative enzyme activity and capillarisation or reduced physical activity and training with ageing (Rofers and Evans 1993). Why there is an age-related decline in fat oxidation resulting in IMCL accumulation and insulin resistance cannot be entirely explained by a decline in physical activity or availability of free fatty acid during exercise. Animal studies show that the rate of fatty acid oxidation in perfused working hearts was lower in aged compared to young (Abu-Erreish *et al.* 1977). As an inverse relationship exists between insulin sensitivity and triglyceride accumulation in muscle (Johannesen *et al.* 2012), the capacity of fat oxidation in aged muscle may be of critical importance to development of metabolic disease in ageing.

IMCL-derived fatty acid oxidation during exercise and how this affects lipid content and glucose uptake in muscle in older people have also not been investigated in detail. Moreover,

distinct pools of IMCL droplets within the intermyofibrillar (IMF) and subsarcolemmal (SSL) regions of the muscle cell have been identified (Nielsen *et al.* 2010; Crane *et al.* 2010, Chomentowski *et al.* 2011 Jonkers *et al.* 2012;), but their contribution to IMCL oxidation and utilisation during exercise has not been elucidated. A 3-fold higher volume of lipid droplets in the SSL region has been identified in people with T2DM compared to obese and high-endurance athletes (Nielsen *et al.* 2010). SSL lipid droplet accumulation in particular have been implicated in the development of insulin resistance, perhaps as a result of lipid droplet metabolites on insulin action, due to its proximity to muscle fibre nuclei and signalling pathways of the sarcolemma via DAG and ceramides (Coen and Goodpaster 2012). Moreover, change in the number of lipid droplets post-exercise in SSL and IMF differ but have only been investigated in healthy middle-aged sedentary normal-weight, (Malenfant *et al.* 2001a and b, He *et al.* 2004; Li *et al.* 2014) overweight (Malenfant *et al.* 2001, Li *et al.* 2014), T2DM (Nielsen *et al.* 2010) and endurance-trained older subjects (van Loon *et al.* 2003).

As body composition and physical activity changes with age it is important to examine if and how these factors, rather than inherent ageing per se, affect insulin sensitivity, skeletal muscle substrate metabolism and utilisation in healthy older vs. healthy young men. Moreover, no studies to date have characterised skeletal muscle fat metabolism during exercise in older healthy people with skeletal muscle insulin sensitivity. This study compared body composition, substrate metabolism and skeletal muscle lipid accumulation during rest and light-intensity exercise and insulin sensitivity in older and young healthy men with the premise that these parameters are influenced by body composition, rather than ageing. It was also hypothesised that lipid accumulation in ageing is caused by impaired IMCL oxidation during exercise.

3.2 Methods

3.2.1 Human Participants

Seven old lean (69.7±0.9 years, body mass 70.3±2.4 kg), 7 old overweight (68.6±0.8 years, body mass 86.3±1.8 kg) and 7 young (21.1±0.9 years, body mass 74.4±4.0 kg) healthy males were recruited to the study. All participants underwent medical screening, blood testing and gave informed consent as described in the common methods section (Chapter 2). Subjects were excluded if they smoked, had diabetes or other metabolic disorders, cardiovascular disease, blood disorders or abnormal blood tests.

3.2.2 Pre-experimental tests

The incremental maximal oxygen consumption (VO_2 max) test was performed prior to the experimental visits to determine the workload or intensity equivalent to 50% VO_2 max that the participants were required to cycle at during the exercise visits. This low intensity exercise model was chosen as fat oxidation would be expected to be maximal (Romijn *et al.* 1993, Achten 2004), where in vivo mitochondrial function would be challenged and could be assessed, and previous studies have suggested age-related differences (Sial *et al.* 1996).

Participants attended for two experimental visits separated by at least a week, as described below.

3.2.3 Experimental Visits

The first experimental visit involved a resting 3-hour hyperinsulinaemic euglycaemic clamp with infusion of 2DG, a glucose analogue to determine skeletal muscle insulin sensitivity; whereas the following visit involved the fatty acid tracer, [U-^{13}C] palmitate infusion and exercising at 50% of VO_2 max for one hour to measure fat oxidation and metabolism in muscle during light-intensity exercise.

3.2.3.1 Hyperinsulinaemic euglycaemic clamp visit

Participants arrived at the David Greenfield MRC/ARUK Centre for Musculoskeletal Ageing Research laboratory at 0800 after an overnight fast. A DEXA scan (Lunar Prodigy, GE Healthcare, US) was performed to assess body composition. They then rested semi-supine on a bed while cannulae were inserted retrograde into a superficial vein on the back of the hand for arterialised blood sampling and forearm veins for insulin, glucose and 2DG infusions. The hand was placed in a hand-heated box throughout the experiment to allow for arterialised blood sampling. A 3-hour hyperinsulinaemic euglycaemic clamp at 60 mU m^{-2} min^{-1} was carried out to assess insulin sensitivity. 2DG was infused at a rate of 6mg kg^{-1}h^{-1} at the same time. Blood glucose concentrations during the clamp were determined at 5 min intervals using an autoanalyser (Yellow Springs Instrument YSI, US). Arterialised venous blood was also obtained at t= 0 and every 30 min throughout the clamp and centrifuged at 1000g at 4°C for 10 min, and stored at -80°C until subsequent analysis for serum insulin and plasma 2DG. Indirect calorimetry was performed before and during steady state of the hyperinsulinaemic euglycaemic clamp (2 hours after start of clamp) using a GEM (Gas Exchange Measurement, GEMNutrition Ltd, Cheshire, UK) ventilated hood system. Samples for serum insulin were placed in aliquots and frozen at a minimum of -20°C and measured using a solid-phase ^{125}I radioimmunoassay using standard insulin kits (Milipore Human Insulin Assay; Merck Millipore). Muscle biopsy samples using the technique described by Bergstrom were obtained from the *vastus lateralis* muscle of each subject at rest before and immediately after the clamp to determine incorporation of muscle 2DG. 2DG is a glucose analogue and is regarded a robust surrogate marker of glucose uptake by muscle (Yamamoto *et al.* 2009). Post-absorptively, total muscle 2DG accumulates in a linear fashion, and exercise, a known stimulus of skeletal muscle glucose uptake, significantly and consistently increases muscle 2DG uptake.   The linear accumulation of 2DG

suggests that 2DG is neither stimulating (through a "mass effect") nor inhibiting (through hexokinase inhibition) its own uptake. It closely resembles glucose in the characteristics of its transport but is metabolised only to the 6-phosphate derivative, 2DG6P. It is therefore effectively trapped within skeletal muscle where its concentration (and that of 2DG) can be determined as a function of glucose uptake, assuming 2DG uptake to be representative of all hexose uptake.

3.2.3.2 Exercise Visit

At least a week after the resting study visit, participants reported to the laboratory and rested semi-supine on a bed while a cannula was inserted retrograde into a superficial vein for arterialised blood sampling and the other into a forearm vein for infusion of [U-^{13}C] palmitate (99% enriched (Cambridge Isotope Laboratories, Andover, MA, USA) at a concentration of 0.19mg kg^{-1} h^{-1} for 2 hours. Breath samples were collected via one-way valve bags and introduced into breath tubes (BD Biosciences) for subsequent ^{13}CO$_2$ enrichment analysis before the start of the infusion and every hour of the resting period. Plasma samples for palmitate tracer and FFAs were collected hourly during resting. At the end of the second hour, percutaneous biopsy sampling from the *vastus lateralis* muscle was performed before participants went on to cycle on the ergometer at 50% VO$_2$ max equivalent workload. The [U-^{13}C] palmitate concentration was increased to 0.28 mg kg^{-1} hr^{-1} at the onset of exercise for 1 hour. Blood plasma for FFAs, palmitate tracer and breath samples for ^{13}CO$_2$ were obtained every 10 minutes. During the last 10 minutes of exercise, indirect calorimetry was determined (Quark CPET system, Cosmed, Italy). A muscle biopsy was again taken at the end of the 1 hour exercise.
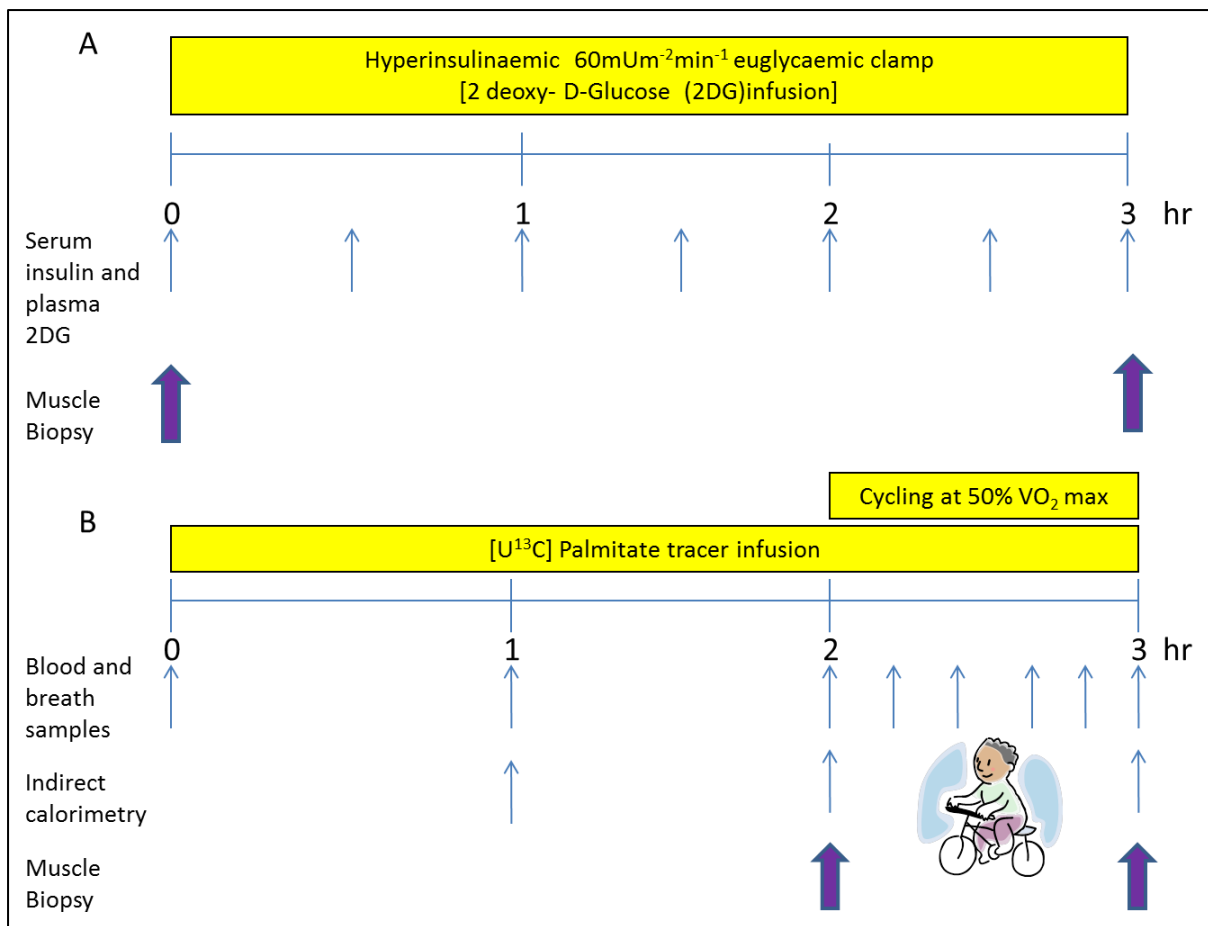
Figure 3.1: Schematic diagram on the experimental visits. Protocol A –Resting visit and Protocol B- Exercise visit

3.2.4 Sample collection and analysis

All DEXA scans were analysed by a single operator to avoid inter-operator variability. The scans were analysed for trunk, leg and arm composition using standardised regions conforming to specifications. Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1000g at 4°C for 10 min. Plasma aliquots were subsequently stored at -80°C until analysis. Blood serum and plasma were collected at baseline and every 30 minutes during the clamp, allowed to clot and then centrifuged (1400 RCF at 4°C for 10 mins) for subsequent storage at -80°C until use for

determination of insulin concentrations and 2DG glucose tracer respectively. Muscle samples were snapped frozen in isopentane and stored in liquid nitrogen, and subsequently analysed for 2DG content.

3.2.5 Palmitate tracer analysis

Palmitate tracer is extracted from plasma after addition of a stable isotope internal standard to allow quantification of concentration, purified by thin layer chromatography and derivatised to their methyl esters. Isotopic enrichment of palmitate is then determined by gas chromatography mass spectrometry (GCMS). This method is superior to older methods of tracing FFAs, such as calorimetry and enzymatic assays. It provides an accurate way of identifying individual and total FFA concentrations observed during an insulin infusion and is specific in determining tracer enrichment. Relatively large amounts of tracer and albumin were previously required to allow detection. Infusion rates can now be reduced with the introduction of combustion isotope ratio mass spectrometry where $^{13}CO_2$ is measured. Of the FFA tracers, palmitate, oleate and linoleate provide estimates of total FFA flux that are within 15% of actual values. Continuous palmitate infusion allows determination of regional kinetics in tissues.

3.2.6 Electron Microscopy analysis

Lipid and mitochondrial analyses were determined using a computerised image analysis system (Image-J), by an operator blinded to subject and age following criteria set to identifying lipid and mitochondria (Crane *et al.* 2013). Lipid droplets and mitochondrial fragments were circled and converted to actual size using a calibration grid. Skeletal muscle samples were first fixed in 1% osmium tetroxide, then dehydrated in ethanol and embedded in Spurr's resin. Thin sections were cut using an ultramicrotome, placed on copper/palladium

(Cu/Pd) grids and stained for 5 minutes in uranyl acetate followed by 2 minutes in lead acetate. 40 longitudinal images were visualised using a transmission electron microscope at a magnification of x4200. Two micrographs from four different muscle fibres were taken at random and sequentially by an operator blinded to subject age. Images were taken from the SSL region adjacent to the nucleus with most of the image containing the intermyofibrillar area. The images were photographed using a 1-s exposure time and digitised using a white light illuminator. Values are reported as mean individual IMCL or mitochondrial size, total number of IMCL droplets or mitochondria per square micron of tissue and percentage IMCL or mitochondrial area density.
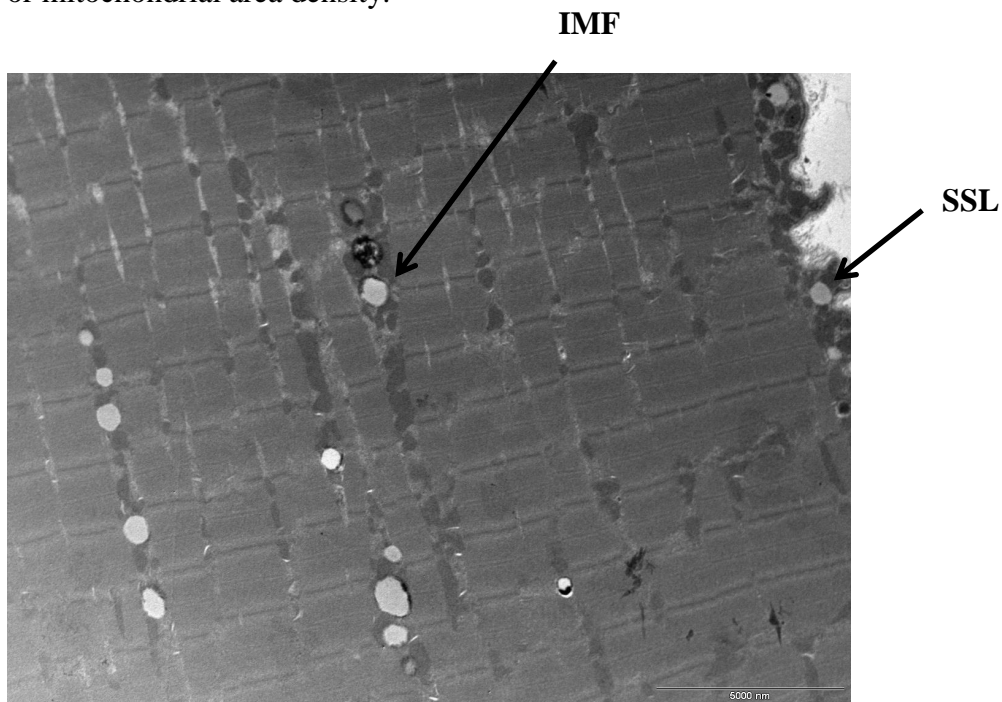
Figure 3.2: Electron micrograph (x4200) of a *vastus lateralis* muscle cell depicting IMF and SSL lipid droplets.

3.2.7 Insulin sensitivity and skeletal muscle 2DG uptake

Insulin sensitivity was measured as the rate of insulin-stimulated glucose disposal (Rd) during a 3hour hyperinsulinaemic (60mU $m^{-2}min^{-1}$) euglycaemic clamp at the same time as infusion of the stable tracer isotope 2DG. The Matsuda's insulin sensitivity index was also

determined from steady state of plasma glucose and serum insulin. Frozen muscle (30–40 mg) was powdered under liquid nitrogen, and transferred to 70% ethanol. The sample was vortex mixed and then centrifuged at 5000g for 10 minutes. The resultant supernatant was used for 2DG/2DG6P analyses using a 2DG uptake measurement kit (Cosmo Bio Ltd, Tokyo). Briefly 2DG accumulates in muscle as 2DG6P and is oxidised by the introduction of glucose-6 phosphate dehydrogenase (G6PDH), resulting in NADPH where it was quantified at 420 nm using a recycling amplification enzymatic-photometric system (Saito K and Minokoshi Y *et al.* 2011). For determination of plasma 2DG, 100uL of plasma was aliquoted and mixed with a standard 10ul of fluorodeoxyglucose (FDG). 1 ml of 100% ice cold ethanol is added to each tube and placed in ice/fridge for 20mins then spun for 2 minutes at 10000 rpm. The supernatant is dried completely in the Techne$^®$ block heater at 90$^°$C for 10minutes. 100uL of oxime (20mg hydroxylamine HCL per 1ml pyridine) is added to each tube, vortex-mixed and incubated at 75$^°$C for 30 minutes in the oven. After cooling at room temperature, 70uL of BSTFA is mixed and incubated for 30 minutes at 70$^°$C. The samples were then run on gas chromatography mass spectroscopy (GCMS), and the first major doublet peak area (2DG) and second major doublet peak area of FDG standard (elutes closely after second 2DG peak) are recorded.

3.2.8 Analysis of lipid metabolites DAG and ceramide

Quantification of intramuscular DAGs and ceramides was performed using high-performance liquid chromatography (HPLC) tandem mass spectroscopy. Tissue homogenates were fortified with internal standards and extracted into a one-phase neutral organic solvent system, evaporated and reconstituted in methanol. Quantitative analysis was performed in a positive multiple-reaction-monitoring mode, based on calibration curves generated by adding to an artificial matrix known amounts of target analytes, synthetic standards and an equal amount of internal standard. DAG and ceramide levels were normalized to total protein

levels. For skeletal muscle DAG and ceramide analyses, 50 ng internal standard (1,3[d5]-15:0 DAG) was added to 5 mg freeze-dried muscle powder, total muscle lipids were extracted in $CHCl_3$:MeOH:H_2O (Folch *et al.* 1957) and the most abundant DAG (diC16:0, C16:0/C18:1, diC18:1) and ceramide (C16:0, C18:0, C18:1, C20:0, C24:1, C24:0) species were quantified using LCMS/MS (Blachnio-Zabielska *et al.* 2012,2013). Briefly, chromatographic separation was performed on a MOS-1 Hypersil C8 column (2.1 x 100 mm; 3 µm); Thermo Scientific at a flow rate of 0.2 ml/min, using a binary gradient (90-99% solvent B; 0-20 mins) with 1.5 mM ammonium formate, 0.1% formic acid in water as solvent A and 2 mM ammonium formate, 0.15% formic acid in methanol as solvent B. DAG and ceramides were monitored in positive electrospray ionisation mode (Quattro Ultima triple quad) as their $[M+NH_4]^+$ and $[M-H_2O]^+$ adducts, respectively. Peak areas (MassLynx 4.0, Micromass Ltd, UK) normalised to the internal standard were converted to absolute concentrations using standard curves constructed for each species undergoing the full extraction procedure.

3.2.9 Determination of skeletal muscle metabolites

Freeze-dried muscle was dissected free of visible blood and connective tissue, pulverised and used for the determination of muscle free carnitine, acetylcarnitine and long-chain acylcarnitine using the radioenzymatic method described previously by Cederblad (Cederblad *et al.* 1990). Approximately 20 mg of wet muscle tissue was used to determine maximal CPT-1 activity using the forward radioisotope assay (McGarry *et al.* 1983). Briefly, muscle was homogenised in 50 mm Tris/HCl buffer (pH 7.5) and immediately used to determine malonyl-CoA (10 µm)-sensitive [14C] palmitoylcarnitine production from 100 µm palmitoyl-CoA, 1 mm L-carnitine and 0.05 µCi l-[14C]carnitine, which was normalised to total protein content using the Bradford assay.

Muscle phosphocreatine (Pcr), lactate and glycogen were determined on freeze-dried muscle using the spectrophotometric method of Harris (Harris *et al.* 1974). Muscle total creatine content was calculated as the sum of free creatine and PCr. In addition, maximal citrate synthase activity was determined spectrophotometrically on whole muscle homogenates based on the methods of Opie & Newsholme (1967) and Zammit & Newsholme (1976) and expressed as mmol min^{-1} (kg wet muscle)$^{-1}$.

3.2.10 Calculations

Total fat and carbohydrate oxidation rates were calculated using the non-protein respiratory quotient (Frayn 1983) and described in detail in the common methods section of this thesis.

Fat oxidation rate =1.695 x VO$_2$+ 1.701 x VCO$_2$ g/min

Carbohydrate oxidation rate = 4.585 x VCO$_2$ -3.226 x VO$_2$ g/min

EE = 15.9 x VO$_2$+ 5.2 x VCO$_2$ J/min

Where VO$_2$ and VCO$_2$ are expressed as litres per min and oxidation rates as grams per minute.

Breath and plasma enrichments are expressed as the tracer/tracee ratio (TTR);

TTR = (^{13}C/^{12}C)sa - (^{13}C/^{12}C)bk

where sa denotes the sample and bk the background value.

The rate of appearance (Ra) and rate of disappearance (Rd) of palmitate was calculated using the single-pool non steady- state Steele equations (1959) adapted for stable isotope methodology as described by Wolfe & Jahoor 1990.

$$\mathbf{Ra = \frac{F - V[(C_2 + C_1)/2][(E_2 - E_1)/(t_2-t_1)]}{(E_2 + E_1)/2}}$$

$$\mathbf{Rd = Ra - \frac{V(C_2-C_1)}{t_2-t_1}}$$

where F denotes the infusion rate (μmol kg^{-1} min^{-1}), V is the distribution volume for palmitate (40 ml kg^{-1}), C_1 and C_2 are the palmitate concentration (mmol l^{-1}) at times 1 (t_1) and 2 (t_2), respectively, and E_1 and E_2 are the plasma palmitate enrichments (TTR) at times t_1 and t_2, respectively. $^{13}CO_2$ production (Pr$^{13}CO_2$; mol min^{-1}) from the infused palmitate tracer was calculated as:

Pr^{13}CO2 = (TTRCO$_2$ X VCO$_2$)/(k)

where TTRCO$_2$ is the breath $^{13}C/^{12}C$ ratio at a given time point, k is the volume of 1 mol of CO2 (22.4 l mol^{-1}).

Plasma palmitate oxidation (Rox; mol min^{-1}) can subsequently be calculated as:

Rox palmitate = Rd palmitate x (Pr^{13}CO2/F x 16)

where Rd palmitate is the rate of disappearance of palmitate (mol min^{-1}), F is the palmitate infusion rate (mol min^{-1}) and 16 is the number of carbon atoms in palmitate. Total plasma FFA oxidation was calculated by dividing palmitate oxidation rate by the fractional contribution of plasma palmitate to total plasma FFA concentration.

The contribution of plasma FFA oxidation to total fat oxidation was determined by assuming that the molecular mass of triglyceride is 860gmol^{-1} and every TG molecule contains three

fatty acids. The contribution of other fat sources was hence calculated by subtracting plasma

FFA oxidation from total fat oxidation.

Insulin sensitivity during the clamp was calculated using the equation

$$SI_{Clamp} = M/(G \times \Delta I)$$

where M is normalized for G (steady-state blood glucose concentration;mmol^{-1}) and ΔI (difference between fasting and steady-state plasma insulin concentrations, mUl^{-1}).

5.2.11 Power Calculations

The repeated measures coefficient of variation for the insulin clamp technique is 10%; therefore a 15-20% difference in insulin sensitivity should be able to be detected in 7 participants. Therefore the aim was to recruit 10 volunteers for each group (older lean, older overweight and young lean) to allow for a 30% dropout. Both older lean and overweight volunteers were subsequently investigated for the effects of carnitine on muscle metabolism and insulin sensitivity (Chapter 6).

3.2.12 Statistical analysis

Values presented in text, tables and figures are expressed as mean ± the standard error of mean (s.e.m). Analyses were performed using the statistical program (GraphPad Prism 6.0, GraphPad Software Inc, USA) to detect differences between the three groups at rest using a one-way ANOVA and within and between groups during exercise using a two-way ANOVA (time and treatment factors). When a significant effect was observed, Tukey's and Sidak's post-hoc test was performed, respectively, to identify individual differences. Statistical significance was set at $P < 0.05$.

3.3 Results

3.3.1 Participant Characteristics

10 participants for each group were initially recruited (20 older and 10 young); however there were a total of 6 and 3 drop-outs from the older and young groups respectively, due to several reasons (Appendix 1). Table 3.1 shows characteristics of study participants. Body mass, body mass index (BMI) and lean body mass (LBM) were not different in older lean and young groups at baseline. When total body fat mass was divided into regions, older overweight (OO) had higher mean BMI and body mass, greater trunk, arm and leg fat mass compared with older lean (OL) and young lean (YL) participants (Figure 3.1). HOMA-IR was also greater in OO than OL ($P<0.001$). Both OO and OL were well-matched in terms of age and both absolute (ml/min) and relative (ml/kg LBM/min) VO_2max. OO and OL achieved lower absolute and relative VO_2max and corresponding 50%VO_2max workloads compared to YL. Self-reported habitual physical activities were similar between the young and older lean participants and lower in older overweight compared to older lean ($P<0.001$).

Table 3.1: **Characteristics of Participants**

	Young lean	Old lean	Old overweight
n	7	7	7
Age (y)	21.5 ± 1.0	69.7 ± 0.9[+++]	68.6 ± 0.8[***]
Statin use (n)	0	3	4
Body mass (kg)	71.8±3.6	70.3±2.4	86.3±1.8[***,†††]
BMI (kg/m^2)	22.4 ± 0.7	24.0 ± 0.6	29.0 ± 0.7[***,†††]
Fat free mass (kg)	55.9 ± 3.2	51.3 ± 1.5	55.6 ± 1.9
Arm lean mass (kg)	7.6 ± 0.4	6.7 ± 0.3	7.4 ± 0.3
Leg lean mass (kg)	21.8 ± 0.4	18.3 ± 0.6	19.6 ± 0.7
Trunk fat mass (kg)	4.6 ± 0.8	7.2 ± 1.1	16.0 ± 0.8[***,†††]
Arm fat (kg)	0.9 ± 0.1	1.5 ± 0.2	2.6 ± 0.3[***,†††]
Leg fat (kg)	4.7 ± 0.4	4.9 ± 0.4	7.4 ± 0.6[†]
Fasting glucose (mmol/L)	4.5±0.1	4.7±0.1	5.0±0.1[*]
Fasting insulin (mIU/L)	10.6±1.4	7.4±1.6	12.6±1.2[†]
HOMA IR	2.14 ± 0.32	1.60 ± 0.36	2.81 ± 0.25[†††]
VO$_2$max (l/min)	3.19 ± 0.19	2.26 ± 0.15[+++]	2.19 ± 0.13[***]
VO$_2$max (ml/kg LBM/min)	57.4 ± 2.4	43.8 ± 1.9[+++]	39.9 ± 1.5[***]
Workload at 50% VO$_2$max (W)	93.0 ± 5.9	55.9 ± 5.8[+++]	46.3 ± 6.7[***]
Heart rate at 50% VO$_2$max (beats/min)	137 ± 2	102 ± 7[+++]	102 ± 6[***]
Physical activity frequency (occasions/week)	3.5 ± 0.5	5.1 ± 1.2	1.8 ± 0.7[†††]
Incremental shuttle walk test (ISWT) (m)		622.9±16.1	498.6±23.2[†††]

One-Way ANOVA with Tukey post-hoc test apart from unpaired t-test for ISWT

All values (n=7) are means ± standard error of mean (SEM).*P<0.05, ***P<0.001, Old overweight different to corresponding Young lean value. †P<0.05, †††P<0.001, Old overweight different to corresponding Old lean value. $^{+++}$P<0.001, Old lean different to corresponding Young lean value.
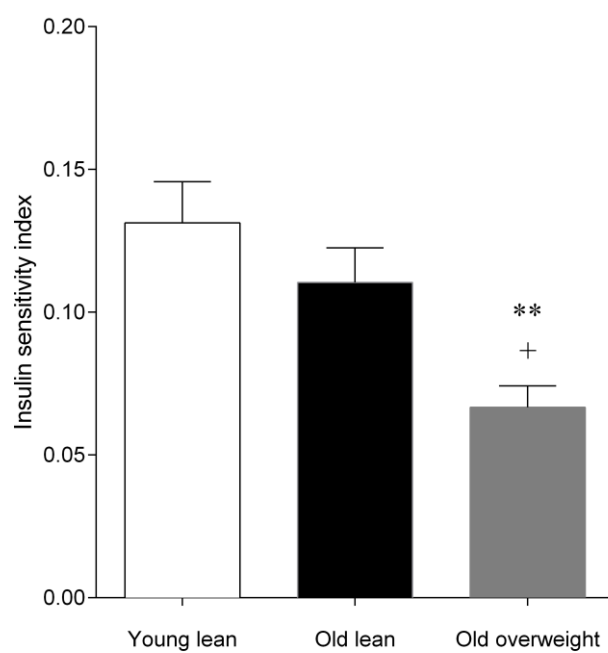
Figure 3.3: Insulin sensitivity index of young lean (n=7), old lean (n=7) and old overweight (n=7). $^{+}$P<0.05 different to Old lean value, **P<0.01 different to Young lean value.
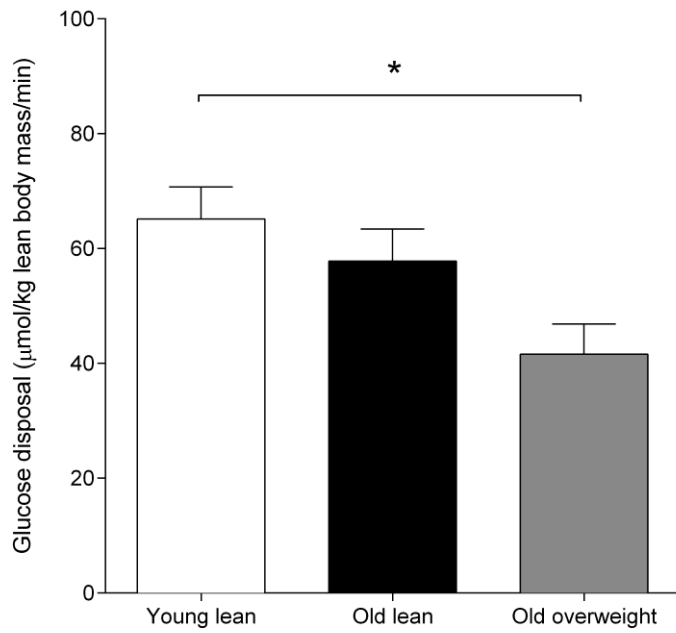
Figure 3.4: Steady state glucose disposal rates during the resting hyperinsulinaemic 60mu m$^-$^{2}min^{-1} euglycaemic clamp in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) participants. *P<0.05 different to Young lean value.
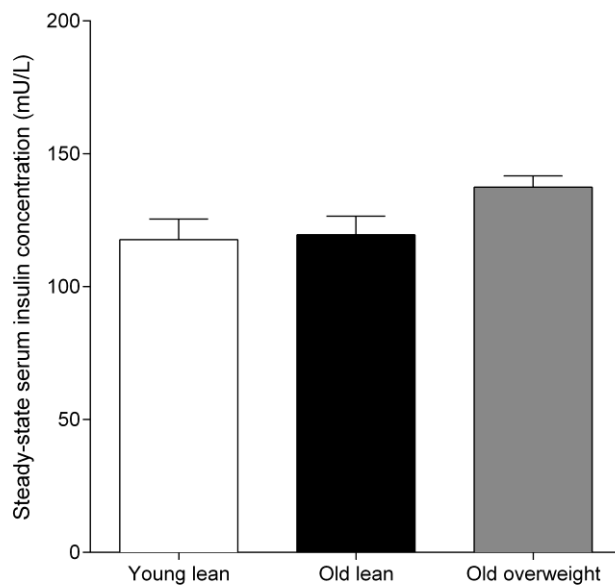
Figure 3.5: Steady state serum insulin concentrations during the hyperinsulinaemic 60mu m$^-$^{2}min^{-1} euglycaemic clamp in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) participants.
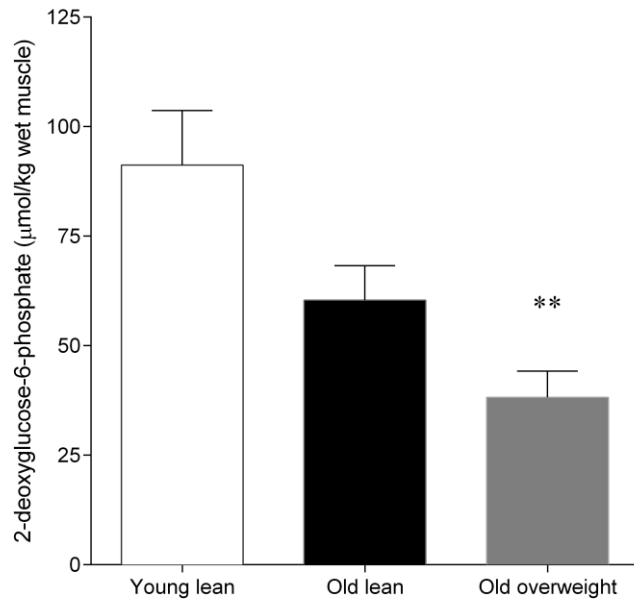
Figure 3.6: 2DG6P uptake in skeletal muscle during steady state of the hyperinsulinaemic 60mum^{-2}min^{-1} euglycaemic clamp in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) participants. **P<0.01 different to Young lean value.
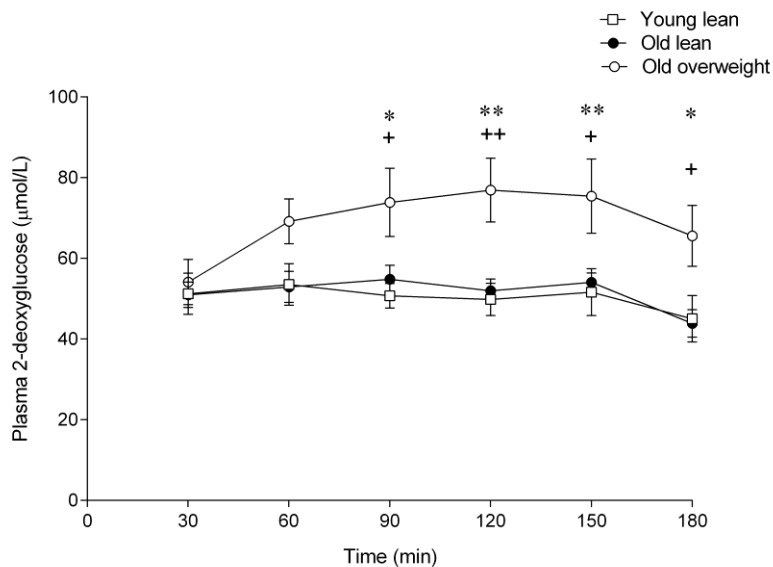
Figure 3.7: Plasma 2DG during the resting 3hr hyperinsulinaemic 60mu m^{-2}min^{-1} euglycaemic clamp in young lean (n=7), old lean (n=7) and old overweight (n=7) participants. *P<0.05, ** P<0.01, Old overweight different to corresponding Young lean value. $^{+}$P<0.05, $^{++}$P<0.01, Old overweight different to corresponding Old lean value.
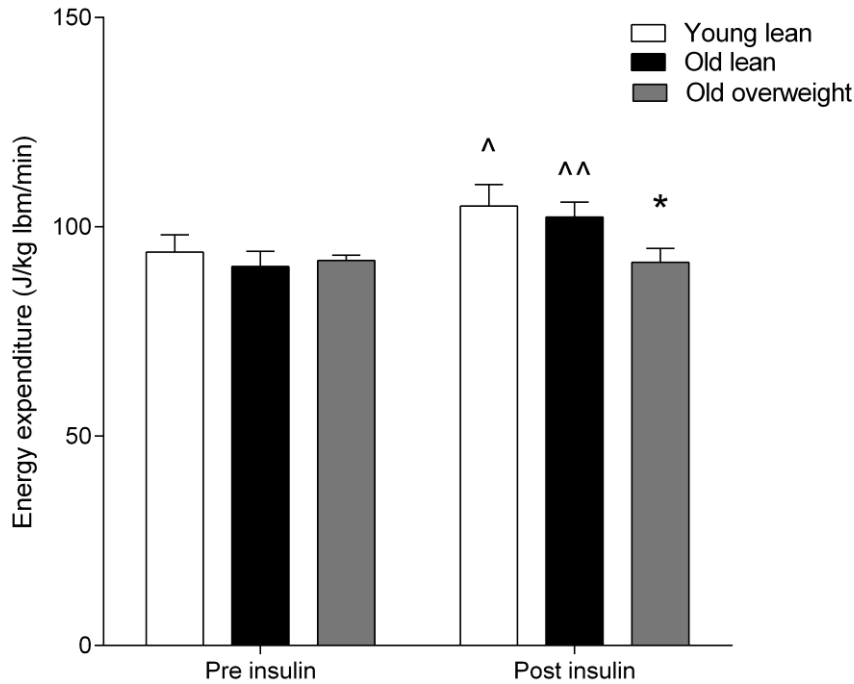
Figure 3.8: Resting energy expenditure pre and post insulin during the 3 hr hyperinsulinaemic 60mu m^{-2}min^{-1} euglycaemic clamp in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) participants. *P<0.05, Old overweight different to corresponding Young lean value. ^P<0.05, ^^P<0.01, different to corresponding pre-insulin values.
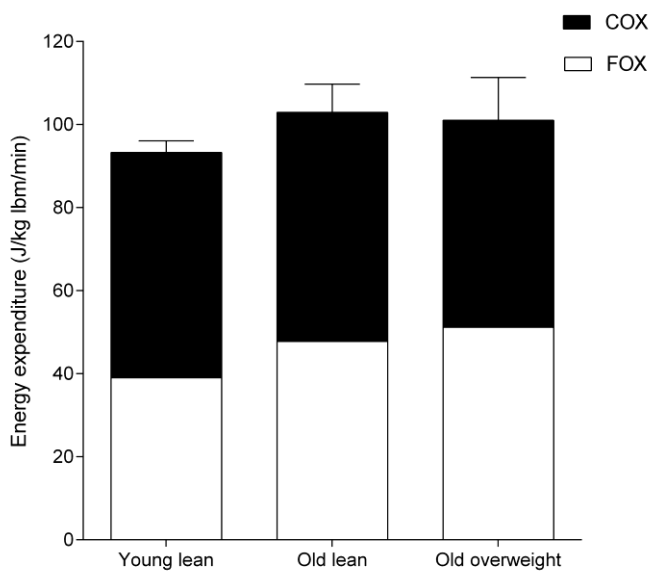
Figure 3.9: Relative contribution of carbohydrate (COX) and fat (FOX) oxidation rates to total resting energy expenditure during the 3 hr hyperinsulinaemic 60mu m^{-2}min^{-1} euglycaemic clamp in Young lean (n=7), Old lean (n=7) and Old overweight (n=7).

3.3.2 Insulin sensitivity

Fasting glucose was greater in OO (5.0±0.1 mmol/L; P<0.05) than YL (4.5±0.1 mmol/L; P<0.05), with a trend of this being greater than OL (4.7±0.1 mmol/L; P=0.07), Table 3.1.

Whilst there was no difference in fasting insulin concentrations between OO (12.6±1.2 mIU/L) and YL (10.6±1.4 mIU/L), OO had greater fasting insulin concentration than OL (7.4±1.6 mIU/L; P<0.05), Table 3.1.

Matsuda's Insulin Sensitivity Index (SI) was similar between YL (0.13±0.01) and OL (0.11±0.01; P<0.05) and both groups in turn have greater SI than OO (0.07±0.01; P<0.01 in YL, P<0.05 in OL); Figure 3.3.

Insulin sensitivity as determined from glucose disposal rates via the 3 hr hyperinsulinaemic 60mU $m^{-2}min^{-1}$ euglycemic clamp at rest in OO was lower than OL and YL (41.6 ± 5.3 µmol kg lbm^{-1} min^{-1} vs. 57.8 ± 5.9 µmol kg $lbm^{-1}min^{-1}$; P<0.05 and 65.2 ± 5.6 µmol kg $lbm^{-1}min^{-1}$, P<0.01; respectively), Figure 3.4.

Steady-state mean serum insulin levels during the clamp did not differ between groups; Figure 3.5. The accumulation of 2DG6P in skeletal muscle was greater by two-fold in YL compared with OO during the glucose clamp (91.2 vs. 38.2 µmol kg^{-1} wet muscle; P<0.01) but not different in OL; Figure 3.6

Steady –state mean plasma 2DG concentrations during the 3 hr insulin clamp were greater in OO compared to OL and YL (72.6 ± 2.2 vs. 50.0 ± 1.9 and 48.8 ± 2.2 $µmolL^{-1}$; P<0.05) respectively; Figure 3.7.

Insulin stimulated energy expenditure increased at rest from 94.0 to 105.0 J kg $lbm^{-1}min^{-1}$ (P<0.05) and from 90.5 to 102.4 J kg $lbm^{-1}min^{-1}$ (P<0.01) in YL and OL respectively, but not in OO; Figure 3.8. However there was no difference in the relative contribution of

carbohydrate (COX) and fat (FOX) oxidation rates to total resting energy expenditure during the 3 hr hyperinsulinaemic 60mu m^{-2}min^{-1} euglycaemic clamp; Figure 3.9.

3.3.3 Muscle Phenotype

3.3.3.1 IMCL volume and droplets in the SSL and IMF regions

At rest the percentage area of SSL region of skeletal muscle covered by lipid droplets in OO was almost 3-fold greater compared with YL ($4.29 \pm 1.23\%$ vs. $1.43 \pm 0.19\%$; P<0.05) and there was a tendency for a 2- fold greater percentage area covered by lipid in OO than OL (4.29 ± 1.23 vs. $1.99 \pm 0.45\%$; P=0.06) respectively (Figure 3.10). SSL lipid area in OO was also greater than YL (P<0.01) and OL (P<0.05) post-exercise, predominantly due to a 25% increase in mean SSL lipid size (Figure 3.11). In contrast there was no difference in the percentage area of IMF fibre covered by droplets between groups; Figure 3.12. The percentage of IMF fibre area covered by lipid droplet during exercise decreased by 40% in YL (0.05; Figure 3.13). Average IMF LD size was 45% greater in OO compared to OL and YL post-exercise (both P<0.01; Figure 3.14).

3.3.3.2 Post-exercise skeletal muscle lipid content

Post exercise the percentage area of SSL covered by lipid droplets in OO was almost 3-fold compared with YL (5.06 ± 0.96 vs. $1.26 \pm 0.27\%$; P<0.01) respectively and more than 2-fold compared with OL (5.06 ± 0.96 vs. $2.05 \pm 0.26\%$; P<0.05); Figure 3.10. This was associated with an increase in lipid droplet size in OO (0.40 ± 0.06 to 0.50 ± 0.07 μm^2; P<0.05) but not in OL or YL; Figure 3.11. A decrease in the percentage of IMF area covered by droplets was seen after exercise in YL (0.65 ± 0.11 to $0.40 \pm 0.09\%$; P<0.05) but not in OL or OO; Figure

3.12. A reduction in droplet number in OO was seen (23.1 ± 3.8 to 16.7 ± 2.7; P<0.05).

Average droplet size remained unchanged post-exercise across the groups; Figure 3.13.
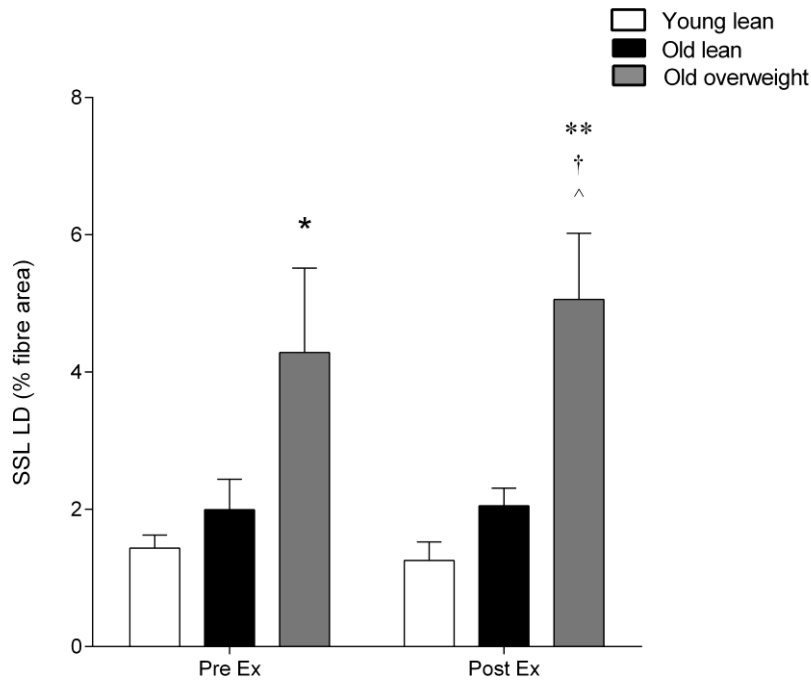
Figure 3.10: SSL lipid droplet % fibre area in young lean (n=7), old lean (n=7) and old overweight (n=7) pre and post-exercise. *P<0.05, ** P<0.01, Old overweight different to corresponding Young lean value. †P<0.05, Old overweight different to corresponding Old lean value. ^P<0.05, different to corresponding pre exercise value.
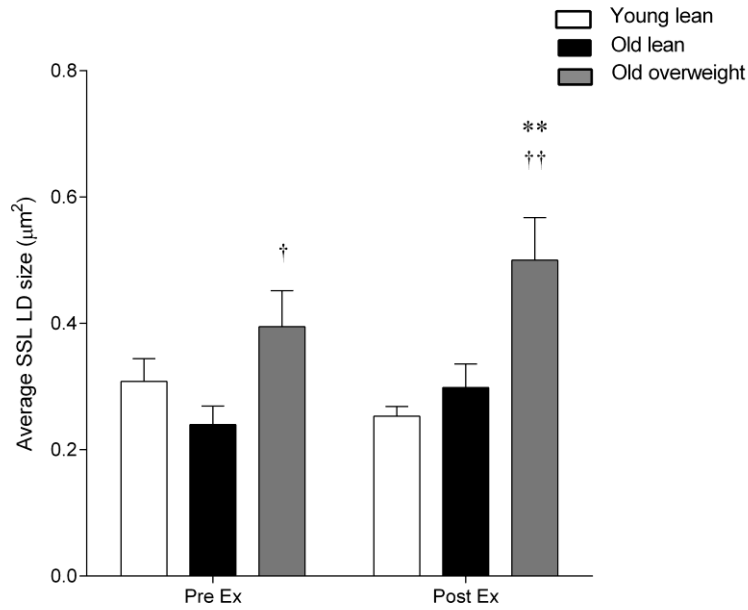
Figure 3.11: Average SSL lipid droplet size per fibre area in young lean (n=7), old lean (n=7) and old overweight (n=7) pre and post-exercise. ** $P<0.01$, Old overweight different to corresponding Young lean value. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, Old overweight different to corresponding Old lean value.
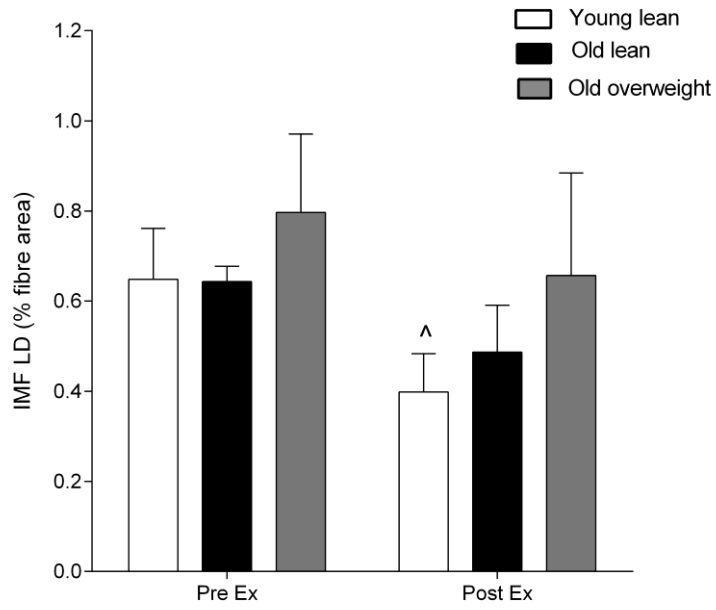
Figure 3.12: Intramyofibrillar lipid droplet percentage fibre area in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) pre and post-exercise. ^$P<0.05$, different to corresponding pre exercise value.
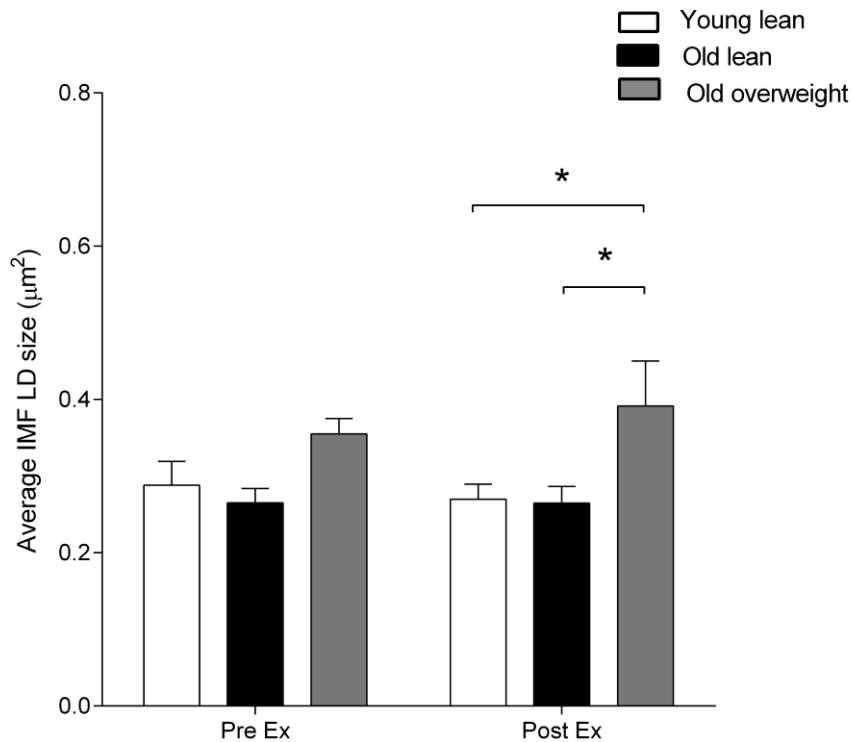
Figure 3.13: Average intramyofibrillar lipid droplet size per fibre area in Young lean (n=7), old lean (n=7) and Old overweight (n=7) pre and post-exercise. *P<0.05 different from corresponding values in Young lean and Old lean values.

3.3.3.3 Skeletal muscle lipid metabolites

There was no difference in skeletal muscle DAG species apart from diC18:1, which was greater in OO compared to OL and YL (42.6 ± 3.3 vs. 29.8 ± 4.2 and 29.4 ± 3.0 ug mg^{-1} protein; P<0.05) respectively; Figure 3.14. There was also no difference in the skeletal muscle ceramide content with the exception of C20:0, which was greater in OO compared to YL (0.49 ± 0.03 vs. 0.31 ± 0.02ug mg^{-1} protein; P<0.01); Figure 3.15. DAG and ceramide analyses were carried out in only 7 OO vs. 5 OL and YL participants who had sufficient muscle samples for analyses.
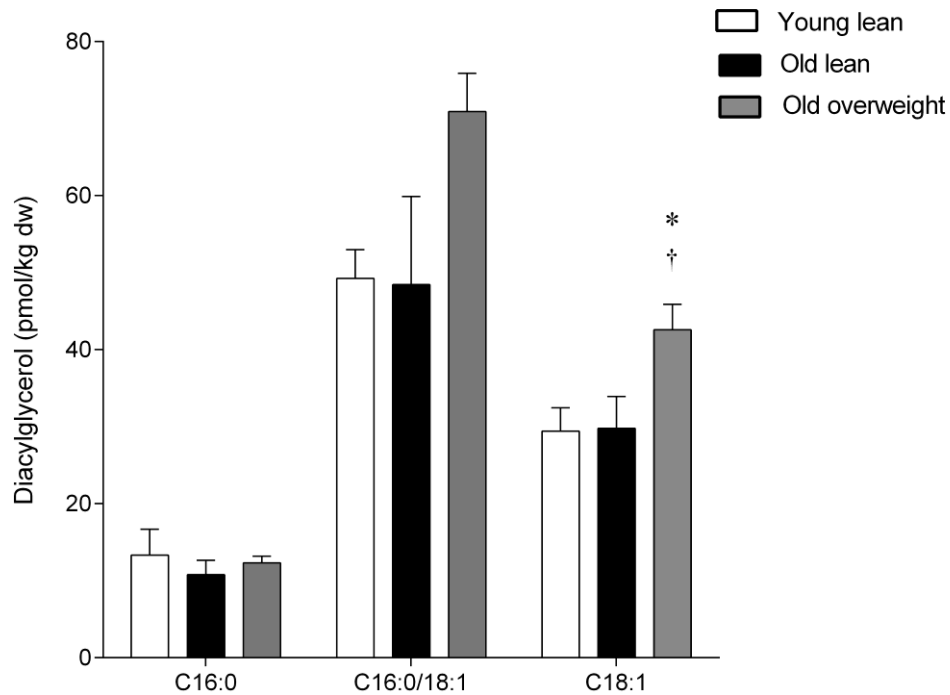
Figure 3.14: DAG species of resting skeletal muscle in Young lean (n=5), Old lean (n=5) and Old overweight (n=7). *P<0.05 vs. Young lean, [†] P<0.05 vs. Old lean.
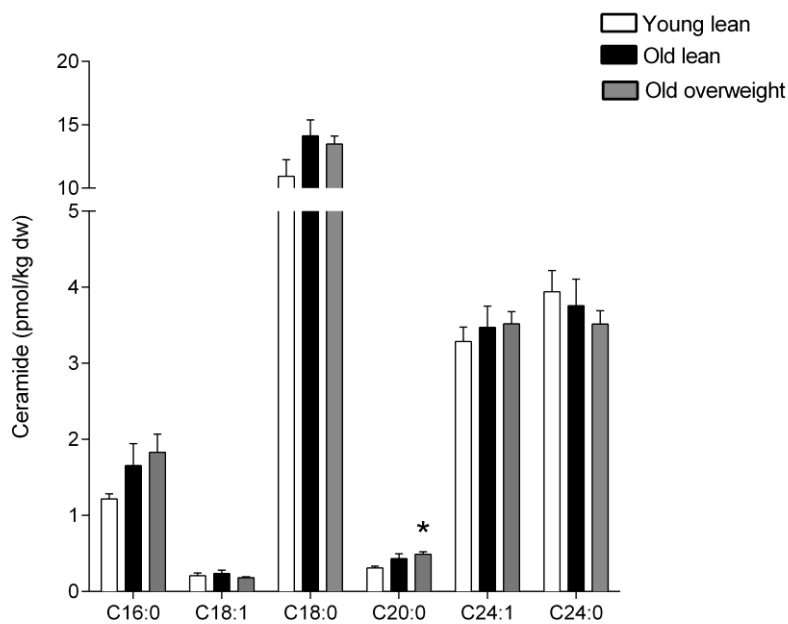
Figure 3.15: Ceramide species of resting skeletal muscle in young lean (n=5), old lean (n=5) and old overweight (n=7). *P< 0.05 vs. corresponding young lean values.

3.3.3.4 Citrate synthase (CS) and CPT-1 activity

There was a trend of lower CS activity in OO compared to OL (96.8 vs. 125.2 $mmol^{-1}mg$ protein min^{-1}; P=0.08) respectively. There was no difference in CPT-1 activity (1.84 ± 0.19, 2.05 ± 0.12 and 2.58 ± 0.28 % of CS activity, respectively) between YL, OL and OO, respectively; Figures 3.16A and B.
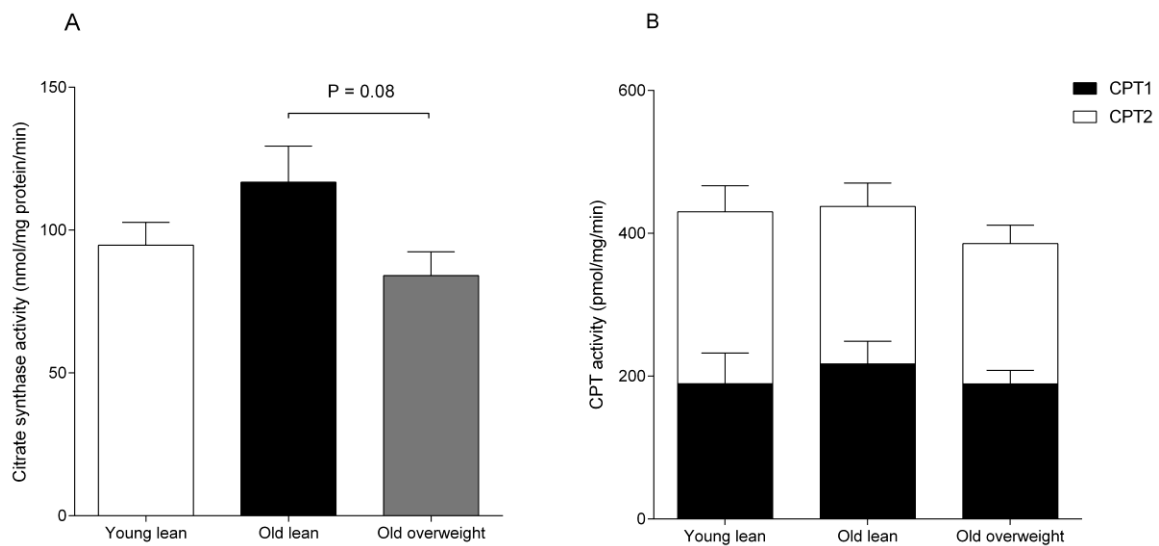
Figures 3.16A and B: Citrate synthase and CPT activity analyses from muscle of Young lean (n=7), Old lean (n=7) and Old overweight (n=7).

3.3.4 Exercise fuel metabolism

There was no difference in the relative contribution of total fat to total energy expenditure during exercise at 50% VO_2max between OO, OL and YL (40.0 ± 4.6 vs. 42.4 ± 3.1 and 43.9 ± 7%, respectively; Figure 3.17). Other fat oxidation (IMCL) was greater in YL (199.0 ± 36.4J kg lbm^{-1} min^{-1}) compared to OL and OO (76.7±13.9 and 73.8 ± 13.1 J/kg lbm min^{-1}; P<0.05) respectively. Energy expenditure was greater in YL compared to OL and OO

(622.2 ± 37.7 vs. 498.0 ± 32.4 and 439.2 ± 41.1 J kg lbm^{-1} min^{-1}; $P<0.05$) respectively. There was no difference in the relative contribution of carbohydrate oxidation during exercise at 50% VO$_2$ max between groups. The difference between plasma fatty acid rate of disappearance and oxidation rates was also not different between OO, OL and YL (8.01 ± 1.62, 7.30 ± 1.17 and 4.97 ± 1.02 µmol kg lbm^{-1}min^{-1}) respectively; Figure 3.18A. Similarly there was no difference in palmitate oxidation rates across the groups, 1.34 ± 0.21, 2.16 ± 0.29 and 2.06 ± 0.49 µmol/kg/min in OO, OL and YL respectively; Figure 3.18B.

3.3.5 Exercise IMCL utilisation

The relative contribution of IMCL to total fat oxidation in OO and OL was lower than that of YL (45.0 ± 7.9 and 38.7 ± 7.7 vs. $71.9 \pm 3.3\%$ respectively; $P<0.01$ Figure 3.17).
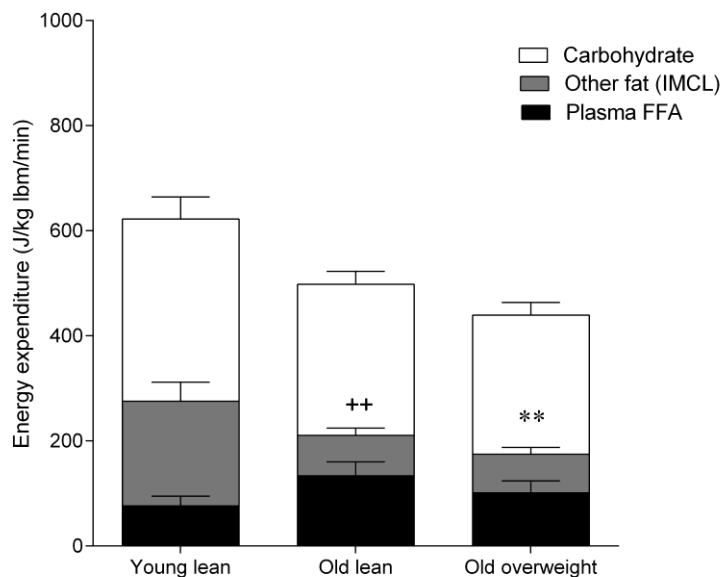
Figure 3.17: Total energy expenditure and relative contribution of fat and other fat (IMCL) to energy expenditure during 1 hour 50% VO$_2$ max exercise in young lean (n=7), Old lean (n=7) and Old overweight (n=7). **$P<0.01$ vs.corresponding Young lean values (total energy expenditure). $^{++}$ $P<0.01$ vs. corresponding Young lean values (other fat).
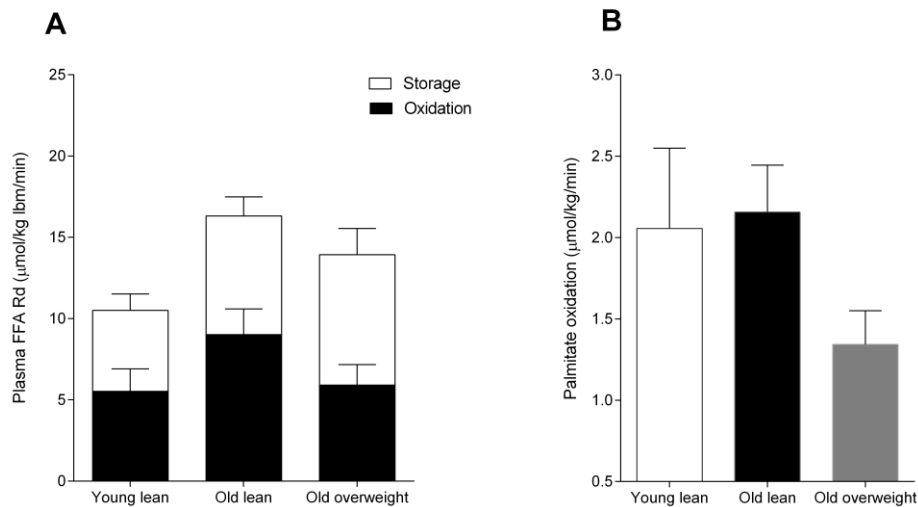
Figures 3.18A and B: Plasma FFA rate of disappearance (Rd) and contribution of fat oxidation (A) and palmitate oxidation (B) during 1 hr exercise at 50% VO$_2$ max in Young lean (n=7), Old lean (n=7) and Old overweight (n=7).

3.3.6 Exercise systemic metabolism

From similar baseline concentrations, plasma noradrenaline rose to a similar steady-state in OL and OO throughout 1 hr of exercise, and was around 1.5-fold greater than the steady-state concentration achieved in YL (both P<0.05 respectively; Figure 3.19). There was no difference in plasma adrenaline between groups at baseline, however at steady state OL and YL have greater levels than OO (0.79 ± 0.23 and 0.74 ± 0.10 nmol/L vs. 0.58 ± 0.16, P<0.05; respectively, Figure 3.20).

Steady state plasma FFA concentrations were greater in the older group (both OO and OL) compared to YL, P<0.05; Figure 3.21. Blood lactate concentrations initially increased in the first 10 minutes of exercise (0.71 ± 0.06 to 1.50 ± 0.29, 0.97 ± 0.13 to 2.05 ± 0.31, and 0.87 ± 0.09 to 1.73 ± 0.28 mmol/L in OL, OO and YL respectively. During steady state, plasma lactate concentration in OO was greater than of OL and YL, P<0.05; Figure 3.22).

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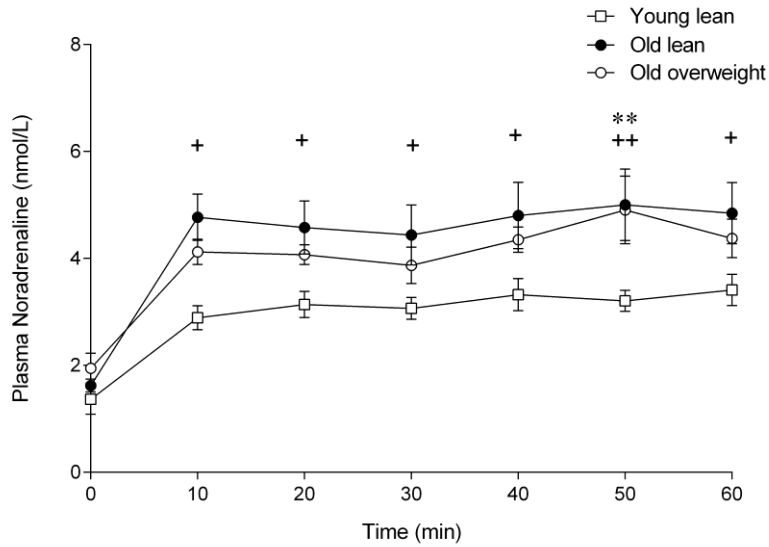
Figure 3.19: Plasma noradrenaline during 1 hr exercise at 50% VO_2 max in young lean (n=7), old lean (n=7) and old overweight n=7). ** P<0.01, Old overweight different to corresponding Young lean value. [+]P<0.05, [++]P<0.01, Old lean different to corresponding Young lean value.
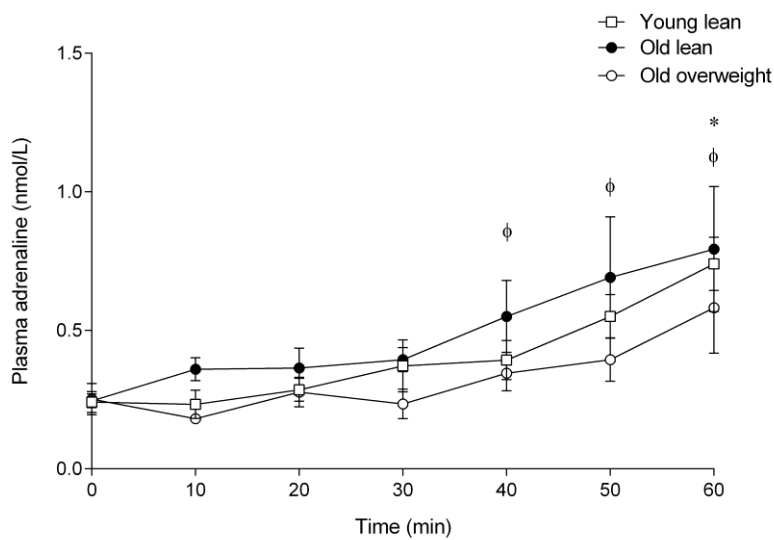

Figure 3.20: Plasma adrenaline during 1 hr exercise at 50% VO_2 max in Young lean (n=7), Old lean (n=7) and Old overweight (n=7) [φ]P<0.05, Old overweight different to corresponding Old lean value. *P<0.05, Old overweight different to corresponding Young lean.
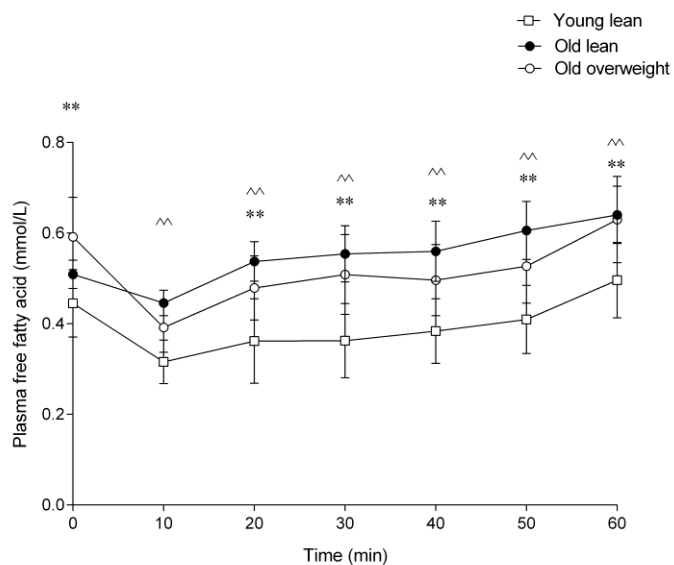
Figure 3.21: Plasma FFA concentrations during 1 hr exercise at 50% VO$_2$ max in Young lean (n=7), Old lean (n=7) and Old overweight (n=7). **P<0.05 in Old lean compared to corresponding old overweight values, ^^P<0.05 in Young lean compared to corresponding old overweight values.
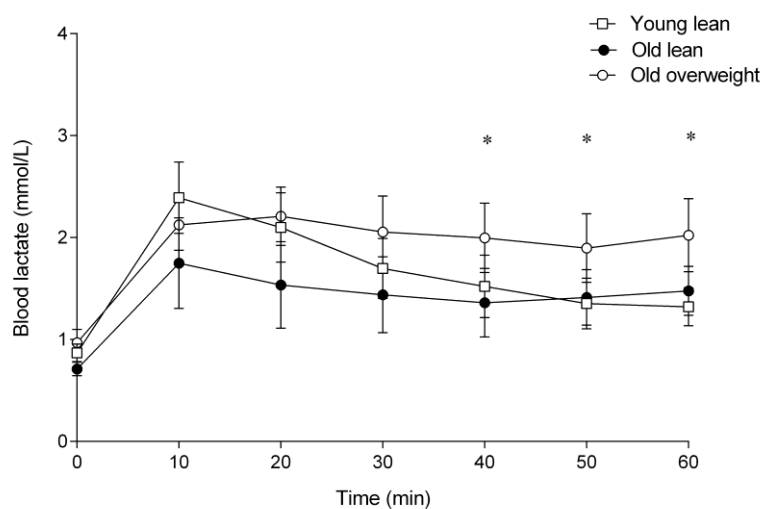
Figure 3.22: Plasma lactate during 1 hr 50% VO$_2$ max exercise in Young lean (n=7), Old lean (n=7) and Old overweight (n=7), *P<0.05 Young lean and Old lean compared to corresponding Old overweight values.

3.3.7 Exercise muscle metabolism

OO had the lowest resting skeletal muscle glycogen content compared to OL and YL (235.7 ± 22.6 vs. 329.7 ± 29.3 and 316.5 ± 22.9 mmol kgdm^{-1}; P<0.05) respectively, Figure 3.23. There was a trend of an increase in skeletal muscle glycogen content in YL post exercise (231.8 ± 37.8 to 316.5 ± 22.9 mmol kgdm^{-1}, P=0.09. There were no differences post-exercise in OL and OO (288.6 ± 48.3 to 329.7 ± 29.3 and 228.6 ± 42.0 to 235.7±22.6 mmol kgdm^{-1}) respectively.

Skeletal muscle acetylcarnitine content increased in YL and OL post-exercise (YL 1.7 ± 0.4 to 4.8 ± 1.1; P<0.05, OL 1.0 ± 0.2 to 7.5 ± 1.5 mmol kgdm^{-1}; P<0.01, respectively) Figure 3.24.

Skeletal muscle Pcr content was lower in OO compared to OL and YL at rest (60.1±5.1 vs. 75.4 ± 2.4; *P<0.05 and 75.5 ± 2.6 mmol kgdm^{-1}; [†]P<0.05) respectively and this was also seen post-exercise (58.9 ± 5.4 vs. 73.7 ± 4.2, *P<0.05 and 77.6 ± 3.7 mmol kgdm^{-1}; [†]P<0.05) respectively, Figure 3.25.

Skeletal muscle lactate content more than doubled in OO (4.6±1.1 to 11.1 ± 1.3 mmol kgdm^{-1}; P<0.05) post-exercise compared to corresponding value pre-exercise, Figure 3.26.
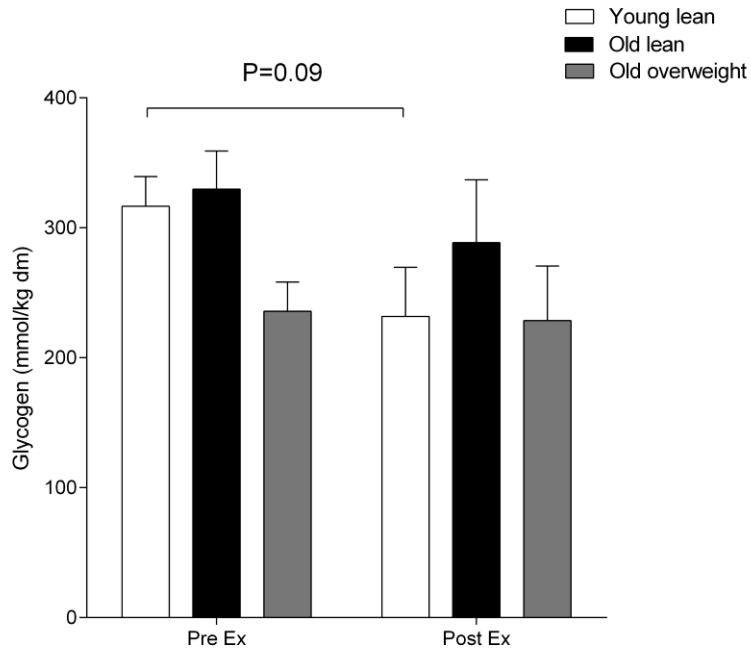
Figure 3.23: Skeletal muscle glycogen content pre- and post-exercise at 50% VO$_2$ max in young lean (n=7), old lean (n=7) and old overweight (n=7).
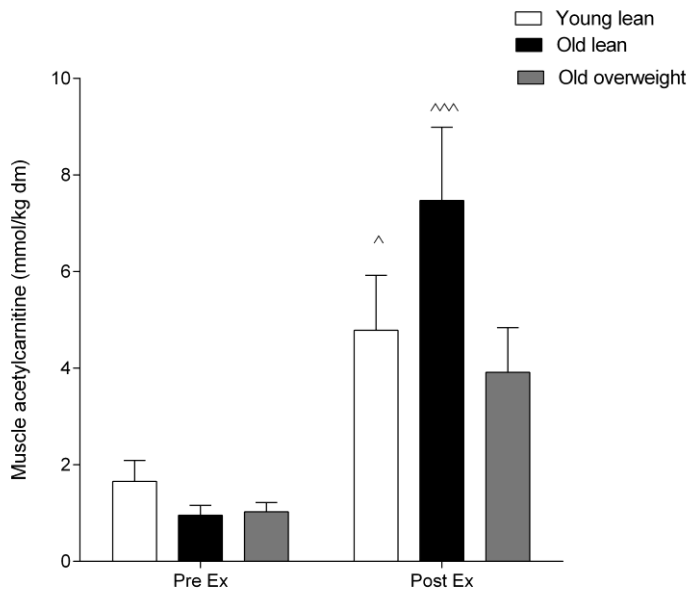
Figure 3.24: Skeletal muscle acetylcarnitine content pre and post exercise at 50% VO$_2$ max in young lean (n=7), old lean (n=7) and old overweight (n=7). ^P<0.05, ^^^ P<0.001 vs. corresponding pre-exercise values.
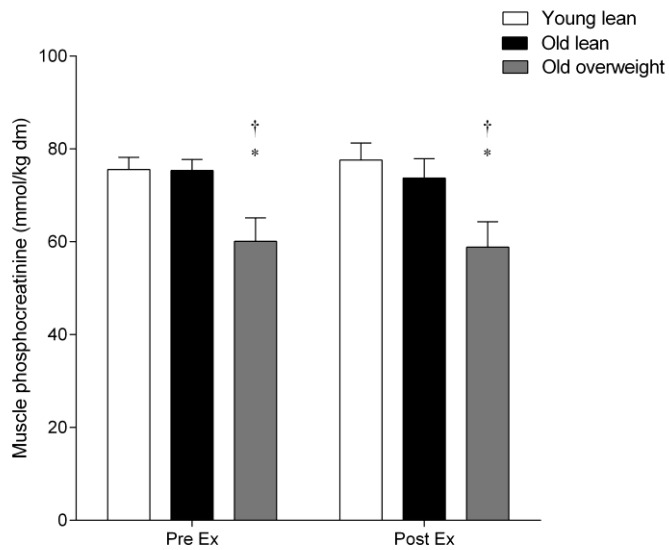
Figure 3.25: Skeletal muscle phosphocreatinine content pre and post exercise at 50% VO_2 max in young lean (n=7), old lean (n=7) and old overweight (n=7). *P<0.05, Old overweight different to corresponding Young lean value. †P<0.05, Old overweight different to corresponding Old lean value.
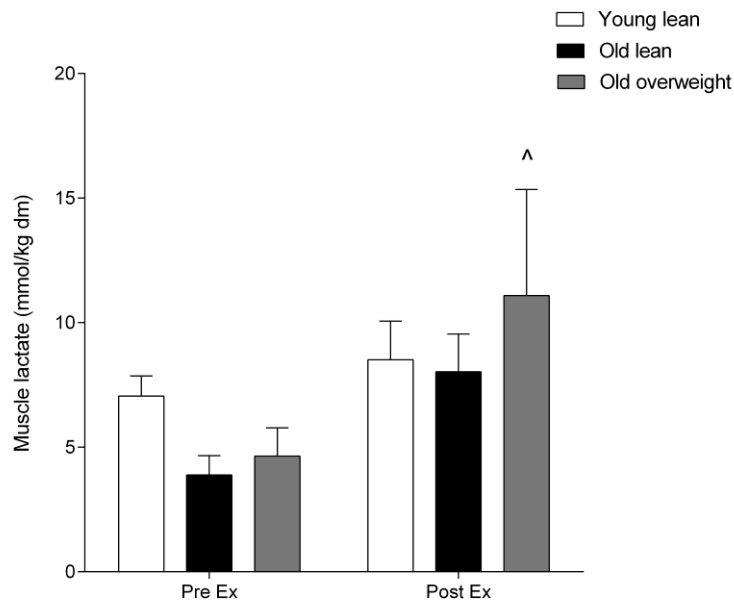
Figure 3.26: Skeletal muscle lactate content pre and post exercise at 50% VO_2 max in young lean (n=7), old lean (n=7) and old overweight (n=7). ^P<0.05 vs. corresponding pre exercise value.

Table 3.4: Summary of differences between young lean, old lean and old overweight healthy males pre-intervention (Week 0).

	Young lean (n=7)	Old lean (n=7)	Old overweight (n=7)
Body fat	Low	Low	High
BMI	Low	Low	High
VO$_2$ max	High	Low	Low
Physical activity levels (based on self-reported activity levels)	Moderate to high	Moderate to high	Low to moderate
EE upon insulin stimulation at rest	Increased	Increased	Unchanged
Relative contribution of total fat oxidation to EE during light-intensity exercise	Similar	Similar	Similar
Relative fatty acid oxidation rates during light-intensity exercise		Lower compared to young lean	Lower compared to young lean
IMCL utilisation during exercise		Lower compared to young lean	Lower compared to young lean
SSL Lipid accumulation post-exercise	Unchanged	Unchanged	Increased
IMF Lipid accumulation post-exercise	Decreased	Unchanged	Unchanged

3.4 Discussion

Ageing may be influenced by various factors in its association with a decline in insulin sensitivity. This study demonstrated differences in body composition, VO_2 max, substrate oxidation at rest and exercise, skeletal muscle metabolism and lipid content between the older and young groups. Self-reported physical habitual activities were similar between young and old lean, but lower in old overweight groups. This study supports the notion that skeletal muscle insulin sensitivity is reduced in older people compared to young, with greater insulin resistance in the older overweight compared to older and young lean, and is associated with reduced IMCL utilisation during light-intensity exercise. This suggests a discordant reduction in muscle fat oxidation, as FFA availability and uptake were not limiting, resulting in an increase in muscle lipid accumulation particularly in the SSL region of muscle in older overweight people. This is not entirely due to age per se, but associated with accumulation of fat mass and possibly activity levels. Skeletal muscle insulin resistance also appears to be associated with lipid accumulation in the SSL region. This is the first study to directly investigate whether IMCL oxidation is impaired during exercise in older insulin resistant individuals, taking into account other factors such as adiposity and physical activity.

3.4.1 Body composition

When body composition was differentiated into overweight (BMI≥26) and lean (BMI≤25), a difference was seen in older individuals and thus they were separated post-hoc into groups of older lean and older overweight. It is well documented that changes in body composition, often without concomitant changes in body weight and BMI occur as part of the normal biology of aging (St-Onge 2005). Fat mass increases and fat-free mass decreases (Cohn *et al.*

1980, Flynn *et al.* 1989) in line with features of sarcopenic obesity (Prado *et al.* 2008) seen in ageing. The average male aged 65-70 has 12 kg less lean body mass than at age 25 (Forbes and Reina 1970). Over a 2 year follow-up study in older men, there were significant losses in leg muscle mass and appendicular skeletal muscle mass, calculated as the sum of arm and leg fat-free soft tissue (Zamboni, Zoico *et al.* 2003). This may be as a result of a general decline in physical activity with ageing, however over a three year-period, a mild but significant decline in muscle mass and body fat accumulation were seen in healthy old participants despite leisure time physical activity (Raguso 2006). Computed tomography of muscles show that after the age of 30, there is a decrease in cross-sectional area of the thigh and density associated with increased intramuscular fat (Borkan *et al.* 1983). Although the exact underlying cause is not known, it would appear that there may be other mechanisms including physical inactivity that promotes accumulation of lipid and/or reduction in fat oxidation with ageing. Moreover, this study has shown reduced insulin sensitivity levels in older overweight compared to older and young lean subjects. Older overweight subjects also reported lower physical activity time per week.

3.4.2 Maximal oxygen consumption (VO$_2$ max)

Older partipants recruited to the study achieved lower maximal oxygen consumption (VO$_2$ max) than young participants during the incremental exhaustive exercise test regardless of body composition or physical activity levels. This study demonstrated around a 40% difference in mean VO$_2$ max levels between older and young in concert with evidence supporting a 10% per decade decline in VO$_2$ max in men and women (Heath *et al.* 1981) irrespective of activity level (Hawkins and Wiswell 2003). This age-related decline may be due to adaptations of the body to cardiovascular (reduction in maximal heart rate) and body composition changes (lean body mass reduction) (Hawkins and Wiswell 2003) and decrease in vigorous physical activity (Rosen, Sorkin *et al.* 1998).

In a longitudinal study based on the Baltimore Longitudinal study in ageing (Flegg and Laketta 1988), peak VO_2 max decelerated with each increasing decade particularly after the age of 70. Although greater levels of habitual physical activity increase the absolute peak $\dot{V}O_2$ at any age, this does not appear to prevent the accelerated decline with advancing age. Functional capacity as assessed by ISWT in this study revealed a distinct difference between older lean and older overweight with shorter distances walked by overweight men. This demonstrates that ageing per se does not influence functional capacity as much as adiposity or body composition.

3.4.3 Insulin sensitivity

A modest increase in mean fasting plasma glucose of 1mg/dL (0.1mmol/L) is seen per decade (Davidson 1979). Using the hyperinsulinaemic euglycaemic clamp in the present study there was a significant difference ($p<0.01$) in glucose disposal rates between older and young participants. A significant difference in fasting insulin sensitivity index was also seen between older and young participants (higher fasting glucose but similar fasting serum insulin levels). When divided into older overweight, older lean and young lean, insulin sensitivity index was lowest in the older overweight, followed by older lean and young lean. Although there are numerous studies on ageing and insulin resistance, there are not as many studies cited in literature that have primarily evaluated and compared glucose disposal rates in older and young healthy volunteers using the hyperinsulinaemic euglycaemic clamp, and even fewer have examined the difference on older participants based on body composition. Older volunteers were shown to have 30% lower mean glucose disposal rates compared to young despite similar serum insulin levels at steady state. In line with the development of sarcopenic obesity in ageing, it is unclear if the increasing glucose intolerance in aging is caused by an

age-related decline in muscle mass or fat regional distribution, or indeed whether the increasing insulin resistance in ageing contributes to functional decline of muscle. The insulin infusion or glucose disposal rates were based on lean body mass in all groups. One of the more pertinent studies utilising the insulin clamp showed that glucose disposal rates were significantly decreased by 30-35% in the elderly group compared with the non-elderly group at all steady state plasma glucose concentrations at a fixed insulin concentration (Fukagawa, Minaker *et al.* 1988). However body composition was not analysed in greater detail. The authors concluded that the reduced insulin sensitivity was due to a reduction in the maximal capacity of glucose utilization with aging. Glucose and protein homeostatic responses to different insulin infusion rates during a euglycaemic clamp showed lower glucose disposal rates adjusted for lean body mass in elderly men compared to young at all infusion rates apart from the highest rate ($400mU/m^2/min$)(Krebs and Roden 2005). The effect of non-insulin-mediated glucose disposal (which is responsible for the majority of basal glucose uptake) was examined in old versus young healthy men using a euglycaemic clamp and showed a significantly lower glucose disposal in older men at baseline (Meneilly, Elahi *et al.* 1989) This may translate to why fasting glucose increases with age.

Another plausible cause for the observed reduced glucose disposal rates in older people could be explained by skeletal muscle insulin resistance, as this increase with age through the accumulation of IMCL and associated metabolites and altered muscle mitochondria. Skeletal muscle acts as a pool for insulin-mediated glucose uptake so it would be reasonable to hypothesise that perturbations to muscle such as changes seen with ageing would affect glucose uptake and thus insulin sensitivity. Indeed skeletal muscle uptake of 2DG was greatest in young, followed by old lean and old overweight.

3.4.4 Substrate oxidation at rest under fasting and insulin-stimulated conditions

At rest and under fasting conditions there was no significant difference between older and younger volunteers with regards to carbohydrate and fat oxidation rates and respiratory exchange ratios (RER). There was a trend towards increased energy expenditure in the younger group when expressed per kg lean mass. Upon insulin stimulation, carbohydrate oxidation rates increased significantly and a trend towards a rise in RER was seen in younger participants. A trend towards an increase in carbohydrate oxidation was seen in the older group whilst no changes were seen in fat oxidation rates, RER and energy expenditure.

The effect of insulin on carbohydrate oxidation at baseline was significant in younger participants compared to older participants, with younger individuals achieving a relative 13% increase in carbohydrate oxidation compared to older individuals. Studies examining substrate oxidation in ageing tended to focus on the effects of diet and physical activity.(Davy, Horton *et al.* 2001; Melanson, Donahoo *et al.* 2007). Of the few pertinent studies concerning fat oxidation in the fasting state, some have shown a decline (Calles-Escandon *et al.* 1995) and an increase in fat oxidation with ageing (Bonadonna *et al.* 1994). A possible reason for the inconsistencies may lie with the influence of body composition. Melanson *et al.* 1997 observed no significant effects of age per se on fasting fat oxidation, but a significant negative effect of body fat was seen when the best-fitting regression multiple analysis was applied. Others supporting this observation have shown low basal fat oxidation rates in older obese individuals and that oxidation rates are altered with changes in body composition (Wohl, Girman *et al.* 2004). In the post-prandial state, lower fat oxidation rates were seen in elderly women following a moderately large meal (Melanson *et al.* 1998). The blunted response of energy expenditure to insulin stimulation observed in older participants in this study is in concordance with data seen in that of T2DM (van de Weijer, Sparks *et al.* 2013). In insulin resistant individuals, capacity to increase skeletal and whole body glucose

oxidation and storage during the clamp is impaired with little or no change in respiratory quotient (RQ) (van de weiger 2013, Rowe, Minaker *et al.* 1983; Kelley and Mandarino 2000).

The lower mean glucose disposal rates and impaired substrate utilization upon insulin stimulation in older overweight participants shown in this study imply that insulin sensitivity is impaired in overweight and obese individuals. As skeletal muscle accounts for the majority of insulin-mediated disposal of glucose (under hyperinsulinaemic clamp conditions) (De Fronzo *et al.* 2009), perturbations to this tissue may aggravate insulin resistance. Mitochondrial abnormalities impair lipid oxidation and consequently increase lipid accumulation in muscle (Boden, Chen *et al.* 1994). This imbalance further impairs insulin signalling and increases lipotoxic intermediates leading to a decline in glucose uptake in muscle (Randle *et al.* 1963, Defronzo 2004, Kashap *et al.* 2003, Schrauwen *et al.* 2010). Impaired in vivo mitochondrial function has also been shown to be the single most important predictor of basal RER, in contrast insulin-stimulated RER was largely influenced by glucose disposal rate suggesting that mitochondrial capacity mainly affects basal substrate utilization but does not necessarily impact on skeletal muscle to switch substrates.

3.4.5 Skeletal muscle lipid

Studies of the role of IMCL subcellular fractions and distribution of important muscle determinants of insulin sensitivity such as lipid, mitochondria, glycogen and nuclei are extremely limited. Post-exercise, the overweight and older lean participants in the present study showed a 3-fold and a trend towards a 2-fold accumulation of lipid in the SSL region of muscle, respectively. Greater IMCL accumulation in this region in older people during exercise has been attributed to a need for rapid energy source in conditions of continuous supply of lipid (Skovbo *et al.* 2008). If unaccompanied by a concomitant rise in fat oxidative

capacity, as seen in conditions of insulin resistance this may result in a surplus and thus overspill of lipid in muscle. In a pertinent study evaluating lipid content of skeletal muscle in ageing, older men exhibited larger number of IMCL droplets in total muscle area, greater IMCL content in the SSL region, lower number of mitochondria, and reduced number of lipid droplets in contact with mitochondria at rest (Blaak and Wagenmakers 2002). The findings in this study also reflect that seen in people with T2DM who showed a 3- fold higher volume of lipid in the subsarcolemmal region and similar volume density of IMF lipids in the pre-training state compared with BMI-matched control subjects and highly endurance-trained subjects. Endurance exercise halved lipid content in the SSL region but no differences in IMF lipids among groups were found (Nielsen *et al.* 2010). One of the novel findings of the present study is that lipid was found to accumulate to a greater extent in the SSL regions of muscle in older people, albeit older overweight during light-intensity exercise and this is likely as a result of blunting of IMCL oxidation capacity. Figure 3.8A shows reduced relative IMCL (other fat) to total fat oxidation during exercise in older overweight compared to older lean and young subjects. This was despite similar self-reported activity levels. Over a period of time this may affect skeletal muscle insulin sensitivity as a result of increasing IMCL stores (Krssak *et al.* 1999, Pan *et al.* 1997). In healthy young individuals, IMCL depots have been shown to decrease in response to acute exercise (Nielsen *et al.* 2000), but very little is known of the effects of exercise on IMCL depots in older individuals. Lipid flux in the SSL regions appears to depend on the degree of uptake and oxidation of fat as aerobic training decreased SSL content of people with T2DM by almost two-fold, approaching levels observed in normal healthy controls (Ritov *et al.* 2005). Moreover there was a strong inverse relationship between pre-training levels of SSL lipid and insulin sensitivity in these subjects. Taken together this demonstrates an abnormality of SSL lipid accumulation in T2DM, and

this could also be an important factor contributing to the regulation of skeletal muscle insulin sensitivity in otherwise healthy older people.

Compartmentalisation of muscle into distinct regions has further enabled examination of physiological differences between IMF and SSL content including the localisation of key proteins involved in the insulin signalling pathways and metabolism. Changes in mitochondrial content (Ritov *et al.* 2005, Happelar *et al.* 2003), GLUT-4 vesicle translocation (Lauritzan *et al.* 2008) and glycogen metabolism (Marchand *et al.* 2007) appear to be location-dependent in response to stimuli such as endurance training (Marchand *et al.* 2007) and diets (Lauritzan *et al.* 2008). Likewise it could also be that there is a predilection for particular lipid intermediates such as DAG or ceramide species to accumulate in the SSL region, so causing a deleterious effect on insulin signalling pathways. However studies on the effect of lipid metabolites on insulin sensitivity are limited and have yielded inconsistent findings and not many have investigated its effect on insulin sensitivity in older people. Whilst lipid infusion studies have provided insights into IMCL accumulation and its effect on insulin-signalling pathways (Hoy *et al.* 2009), others have shown that it may not be IMCL per se affecting insulin sensitivity but increased lipid metabolites (DAGs, acyl-coA, ceramides) causing insulin resistance (Adamas *et al.* 2004, Moro *et al.* 2009, Coen *et al.* 2010). This study did not show a difference in total DAG or ceramide content between old and young, pre and post exercise, but DAG species C18:1 and ceramide species C20:0 were elevated in the older overweight. The significance of this is uncertain and may not entirely explain the difference in insulin-sensitivity in older overweight, older lean and young. In contrast to other studies showing either higher total intramyocellular DAG content or low ceramide content associated with higher insulin sensitive muscle (Dube *et al.* 2011), total DAG content in the present study was comparable between the groups despite greater glucose disposal rates in young subjects. This could be because that only certain DAG moieties are

associated with insulin sensitivity or that DAG and ceramide are not the primary determinants of lipid metabolites that cause insulin resistance in old muscle. This is in accord with other studies where similar ceramide content were seen regardless of differing insulin sensitivities of obese, lean and endurance trained subjects. Aerobic capacity did not appear to influence muscle ceramide content in these groups (Pan *et al.* 1997).

As fatty acid availability or beta-adrenergic stimulation in both older lean and overweight subjects were not limiting the reduction in skeletal muscle fat oxidation may be the result of an age-related decline in the capacity of skeletal muscle to oxidize fatty acids at the expense of lipid being diverted to greater lipid storage in muscle. Factors implicated with this decline include diminished content of muscle oxidative enzymes and capacity, possible adrenergically-mediated reduced activation of fatty acid transport, increased glycolytic flux inhibiting fatty acid transport into mitochondria (van Loon *et al.* 2005) and reduction in the number and skeletal muscle content of mitochondria (Crane *et al.* 2010, Blaak *et al.* 2000). This may, over time have a deleterious effect on muscle mass and metabolism, leading to a progressive decline in muscle metabolite function. These changes may eventually contribute to the development of a decline in muscle mass, strength and increase in body fat content in line with sarcopenia and skeletal muscle insulin resistance. Endogenous carbohydrate oxidation rates during the 50% VO_2 max exercise were not dissimilar between the groups. However during exercise, there was little muscle glycogen use and a trend towards an increase in muscle lactate accumulation post exercise in older overweight subjects. Other skeletal muscle metabolites such as muscle total creatine, acetylcarnitine and PCr were lowest in the older overweight. Muscle oxidative capacity as measured by citrate synthase activity also tended to be lower in muscle of the older overweight, although there was no difference in CPT-1 and CPT-2 activity.

3.4.6 Substrate oxidation during light-intensity exercise

No difference in relative contribution of total fat to total energy expenditure during exercise at 50% VO_2 max was seen between the groups, however the relative contribution of intramyocellular to total fat utilisation in older overweight men was lower than that of older lean and young men despite increased FFA availability (Figure 3.9) and greater catecholamine response compared to young (Figure 3.10A). The relative contribution of plasma FFA availability to oxidation was also lower in older individuals. Taken together, this suggests that IMCL and plasma oxidation are impaired in older individuals despite greater availability.

The impaired total fat oxidation to energy expenditure in older people are consistent with findings of others. These studies did not however compare body composition and IMCL oxidation during exercise between participants in detail. Although fatty acid oxidation was lower in older compared to young healthy males during exercise performed at a similar relative intensity (56% of maximal VO_2 max)(Sial and Coggan 1996) the rate of appearance (Ra) of FFA was found to be lower in older subjects. Higher catecholamine concentration levels in response to exercise and meals appear to promote FFA availability (Coggan and Spina 1997) leading to increased 24 hour fat oxidation (Melanson 2007). Older people appear to have a blunted lipolytic response to FFA availability and beta-adrenergic stimulation (Blaak 2000), and muscle lipid are insensitive to catecholamines (Galbo *et al.* 1975). In this study, although plasma FFA Rd in older individuals was at a level that was even higher than in the young, the relative IMCL utilisation to total fat oxidation during exercise was lower. The present finding of reduced fat oxidation between old and young during exercise is in concert with Sial *et al* who examined substrate utilisation during exercise performed at absolute and relative intensities (Sial *et al.* 1996). In line with findings from the present study FFA availability and lipolysis were not limiting. In fact greater fatty acid flux was seen in

older subjects.This suggests that although fatty acid uptake can be increased, the ability to utilise IMCL through some unknown mechanism is impaired with ageing. This could perhaps be related to altered skeletal muscle metabolism. Although no difference was seen in plasma fatty acid oxidation, the difference between plasma rate of disappearance and oxidation (rate of storage) in older subjects in this study was significant implying greater ectopic fat deposition, presumably in muscle; as fat serves as the primary fuel in skeletal muscle metabolism during low to moderate intensity exercise (Romijn 1993). It could be that healthy individuals muscle appear to possess the 'flexibility' or ability to adapt to increased fat delivery by improving fat oxidation or increasing mitochondrial oxidative capacity during exercise training, facilitating the oxidation of fat over carbohydrate ,whereas the opposite is seen in insulin-resistant states.

3.4.7 Exercise metabolism

There is little disagreement with the major metabolic effects observed after endurance exercise in older people even at submaximal VO_2max. These are slower utilisation of muscle glycogen and blood glucose, greater reliance on fat over carbohydrate oxidation and less lactate production during exercise at a given intensity. Skeletal muscle metabolic response to exercise appear to decline with age and are thought to be due to a reduction in the number of mitochondria , muscle oxidative capacity, increased IMCL , increased fat mass and decline in physical exercise with ageing (Holloszy and Coyle 1984). However endurance and resistance training appear to minimise these changes. The results of the present study mostly support the findings of others, in particular a greater accumulation of muscle lactate and less glycogen utilisation during exercise in older healthy men compared to young subjects. In particular glycogenolysis was lowest in older overweight participants. Muscle PCr and total creatine were also lower in older overweight compared to young subjects.

The older overweight participants in this study were noted to have lower CS levels compared to older lean, and there was no difference between older lean and young participants. The mitochondrial respiratory capacity of skeletal muscle as measured by citrate synthase activity have been found by some to be no different in older or young individuals (Orlander *et al.* 1978, Larsson *et al.* 1978, Grimby *et al.* 1982). In contrast, Essen-Gustavsson and Borges 1986, Coggan *et al.* 1993 and Meredith *et al.* 1989 showed lower CS activity levels in older compared to young individuals. This difference in findings may be because the early Scandinavian studies were confounded by physical activity levels of subjects whereas the latter studies were in truly sedentary individuals. Thus it is unclear from these studies if ageing per se or the influence of physical activity affects the respiratory capacity of skeletal muscle.

3.4.8 Underlying mechanisms

Several mechanisms may explain the observed inability of old overweight men to oxidise excess FFA Rd. Individuals predisposed to familial longevity were shown to have lower IMCL content and increased peripheral insulin sensitivity regardless of physical activity and body composition (Wijsman *et al.* 2012) and endurance-trained young lean subjects also accumulate IMCL (Goodpaster 2001). This suggests that in these individuals, either FFA levels were not elevated and/or fatty acid oxidation was not impaired. Whilst mitochondrial activity and function were not assessed in Wijsman's study, muscle oxidative capacity was shown to be greater in insulin-sensitive endurance-trained athletes despite higher IMCL content (Wijsman 2012). Compared with young, IMCL content was increased by up to 40%, and reduction in mitochondrial oxidative and phosphorylation activity was seen in older people through [1]H Nuclear Magnetic Resonance Spectroscopy (^{1}HNMRS) (Petersen 2003) . However, although participants were matched in body composition habitual activity levels were not considered. These studies point towards impaired mitochondrial function as a cause

of impaired fatty acid oxidation and insulin resistance in older people. Findings elucidated from this study show that impairment of IMCL oxidation and subsequent skeletal muscle insulin resistance may be due to perturbations to mitochondrial content and function in overweight or obesity as there was a trend towards lower CS, CPT-1, relative contribution of fat to total energy expenditure or muscle metabolic response to exercise. Although this may explain the difference in older overweight participants in the present study, fatty acid oxidation impairment is more likely as a consequence of greater FFA Rd relative to energy expenditure. This study has shown greater lipid accumulation in SSL region of older overweight as a result of impaired IMCL oxidation, whilst no difference was seen in older lean and young. This is in contrast to others (Crane *et al.* 2010) who have shown greater SSL lipid accumulation in older individuals irrespective of body composition, implying that age is a predictor of IMCL content. The difference may be because older participants in Cranes' study were not matched for physical activity.

3.4.9 Conclusion

In conclusion, this study demonstrates that several parameters change with age, such as increased truncal fat mass, increased levels of fasting glucose, decreased VO$_2$ max, impaired skeletal muscle insulin sensitivity and fat oxidation rates during light-intensity exercise, compared to young. However differences in body composition and habitual physical activity appear to have a greater influence on skeletal muscle insulin sensitivity and lipid accummulation. Further, lipid droplet accumulation in the subsarcolemmal region of muscle of older overweight could further explain a cause for the development of insulin resistance in older insulin resistant people as this is the site of insulin action. The greater FFA availability in old (both lean and overweight) and noradrenaline concentrations during exercise suggest an exaggerated response that develops with ageing per se. Future studies should focus further

on unraveling the mechanisms involved at the SSL region of muscle in healthy older people and compare this with people with diabetes. Certain micronutrients shown to increase fat oxidation should be investigated to see if this could improve insulin sensitivity. This will be tested and discussed in Chapter 5. Figure 3.11 illustrates the interplay between adiposity, reduced physical activity and skeletal mucle insulin resistance and T2DM.
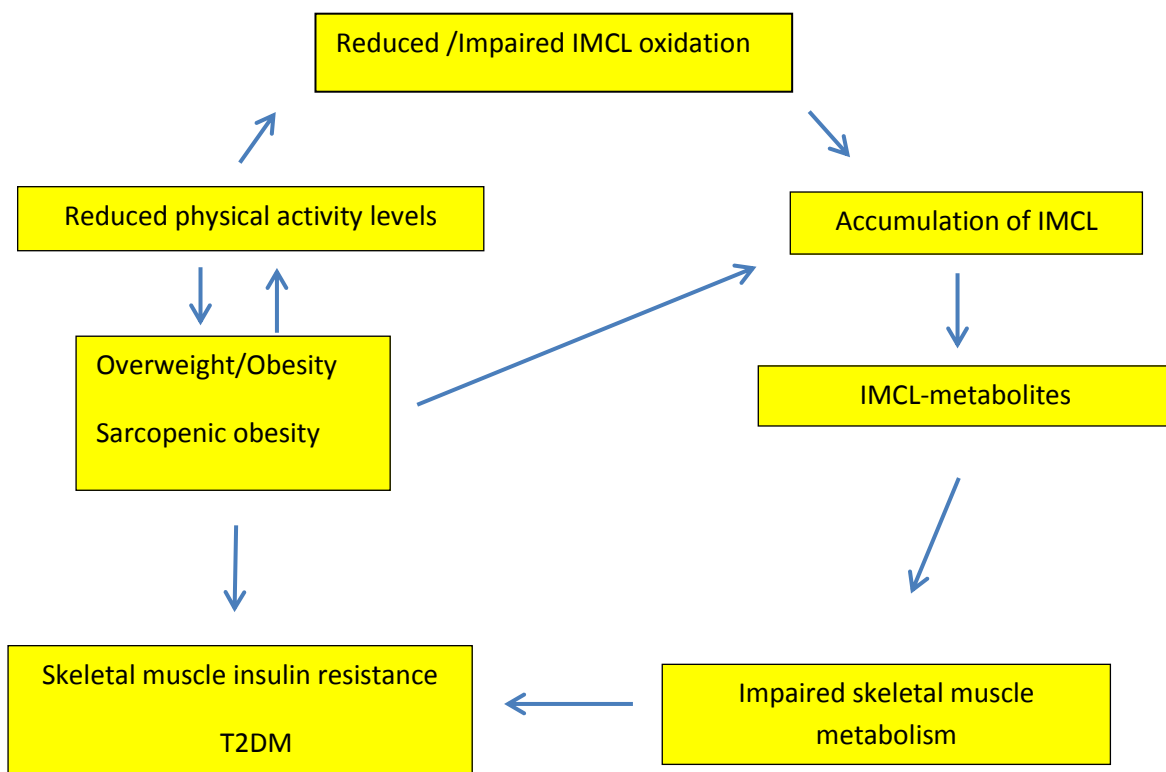
Figure 3.11: The relationship between impaired IMCL oxidation and adiposity in promoting skeletal muscle insulin resistance.

Chapter 4: STUDY 2

Investigating the effects of an acute increase in lipid availability on insulin and amino acid stimulated protein metabolism.

4.1 Introduction

Anabolic resistance or the inability of skeletal muscle to synthesise new protein in response to anabolic stimuli such as amino acid ingestion or exercise has been implicated in muscle loss seen in ageing (Volpi, Mittendorfer *et al.* 2000; Cuthbertson, Smith *et al.* 2005), sarcopenia (Rasmussen, Fujita *et al.* 2006), insulin resistance, critical illness (Rennie 2009) and lipotoxicity. The issues concerning anabolic resistance have gathered momentum in recent years in line with recognition of increasing muscle loss and strength with ageing, and its impact on the older population as a main contributor to disability, risk of falls and quality of life (Evans 1995; Melton, Khosla *et al.* 2000). Furthermore as there is a strong association between anabolic resistance and insulin resistance, examining the underlying mechanisms involved can provide important insights into elucidating the protein and insulin signalling pathways affected.

4.1.1 Insulin stimulated protein metabolism

Insulin is widely described as a potent stimulus for muscle protein anabolism, indeed hyperinsulinaemia can increase muscle protein synthesis particularly when muscle amino acid availability is also increased (Bennet, Connacher *et al.* 1990). However insulin has been regarded to have a permissive rather than a modulatory role in MPS, and it is the availability of amino acids rather than insulin that appears to regulate rate of protein synthesis (Cuthbertson *et al.* 2005).

Insulin induces protein synthesis by activating translational factors and increasing cellular ribosomes mediated primarily through phosphoinositide 3-kinase and activation of protein kinase B. This allows for overall activation of protein synthesis through its regulation of mammalian target of rapamycin (mTOR) and 4E-BP1, both of which are mainly involved in synthesis of protein (Proud 2006). mTOR complex 1 also targets and activates kinases such as S6K1 that ultimately results in ribosomal biogenesis and translation. This has been discussed in detail in chapter 1.
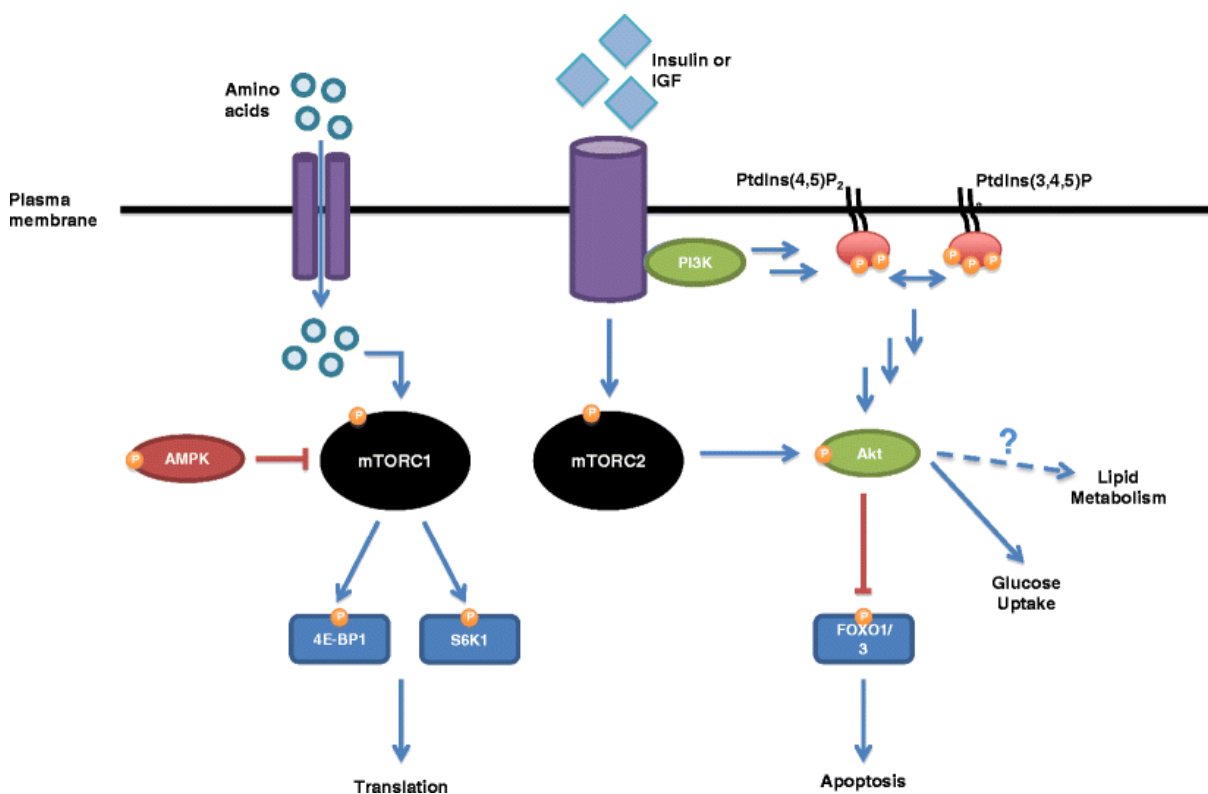
Figure 1 from (Haran, Rivas *et al.* 2012). Anabolic stimuli (amino acids, growth factors (insulin, IGF-1), and exercise (not shown) act through the mTOR and Akt signalling pathways. mTORC1 is involved in the phosphorylation and activation of S6K1 and phosphorylation and inactivation of 4E-BP1, resulting in ribosome biogenesis, increase in translational efficiency, and heightened MPS. The energy sensor AMPK inhibits this pathway, and is active when the AMP/ATP ratio is high. Growth factor stimulates activation of the PI3K pathway as well as mTORC2; both eventually activate Akt,

which can inhibit stress signalling and apoptosis through inactivation of FOXO1/O3 transcription factors. Akt also plays a role in carbohydrate metabolism.

P- phosphorylation, activation or inactivation; PI3k- phosphoinositide 3 kinase; Akt-protein kinase B; FOXO- forkhead protein box O; AMPK -AMP-activated kinase; 4E-BP1- eIF4E-binding protein 1; S6K1- S6 kinase 1

4.1.2 Lipid excess and anabolic resistance

Diet-induced obesity, a major risk factor in the development of insulin resistance, was found to cause reduced skeletal MPS in mice (Anderson, Gilge *et al.* 2008). There was also an association between impaired protein metabolism and reduced post-absorptive protein turnover in obese individuals (Guillet, Delcourt *et al.* 2009). Although there exists compelling evidence to link obesity and reduced protein metabolism, the underlying mechanisms to explain this association are not clear. Increased activation of mTOR pathway and its downstream effectors have been implicated as contributors to insulin resistance stemming from obesity (Le Bacquer, Petroulakis *et al.* 2007).

Increased basal expression of proinflammatory mediators such as NFκB, TNFα and IL-6 in older people (possibly due to increased fat accumulation in ageing) inhibits muscle anabolism by interfering with mTOR signalling (Cuthbertson *et al.* 2005). The same mediators are associated with lipid accumulation in non-fatty tissue sites.

Accumulation of lipids, in particular sphingolipids are well-known to interfere with insulin-signalling pathways thereby contributing to the development of insulin resistance (Hannun *et al.* 2002; Summers *et al.* 2005). Similarly lipids such as ceramides cause diminished amino acid availability and reduced phosphorylation of translational regulators downstream of

mTOR in vitro. Ceramides can activate protein phosphatase 2A which targets and inhibits Akt and S6K1 by dephosphorylation (Hannun *et al.* 2002).

Other than ceramides, triglycerides are also thought to be detrimental to anabolic signalling in skeletal muscle. Age-induced intramuscular triglyceride infiltration is associated with an increase in several lipogenic regulators including sterol regulatory element-binding protein, fatty acid synthase, acetyl CoA carboxylase, and stearoyl CoA desaturase. It has been demonstrated that AMPK activation and concentration are heightened in aged skeletal muscle, even in animals that were subjected to anabolic stimulus in the form of chronic muscle overload (Hannun *et al.* 2002). This would suggest that intramuscular lipid accumulation, especially triglycerides blunts anabolic signalling pathways.

4.1.3 Increasing lipid availability to induce insulin resistance

The studies described so far have been performed in chronic settings of obesity and insulin resistance; however other contributing factors such as physical activity and diet may influence anabolic sensitivity to amino acids. Under these conditions it is difficult to determine the effects of insulin resistance per se on the mechanisms involved in protein turnover. The effect of excess lipid on insulin and amino acid stimulated skeletal muscle protein synthesis and the associated signalling pathways in vivo in humans is important but not frequently studied.

Intravenous infusion of a lipid emulsion with heparin is used to elevate free fatty acid availability to allow investigation of the acute effects of lipid-induced insulin resistance on insulin signalling pathways and its subsequent impairments in glucose uptake, storage and oxidation (Boden, Jadali *et al.* 1991; Chokkalingam *et al.* 2007). Several studies have shown

that increasing free fatty acid acutely via this method had little effect on muscle protein synthesis and actually improved net muscle protein balance by inhibiting protein breakdown in a dose dependent manner (Ferrannini, Barrett *et al.* 1986; Tessari, Nissen *et al.* 1986; Wicklmayr, Rett *et al.* 1987; Gormsen, Gjedsted *et al.* 2008; Katsanos, Aarsland *et al.* 2009). In particular the study by Katsanos *et al.* observed no effect on the normal muscle protein synthetic response to ingestion of essential amino acids during a 13.5 hour intravenous lipid infusion.

This present study examined the effect of acutely elevating fatty acid availability to a concentration found to induce insulin resistance, on muscle signalling and protein synthetic response to amino acid ingestion in the presence of steady-state circulating insulin concentration in humans. The study also aimed to elucidate the protein signalling pathway involved in altered protein metabolism under these conditions.

4.2 Methods

Seven healthy males (23.0 ± 0.8 yrs, body mass 78.5 ± 3.8 kg, BMI 24.5 ± 0.9 kg/m^2) participated in the study.

They presented to the laboratory fasted and abstained from strenuous exercise for the previous 48 hours. In a rested supine position, cannulae were inserted into veins for arterialised-venous blood sampling, infusion of insulin, 20% dextrose and [-ring^{2}H$_5$] phenylalanine (Cambridge Isotopes Limited, Cambridge,MA, USA) and the contralateral forearm for infusion of a lipid emulsion (Intralipid; Fresenuis Kabi,Germany) or 0.9% saline. On each visit a 7 hour [ring-^{2}H$_5$]-phenylalanine infusion (0.5/kg/hr) was performed in combination with 10% Intralipid (Lipid) or saline (Control) at a rate of 100mls/hr. Heparin sodium was also infused at a rate of 600U hr^{-1} to elevate plasma non-esterified fatty acid availability. After a 4 hours basal period, a 21 g bolus of amino acids (except phenylalanine

and tyrosine; Tyrosidon, SHS International Ltd, UK) was administered in a 440mls solution via a nasogastric tube to avoid issues with palatability. This amount of amino acids was selected to provide 10 grams of essential amino acids including 2.3 grams of leucine. This dose was determined as sufficient to provide a robust stimulation of muscle protein synthesis rates in young men under normal conditions (Cuthbertson *et al.* 2005). At the same time, a 3 hour hyperinsulinaemic euglycaemic clamp was carried out at a rate of $50\text{mUm}^{-2}\text{min}^{-1}$ (fed period). This rate was chosen as it was previously demonstrated to suppress endogenous hepatic glucose production under insulin resistant conditions known to alter substrate metabolism (Chokkalingam *et al.* 2007; Soeters, Sauerwein *et al.* 2009). The variable glucose infusion rate required to maintain euglycaemia was equivalent to peripheral glucose disposal and therefore peripheral insulin sensitivity.

4.2.1 Sample collection and analysis

Arterialised-venous blood was obtained at t=0, 1.5 hr and every 30 minutes thereafter. Plasma was treated with tetrahydrolipostatin (THL) and analysed for NEFA as described in the common methods chapter. Plasma separated from EGTA treated blood was analysed for insulin concentration by ELISA. After deproteinisation on ice with dry 5-sulfosalicylic acid the same plasma was used to analyse phenylalanine and leucine concentrations and enrichment by GC-MS after derivatisation with tert-butyl dimethylsilyl (TBDMS) as described (Gorissen, Burd *et al.* 2014). Muscle samples were obtained from the vastus lateralis muscle using the Bergstrom needle biopsy technique. Samples were analysed for intracellular tissue free phenylalanine enrichments in the similar manner as the plasma samples. Amino acids were purified from the remaining pellet as described previously and used to determine the phenylalanine enrichments in mixed muscle protein GCMS. Total muscle protein homogenates were extracted from another muscle sample portion obtained at t= 4 and 7 hr by homogenisation in a HEPES phosphatase buffer in the presence of protease

and phosphatase inhibitors. The total muscle protein content of total and phosphorylated Akt, mTOR and 4E-BP1 were determined by western blot analysis and normalised to alpha actin to control for loading. A further third sample was analysed for activation status of the pyruvate dehydrogenase complex.

4.2.2 Calculations

Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in protein bound [ring-^{2}H$_5$]-phenylalanine by the enrichment of the precursor. Plasma and muscle free phenylalanine enrichments were used to provide an estimate of the lower and higher boundaries of true FSR respectively. The formula used was:

$$\textbf{FSR} = \frac{\Delta \textbf{Ep} \quad \textbf{x} \quad \textbf{t}}{\textbf{Eprecursor}} \quad \textbf{X} \quad \textbf{100}$$

Where ΔEp is the delta increment of protein bound [^{2}H$_5$] phenylalanine during incorporation periods, Eprecursor is the enrichment of the precursor used during the time period for amino acid incorporation determination, and t denotes the time duration (hr) between biopsies. The equation is multiplied by 100 to express FSR as percentage per hour.

4.2.3 Statistics

A two-way ANOVA was performed to detect differences within and between treatment groups for all measures described. When a significant effect was observed, a Student's t-test with Bonferroni correction was performed to locate differences. Statistical difference was declared at $P<0.05$. All values presented in texts and figures represent mean $\pm$ the standard error of mean s.e.m.

4.3 Results

4.3.1 Insulin resistance of glucose metabolism

Lipid with heparin infusion caused an elevation of steady-state plasma NEFA concentrations throughout the basal period compared to Control ($P< 0.001$; Figure 1A). Plasma NEFA concentrations were suppressed ($P<0.001$) by insulin and amino acid administration to a similar degree (delta 0.38 ± 0.01 vs. 0.39 ± 0.01 mmol l^{-1}; Figure 1A) such that steady-state NEFA concentration remained greater in lipid throughout the fed period ($P<0.001$; Figure 1A) where it was maintained at a fasting concentration. Steady state plasma insulin concentrations were similar between the two groups during the fed period (104 ± 5 vs 99 ± 3 mU l^{-1}, respectively; Figure 1B). However, despite this similar circulating insulin concentration, there was a $19.9 \pm 6.2\%$ lower average glucose disposal during the final hour of the fed period in Lipid compared to control (**$P<0.01$; Figure 1C) and a $56\pm12\%$ lower PDCa by the end of the fed period at 7 hrs (*$P<0.05$;Figure 1D).
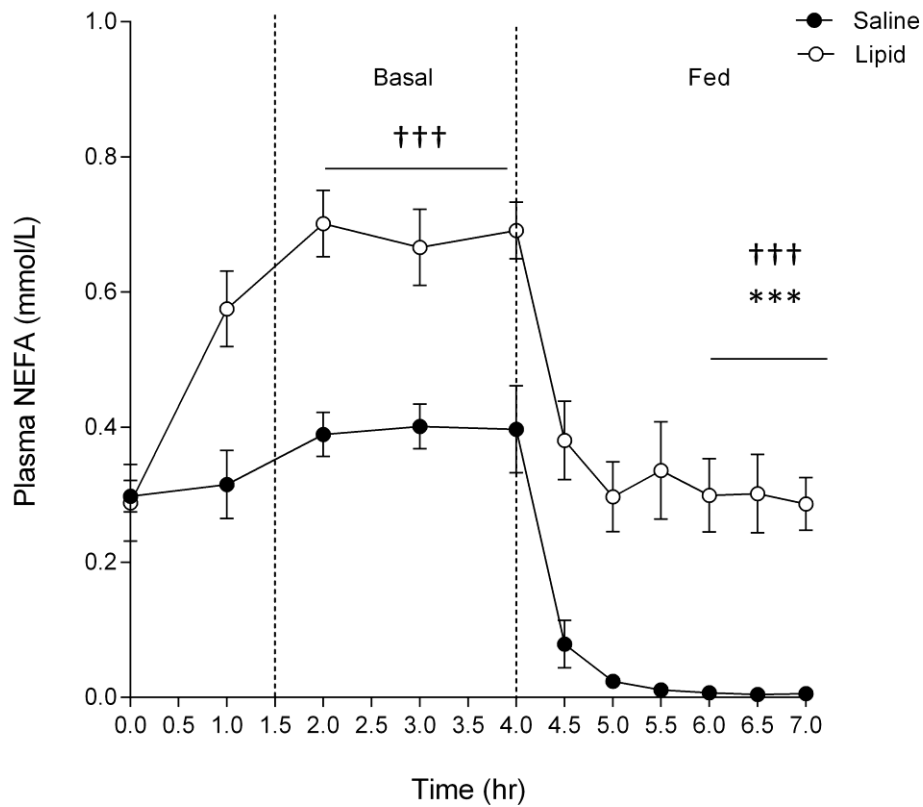
Figure 1A: Plasma NEFA concentration before (Basal 1.5-4 h) and after (Fed 4-7 h) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (100 mU/L) clamp during 7 hr intravenous infusion of saline (Control; black circles) or 10% Intralipid (Lipid; white circles) at a rate of 100 ml/hr. Values represent means ± SEM. [†††] $P<0.001$, Lipid significantly different from corresponding Control value. *** $P<0.001$, Control and Lipid during Fed significantly different from corresponding Basal steady-state.
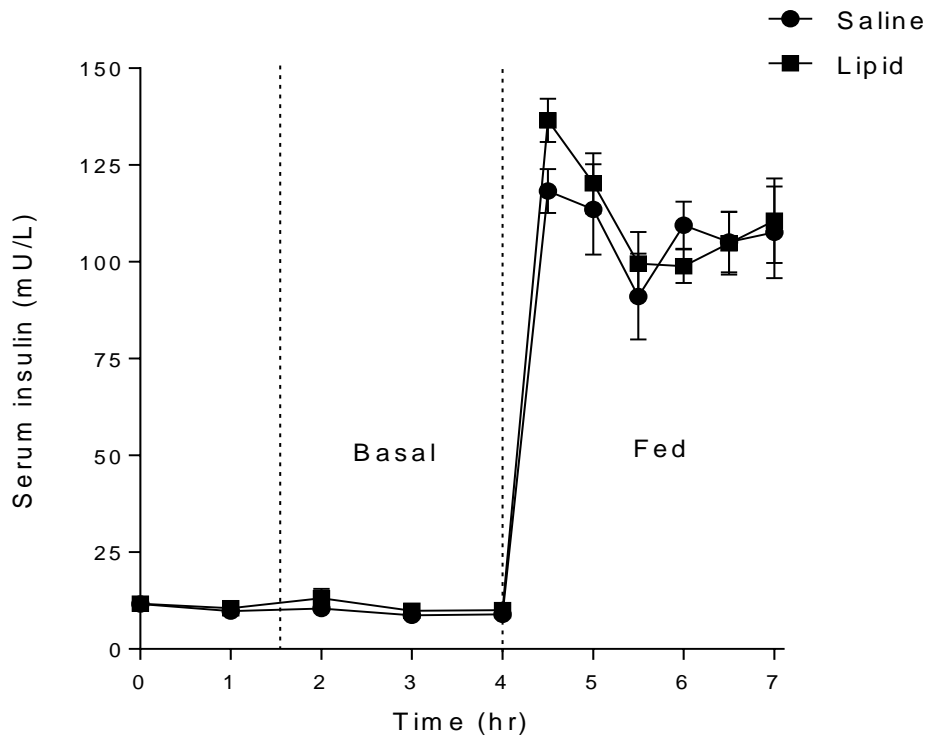
Figure 1B: Serum insulin concentration before (Basal 1.5-4 h) and after (Fed 4-7 h) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (~100 mU/L) clamp during 7 hr intravenous infusion of saline (Control; black squares) or 10% Intralipid (Lipid; black circles) at a rate of 100 ml/h. Values represent means ± SEM.

Figure 1C: Whole-body glucose disposal after (Fed 4-7 hr) the administration of 21 g of amino acids and during the 3 hr euglycaemic hyperinsulinaemic (~100 mU/L) clamp during 7 hr intravenous infusion of saline (Control; white circles) or 10% Intralipid (Lipid; black circles) at a rate of 100 ml/hr. Values represent means ± SEM. ** $P<0.01$, Lipid significantly different from corresponding Control value.

Figure 1D: PDCa before (Basal 1.5-4 h) and after (Fed 4-7 h) the administration of 21 g of amino acids and a 3 h euglycaemic hyperinsulinaemic (~100 mU/L) clamp during 7 hr intravenous infusion of saline (white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/hr. Values represent means ± SEM. *P<0.05, Lipid significantly different from corresponding Control value.

4.3.2 Amino acid metabolism

No effect was seen during the basal period following lipid infusion on plasma leucine or phenylalanine concentrations compared to control which were both maintained at fasting concentrations throughout (Figure 2A and 2B). Similarly plasma phenylalanine enrichments remained at the same steady state levels in Lipid and control (Figure 2C) resulting in the same phenylalanine Ra (46.6±1.8 vs. 48.2 ±2.7 $\mu molkg^{-1}min^{-1}$) in lipid and control respectively. Insulin and amino acid administration resulted in a similar peak in plasma

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leucine concentration in Lipid and control after 30 minutes of the fed period (P<0.001; Figure 2A). However insulin and amino acid administration caused a steady decline in plasma phenylalanine concentration during control such that it was 49± 3% lower during the final hour of the fed period compared to basal (P<0.001; Figure 2B). The degree of reduction in plasma phenylalanine concentration in response to insulin and amino acid administration was greater in Lipid (65± 3%; P<0.001) when compared to control, such that the steady-state phenylalanine concentrations were lower (P<0.001; Figure 2B). However, this did not result in greater enrichment during the final hour of the fed period in lipid compared to control (Figure 2C) or a difference in whole body phenylalanine Ra (28.9 ± 1.0 vs. 32.9±1.6 µmol kg/min) in lipid and control respectively.
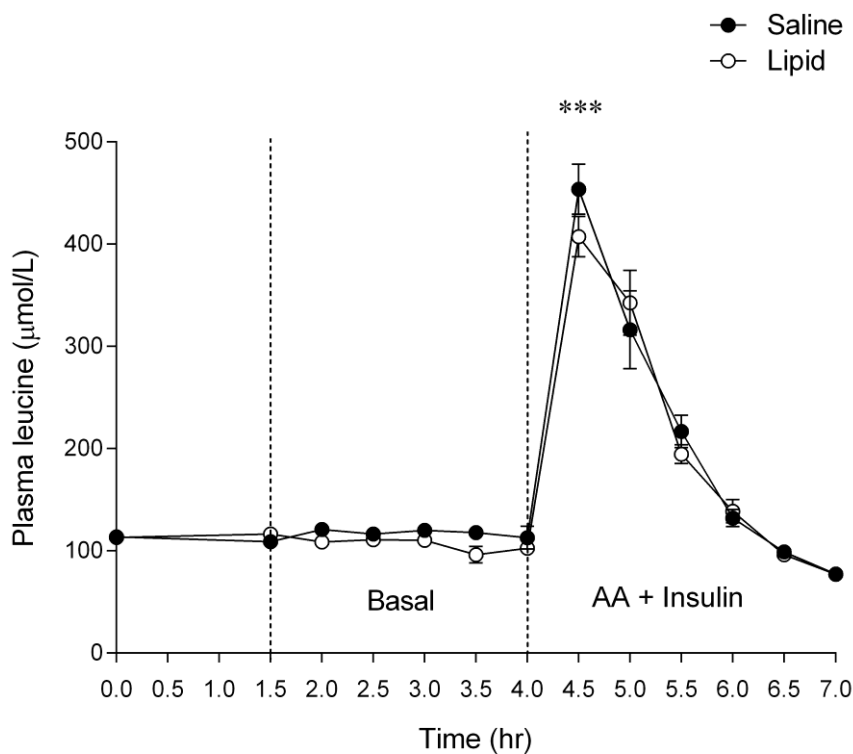
Figure 2A: Plasma leucine before (Basal 1.5-4 hr) and after (AA + Insulin) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (~100 mU/L) clamp during 7 hr intravenous infusion of saline (Control) or 10% Intralipid (Lipid) at

a rate of 100 ml/h. Values represent means ± SEM. *** P<0.001, Control and Lipid during

AA + Insulin significantly different from corresponding basal values.
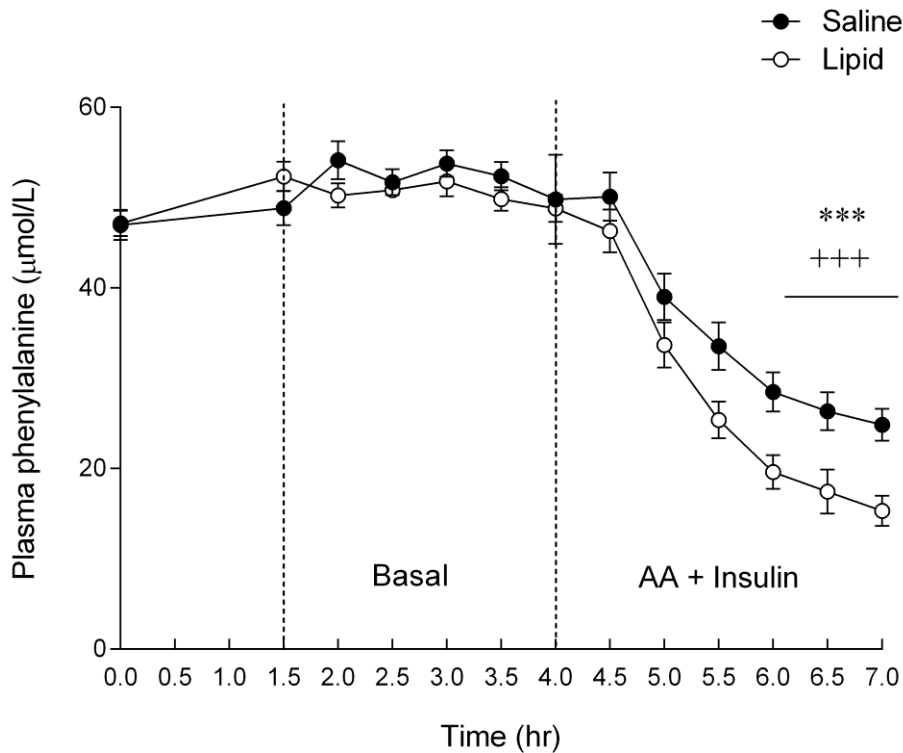
Figure 2B: Plasma phenylalanine concentration before (Basal 1.5-4 h) and after (AA +

Insulin) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic

(~100 mU/l) clamp during 7 hr intravenous infusion of saline (Control) or 10% Intralipid

(Lipid) at a rate of 100 ml/h. Values represent means ± SEM. [+++] P<0.001, Lipid significantly

lower corresponding Control value. *** P<0.001, Control and Lipid during AA + Insulin

significantly different from corresponding basal values.
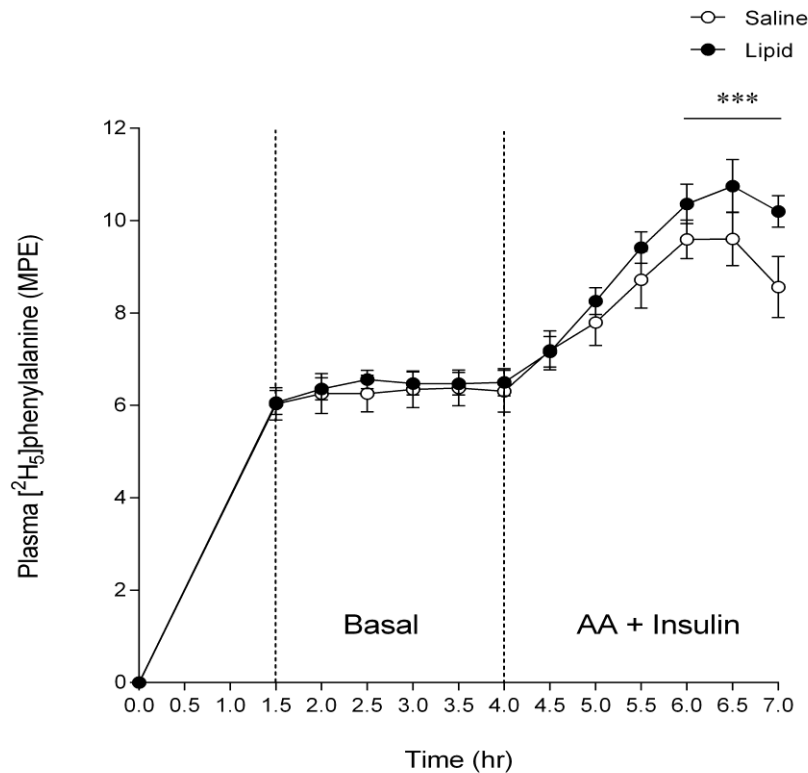
Figure 2C: Plasma phenylalanine enrichment before (Basal 1.5-4 h) and after (AA + Insulin) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (~100 mU/l) clamp during 7 hr intravenous infusion of saline (Control) or 10% Intralipid (Lipid) at a rate of 100 ml/h. Values represent means ± SEM. *** $P<0.001$, Control and Lipid during AA + Insulin significantly different from corresponding basal values.

There was no effect of lipid infusion on basal mixed muscle FSR compared to control calculated using muscle free (Figure 3A) phenylalanine enrichment as the precursor pool. However whereas mixed muscle FSR increased from the basal to fed period in control, it did not respond to insulin and amino acid administration in lipid such that it was significantly lower than Control ($P<0.05$; Figure 3A).There were no significant differences between lipid and control in the plasma (9.4±0.3 vs 8.6±0.5 mole percent excess (MPE), respectively or muscle free (9.4±0.3 vs.8.6±0.5 MPE, respectively) precursor pools following insulin and amino acid administration.
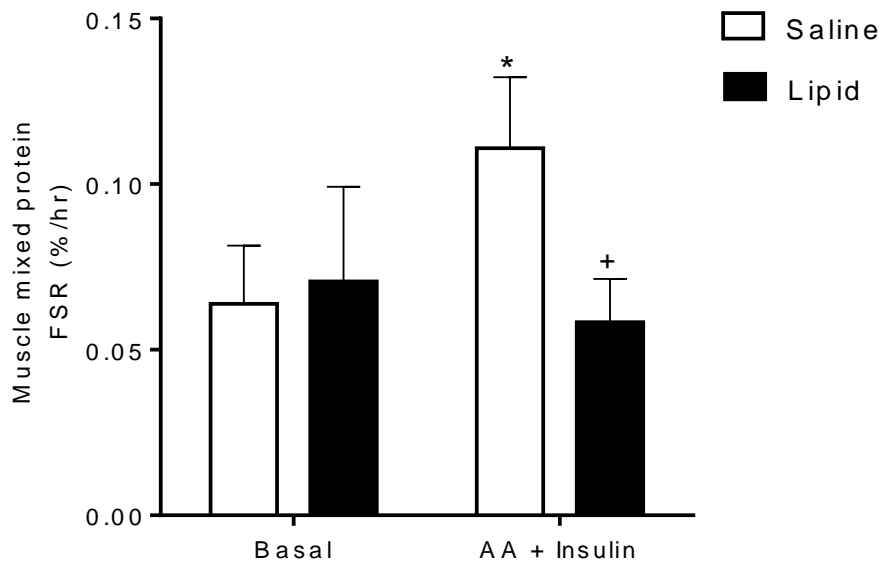
Figure 3: Skeletal muscle mixed protein fraction synthetic rate (FSR) calculated from skeletal muscle [-ring^{2}H$_5$]phenylalanine precursor pools before (Basal) and after (AA + Insulin) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (~100 mU/L) clamp during 7 hr intravenous infusion of saline (Control; white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means ± SEM. [+] P<0.05, Lipid significantly lower than corresponding Control value. * P<0.05, AA + Insulin significantly greater from corresponding basal values.

4.3.3 Associated signalling pathways

Lipid infusion did not affect the phosphorylation status of Akt, mTOR or 4E-BP1 compared to control during the basal period. Insulin and amino acid administration increased the phosphorylation of Akt (P<0.05; Figure 4A), mTOR (P<0.01; Figure 4B) and 4E-BP1 (P<0.01; Figure 4C) by 1.9, 1.7 and 2.9-fold respectively compared to basal in control. However whereas insulin and amino acid administration also increased mTOR phosphorylation by 1.5 fold from basal in Lipid (P<0.05; Figure 4B) the 1.8 fold increased in Akt phosphorylation was not significantly different (P>0.05; Figure 4A). Furthermore lipid

infusion had no effect on 4EBP1 phosphorylation at all such that it was less than half that of

control at the end of the fed period (P<0.01; Figure 4C).
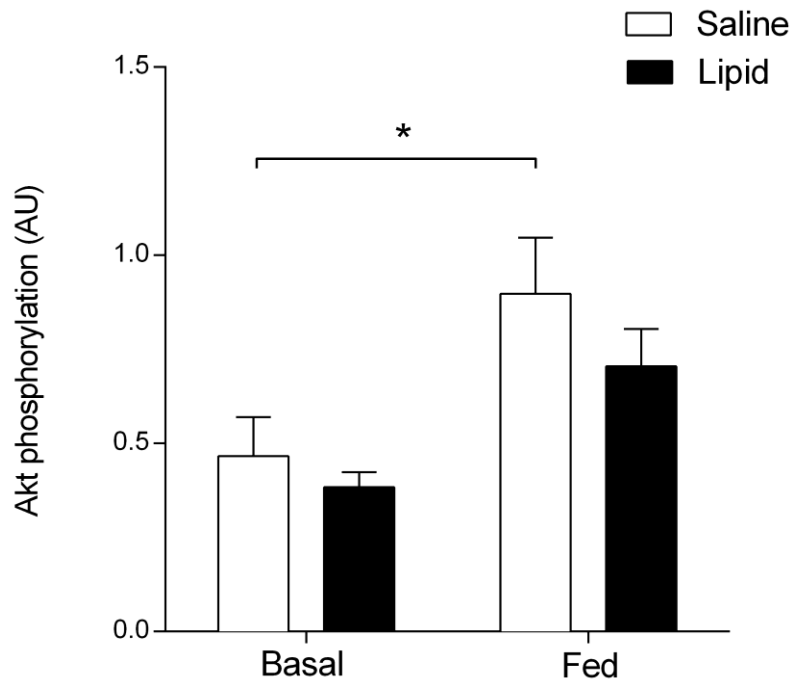
Figure 4A: Skeletal muscle Akt serine 473 phosphorylation before (Basal) and after (Fed)
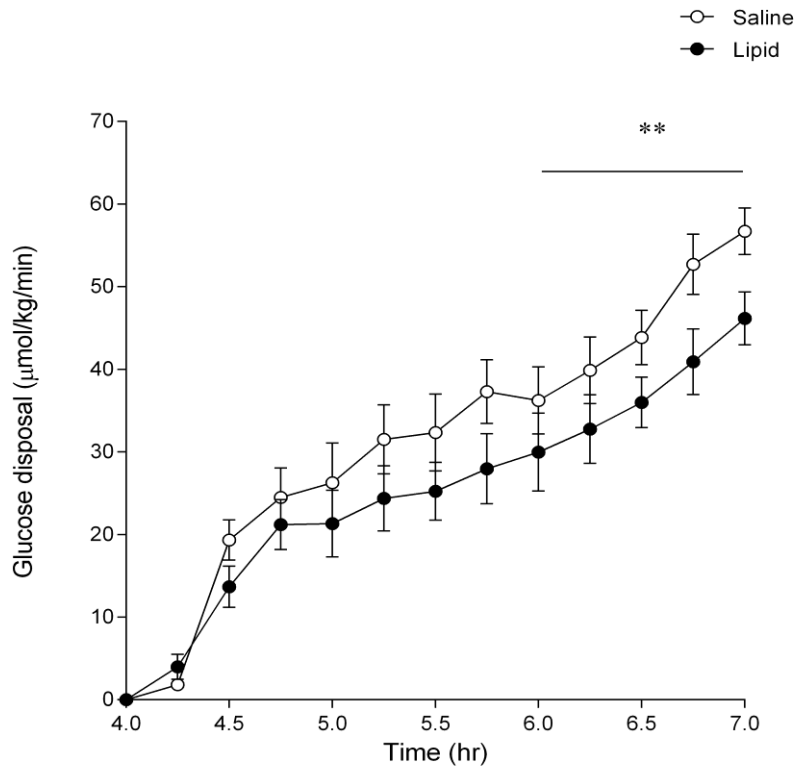
the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (100

mU/L) clamp during 7 h intravenous infusion of saline (Control; white bars) or 10%

Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means ± SEM. * P<0.05,

Fed significantly greater from corresponding Basal values.
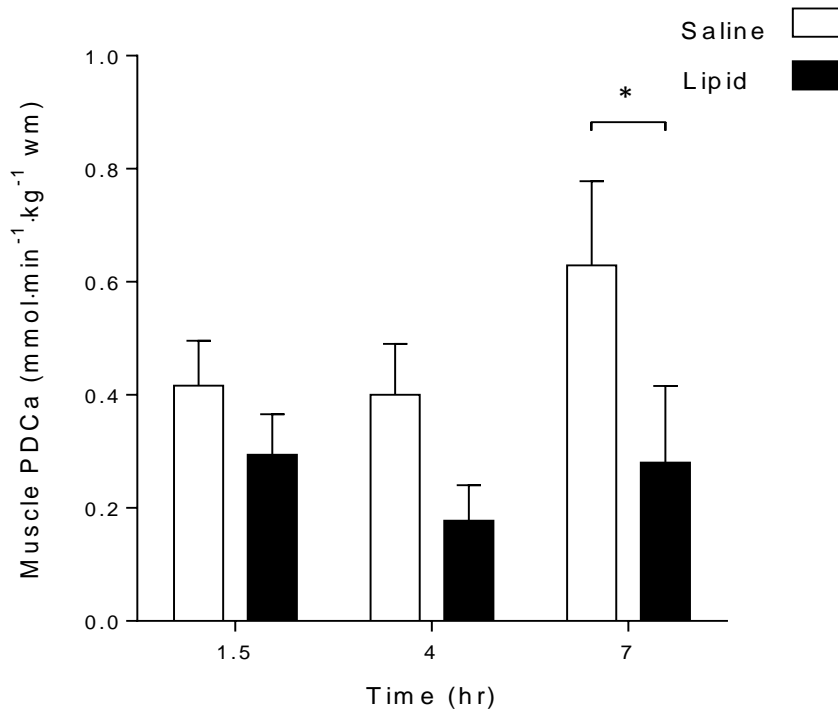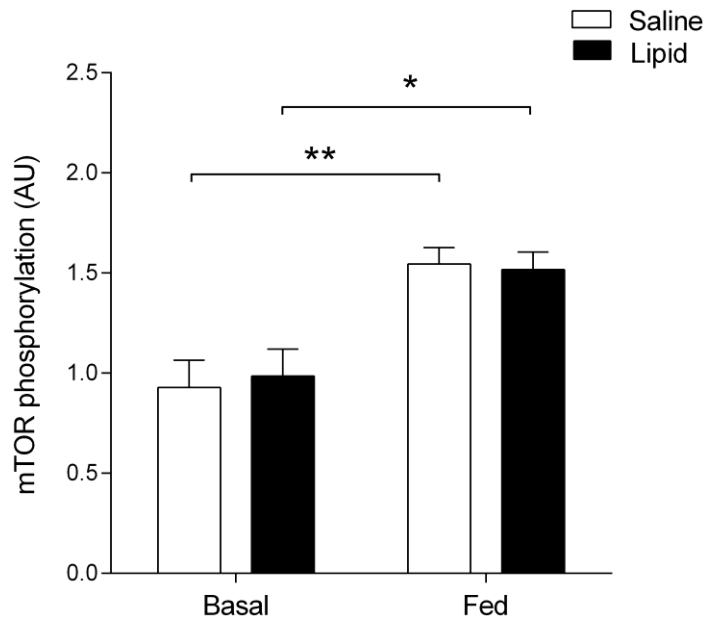
Figure 4B: Skeletal muscle mTOR serine 2448 phosphorylation before (Basal) and after (Fed) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (100 mU/L) clamp during 7 h intravenous infusion of saline (Control; white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means ± SEM. ** P<0.01, * P<0.05, Fed significantly greater from corresponding Basal values.
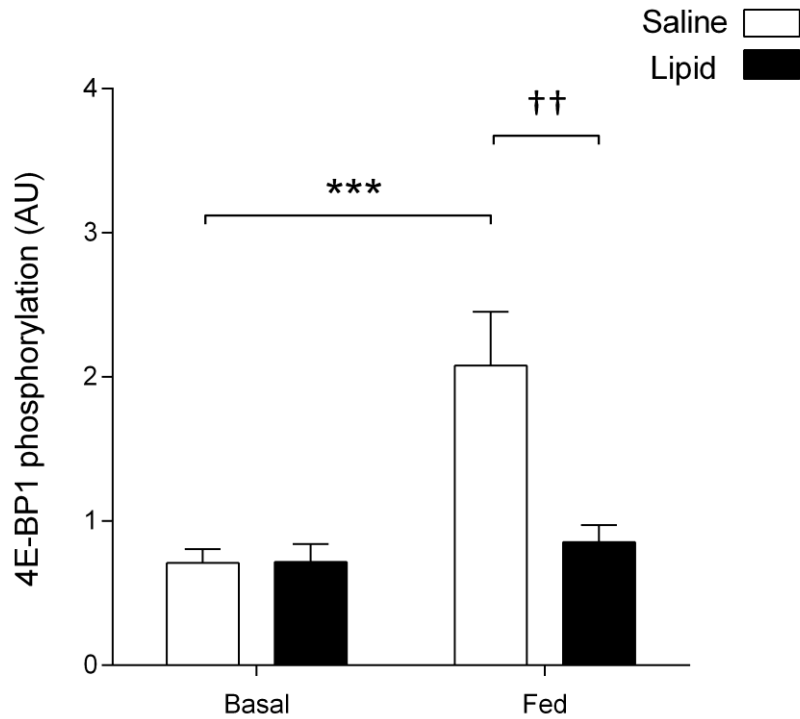
Figure 4C

Figure 4: Skeletal muscle 4E-BP1 threonine 37/46 phosphorylation before (Basal) and after (Fed) the administration of 21 g of amino acids and a 3 hr euglycaemic hyperinsulinaemic (100 mU/L) clamp during 7 h intravenous infusion of saline (Control; white bars) or 10% Intralipid (Lipid; black bars) at a rate of 100 ml/h. Values represent means ± SEM. ††
$P<0.01$, Lipid significantly lower than corresponding Control value. *** $P<0.001$, Fed significantly greater from corresponding Basal values.

4.4 Discussion

Anabolic resistance has been associated with ageing, insulin resistance and sarcopenia. Mechanisms underlying perturbations to anabolic signalling pathways are increasingly studied and unravelled. However, confounding factors such as physical inactivity (commoner with ageing) or obesity and diet (insulin resistance and ageing) may distract from establishing the cause of anabolic resistance per se. Therefore, the aim of this study was to examine the effect of acutely elevating fatty acid availability so as to induce insulin resistance of glucose metabolism on skeletal muscle signalling and protein synthesis in response to amino acid ingestion in the presence of a controlled, steady-state circulating insulin concentration. Under these conditions the present study showed that the insulin resistance observed following intravenous lipid infusion was associated with the inability of skeletal muscle to increase protein synthesis in response to insulin and amino acid administration ie anabolic resistance. This appeared to be partly mediated through the repression of translation initiation at the level of 4E-BP1.

The association between insulin resistance and anabolic resistance has long been established and observed in animal models. Genetically obese, insulin-resistant rats exhibited decreased muscle protein synthesis in response to nutritional (Dunn and Hartsook 1980; Shargill, Ohshima *et al.* 1984; Chan, Hansen *et al.* 1985) and exercise stimuli (Friedman, Lemon *et al.* 1990). Diet-induced obesity in mice (Anderson, Gilge *et al.* 2008) and rats (Masgrau, Mishellany-Dutour *et al.* 2012) was shown to impair the activation of skeletal muscle protein synthesis in response to feeding, particularly in glycolytic muscle where there was chronic lipid infiltration (Masgrau *et al.* 2012).

Whole body protein anabolic response to hyperinsulinaemia and hyperaminoacidaemia is blunted in obese women compared to lean (Chevalier, Marliss *et al.* 2005), and skeletal

muscle protein synthesis in response to insulin and amino acids appears to be negatively-correlated to whole body fat mass in humans (Guillet, Delcourt *et al.* 2009). However the contribution of excess lipid and insulin resistance to anabolic resistance is unlikely to be determined from these studies as confounding factors such as physical inactivity or diet can also induce anabolic resistance (Friedman *et al.* 1990, Wall, Snijders *et al.* 2013). This study has demonstrated that an increase in fat availability or lipid-induced insulin resistance per se can induce protein synthesis resistance in response to insulin and amino acid ingestion independent of confounding factors.

The ingestion of 21 grams of amino acids containing 10 g of essential amino acids including 2.3g of leucine increased mixed muscle protein synthesis by 60% over 3 hours post-prandial, in concert with other studies (Cuthbertson *et al.* 2005; Luzi, Castellino *et al.* 1996). In the presence of elevated lipid availability this response was completely suppressed (Figure 3). In contrast, the study by Kotsanos *et al.* 2009 showed no blunting of the 50% increase in muscle protein synthesis in response to 7 g of essential amino acids, despite higher doses of lipid (nearly 4 times the dose of lipid used in this study). This may be because the lipid infused in Kotsanos's study (20% Liposyn at 90 ml/h for 13.5 hr) had an insulin secretagogue effect resulting in a 4-fold higher circulating insulin concentration before and after amino acid ingestion compared to saline infusion. This level of insulin concentration could overcome any effect of insulin resistance on amino acid metabolism. When insulin was maintained at steady-state concentration above that known to physiologically stimulate muscle protein anabolism, in both the lipid and control arms of the present study, the lipid infusion resulted in a lower muscle protein synthetic rate in response to amino acid ingestion compared to control. This suggests that lipid per se can induce anabolic resistance of amino acid metabolism. Lipid infusion also reduced insulin stimulated peripheral glucose disposal and skeletal muscle PDC activation in the present study by 20 and 50% respectively, in concert

with studies administering a similar dose of lipid infusion (Stephens, Mendis *et al.* 2014) reflecting insulin resistance at the level of skeletal muscle. This raises the pertinent question as to whether a similar pathway in muscle exists that may cause lipid-induced insulin resistance and anabolic resistance.

This and previous studies (Greenhaff, Karagounis *et al.* 2008) have shown that insulin and amino acid administration per se increased phosphorylation of skeletal muscle Akt, mTOR and 4E-BP1. However, despite causing impaired glucose uptake and protein synthesis, lipid infusion in this study did not appear to impair the phosphorylation of Akt or mTOR. This lack of effect on Akt phosphorylation has been reported before (Kruszynska, Worrall *et al.* 2002) and may explain why there was no inhibitory effect of lipid infusion on phosphorylation of mTOR, a key regulator of protein synthesis and substrate for Akt. A major finding from this study was that lipid completely suppressed the ability of insulin and amino acid administration to phosphorylate 4EBP-1, which is normally essential to allow active eIF4F complex and translation initiation to occur. This indicates that an intracellular-signalling defect causing lipid-induced anabolic resistance independent of the Akt-mTOR axis exists, but the exact mechanisms are not known. It may be that there is an alternative insulin or amino acid sensitive protein synthesis pathway that is impaired with lipid. It is also possible that the acute inhibition of protein synthesis in response to elevated NEFA is independent of insulin resistance. As seen in this study, there was no effect of lipid on protein synthesis under basal conditions in the absence of insulin stimulation emphasising the importance of insulin availability in protein synthesis. Ceramide lipids have been demonstrated to impair amino acid-stimulated protein synthesis in L6 cells at the level of translation initiation as well as insulin-stimulated amino acid uptake via an independent mechanism from ceramide-induced insulin resistance of glucose uptake (Lang 2006).

Although muscle protein breakdown was not assessed, there was no effect of lipid on whole-body protein breakdown (phenylalanine Ra) during the basal or fed period of the present study. These findings suggest that muscle loss occurring in conditions of elevated lipid availability and insulin resistance is likely due to reductions in the ability to synthesise muscle protein in response to anabolic stimuli, rather than accelerated muscle protein breakdown (Rennie, 2009; Wall *et al.*, 2013).

4.5 Conclusion

This study has demonstrated that insulin resistance via increased lipid availability can induce anabolic resistance of skeletal muscle protein synthesis in humans in response to amino acid ingestion under steady state hyperinsulinaemic conditions without affecting post-absorptive muscle protein synthesis. This impairment appears to be located downstream of the Akt-mTOR signalling pathway at the level of translation initiation, as phosphorylation of 4E-BP1 in response to feeding was completely suppressed. Future studies should examine the effects of insulin resistance in overweight/obesity from long-term fat overfeeding and ageing on skeletal MPS.

Chapter 5: STUDY 3

Investigating the effects of fat and carbohydrate overfeeding on liver fat and insulin sensitivity.

5.1 Introduction

One of the more pertinent issues of the 21st century concerns the challenge of tackling growing obesity rates. In 2014, more than 1.9 billion adults were overweight. Of these over 600 million were obese (World Health Organisation, WHO). Current estimates are a 33% increase in obesity prevalence and a 130% increase in severe obesity prevalence over the next two decades (Finkelstein 2012). As the obesity rates escalate so will the number of people with insulin resistance and hence T2DM and related metabolic disease. This is clearly a huge public health concern.

Ethan Sims' well-known study of overfeeding prison inmates in Vermont (Sims *et al.* 1970) sparked interest in examining the effects of overfeeding nutrients on human metabolism. His study primarily investigated changes in adipose tissue as a result of overfeeding and will perhaps never be replicated (in-mates were overfed for up to 200 days until they gained up to 25% excess of their initial body weight), but has provided insight and impetus to others in exploring metabolic adaptability and changes in response to increased energy intake and expenditure.

A chronic positive energy balance is clearly the primary driver of obesity, but there may be an additional effect of dietary composition on weight gain and insulin resistance. Overconsumption of high energy nutrients such as fat and a sedentary lifestyle not only results in expansion of adipose tissue (Guilherme *et al.* 2008) but may also lead to fat accumulation in non-adipose tissues (visceral fat) such as skeletal muscle, liver and heart

(Bachmann *et al.* 2001; Schrauwen-Hinderling *et al.* 2005; van Herpen *et al.* 2011). Moreover, insulin resistance is not always accompanied by increased adiposity, such as in people with highly-active antiretroviral therapy (HAART), a condition characterised by insulin resistance, lack of subcutaneous tissue and increased liver fat (Yki-Jarvinen 2005). Mechanisms by which visceral fat is thought to modulate insulin action include increased portal release of FFAs (Bjorntorp P, 1990; Williamson JR *et al.* 1966) and abnormal expression and secretion of fat-derived peptides such as leptin and TNF-α (Hotamisligil *et al.* 1996).

Skeletal and hepatic insulin resistance occur following excess overfeeding (Wang *et al.* 2001), whilst insulin sensitivity is enhanced following energy restriction (Kelley *et al.* 1993) and weight loss (McAuley *et al.* 2006), indicating that energy excess promotes adiposity and insulin resistance. Conversely, dietary composition, rather than total energy intake and body mass have been shown to have a significant influence on insulin sensitivity (Salans *et al.* 1974; Krishnan *et al.* 2007; Villegas *et al.* 2007). This is also exemplified in the San Luis Valley Diabetes Study, where high total and saturated fat intake were shown to increase insulin resistance independent of weight, physical or sedentary lifestyles in a non-diabetes population (Marshall *et al.* 1997).

Diets high in either fat or carbohydrate have been shown to be associated with development of insulin resistance, increased hepatic glucose production and T2DM (Marshall *et al.* 1991; Pereira *et al.* 1997; Schulze *et al.* 2004). The associated studies were presented in chapter 1. The issue of whether macronutrient consumption has a secondary influence on liver fat and insulin sensitivity remains contentious. Is it the total energy consumption or individual macronutrients that are the real protagonists in the development of increased fat mass and insulin resistance? Are there differential effects of dietary carbohydrate or fat on

liver fat and insulin sensitivity? These questions provided impetus to the objectives of this study.

5.2 Aims

The present study compared the effect of high-fat versus high-carbohydrate hyperenergetic feeding on liver fat content and whole body and liver insulin sensitivity in healthy overweight participants. The primary objective was to test the hypothesis that a diet in excess of energy (+25%) will increase intrahepatic lipid (IHL) content and thus hepatic insulin resistance. The study further explored the hypothesis that a hyperenergetic high-fat diet (+25% energy in the form of fat) will increase IHL content, IMCL and thus hepatic and skeletal muscle resistance to a greater degree than a hyperenergetic high-carbohydrate diet (+25% excess energy in form of carbohydrate) as a result of an inability to increase resting energy expenditure.

Given that the effect of high energy overfeeding is seen within 7 days in the majority of previous studies, it seemed likely that 2 weeks of 25% excess energy high fat or carbohydrate diets would be sufficient to produce effects on liver fat and insulin sensitivity. Overweight or obese healthy participants were recruited as they were more likely to have an initial degree of elevated liver fat and insulin resistance compared to healthy lean participants which is then more likely to be altered by dietary change.

Based on studies seen in the current literature this is the first study to provide a comprehensive assessment of the effects of either hyperenergetic high fat or high carbohydrate on liver fat, whole body and peripheral insulin sensitivity (liver), lipid and liver metabolism.

5.3 Methods

5.3.1 Participants

24 healthy overweight/obese male participants were initially screened and consented to participating in the study according to the Declaration of Helsinki. Anyone who drank excessive alcohol (>28 units per week) or had elevated LFTs (AST or ALT three times above the upper range of normal) were excluded from participation. Participants completed a three day diet diary and International Physical Assessment Questionnaire (IPAQ) to determine their estimated daily total energy expenditure. They then consumed an isoenergetic diet meeting their usual total energy expenditure for a week before attending their first experimental visit.

5.3.2 Experimental Visit

Volunteers fasted from 2200 before the morning of the visit and first attended the Sir Peter Mansfield MR Centre, University Park, University of Nottingham at 0800 for scanning of their liver, abdominal subcutaneous tissue and thigh muscle. They then attended the MRC/ARUK David Greenfield Human Physiology Unit where they were weighed and underwent anthropometric assessments (callipers and bioelectric impedance analysis). At the start of the experimental study volunteers rested in a semi-supine position while cannulae were inserted into a superficial dorsal hand vein for arterialized-venous blood sampling and in both forearm veins for insulin, glucose and stable isotope infusions. Baseline blood was taken to measure liver transaminases, lactate, uric acid, beta-hydroxybutyrate, adipocytokines, inflammatory cytokines, lipid profile and free fatty acids. A 4.5 hour primed (4mg/kg) continuous infusion of [6, 6 ^{2}H$_2$] glucose (40µg/kg/min) was infused from the start of the study to measure hepatic glucose production and thus assess hepatic insulin sensitivity.

Blood samples were taken every hour for the first 2 hours and every 30mins for the last 2.5 hours to determine plasma [6,6^{2}H$_2$] glucose to allow subsequent calculation of endogenous hepatic production of glucose. Further blood samples were taken every 30 minutes for measurement of insulin and glucagon, and every 1 hour for measurement of C-peptide, plasma lipids, FFAs, lactate, uric acid, β-OHB, adipocytokines, CRP, IL-6 and TNF-α.

After 2 hours of tracer infusion, the hyperinsulinaemic (30mu/m^2/min) euglycaemic clamp was commenced for 2.5 hours. This rate of insulin infusion was chosen based on a previous study investigating the effects of either high fructose or glucose on hepatic insulin sensitivity (Johnston *et al.* 2013), where it was shown that this dose of insulin will partly but not completely suppress hepatic glucose output.

Respiratory exchange measurements (oxygen consumption and carbon dioxide production) were recorded using a ventilated hood attached to a metabolic cart for 15 minutes at baseline, before the insulin clamp and 2.5 hours from the start of the clamp in order to indirectly calculate REE and RER to determine whole body carbohydrate and lipid oxidation rates.
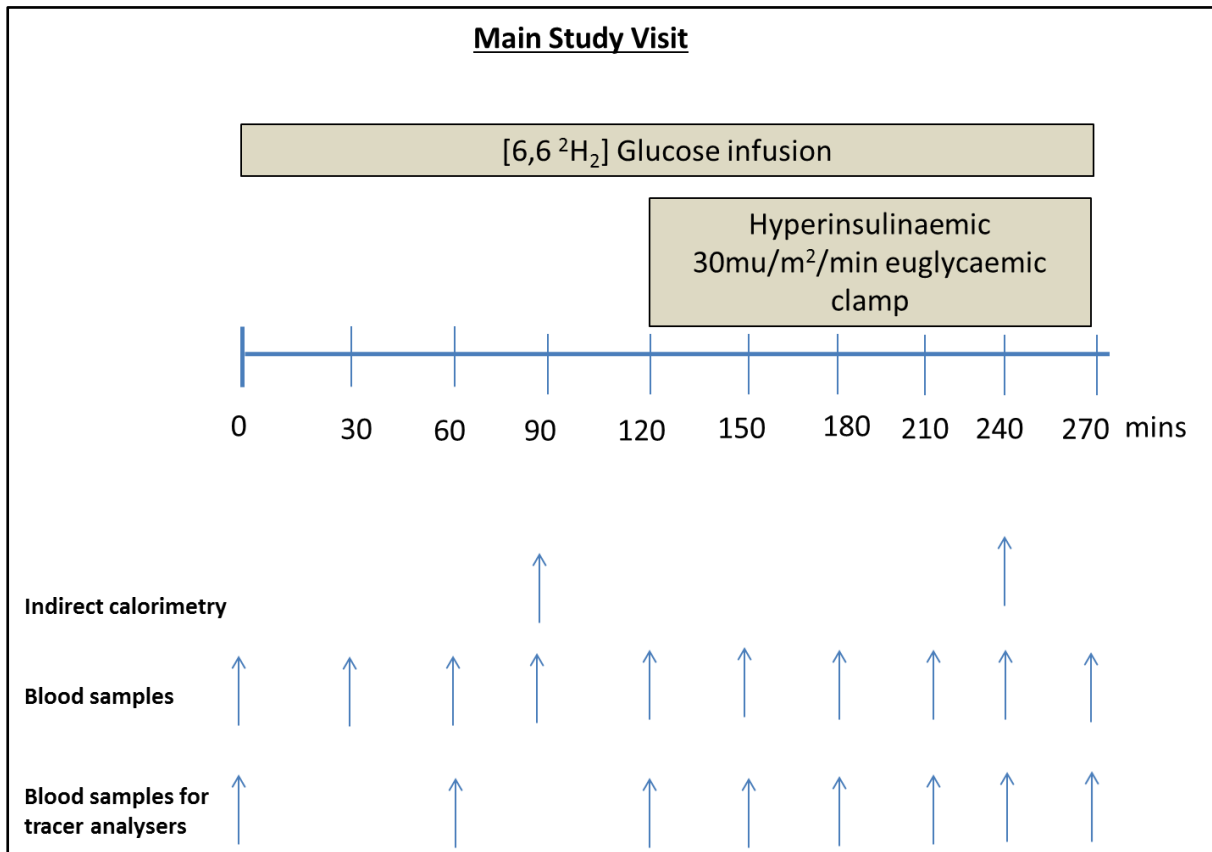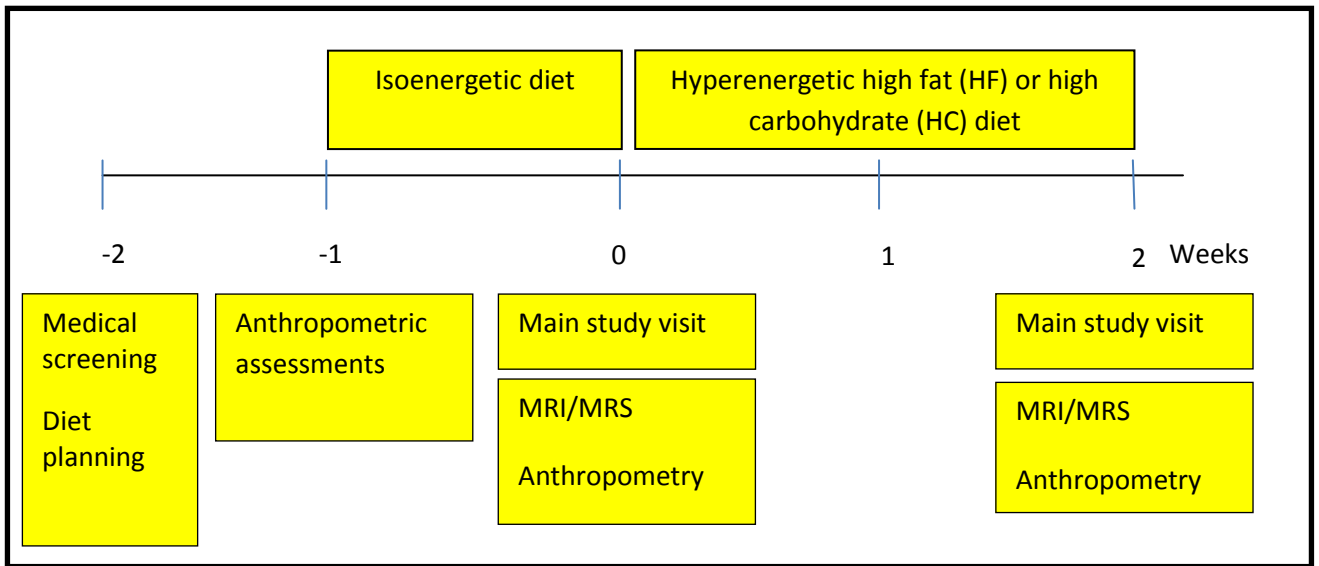
Figure 5.1: Schematic design of High Fat High Carbohydrate (HFHC) protocol

5.3.3 Energy requirements

Estimated energy intake for each subject was calculated using the Oxford (Henry-modified) equation. This method was chosen over the Schofield and Mifflin-St Jeor prediction equations for a number of reasons. The Schofield equation has been found to overestimate BMR in several populations (Henry C, 2005), and although this overestimate is small (<5%), the Oxford equation has been most vigorously tested and its use has been recommended by the Scientific Advisory Committee on Nutrition (SACN) (Dietary reference values for energy, SACN 2011) to predict estimated energy requirements in healthy populations. The use of the modified Henry equation is warranted particularly in the current growing population of overweight and obesity, where other prediction equations may underestimate energy requirements. Resting energy expenditure (REE) is influenced by body composition, thus in overweight people equations of REE may not give a true estimate. Although the Mifflin-Jeor equation appears to predict REE better than other equations in obese subjects (Frankenfield *et al.* 2003; Dobratz *et al.* 2007; Weijz *et al.* 2008 and 2010; de Oliviera *et al.* 2012), values may deviate > 10% in as much as 30% of obese (Madden 2014). Other than REE, the variation of physical activity may also influence and impact on TEE. TEE may be estimated by multiplying the REE with the physical activity level (PAL) which itself may be determined from population reference data (SACN 2011) or by individual assessment. The UK reference for PAL in adults is 1.63 (SACN 2011), assuming >60% of the population are overweight or obese and who participate in typical activity levels.

Henry Equation

Males

Age 18-30: REE (kcal/d) = 16.0W + 545

Age 30–60: REE (kcal/d) = 14.2W + 593

Where REE is in kcal/day and W denotes weight in kg

Estimated TEE = REE estimated using the Henry Equation X PAL (1.63)

5.3.4 Dietary plan

24 healthy but overweight/obese (BMI 26-37 kg/m^2) males aged 18-55 initially went on a 7 day isoenergetic diet matched to the individual's predicted TEE calculated using the Henry Equation (Henry 2005) and PAL questionnaire. They were then randomized to two groups, each of which received a high energy (+25% energy excess) diet with either high fat (HF - 48-50% fat, 37% carbohydrate CHO, 13%-15% protein) or high-carbohydrate (HC - 24-26%-28% fat, 59%-61% CHO and 13-15% protein) for 2 weeks before returning for the second experimental protocol visit. The HC group received extra carbohydrate in the form of a maltodextrin drink daily such that they were supplemented with an extra 25% excess energy daily. Similarly the HF group received 25% excess calories in the form of double cream that could be ingested with their desserts in the evening. Participants were also requested to complete a 3 day diet diary to determine actual total energy consumed per day and patterns of food consumption. This was done using "Microdiet" (Downlee Systems Ltd), a nutrient analysis software programme designed to analyse and calculate nutrient totals and contribution to meals designed for the study. An example of the contribution of energy from the nutrients for a calculated TEE as calculated using "Microdiet" can be found in the appendices section.

5.3.5 Example menu (See appendices)

Menus were developed for each day for the 3 weeks with input by a dietitian. Breakfast consisted of cereals that were weighed (50 grams) at the physiology lab and provided to participants prior to the start of each week of the study. Skimmed milk powder (weight pre-determined to balance protein contribution) was also measured and distributed to volunteers prior to the study. The rest of the food on the menu was delivered by a commercial supermarket one or two days prior to the start of the week of feeding.

5.3.6 Measurable endpoints/statistical power of the study

The primary endpoint of the study was liver fat content. Hepatic and peripheral insulin sensitivity (through deuterated glucose and glucose disposal during clamps respectively) was also examined. The repeated measures coefficient of variation for the insulin clamp technique is 10%. Based on isoenergetic high-fat feeding studies it was predicted that there would be a greater than 20% difference in glucose disposal from the high fat versus high carbohydrate diet. 8-10 participants would give an 80% chance of detecting a difference in insulin resistance equivalent of 1 standard deviation for that variable at p value <0.05. 12 participants for each group (HCHF or HCHC) were therefore recruited to allow for dropouts of 2-4 participants per group.

5.4 Results

5.4.1 Participant demographics

A total of 24 male White Europeans were recruited to the study, and randomised to either the high-fat (HF) or high carbohydrate (HC)-fed groups (12 per group). One subject from the high fat group did not complete his second hyperinsulinaemic euglycaemic clamp visit, therefore evaluations of peripheral insulin sensitivity and other metabolic data after the 2

week intervention period were performed on 11 participants in the HF group and 12 in the HC group. ^{1}H MRS and MRI abdomen was only performed in 22 participants, 12 from the HF group, and 10 from the HC group. The 2 participants from the HC group were excluded due to contraindications to MRI (See Appendices section).

All participants were overweight or obese with a mean body mass index of 30.6 ± 0.6 kg/m^2 ranging from 26.2 to 37.3 kg/m^2. Percentage body fat as calculated using skin-fold thickness and the SIRI formula averaged 33.7%, ranging 22.9-42.0%. The participants were well-matched in all parameters (see Table 5.1) bar skinfold thickness of biceps, and mean estimated TEE was 3200 kcal/day.

5.4.2. Baseline Anthropometrics

Table 5.1. Participant anthropometrics

	Total (n=23)	High Fat (n=11)	High Carbohydrate (n=12)	P value
Age (years)	40.6 ± 1.6	39.1 ± 2.1	42.3 ± 2.4	0.34
Weight (kg)	98.9 ± 2.0	100.2 ± 3.0	97.2 ± 2.6	0.51
BMI (kg/m^2)	30.6 ± 0.6	30.7 ± 0.9	30.1 ± 0.9	0.88
Hip circumference (cm)	105.8 ± 1.0	106.1 ± 1.5	105.4 ± 1.5	0.78
Waist circumference (cm)	105.8 ± 1.4	105.5 ± 2.2	105.9 ± 1.8	0.79
Waist > 102cm (n participants)	16/23	8/11	8/12	0.70

Waist to hip ratio	0.99±0.01	0.99±0.01	1.00±0.01	0.45
Fat (kg)	24 ± 1.2	25.0 ± 2.0	22.7 ± 1.4	0.37
Lean (kg)	74.7 ± 1.4	74.8 ± 1.8	74.5 ± 2.3	0.93
Biceps (mm)	8.5 ± 0.6	9.9 ± 0.8	6.9 ± 0.5	<0.01
Triceps skinfold thickness(mm)	17.3 ± 1.6	18.0 ± 2.2	16.5 ± 2.5	0.66
Subscapular skinfold thickness(mm)	26.5 ± 3.1	30.4 ± 5.3	21.7 ± 2.2	0.18
Iliac crest skinfold thickness(mm)	15.5 ± 1.0	16.3 ± 1.5	14.5 ± 1.1	0.37
Abdominal skinfold thickness (mm)	36.4 ± 1.4	37.5 ± 2.2	35.1 ± 1.8	0.43
Bio-Impedance Analysis (BIA) (% Fat)	33.7	36.2	31.1	0.08

5.4.3 Baseline MRI of abdomen

Table 5.2. Visceral and subcutaneous abdominal tissue

	Total (n=22)	High fat (n=12)	High carbohydrate (n=10)	P value
Visceral abdominal tissue (VAT) volume (ml)	1080.4±130	949.9 ±149.8	1223.9±115.1	0.17
Abdominal volume (ml)	7171.9±262.8	6998.7±273.1	7362.4±292.1	0.37
Subcutaneous abdominal tissue (SAT) (ml)	2759.3±154.7	2745.6±238.1	2775.7±199.1	0.95
Ratio (VAT/Abdominal volume)	0.15±0.01	0.13±0.02	0.16±0.01	0.15
Ratio (VAT) / (SAT)	0.28±0.02	0.25±0.03	0.30±0.02	0.24

5.4.4 Intrahepatic-cellular lipid (IHCL)
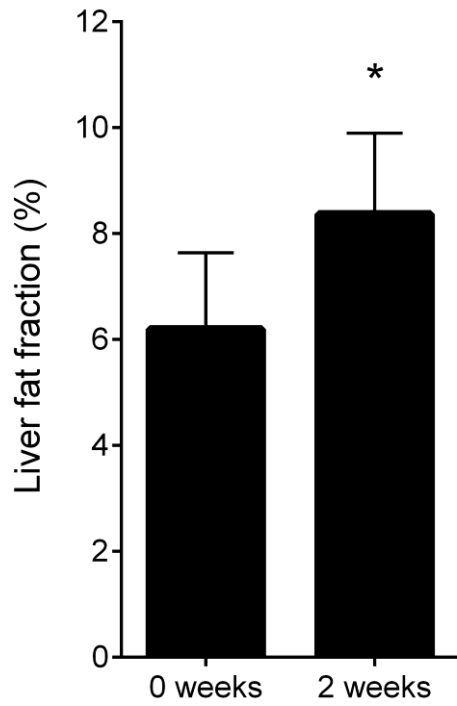
Figure 5.2: Percentage liver fat fraction at 0 weeks and following overfeeding 25% excess

energy of diets high in carbohydrate (n=10 subjects) and fat (n=11 subjects) at 2 weeks.

*P<0.05 from 0 weeks.
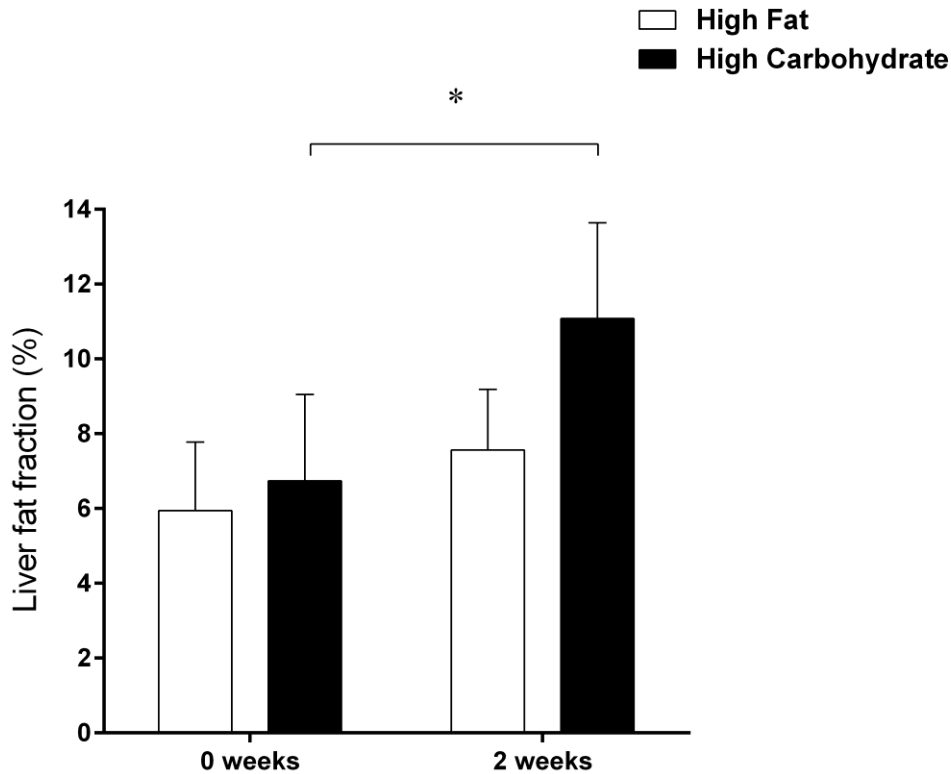
Figure 5.3: Absolute liver fat fractions (%) in the high fat (n=11) and high carbohydrate groups (n=10) at 0 weeks and following 2 weeks of 25% excess energy of either high fat of high carbohydrate. *P < 0.05 from 0 weeks.

Of the 21 participants who had MRI scan of their liver, 7/21 had fatty liver (defined as >5.6% liver fat content (Szczepaniak et al. 2005) (4 from HF, 3 from HC) at baseline. No difference in liver fat was seen in both groups following the 7 days of isoenergetic diet. Following 2 weeks of excess energy feeding, 12/25 had fatty liver (6 participants from each group, 7 with pre-existing fatty liver) and there was an overall 34% increase in liver fat fraction in the whole group from 6.4 ± 1.5 to $8.6 \pm 1.6\%$ (P<0.05). There was no difference in baseline liver fat fractions between the high fat vs. high carbohydrate groups (6.1 ± 2.1 vs.$7.4 \pm 2.4\%$). After 2 weeks, liver fat fractions increased to $7.8 \pm 1.8\%$ and $11.0 \pm 2.7\%$ in the high fat and high carbohydrate groups respectively. There was a significant increase of 49% in liver fat fraction at 2 weeks in the high carbohydrate group ($+3.5 \pm 1.5\%$; P<0.05) compared to the high fat

group ($+1.7 \pm 0.8\%$). No difference in changes between the high fat and carbohydrate groups were seen (P=0.3).

5.4.5 Hepatic insulin sensitivity

5.4.5.1 Fasted and post-prandial hepatic glucose production

Table 4.3 Hepatic glucose production (mg/kg/min) fasted and steady state of hyperinsulinaemic ($30mu/m^2/min$) euglycaemic clamp at 0 weeks and after 2 weeks of high fat or high carbohydrate overfeeding.

		Whole group n=18	High Fat n=10	High carbohydrate n=8	P value Difference between groups
Baseline mg/kg/min	Fasted	2.27±0.14	2.22±0.18	2.35±0.19	0.4
	Steady state insulin	1.02±0.24	0.83±0.15	1.25±0.51	
2 weeks mg/kg/min	Fasted	2.18±0.08	2.26±0.38	2.08±0.10	0.5
	Steady state insulin	0.96±0.21	0.73±0.33	1.25±0.22	

Rates of fasting hepatic glucose production remained unaltered in the whole group after 2 weeks of overfeeding 25% excess energy. There was no difference in fasted rates between the fat and carbohydrate overfeeding groups after 2 weeks (Table 4.3). In the whole group, hepatic glucose production was suppressed by 55% and 56% by the end of the clamp at baseline and following 2 weeks of overfeeding respectively. There was no difference in

hepatic glucose production suppression rates at baseline and after 2 weeks of fat overfeeding (62% vs. 68%), whereas hepatic glucose production was suppressed by 47% and 40% at baseline and after 2 weeks of carbohydrate feeding.

5.4.6 Baseline insulin sensitivity

Table 5.4: Baseline measurement of insulin sensitivity

	Total n=22	High Fat n=10	High Carbohydrate n=12	Difference between groups
GDR (µmol/kg mass/min)	25.0±1.7	26.1±2.7	24.2±2.3	0.6
Fasting Glucose (mmol/L)	4.4±0.1	4.6±0.1	4.2±0.1	0.01
Fasting Insulin (mU/L)	11.7±1.2	11.6±1.5	11.8±1.8	0.9
HOMA-IR	2.1±0.2	2.2±0.4	2.1±0.2	0.3
C-peptide (ng/mL)	0.83±0.09	0.82±0.12	0.84±0.14	0.9
Insulin/C-peptide ratio	0.0021±0.0002	0.002±0.0003	0.002±0.0002	0.39
Glucagon (pg/ml)	112.6±6.2	105.1±9.0	119.5±7.9	0.25

5.4.7 Baseline lipid, liver and inflammatory markers

Table 5.5: Baseline lipid, liver and inflammatory markers

	Total n=21	High fat n=10	High carbohydrate n=11	Difference between groups
FFA (mmol/L)	0.50±0.03	0.46±0.05	0.53±0.05	0.27
Cholesterol (mmol/L)	5.0±0.2	5.1±0.3	5.0±0.3	0.8
HDL(mmol/L)	1.01±0.04	1.01±0.04	1.01±0.05	1.0
LDL(mmol/L)	3.39±0.21	3.46±0.29	3.32±0.28	0.74
Apolipoprotein A1 (g/L)	1.11±0.03	1.09±0.04	1.14±0.06	0.7
Apolipoprotein B (g/L)	1.02±0.06	0.96±0.09	1.05±0.07	0.8
AST (U/L)	24.3±1.7	21.6±1.5	26.8±2.6	0.1
ALT (U/L)	15.6±1.9	14.2±2.2	16.9±2.9	0.47
TAG (mmol/L)	1.37±0.11	1.26±0.15	1.47±0.16	0.34
CRP (mg/L)	2.28±0.69	2.27±0.9	2.29±0.93	0.99
IL-6 (pg/mL)	4.9±1.7	6.6±3.1	3.3±1.4	0.3
Lactate (mmol/L)	1.1±0.1	0.9±0.1	1.2±0.1	0.22
TNF-α (pg/mL)	6.6±2.4	8.6 ±4.3	4.7±2.3	0.4

5.4.8 Effect of high fat or carbohydrate overfeeding after 2 weeks

Table 5.6: Insulin sensitivity and lipid, liver and inflammatory markers at baseline and after 2 weeks of high fat and high carbohydrate diets. (Where specifically indicated, total n=22, high fat n=10 and high carbohydrate n=12). *P<0.05; **P<0.01; ^P= 0.07; [†]P=0.09 compared to week 0.

	Total n=22		High Fat n=10		High Carbohydrate n=12		Differential effects P value
	0 weeks	2 weeks	0 weeks	2 weeks	0 weeks	2 weeks	
GDR µmol/kg mass/min)	25.0±1.7	23.7±1.9	26.1±2.7	24.5±2.3	24.2±3.0	23.1±2.9	0.59
M Value (mg/kg mass/min)	4.51±0.31	4.44±0.37	4.70±0.48	4.89±0.51	4.35±0.40	4.08±0.52	0.38
Fasting Glucose (mmol/L)	4.4±0.1	4.5±0.1	4.6±0.1	4.5±0.1	4.2±0.1	4.5±0.1	0.04

Fasting Insulin (mU/L)	10.2±0.9	10.5±1.1	10.4±1.7	10.8±2.4	10.0±0.8	10.2±0.5	0.85
HOMA-IR	2.0±0.2	2.1±0.2	2.2±0.4	2.2±0.5	1.9±0.2	2.0±0.1	0.49
C-peptide (ng/ml)	0.83±0.09	0.83±0.11	0.82±0.13	0.84±0.16	0.84±0.14	0.82±0.17	0.78
Insulin/C-peptide ratio	0.002±0.0002	0.002±0.0003	0.002±0.0003	0.002±0.0005	0.002±0.0002	0.002±0.0002	0.31
Glucagon (pg/ml)n=21	114.3±5.9	108.9±6.4	105.1±9.4	110.5±14.1	119.5±7.9	114.5±9.4	0.48
FFA (mmol/L)	0.50±0.03	0.44±0.03	0.46±0.05	0.45±0.03	0.53±0.05	0.43±0.05^	0.65
Leptin (ng/mL)	9.9±1.0	11.5±1.7*	9.1±1.1	10.0±1.1	10.6±1.6	12.8±2.1^	0.49
Cholesterol (mmol/L)	5.0±0.2	5.1±0.2	5.0±0.3	5.2±0.3	5.0±0.3	5.1±0.3	0.8
HDL (mmol/L)	1.01±0.03	1.01±0.05	1.02±0.04	1.09±0.07	1.01±0.05	0.96±0.05	0.3
LDL (mmol/L)	3.3±0.2	3.5±0.2	3.3±0.3	3.5±0.3	3.3±0.3	3.5±0.3	0.5
Apolipoprotein A1 (g/L)	1.10±0.03	1.18±0.02**	1.09±0.04	1.22±0.03*	1.14±0.06	1.17±0.04**	0.79

Apolipoprotein B(g/L)	1.01±0.06	1.08±0.05**	1.00±0.09	1.10±0.09*	1.03±0.07	1.07±0.06**	0.98
AST (U/L)	24.3±1.7	26.7±2.4	21.6±1.5	22.5±1.7	26.8±2.6	30.5±3.9	0.2
ALT (U/L)	15.6±1.9	17.7±1.5	14.2±2.2	15.7±2.2	16.9±2.9	19.5±2.0	0.3
TAG (mmol/L)	1.37±0.11	1.72±0.16*	1.23±0.15	1.47±0.16	1.47±0.16	1.95±0.25*	0.49
CRP (mg/L)	2.3±0.7	1.9±0.4	2.3±0.9	1.6±0.4	2.1±0.9	2.0±0.7	0.91
IL-6 (pg/mL)	4.6±1.5	4.7±1.2	6.6±3.1	6.8±2.3	3.1±1.3	3.0±0.9	0.9
Lactate (mmol/L)	1.07±0.07	1.22±0.09*	0.98±0.10	1.05±0.08	1.15±0.09	1.37±0.13†	0.08
TNF-α (pg/mL)	6.6±2.4	7.3±2.5^	8.6±4.3	9.4±4.5	4.4±2.2	5.0±2.4†	0.38
Uric acid	350.5±14.6	348.1±14.1	331.5±21.4	333.3±15.9	369.5±21.4	362.8±25.0	0.24
β-OH butyrate n=21	0.18±0.02	0.17±0.02	0.15±0.03	0.18±0.02	0.21±0.03	0.17±0.04	0.38

Markers of insulin sensitivity in the high carbohydrate and high fat-fed groups at baseline were matched apart from fasting glucose. There were no changes in fasting plasma glucose, serum insulin, glucagon, glucose disposal rates, HOMA-IR or adipose tissue-IR following 2 weeks of energy excess. There were also no differential effects between dietary groups at 2 weeks.

5.4.9 Lipid, liver and inflammatory markers

Lipid profile, liver function and inflammatory markers were well-matched between the two groups at baseline. Fasting TAG increased in the whole group following 2 weeks of excess energy intake. This was particularly noted in the carbohydrate-fed group where TAG increased by 23% from week 0. Fasting liver enzymes (AST, ALT), total, high density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol did not change following 2 weeks of excess energy in the HF and HC diets. After 2 weeks of energy excess, there were increases in Apolipoproteins A (Apo A) and B1 (Apo B1) in the whole group, but this was only significant in the HF group. Fasting serum leptin increased following 2 weeks of excess overfeeding and a trend was seen (P=0.07) after consumption of a high carbohydrate diet. Plasma lactate and TNF-α increased in the group as a whole after 2 weeks of excess energy, with a trend of an increase seen with both (P=0.09) in the high carbohydrate fed group. No difference was seen with the systemic inflammatory markers CRP and IL-6, uric acid and beta-hydroxybutyrate across the groups.

5.4.10 Plasma measurements during the hyperinsulinaemic euglycaemic clamp

5.4.10.1 Insulin
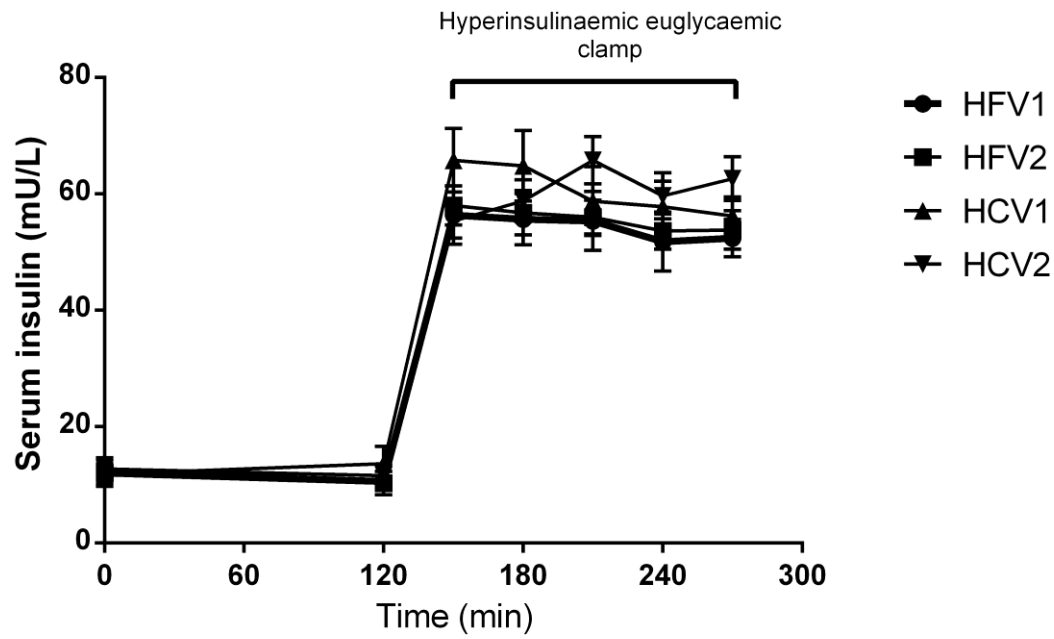
Figure 5.4: Serum insulin before and during the hyperinsulinaemic ($30mu/m^2/min$)

euglycaemic clamp

In the first 2 hours of the study (pre-insulin), fasting serum insulin averaged 11.8mU/L.

During the hyperinsulinaemic euglycaemic clamp, serum insulin rose to and plateaued at

59mU/L for the following 2.5 hours.

5.4.10.2 Free fatty acids
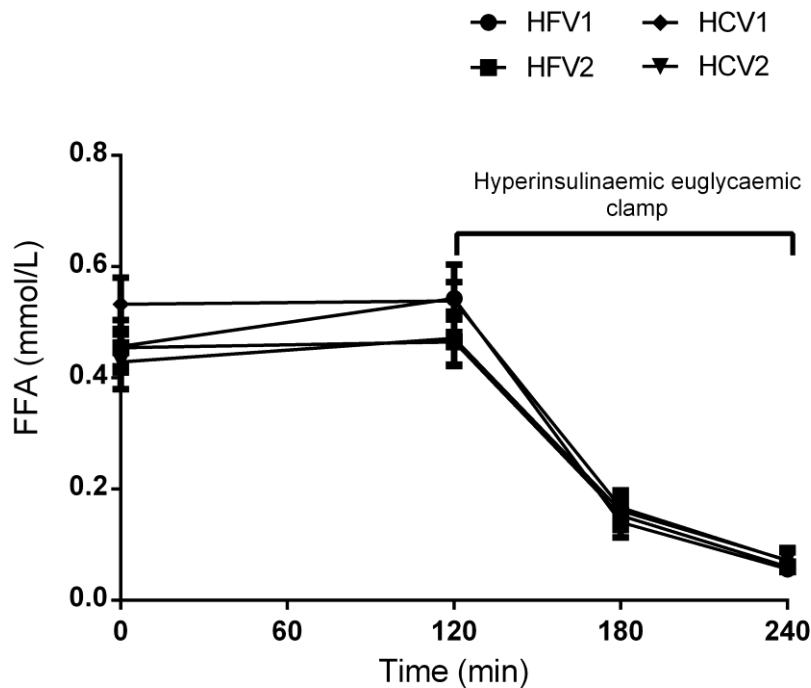
Figure 5.5: Serum FFA before and during the hyperinsulinaemic ($30mu/m^2/min$)

euglycaemic clamp

During the 2 hours pre-insulin, whole group fasting FFA concentrations averaged 0.50 ± 0.03

mmol L^{-1} at baseline with no difference after 2 weeks of overfeeding at 0.44 ± 0.03 mmol L^{-1}.

Post-insulin, FFA levels were suppressed at 0.06 ± 0.01 and $0.07 \pm 0.01 mmolL^{-1}$ at baseline and

after 2 weeks respectively. There were no differential effects of carbohydrate and fat

overfeeding on FFA levels.

5.4.11 **Indirect Calorimetry**

Table 5.7: Indirect calorimetry fasted and at steady-state insulin at 0 weeks and after 2 weeks of high carbohydrate or fat.

Data from only 7 and 11 participants from the high fat and high carbohydrate groups respectively were obtained due to technical/incomplete data retrieval. *P<0.05 different from fasting values, week 0

	High Fat n=7				High Carbohydrate n=11			
	Week 0 (Fasting)	Week 0 Post-clamp	Week 2 Fasting	Week 2 Post-clamp	Week 0 Fasting	Week 0 Post-clamp	Week 2 Fasting	Week 2 Post-clamp
Cox (J/kg/min)	6.6 ± 1.1	17.6±4.4*	8.4± 2.4	14.3±2.9	7.2±2.1	12.0±4.2	10.1±4.1	18.6±2.8
Fox(J/kg/min)	62.6 ± 3.2	52±5.7	58.8±3.3	50.9±2.7	54.5±2.8	49.3±3.6	52.1±4.6	43.6±2.3
EE(J/kg/min)	69.2 ± 2.9	68.8±2.5	66.6±1.8	64.6±1.7	61.8±1.3	62.3± 1.7	61.3±1.6	62.1±0.9
RER	0.73±0.01	0.78±0.02	0.77±0.02	0.78±0.02	0.74±0.01	0.76±0.02	0.75±0.02	0.78±0.01

Carbohydrate oxidation at rest increased post-insulin in the high fat group at 2 weeks. No

significant changes in carbohydrate or fat oxidation, EE and RER were seen after 2 weeks of

either fat or carbohydrate overfeeding.

5.4.12 Intramyocellular and extramyocellular lipid content
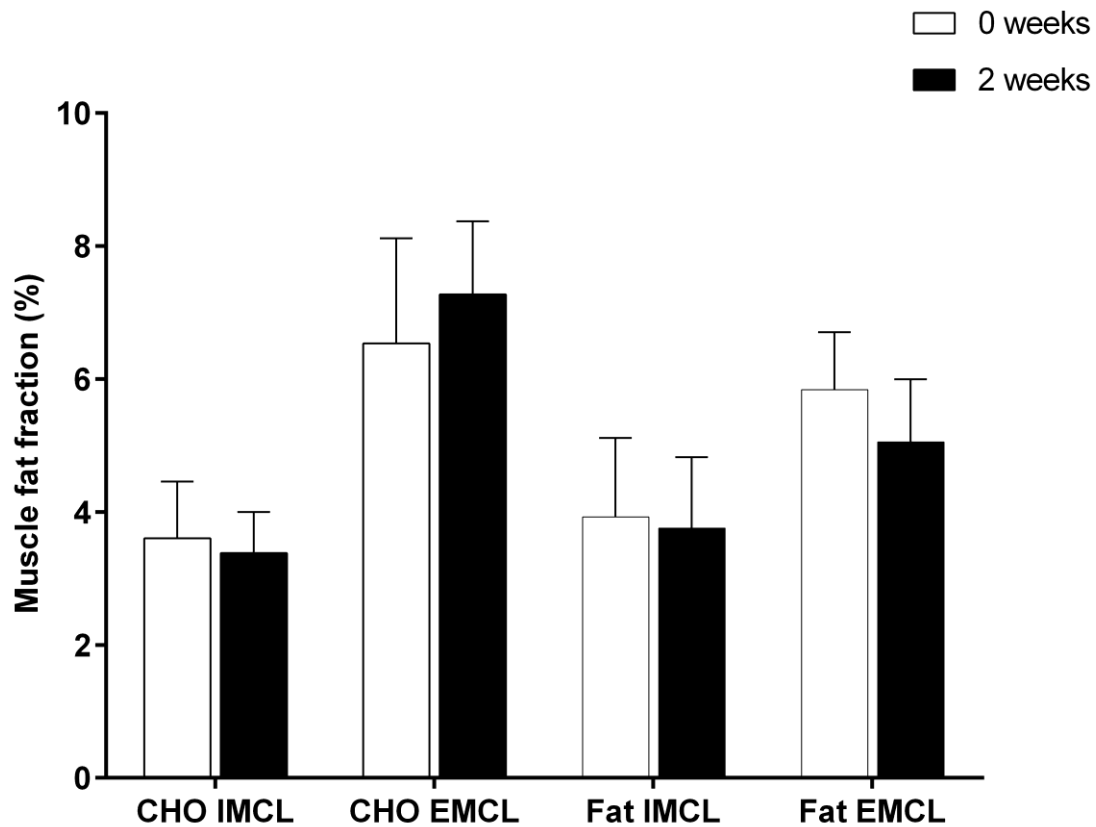
Figure 5.7: Intramyocellular (IMCL) and extramyocellular lipid (EMCL) fractions using

magnetic resonance spectroscopy at 0 weeks and after 2 weeks of fat and carbohydrate

(CHO) overfeeding.

5.4.12.1 Intramyocellular Lipid (IMCL)

IMCL did not change from baseline after 2 weeks of fat or carbohydrate overfeeding, nor were there differences between the dietary groups.

5.4.12.2 Extra-myocellular lipid (EMCL)

There were no significant changes in EMCL with either diet (Figure 5.7).

5.4.13 Intervention outcomes

5.4.13.1 Tolerability/Side-effects

There were no study participant drop-outs. The reasons for incomplete study visits are outlined below (Table 5.8).

No major side-effects from consuming the meals during the overfeeding period were reported by participants. Minor side-effects reported included headache (likely secondary to caffeine or fluid restriction in one participant in the HC group), nausea and vomiting 2 hours after completing the hyperinsulinaemic clamp visit (may be secondary to increased food intake post-clamp), constipation (subject in HC group possibly secondary to changes in dietary intake), bloating and fullness (subject in HC group secondary to increased intake in addition to carbohydrate drink). These effects were self-limiting and did not last beyond the period of overfeeding. No major complications from the cannulation, blood-taking or scanning were reported; any concerns were brought up by participants and communicated to the study investigator and resolved completely.

Table 5.8: Participants excluded from MRI/MRS liver and muscle

Subject ID	Group	Reason
10	High fat	Did not complete second visit of insulin clamp as problems with cannulation and subject was not keen to proceed further
8	High carbohydrate	Did not have MRI /MRS as deemed unsafe to have magnetic imaging given participant's job working with steel and risk of exposure of metal fragments in eyes
23	High carbohydrate	Did not have MRI/MRS because of a metallic implant in subjects' spine-this would have interfered with MR signalling and data interpretation over abdominal area.

5.5 Discussion

5.5.1 Effects of overfeeding on liver fat content

2 weeks of high energy feeding increased percentage liver fat content from baseline in the group as a whole, but no difference in liver fat content between HF and HC groups were seen. There was an increase in liver fat by $3.52 \pm 1.45\%$ from baseline in the HC-fed group. This is consistent with several studies (Ngo Sock *et al.* 2010, Sobrecases 2010, van Herpen *et al.* 2011, Sevastianova *et al.* 2013, Westerbacka 2005) but contradicts others (Le *et al.* 2006, Silbernagel *et al.* 2011). The studies involving carbohydrate feeding and which did not observe increased liver fat content were conducted in lean people (Le *et al.* 2006 and Silbernagel *et al.* 2011). In the present study, overweight or obese males were recruited as they are likely to have an initial degree of liver fat and insulin resistance compared to healthy lean participants. They are therefore likely to have a greater predisposition to metabolic perturbations from dietary change, compared to leaner people.

5.5.2 Effect of hyperenergetic high carbohydrate and high fat diets on hepatic glucose production

Although overfeeding with 25% excess energy increased liver fat content it did not affect hepatic insulin sensitivity or insulin suppression on hepatic glucose production over a 2 week period. However the scatter of hepatic glucose production data (standard deviation) is notable so the possibility of a type 2 statistical error should be considered.

5.5.3 Effects of hyperenergetic high carbohydrate and high fat diets on insulin sensitivity

2 weeks of excess energy of either carbohydrate or fat did not result in changes to whole-body insulin sensitivity as measured by the hyperinsulinaemic–euglycaemic clamp. This may

be the result of several factors. It may be that there is indeed no influence of either diet on insulin sensitivity. In a recent study involving overweight participants over a 12 week period of consuming a high energy high fructose diet, there was a trend for decreased hepatic insulin sensitivity, increased fasting glucose and insulin; however this did not result in whole-body insulin resistance (Tappy et al 2015). Stanhope et al (2009) however showed changes in hepatic insulin sensitivity and liver fat content following a similar period of fructose overfeeding. Changes in whole-body insulin sensitivity may not be appreciable until participants gained significant weight. In this study neither weight gain nor a significant increase in RER were seen using indirect calorimetry. A recent study (Boden *et al.* 2015) involved overfeeding healthy males with a 6000 kcal/day typical Western diet (50% CHO, 35% fat and 15% protein) for 1 week. Participants gained weight (+3.5kg), had raised insulin levels and developed insulin resistance (increased HOMA-IR). It may also be that early changes at a cellular level that alter insulin signalling may occur before manifesting as whole body insulin sensitivity, as seen in the study by Adochio *et al.* 2009. This was not assessed in the present study but calls for further investigation in future studies. It has also been suggested that there may be differences in how individuals respond to overnutrition based on their baseline insulin sensitivity (Adochio *et al.* 2009). In that study there was significant heterogeneity in baseline measures of insulin sensitivity (M-value: 7.68 to 17.71 mg/kg FFM/min).

5.5.4 Effects of hyperenergetic high carbohydrate and high fat diets on FFA, lipids and liver enzymes

The accumulation of fat in the liver is recognised as a major contributor to metabolic disease (Marchesini *et al.* 2001). It has been postulated that when fatty acids exceed the liver's capacity to secrete or oxidise excess fat, these are stored as triglycerides. Net retention of triglycerides is the pre-requisite for development of NAFLD (Yki-Jarvinen, 2013). At

baseline, triglycerides, liver enzymes, cholesterol, HDL, LDL, Apolipoprotein A1 (HDL) and B (non-HDL) did not differ between the two groups. Subsequently following 2 weeks of 25% excess energy, triacylglyceride levels increased by 24% overall, with a greater effect seen in the high-carbohydrate fed group. Apolipoprotein A1 and B increased in the group after 2 weeks of overfeeding with significance in the high-fat group but without concomitant rise in total cholesterol, HDL or LDL. Contrary to Stanhope's study (Stanhope *et al.* 2011), fasting Apolipoproteins A1 and B were increased to a greater degree (p<0.01) following 2 weeks of overfeeding in the high fat-fed participants. High triglyceride levels are commonly seen in consumption of high-carbohydrate diets, from fatty acids generated from DNL that drive hepatic triglyceride overproduction (Nestel *et al.* 1998, Boberg *et al.* 1972). Similar effects are seen in other studies of high carbohydrate, low fat studies (Parks *et al.* 1999, Purkins 2004). Parks utilised a whole-food, high-fibre diet as opposed to others using liquid forms of high carbohydrate or diets high in mono or disaccharides. This study used carbohydrates in the form of maltodextrin (a form of soluble polysaccharide produced from starch through partial hydrolysis), which has been used successfully as a carbohydrate supplement in other human studies (Teunissen–Beekman *et al.* 2013, Detko 2013). Schwartz *et al.* 2013 showed that consumption of a high-fat, low-carbohydrate diet for 5 days did not affect plasma TAG concentrations, however after 5 days of low-fat, high-carbohydrate diet there was a significant 1.5-fold increase in plasma TAG concentrations. The major mechanism proposed for raised TAG as a result of high-carbohydrate feeding observed from Parks's study was reduced clearance of VLDL-TAG from plasma as opposed to increased secretion as determined from plasma palmitate kinetics. It would also appear that the type of carbohydrate (simple vs complex) influences fatty acid synthesis, production and clearance rate. Simple sugars are more effective than complex carbohydrate in stimulating DNL (Hudgins *et al.*1996). Barter and Nestel 1973 showed that the majority of VLDL-fatty acids were not

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derived from NEFA in obese individuals but that elevated BMI correlates with lower contributions of plasma NEFA to VLDL-lipid. Many studies have established previously that high-carbohydrate diets increase TAG concentrations (Nagle *et al.* 2009). In addition to confirming these findings, data from this study, as well as those of others (Schwarz *et al.* 2003, Nagle *et al.* 2009), support the hypothesis that hepatic DNL contributes to this process. When normoinsulinaemic and hyperinsulinaemic participants consume a low-fat, high-carbohydrate diet with more than half of the carbohydrate in the form of simple sugars, in particular fructose, hepatic DNL, plasma and liver TAG concentrations are increased. The underlying mechanisms for increased TAG following high carbohydrate intake include overproduction or reduced clearance of TAG from plasma (Parks *et al.* 1999). Previous studies have shown that carbohydrate rather than energy cause the significant increase in triglycerides. In concert with other studies (Purkins *et al.* 2004) there was no effect on cholesterol levels, and may be because the diets were not maintained for an adequate period of time.

Contrary to studies exhibiting significant effects of diets on liver enzymes, this study did not show an effect of the high fat nor high carbohydrate diets on the transaminases AST and ALT. Purkins *et al.* 2004 showed that 9 days of high energy (doubling of energy requirements) high carbohydrate diet caused marked increases in transaminases compared to isoenergetic high fat diet. A clear relationship to the number of days on the diet was also seen, demonstrating the importance of the carbohydrate component rather than high energy content as a major determinant on liver enzymes. Porkos and Van Itallie (1983) showed that it was the surplus of energy and high sucrose which contributed to the rise in transaminases. However the difference between these studies and the present study is the increased amounts of surplus energy provided in previous studies fed more than a week, and reflects the greater effect of increased energy and weight gain on liver enzymes.

No difference in the contribution of fatty acids to liver and VLDL-TG from differing sources such as systemic NEFA, DNL, or diet was found in the study by Donnelly *et al.* 2005. However incorporation of ^{13}C tracer from the test meal into liver showed (through MRS) that this occurred more rapidly and to a greater extent in diet-controlled T2DM compared with age and BMI-matched controls. This may be that the rapid fluxes of fat into and out of liver protects the body from excessive plasma TAG fluxes in the immediate post-prandial period. Another possible explanation is that the buffering capacity of adipose tissue in insulin resistance is impaired leading to overspill into liver and accumulation of TAG. Previous studies have either shown an increase (Stanhope et al 2009), no increase (isocaloric diet of high fructose) or normal levels of triglycerides in normal weight and obese individuals. In Bravo's study (2013), change in triglyceride levels correlated with a change in weight, BMI and fat mass, with no correlation to change in total energy intake or sugar intake or an effect on liver and muscle fat.

5.5.5 Effect of hyperenergetic HF and HC intake on inflammatory markers and adipokines

Low-grade inflammation is frequently observed in obese adults, which has been proposed as a potential cause of increased risk for T2DM and cardiovascular disease (Emanuela 2012). The present study showed that excess energy consumption increased TNF-α in the whole group. There was also a tendency for TNF-α to increase after carbohydrate overfeeding compared to the high fat-fed group. No effect of the diets on IL-6 or CRP was seen amongst groups. Several studies of carbohydrate and glucose loading have shown an effect on increasing inflammatory markers (Kasim-Karakas 2006). Intravenous glucose administration increased concentrations of inflammatory markers IL-6 and TNF-α (Esposito *et al.* 2002). Direct correlations between carbohydrate overfeeding and IL-6 and CRP were seen and attributed to increased oxidative stress, triacylglycerol production in the liver causing hepatic

steatosis (Solga *et al.* 2004; Kerner *et al.* 2005). The CRP and IL-6 production in this study did not change contrary to the studies above. The adipokine leptin, produced by adipose tissue is a mediator of energy balance and is elevated in the obese (Klok *et al.* 2007). Leptin concentrations in both high-fat and carbohydrate-fed groups in this study increased after 2 weeks of energy excess as expected. 2-8 weeks overfeeding increased adipocyte leptin expression and circulating leptin in healthy human subjects (Kolaczynski *et al.* 1996, Levine *et al.* 1999). A trend for increased levels was seen in the high-carbohydrate group. However this did not correspond to an increase in energy expenditure. High-fat meals are shown to lower 24-hour circulating leptin levels relative to high-carbohydrate meals (Havel 1999).

5.5.6 Effects of overfeeding on IMCL

IMCL is an early marker for the development of insulin resistance (Schrauwen-Hinderling, Hesselink *et al.* 2006). An increased IMCL content is the result of either increased circulating fatty acid delivery or reduced oxidation, possibly mediated through intermediary metabolites of triglycerides (Machann, Haring *et al.* 2004). This study, consistent with several other overfeeding studies has failed to show muscle fat accumulation. This appears to occur in studies of less than 10 weeks duration. Maersk *et al.* (2012) showed that liver fat and muscle fat accumulation increased over 6 months and it may well be that intramuscular fat accumulates long after liver fat accumulation. In concert with the absence of changes seen in intra-abdominal and subcutaneous fat mass seen in Westerbacka's study (2005) and this present study suggest that the liver may be the first organ to store excess fatty acids.

5.5.7 Effects of overfeeding on EMCL

The extra-myocellular lipid (EMCL) content was measured as part of the process of determining IMCL content. EMCL is ectopic lipid stored within adipocytes which are interspersed between muscle fibres and reflects total body adiposity (Machann, Haring *et al.*

2004). EMCL was unlikely to change as there were no changes in weight or adiposity in this study.

5.5.8 Study limitations

5.5.8.1 Participants and anthropometrics

Participants divided into the two groups were well-matched at baseline apart from a difference in skinfold biceps thickness; this did not appear to influence overall percentage total body fat calculated using the Siri formula. Moreover, despite the tendency towards a difference in percentage body fat between both groups there was no evidence to suggest that this impacted on study outcomes as no difference was seen in metabolic measures at baseline.

Although baseline measurements of anthropometric, metabolic, and insulin clamp were obtained from all 24 participants, data from the insulin clamp were only possible from 10 vs 12 participants in the high fat and high carbohydrate groups after the 2 week intervention period, respectively. This was due to a subject not completing the post 2 week intervention insulin clamp, and another where there was a technical issue with the insulin pump and thus data from the clamp study were likely unreliable. ^{1}H MRS of the liver and muscle were performed in a total of 22 participants, 12 in the HF group and 10 in the HC group. The 2 participants from the HC group did not proceed for scanning as they had contraindications to MRI. Participants were recruited through mailshots sent locally and advertisements in the local newspaper. Only White European males were recruited to exclude variations in liver fat content at baseline that may be associated with ethnicity and that may confound results. Browning *et al.* reported a significant difference in the incidence of hepatic steatosis depending on ethnic origin from the multi-ethnic Dallas population Heart Study (Browning *et al.* 2004). Furthermore there were differences in intra-abdominal fat accumulation, insulin resistance, and lipid metabolism (Guerrero *et al.* 2009). Another study showed that a high

energy high fat diet rapidly induced insulin resistance in healthy South Asians compared to White European men (Bakker *et al.* 2014).

5.5.8.2 Study design

Some issues of this study will need to be given cautious consideration prior to the interpretation of the data obtained. As with human nutritional studies, factors such as compliance, subject phenotype, total number of participants, environmental control and diets prescribed and when these are taken during the day may influence outcomes and data interpretation.

The variability of baseline liver fat may have also affected overall liver fat accumulation and hepatic glucose production post-intervention, thus a greater number of participants should be considered in future studies to reduce the scatter. Compliance will also need to be considered as a major contributor particularly in the outpatient/community setting. Although participants were requested to note down any failure to consume the food provided, it is difficult to entirely control total dietary intake and changes in activity in the absence of a controlled environment. Markers of overfeeding include weight gain, change in TAG levels and fasting carbohydrate oxidation rates. 25% excess energy in the form of either carbohydrate or fat after 2 weeks was expected to increase weight in participants. Contrary to expectations, this only occurred in half of the participants. 6 of 11 and 6 of 12 participants gained weight in the high-fat and high-carbohydrate groups respectively. However triacylglycerols increased after 2 weeks of overfeeding in the whole group (16/24), more so in the HC group (9/12) indicating a degree of overfeeding success. However no changes in fasting carbohydrate and fat oxidation rates were seen at 2 weeks compared to baseline.

The relatively small change in weight and individual variation in the deposition of fat during overfeeding may have contributed to the variation of weight after 2 weeks. Sims' overfeeding

study showed large variations in weight gain amongst subjects despite being fed similar amounts of energy (Sims *et al.* 1978). Nonetheless, overall little or no weight gain was seen in this study and may have been a result of several factors. Underreporting of dietary intake, changes to activity levels, concerns with weight gain and even errors with weight measurement may have influenced the apparent lack of weight gain in this study's subjects. The absence of changes seen in intra-abdominal and SC fat mass seen in Westerbacka's study (2005) and this present study suggest that the liver may be the first organ to store excess fatty acids. Although this postulation cannot yet be substantiated by other studies as similar experiments are relatively scarce, this finding parallels that seen in animal studies. Rats fed a high fat diet had increased hepatic triglycerides that induced hepatic insulin resistance but not muscle insulin resistance. No changes in weight or SC fat were seen (Samuel *et al.* 2004).

Carbohydrate and lipid oxidation rates, and FFAs remained unchanged after 2 weeks and these findings were also seen in studies of Westerbacka *et al.* 2005 and Bischop *et al.* 2001.

Although most of the participants in this study had baseline IHCL values at >95th percentile for the general population (>5.6%; Szczepaniak *et al.* 2005), data generated from this relatively small study with generally healthy but overweight males may not be truly representative of the disease pathology seen in the population at risk of NAFLD. The overfeeding period was relatively short considering a median follow up of 3 weeks for other high energy studies (see Chapter 1, Table 1.1) and thus may not have been long enough for an appreciable effect to be seen in liver or muscle. However based on current studies observed in literature, 2 weeks have, in the majority of cases, been found to produce effects on liver and muscle fat. Assessing fatty liver via liver biopsies was also not utilised, however this method is invasive and poses a greater risk of complications and will not appeal to many subjects. A liver biopsy is also primarily used in clinical practice for diagnostic purposes and is unable to measure liver fat content to the degree of detail as MRS can. Finally to eliminate

any possible effects of the isoenergetic diet on baseline liver fat and exclude subjects with low liver fat content, subjects should have undergone baseline MRI of their liver before the isoenergetic feeding period, however this was not feasible from a logistics point of view. Participants resting metabolic rate should also be ideally quantified using indirect calorimetry to estimate energy requirements when developing their diets. As with most nutrition and feeding studies total compliance with food intake and activity levels may not have been strictly controlled in free-living conditions. Ideally studies such as this should be undertaken under controlled laboratory environment; however this is not always feasible, requires intensive research staffing levels and deviates from studying individuals in their normal environment.

5.6 Conclusion

In conclusion, this study has shown that 25% excess energy intake over a 2-week period can increase liver fat content but there was no differential effect between the overfed carbohydrate and high fat groups. There was no effect on IMCL for both groups. The metabolic effects of moderate excess energy consumption per se are therefore greater than any differential effects of fat vs. carbohydrate overfeeding. Although liver fat increased from baseline in the high carbohydrate arm, 2 weeks of overfeeding did not affect hepatic or whole body insulin insensitivity. There was no significant liver fat accumulation following high-fat overfeeding. Future studies should investigate the effects of high fat vs. high carbohydrate on liver fat and skeletal muscle in people with pre-diabetes or impaired glucose tolerance (IGT), and examine further the underlying mechanisms of the association between macronutrients and insulin sensitivity.

Chapter 6: STUDY 4

Investigating the effect of carnitine on fatty acid oxidation and insulin sensitivity in older healthy males.

6.1 Introduction

As alluded to in chapter 3, IMCL oxidation during exercise is impaired in older compared to young individuals, albeit these are overweight subjects. Therefore one of the underlying factors affecting insulin sensitivity in the elderly may be reduced fat oxidation capacity.

Improving understanding in mechanisms involved in ageing, fatty acid oxidation, insulin resistance and strategies to improve insulin sensitivity in this age group is clearly warranted. Energy restriction and weight loss have been shown to reduce insulin and glucose concentrations (Wing, Blair *et al.* 1994) whereas increasing physical activity is clearly of benefit to reducing the risk of insulin resistance in ageing (Mayer-Davis, D'Agostino *et al.* 1998; Castaneda, Layne *et al.* 2002). In particular exercise training studies involving older individuals appear to improve basal fat oxidation and mitochondrial content and function suggesting an increase in IMCL utilisation (Solomon, Sistrun *et al.* 2008). However many of these training sessions involve exercising at moderate to high intensities (>60% VO_2 max) for many hours per week that may prove unrealistic for the majority of older individuals. Moreover, the benefits of energy restriction in older individuals will need to be weighed against quality of life concerns. For example, individuals subjected to long-term energy restriction encountered issues such as reduction in bone mineral density, muscle mass, and lethargy (Speakman and Hambly 2007). In later stages of life particularly in frail individuals with sarcopenia, energy restriction may even be counterproductive.

Taking all the above into consideration strategies to improve IMCL utilisation and thereby insulin sensitivity in older individuals whilst ensuring an attainable and realistic regimen are ideal.

6.1.2 Rationale behind the hypothesis that carnitine improves insulin sensitivity

Carnitine's primary role in regulating fatty acid metabolism raises prospects that the availability of this quaternary ammonium compound in skeletal muscle could be manipulated to improve insulin sensitivity (Stephens *et al.* 2007).

Carnitine (3-hydroxy-4-N-trimetyl-aminobutyric acid) has been found to play a central role in mitochondria specifically in fatty acid oxidation. Carnitine facilitates transport of long-chain fatty acids into mitochondria for subsequent beta-oxidation under the influence of the rate-limiting carnitine-palmitoyltransferase 1 (CPT-1) reaction (Fritz *et al.* 1959). It is also involved in buffering excess acyl-coA by shuttling these (as acylcarnitines) and other shortened chained products out of the mitochondria formed during conditions of increased pyruvate dehydrogenase complex (PDC) flux such as high intensity exercise. As intracellular accumulation of acyl-coA derivatives have been implicated in the development of insulin resistance it is thought that carnitine supplementation might overcome this. A reduction in muscle lactate content following carnitine infusion and hyperinsulinaemia was associated with decreased pyruvate dehydrogenase activity and increase glycogen synthesis, suggesting a carnitine- mediated increase in fat oxidation (Stephens *et al.* 2006, Mingrone et al 1999). Indeed it has been shown that carnitine deficiency is associated with insulin resistance, diabetes (Tamamogullari *et al.* 1999), ageing and obesity (Noland *et al.* 2009). Carnitine improved insulin-stimulated glucose disposal in mice with genetically and diet-induced diabetes (Power, Hulver *et al.* 2007). In human studies involving acute intravenous carnitine administration in combination with euglycaemic insulin clamp, whole body glucose disposal

improved in normal healthy participants (Mingrone 1999) and in individuals with T2DM (Capaldo *et al.* 1991, Mingrone 1999).

When combined with a beverage containing large quantities of carbohydrate, oral carnitine supplementation in young healthy humans increased muscle total carnitine content and resulted in a significant switch from carbohydrate to fat oxidation, reduction in muscle glycogen utilisation and PDC inactivation during low intensity exercise. The increased fatty acid oxidation was thought to prevent accumulation of fat mass, enhance energy expenditure and exercise performance and upregulate expression of genes related to fat metabolism and insulin signalling (Wall *et al.* 2011, Stephens *et al.* 2013). The rationale behind ingesting oral carnitine and a carbohydrate supplement lies with several studies that have successfully demonstrated increment of total carnitine in plasma and muscle in subjects when serum insulin levels were elevated. Studies involving carnitine supplementation have shown a significant increment and maintenance in total muscle carnitine with carbohydrate supplement compared to those without (Wall *et al.* 2011, Stephens *et al.* 2013). The underlying mechanism could be explained by the premise that carnitine uptake is insulin-mediated. Under conditions where insulin and carnitine levels are elevated, muscle OCTN2 (skeletal muscle carnitine transporter protein) expression and sarcolemmal Na^+/K^+ ATPase pump activity are increased. Na-coupled carnitine transport is thus augmented by its action of stimulating intracellular Na flux (Stephens *et al.* 2006).

Research outcomes thus far strongly suggest that carnitine supplementation can increase fat oxidation and it is further reasonable to speculate that carnitine can influence insulin sensitivity by promoting fat oxidation rates, prevent accumulation of intramuscular lipid and its intermediate metabolites from interfering with insulin signalling pathways.
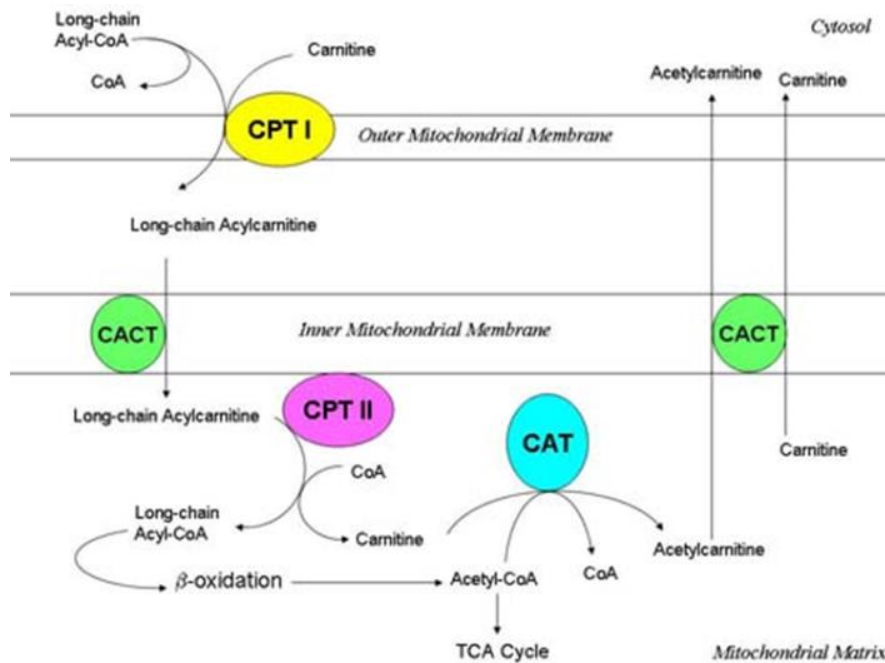
Figure 6.1: Carnitine's role in fatty acid metabolism. Carnitine transports long-chain acyl groups from fatty acids into the mitochondrial matrix by the rate-limiting Carnitine Palmitoyltransferase 1 (CPT1) located on the outer mitochondrial membrane. Acylcarnitine is shuttled inside by carnitine-acylcarnitine translocase (CACT), and then converted to acyl CoA by carnitine acyltransferase II (CPT II). Long chain acyl-CoA subsequently undergoes beta-oxidation before being fed into the TCA cycle.

6.2 Aims of Study

The aim of this study was to examine if muscle total carnitine content of older people could be increased over a 24-week period via ingestion of daily oral L- carnitine in combination with an insulinogenic beverage and twice weekly 1 hour light-intensity exercise. The study also aimed to determine the effects of increasing skeletal muscle carnitine content on body composition, substrate oxidation utilisation during rest and exercise and whole body glucose disposal in older healthy people.

It was hypothesised that skeletal muscle total carnitine (TC) is reduced in older compared to younger healthy men and that chronic L-carnitine in combination with light intensity exercise (as maximal fat oxidation rates are elicited at this intensity (Romijn *et al.* 1993) in older healthy male volunteers can increase skeletal muscle TC to a similar degree seen in studies involving carnitine supplementation to young volunteers. It was also postulated that increasing skeletal muscle carnitine content and improving fat metabolism during light-intensity exercise in older people would improve insulin sensitivity in older people.

Whilst previous studies of carnitine supplementation in young volunteers utilised beverages high in carbohydrate to augment the effects of carnitine, this study used a beverage lower in carbohydrate content but with additional protein to avoid the detrimental effects of high carbohydrate overfeeding in older participants whilst maintaining an insulin response.

6.3 Methods

6.3.1. Human participants and ethical approval:

Fourteen healthy males (69.1 ± 0.6 years, body mass 78.2 ± 2.7kg) were recruited to the study. All participants underwent medical screening and blood testing and gave informed consent as described in the common methods section (Chapter 2).

The incremental maximal oxygen consumption (VO_2 max) test was performed to determine the workload or intensity participants were cycling equivalent to 50% VO_2 max. Participants completed a health related quality of life (QoL) questionnaire (Short Form-36 SF36v2™), incremental shuttle walk test (ISWT) and experimental visits at the start and end of the study. Participants were then randomised in a double-blinded manner to two groups ingesting either 4.5 grams of L-carnitine-L tartrate (Carnipure, Lonza) (CARN) or 4.5 grams placebo (CON) in combination with an insulinogenic beverage (44.4 g carbohydrate, 13.4 g protein; 220mls

Ensure Plus Milkshake, Abbott, Illinois, USA) daily and completed a supervised twice weekly one hour bout of low-intensity exercise at 50% VO_2 max for a total of 24 weeks.

6.3.2 Protocol

6.3.2.1 Resting visit

Participants arrived at the MRC/ARUK Centre for Musculoskeletal Ageing Research laboratory at 0800 after an overnight fast. A dual energy x-ray absorptiometry (DEXA) scan (Lunar Prodigy, GE Healthcare, US) was performed to assess body composition. They rested semi-supine on a bed while cannulae were inserted retrograde into a superficial vein on the back of the hand for arterialized blood sampling and forearm veins for insulin, glucose and 2DG infusions. The hand was then placed in a hand-heated box throughout the experiment to allow for arterialised blood sampling (Gallen & Macdonald, 1990). A 3 hour hyperinsulinaemic euglycaemic clamp at 60 mU kg lean mass $^{-1}$ min^{-1} was carried out to assess insulin sensitivity. 2DG was infused at a rate of 6mg kg^{-1}hr^{-1} at the same time. Blood glucose concentrations during the clamp were determined using an autoanalyser (Yellow Springs Instrument YSI, US). Indirect calorimetry was performed before and during steady state of the hyperinsulinaemic euglycaemic clamp (90 mins from start of clamp) using GEM (Gas Exchange Measurement, GEMNutrition Ltd, Cheshire, UK). Muscle biopsy samples using the technique described by Bergstrom were obtained from the *vastus lateralis* of each participant at rest before and immediately after the clamp to determine total muscle carnitine content.

6.3.2.2 Exercise Visit

At least a week after the resting study visit, participants reported to the laboratory and rested semi-supine on a bed while cannulae were inserted retrograde into a superficial hand vein for arterialised blood sampling and into a forearm vein for infusion of [U-^{13}C] palmitate, at

a concentration of 0.19mg kg^{-1} hr^{-1} for 2 hours. Breath samples were collected via one-way valve bags and introduced into breath tubes for subsequent $^{13}CO_2$ enrichment analysis before the start of the infusion and every hour of the resting period. Plasma samples for palmitate tracer and FFAs were collected hourly during resting. At the end of the second hour, percutaneous biopsy from the *vastus lateralis* muscle was performed before participants went on to cycle on the ergometer at 50% VO_2 max equivalent workload. The [U-^{13}C] palmitate infusion concentration was increased to 0.28 mg kg^{-1} hr^{-1} at the onset of exercise. Blood plasma for FFAs, palmitate tracer and breath samples for $^{13}CO_2$ were obtained every 10 mins. During the last 10 mins of exercise, indirect calorimetry was determined Quark CPET system, Cosmed, Italy). A muscle biopsy of the *vastus lateralis* was taken at the end of the 1 hour exercise.

Preparation of [U-^{13}C] palmitate (99% enriched; Cambridge Isotope Laboratories, Andover, MA, USA) involved dissolving the palmitate in heated sterile water and passed through a 0.2 μm filter into 4.5 % warmed (55°C) human serum albumin to obtain a palmitate to albumin ratio of approximately 3:1 (1.94:0.64 μmol l^{-1}) . The solution was allowed to cool to room temperature. Prior to infusion, sodium [^{13}C] bicarbonate at 0.06375mg kg^{-1} was injected as a bolus to prime the bicarbonate pool (Sidossis *et al.*1996).
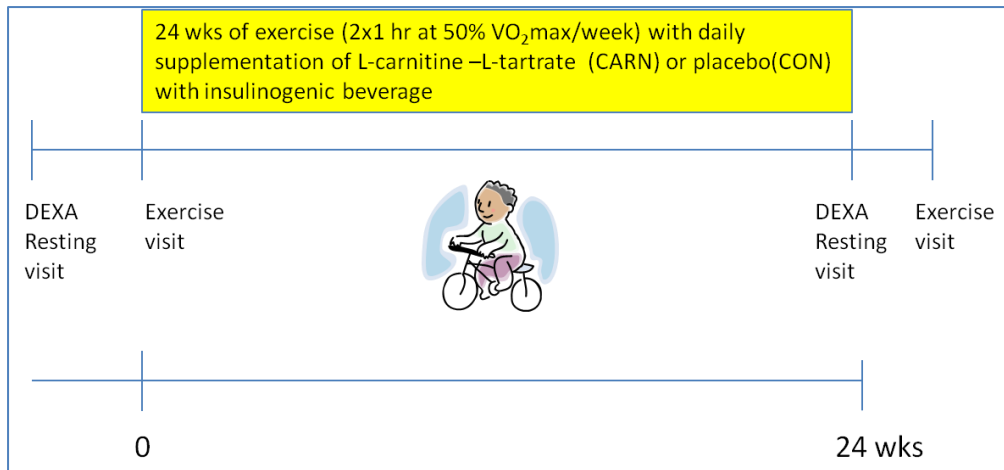
Figure 6.2a: Schematic overview of protocol
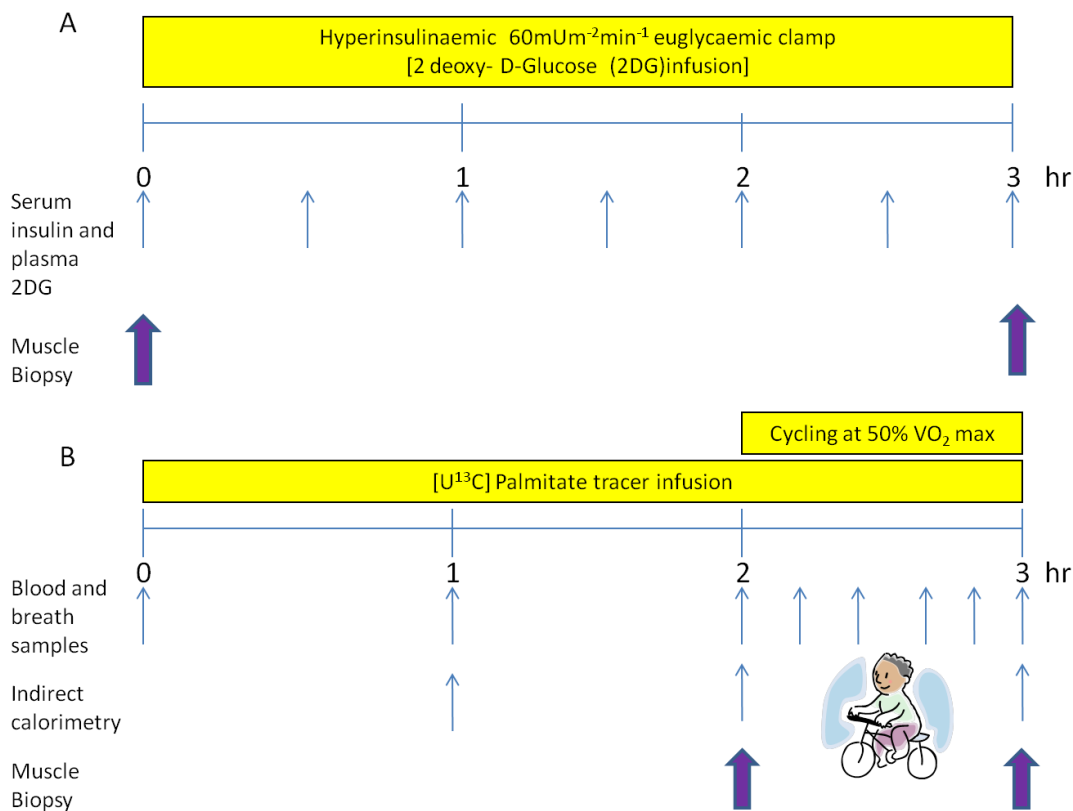
Figure 6.2b: A –Resting visit involved 3 hr hyperinsulinaemic clamp and 2DG infusion, B-Exercise visit involved a 3 hr palmitate tracer infusion and 1 hr cycling at 50% VO_2 max.

6.3.3 Sample collection and analysis

All DEXA scans were analysed by a single operator to avoid inter-operator variability. The scans were analysed for trunk, leg and arm composition using standardized regions conforming to specifications. Blood samples were collected in tubes containing EDTA and centrifuged at 1000g at 4°C for 10 mins. Plasma aliquots were subsequently stored at -80°C until analysis. Blood serum and plasma were collected at baseline and every 30 minutes during the clamp, allowed to clot and then centrifuged (1400 RCF at 4°C for 10 mins) for subsequent storage at -80°C until use for determination of insulin concentrations and 2DG tracer respectively.

Biopsied muscle samples were immediately snap frozen in liquid nitrogen after the biopsy, freeze-dried and stored at -80°C. Muscle was dissected free of visible blood and connective tissue, powdered and used to determine muscle free carnitine, acetylcarnitine and long-chain acylcarnitine using the radioenzymatic method previously described by Cederblad and Lindstedt (Cederblad and Lindstedt 1972).

6.3.4 Calculations

Total fat and carbohydrate oxidation rates were calculated using the non-protein respiratory quotient (Frayn 1983).

Fat oxidation rate = 1.695 VO_2 x 1.701 VCO_2 g/min

Carbohydrate oxidation rate = 4.585 x VCO_2-3.226 x VO_2 g/min

EE = 15.9 x VO_2 + 5.2 x VCO_2 J/min

Where VO_2 and VCO_2 are expressed as litres per min and oxidation rates as grams per minute.

Breath and plasma enrichments are expressed as the tracer/tracee ratio (TTR);

$$\textbf{TTR} = (^{13}\textbf{C}/^{12}\textbf{C})\textbf{sa} - (^{13}\textbf{C}/^{12}\textbf{C})\textbf{bk}$$

where sa denotes the sample and bk the background value.

The rate of appearance (Ra) and rate of disappearance (Rd) of palmitate was calculated using the single-pool non steady- state Steele equations adapted for stable isotope methodology as described by Wolfe & Jahoor 2006.

$$\mathbf{Ra = \frac{F - V[(C_2 + C_1)/2][(E_2 - E_1)/(t_2 - t_1)]}{(E_2 - E_1)/2}}$$

$$\mathbf{Rd = Ra - \frac{V (C_2 - C_1)}{t_2 - t_1}}$$

where F denotes the infusion rate (μmol kg^{-1} min^{-1}), V is the distribution volume for palmitate (40 ml kg^{-1}), C1 and C2 are the palmitate concentration (mmol l^{-1}) at times 1 (t1) and 2 (t2), respectively, and E1 and E2 are the plasma palmitate enrichments (TTR) at times t1 and t2, respectively. $^{13}CO_2$ production (Pr$^{13}CO_2$; mol min^{-1}) from the infused palmitate tracer was calculated as:

Pr^{13}CO2 = (TTRCO2$^{\text{x}}$ VCO2)

where TTRCO2 is the breath $^{13}C/^{12}C$ ratio at a given time point, k is the volume of 1 mol of CO2 (22.4 l mol^{-1}).

Plasma palmitate oxidation (Rox; mol min^{-1}) can subsequently be calculated as:

Rox palmitate = Rd palmitate x (Pr^{13}CO2/F x 16)

where Rd palmitate is the rate of disappearance of palmitate (mol min^{-1}), F is the palmitate infusion rate (mol min^{-1}) and 16 is the number of carbon atoms in palmitate. Total plasma FFA oxidation was calculated by dividing palmitate oxidation rate by the fractional contribution of plasma palmitate to total plasma FFA concentration.

The contribution of plasma FFA oxidation to total fat oxidation was determined by assuming

that the molecular mass of triglyceride is 860 gmol^{-1} and every TG molecules contain three fatty acids.

The contribution of other fat sources was hence calculated by subtracting plasma FFA oxidation from total fat oxidation.

Insulin sensitivity index clamp was calculated using the equation $SI_{Clamp} = M/(G \times \Delta I)$

where M is normalized for G (steady-state blood glucose concentration;mmol^{-1}) and ΔI (difference between fasting and steady-state plasma insulin concentrations,mUl$_{-1}$).

6.3.5 Power calculations

From previous studies on increasing muscle total carnitine content (Wall *et al.* 2011, Stephens *et al.* 2013) a 20% difference in muscle total carnitine was expected to be seen in older and young participants and following 24 weeks of L-carnitine feeding a difference should be able to be detected in 7 older participants with a power of 80% at 5% significance level on a paired t-test basis. The repeated measures coefficient of variation for the insulin clamp technique is 10%, therefore a 15-20% difference in insulin sensitivity should be able to be detected in 7 participants. Therefore we aimed to recruit 10 volunteers for each group (Carnitine and Control) to allow for a 30% dropout.

6.3.6 Statistical analysis

Values presented in text, tables and figures are expressed as mean ± the standard error of mean (S.E.M). The level of significance for statistical tests was set at $P < 0.05$ and analyses were performed using the statistical package Graphpad Prism version 6.0. Differences within and between groups were analysed using two-way ANOVA for repeated measures (time and treatment effects). When a significant time or treatment effect was observed post-hoc analysis using Sidak's multiple comparison test was performed to identify individual differences.

6.4 Results

Table 6.1: Participant characteristics at 0 and 24 weeks in the control (CON) (ingesting 4.5g placebo and insulinogenic beverage 44.4g CHO and 13.8g protein) and carnitine (CARN) groups (ingesting 4.5g L-carnitine L-tartrate and insulinogenic beverage 44.4g CHO and 13.8g protein) in conjunction with twice weekly 1 hour exercise performed at VO_2max 50% workload.

Parameters	Range	CON (n=7)		CARN (n=7)	
		0 weeks	12 weeks	0 weeks	12 weeks
Age (years)	65-73	68.4±1.0		69.9±0.6	
Body mass (kg)	60.2-92.5	77.6±3.7	79.6±3.0	78.9±4.0	78.1±3.6
BMI (kgm^{-2})	21.2-32.6	25.7±1.2	26.5±1.0	27.2±1.2	27.1±1.2
Lean body mass (kg)	42.7-59.1	49.4±1.0	49.3±0.8	51.3±2.3	50.6±2.1
Fat mass (kg)	6.1-33	20.1±3.6	22.0±2.8	19.4±2.2	19.3±2.0
Fasting Glucose ($mmol^{-1}$)	4.2-5.6	4.7±0.1	4.8±0.2	5.0±0.1	4.7±0.2
Fasting Insulin ($mIUL^{-1}$)	2.2-18.1	10.6±1.6	12.0±3.0	9.5±1.9	7.9±1.6
VO_2 max ($ml\ kg^{-1}min^{-1}$)	34.9-49.6	41.2±1.9		41.9±1.9	
Workload at 50% VO_2 max (W)	22-75	53.7±5.7	53.7±5.7	48.4±7.1	48.4±7.1
ISWT (m)	420-690	558.6±36.1	555.7±33.9	562.9±27.9	588.6±17.9
SF36v2™score (%)		89.0	89.6	86.0	87.4

6.4.1 Muscle total carnitine content
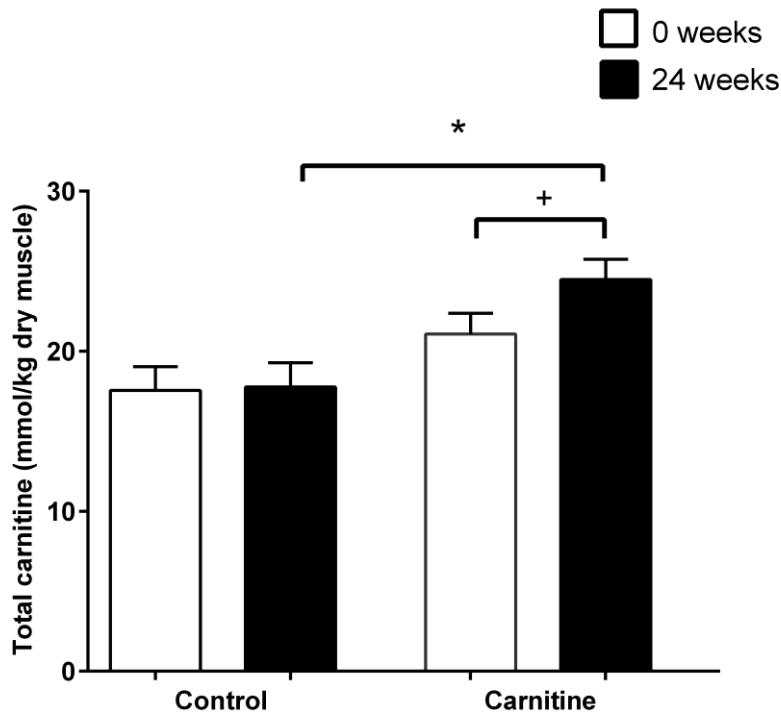
Figure 6.3: Skeletal muscle total carnitine content in control and carnitine groups at baseline and at 24 weeks. All values are expressed as means $\pm$ s.e.m. * P<0.05, $^+$ P<0.05

Skeletal muscle TC content at baseline were 17.5$\pm$ 1.5 and 21.1$\pm$ 1.3 mmol kg^{-1} dm in the control and carnitine groups respectively. These baseline values and skeletal muscle TC content at 24 weeks are presented in Figure 6.3. There were no differences between or within groups at baseline. After 24 weeks muscle TC was 38% greater in the carnitine group compared to control (24.5 $\pm$ 1.3 vs 17.5$\pm$1.5 mmolkg^{-1}dm; *P<0.01).There was a 16% increase in muscle TC from baseline (21.1 $\pm$ 1.3 to 24.5 $\pm$ 1.3 mmolkg^{-1}dm; $^+$P<0.05) in the carnitine group, whilst skeletal muscle TC remained unchanged in the control group (17.5$\pm$1.5 to 17.8$\pm$1.5 mmolkg^{-1} dm).

6.4.2 Subsarcolemmal and intermyofibrillar lipid
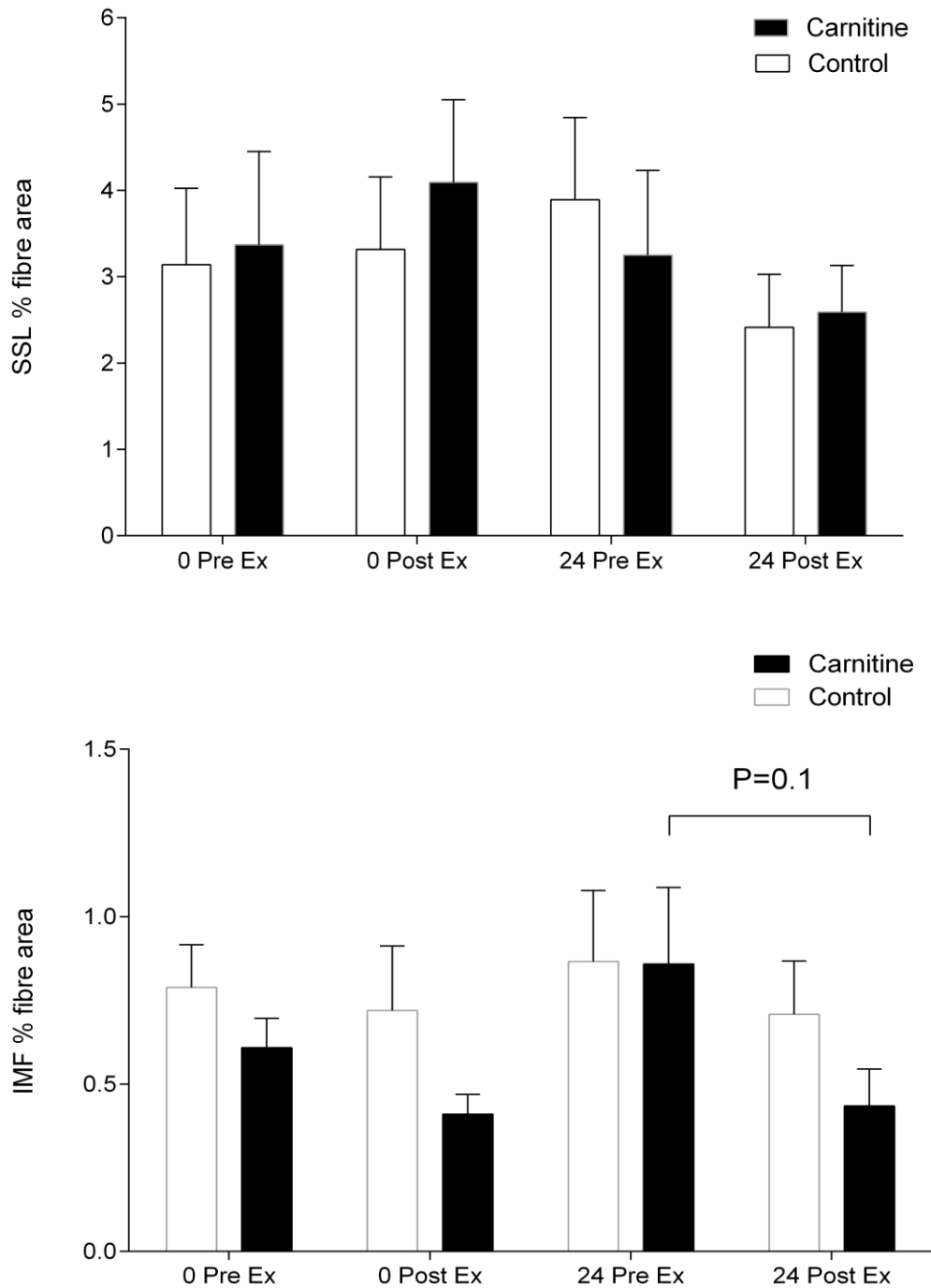
Figure 6.4. Subsarcolemmal (SSL) and intramyofibrillar (IMF) lipid droplet (% fibre area) pre and post exercise at 0 weeks and after 24 weeks of placebo (Control) or carnitine supplement (Carnitine)

SSL lipid content was not different pre and post-exercise in both the control and carnitine groups at baseline; 3.1 ± 0.9 vs.3.3 ± 0.8 % fibre area pre and post-exercise respectively(Control), and 3.4 ± 1.1 vs. 4.1 ± 1.0% fibre area pre and post-exercise respectively (Carnitine). At 24 weeks, SSL lipid content did not decrease post exercise in both the control and carnitine groups; 3.9 ± 1.0 to 2.4 ± 0.6 % fibre area in control compared to carnitine; 3.3 ± 1.0 to 2.6 ± 0.5% fibre area. IMF lipid content was comparable pre and post exercise in the control and carnitine groups at 0 weeks; 0.79 ± 0.11 vs.0.72 ± 0.18 % fibre area pre and post-exercise respectively (Control), and 0.61 ± 0.09 vs.0.41 ± 0.06% fibre area pre and post-exercise respectively (Carnitine). After 24 weeks, there was a tendency for IMF lipid content to reduce post-exercise (0.86 ± 0.23 to $0.44\pm0,11$%; $P=0.1$) in the carnitine group whilst no changes were seen in the control group (0.87 ± 0.20 to 0.71 ± 0.15% fibre area) pre and post-exercise respectively.

6.4.3 Exercise substrate utilisation
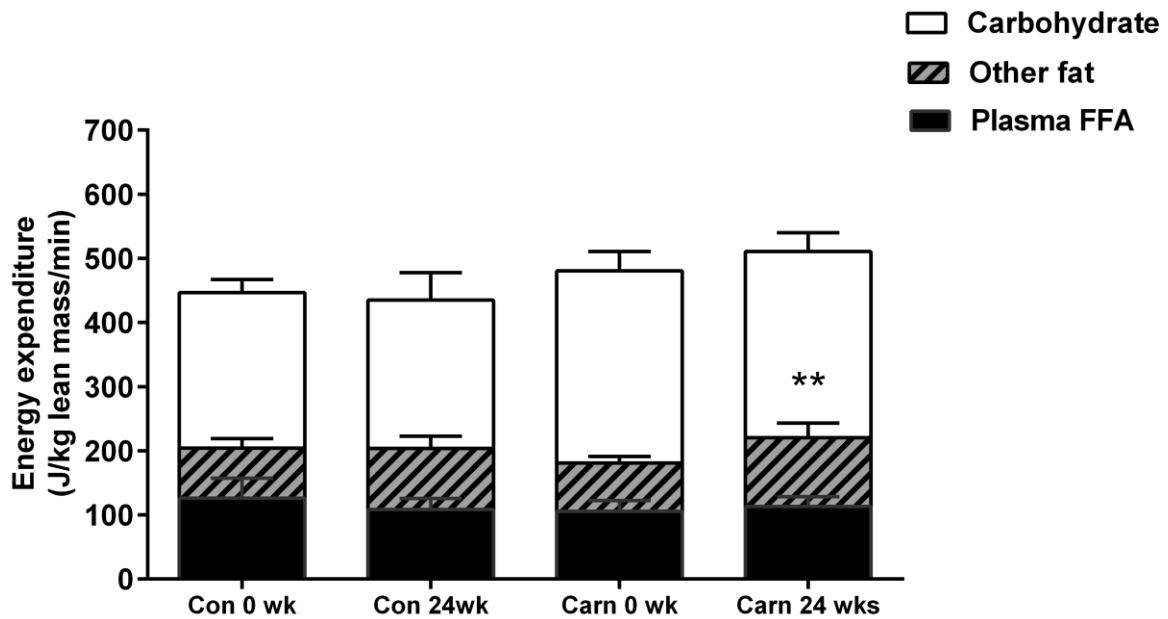
Figure 6.6: Contribution of fat (plasma FFA, black bar; other fat, striped bar) and carbohydrate (white bar) to energy expenditure during exercise at 50% VO_2max in control (Con) and Carnitine (Carn) groups before and after 24 weeks. All values are described as means ± s.e.m. **$P<0.01$, fat oxidation greater than 0 weeks.

There was no difference in the relative contribution of fat to total energy expenditure during exercise (Fig 6.6) at baseline in both groups, and at baseline and 24 weeks in the control group.

However, exercise at 50% VO_2 max and carnitine supplementation after 24 weeks were associated with a 21% ($P<0.01$; Fig 6.6) increase in total fat oxidation during exercise (181.1 ± 15.0 to 220.4 ± 19.6 J/kg lean mass/min), despite no change in plasma fatty acid Rd or oxidation (7.1 ± 1.3 vs. 7.3 ± 1.0 μmol/kg/min at 0 and 24 weeks respectively). Exercise at 50% VO_2 max alone did not alter carbohydrate or fat oxidation rates of older people in the control group.

6.4.4 Exercise metabolism
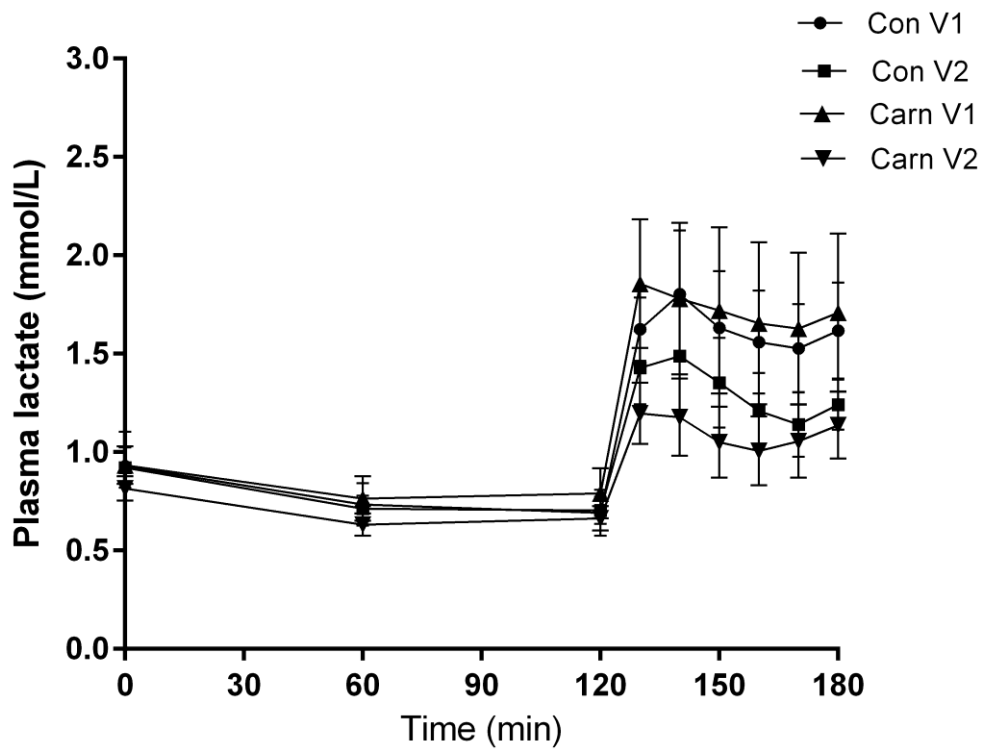
Figure 6.7: Plasma lactate during rest (first 120 minutes) and exercise at 50% VO_2 max (last 60 minutes) of participants in the carnitine group (n=7) at baseline (Carn V1) and after 24 weeks (Carn V2) of once daily 4.5 grams L-carnitine-L tartrate and insulinogenic beverage and twice weekly 1 hour cycling at 50% VO_2 max.
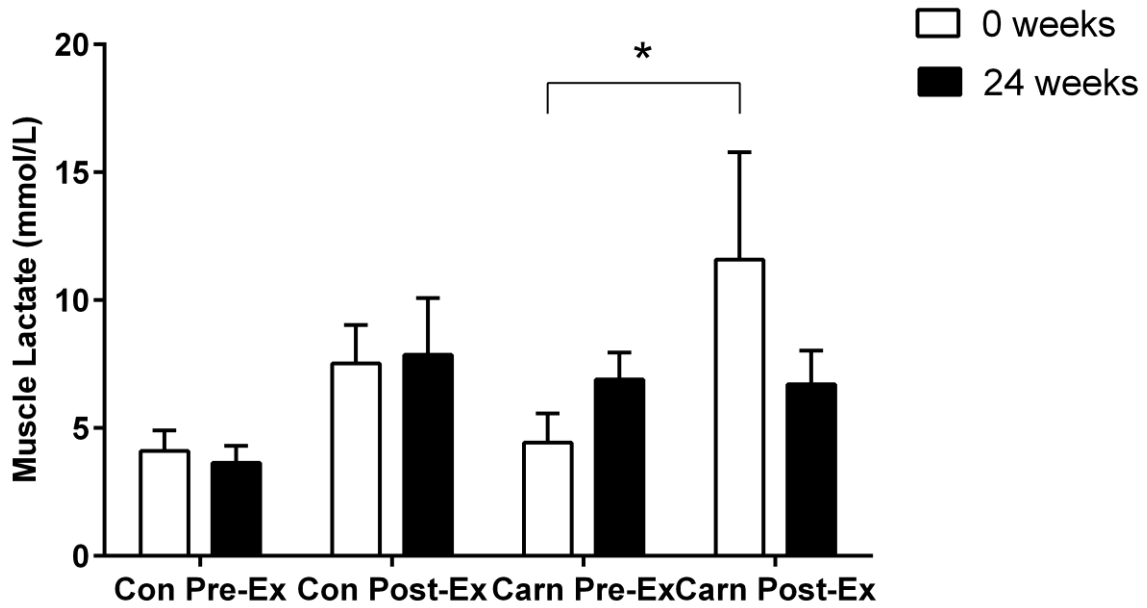
Figure 6.8: Muscle lactate concentrations pre and post-exercise in control (Con; n=7) and carnitine (Carn; n=7) groups at 0 and 24 weeks.*P<0.01 from pre-exercise at 0 weeks.
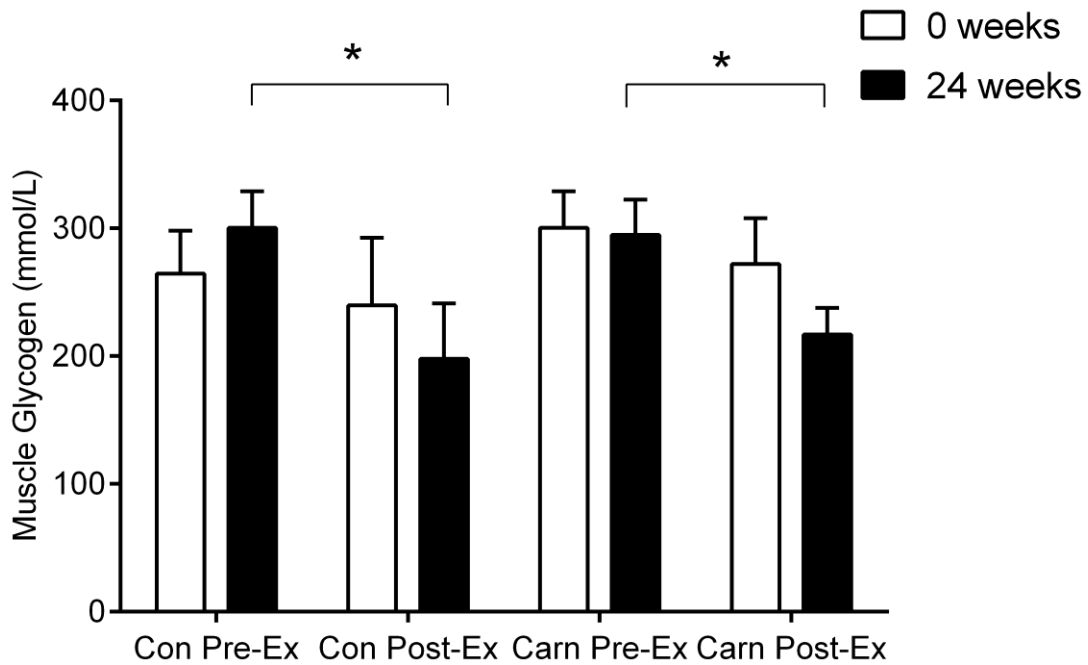
Figure 6.9: Muscle glycogen concentrations pre and post exercise in control (Con; n=7) and carnitine (Carn; n=7) groups at 0 and 24 weeks. *P< 0.05 from pre-exercise at 0 weeks.
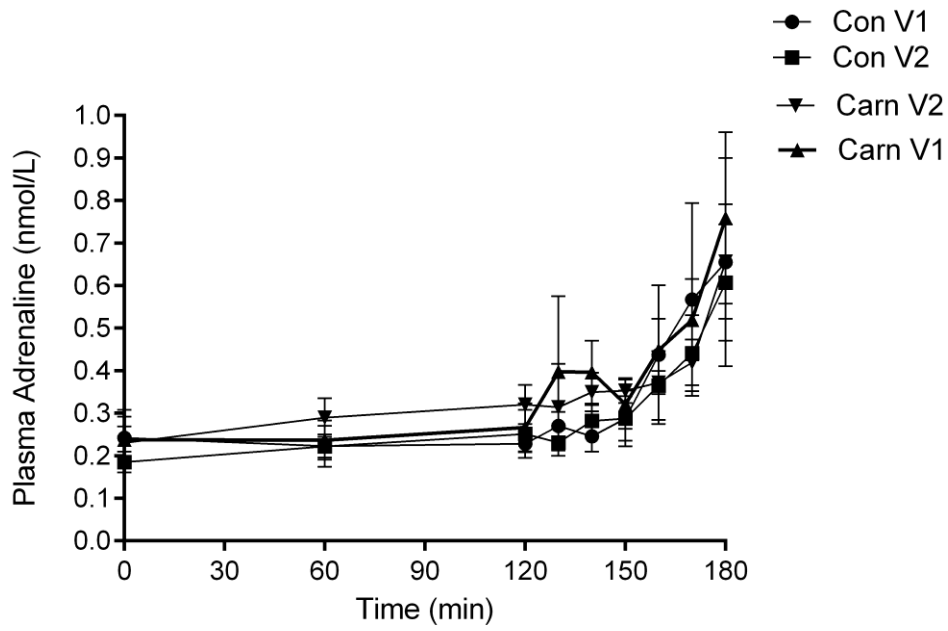
Figure 6.10A
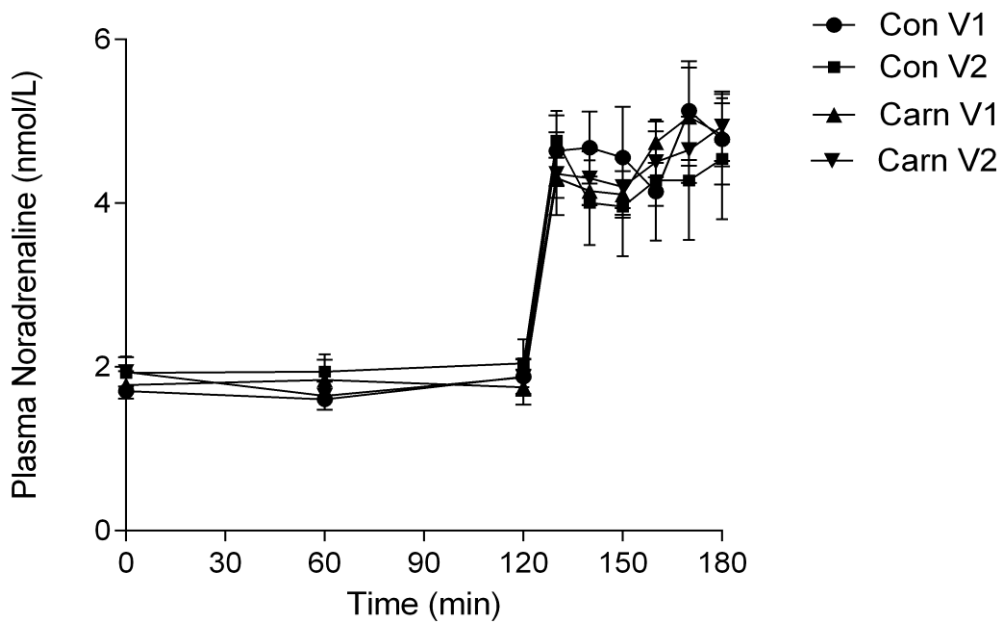
Figure 6.10B

Figures 6.10A and B: Sympathetic activity (noradrenaline and adrenaline concentrations) at rest for first 120 mins and exercise at 50% VO_2 max for the final 60 minutes at 0 and after 24 weeks in carnitine and control groups.

At steady state (last 10 minutes of an hour of cycling at 50% VO_2 max), plasma lactate concentrations in the control group were lower compared to baseline after 24 weeks (delta mean of 0.38 mmol/l; 1.57±0.23 compared with 1.19±0.15mmol/L). This was lower in the carnitine group (delta mean 0.59 mmol/L; 1.69±0.39 compared with 1.10±0.18mmol/L).

Skeletal muscle lactate concentration tended to increase post-exercise in the carnitine but not in the control group at 0 weeks (4.4 ± 1.1 to 11.6 ± 4.2mmol/L; P=0.07 and 4.1 ± 0.8 to 7.5 ± 1.5 mmol/L) respectively. The effect appeared to be obliterated in the carnitine group post-exercise after 24 weeks (6.9±1.1 to 6.7±1.3 mmol/L) as opposed to a tendency to an increase, 3.6 ± 0.8 to 7.8 ± 2.2 mmol/L; P=0.09 in the control group.

After 24 weeks, the degree of muscle glycogen depletion was significant in both carnitine and control groups (295±27.5 to 216.9 ± 20.8 mmol/kg dm and 311±50.3 to 197.8 ± 43.4 mmol/kg dm) respectively.

Sympathoadrenal activity represented by noradrenaline and adrenaline concentrations remained similar during exercise before and after 24 weeks in the carnitine and control groups; Figure 5.10A and B.

6.11 Insulin sensitivity
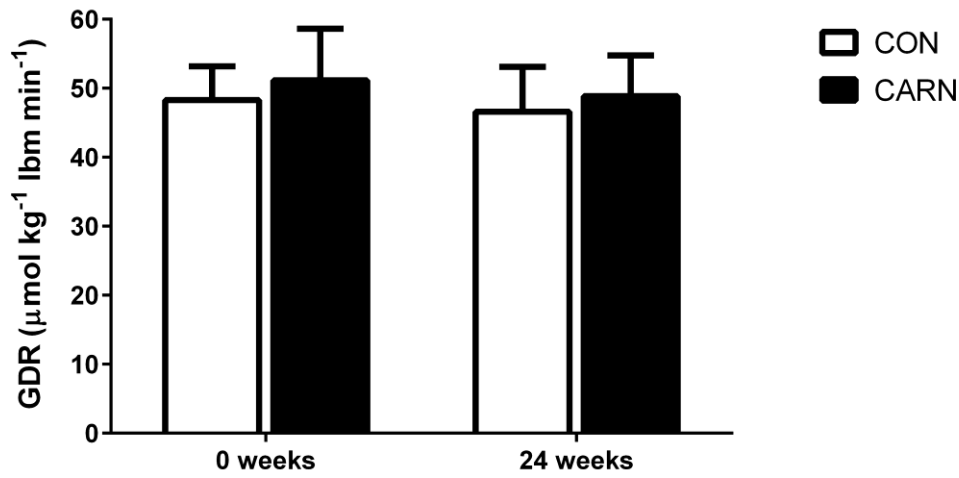
Figure 6.11: Glucose disposal rates (GDR) of participants ingesting either 4.5 g L-carnitine L-tartrate (CARN) or 4.5 g placebo (CON) with insulinogenic beverage (44.4g CHO, 13.6g protein)and twice-weekly 1 hr cycling at 50% VO_2 max at 0 and 24 weeks.
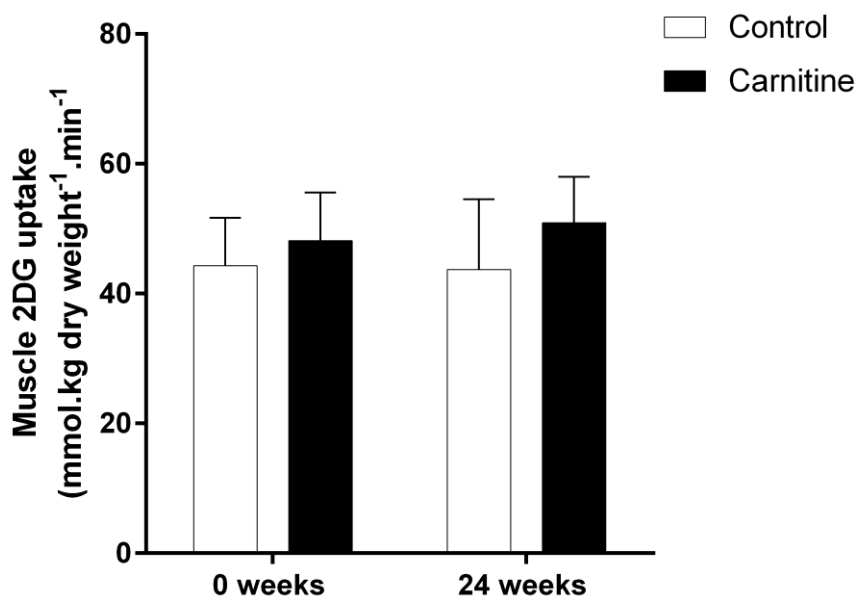
Figure 6.12: Muscle 2DG uptake in control and carnitine groups at baseline and 24 weeks

There was no difference in glucose disposal rates of participants randomised to the Carnitine and Control groups at baseline (51.2 ± 7.5 vs 48.3 ± 4.9 µmol kg^{-1} lean mass min^{-1}). After 24 weeks of treatment, no difference in mean glucose disposal rates were seen in both groups 48.9 ± 5.9 and 46.6 ± 6.5 µmol kg^{-1} lean mass min^{-1} respectively; Figure 6.11. Similarly there was no difference in 2DG uptake in muscle between Carnitine and Control at baseline and at 24 weeks; Figure 6.12. Muscle 2DG uptake was 44.3 and 43.7 mmol.kgdw^{-1}.min^{-1} in the control group and 48.2 and 51.0 mmol.kgdw-1.min-1 in the carnitine group at 0 and 24 weeks respectively.

6.6 Substrate oxidation at rest under fasted and insulin-stimulated conditions

Table 6.13: Carbohydrate and fat oxidation rates, respiratory exchange ratio (RER) and energy expenditure in control (n=5) and carnitine groups (n=6) during the resting hyperinsulinaemic 60mu m^{-2}min^{-1} euglycaemic clamp. *P<0.05 compared to pre-insulin values $^{\phi}$ p=0.06 compared to pre- insulin values.

	Control (n=5)				Carnitine (n=6)			
	0 weeks		24 weeks		0 weeks		24 weeks	
	Pre-Insulin	Post-Insulin	Pre-Insulin	Post-Insulin	Pre-Insulin	Post-Insulin	Pre-Insulin	Post-Insulin
Carbohydrate Oxidation (J/kg/lbm/min)	46.5±8.1	44.8±11.7	63.8±8.4	84.8±4.5	57.0±6.1	70.5±6.6	51.8±2.6	67.8±3.1*
Fat Oxidation (J/kg lbm/min)	42.0 ±7.3	49.7±9.4	34.0±9.9	17.4±3.4	32.7 ±7.3	23.0±4.1	44.1±3.8	30.4±2.9$^{\phi}$
RER	0.85±0.03	0.83±0.03	0.91±0.03	0.94±0.01	0.89±0.02	0.92±0.01	0.86±0.01	0.90±0.01*
Energy Expenditure (J/kg lbm/min)	88.5±4.4	94.5±8.2	97.8±8.2	100.2±5.6	89.8±3.2	93.5±3.1	95.9±2.0	98.2±1.5

At rest, carbohydrate oxidation rates increased by 31% upon insulin stimulation at 24 weeks in the carnitine group whilst there was a trend for a reduction in fat oxidation rates (44.1±3.8 to 30.4 ± 2.9 J kg lean body mass $^{-1}$ min^{-1}, P=0.06). Accordingly, an increase in respiratory exchange ratio (RER) after 24 weeks was seen during the insulin clamp in the carnitine group (0.86±0.01 to 0.90±0.01, P<0.05). There were no associated changes in basal fasted oxidation rates. No changes in fasted or fed states were seen in the control group (Figure 6.13).

6.4.7 Body composition
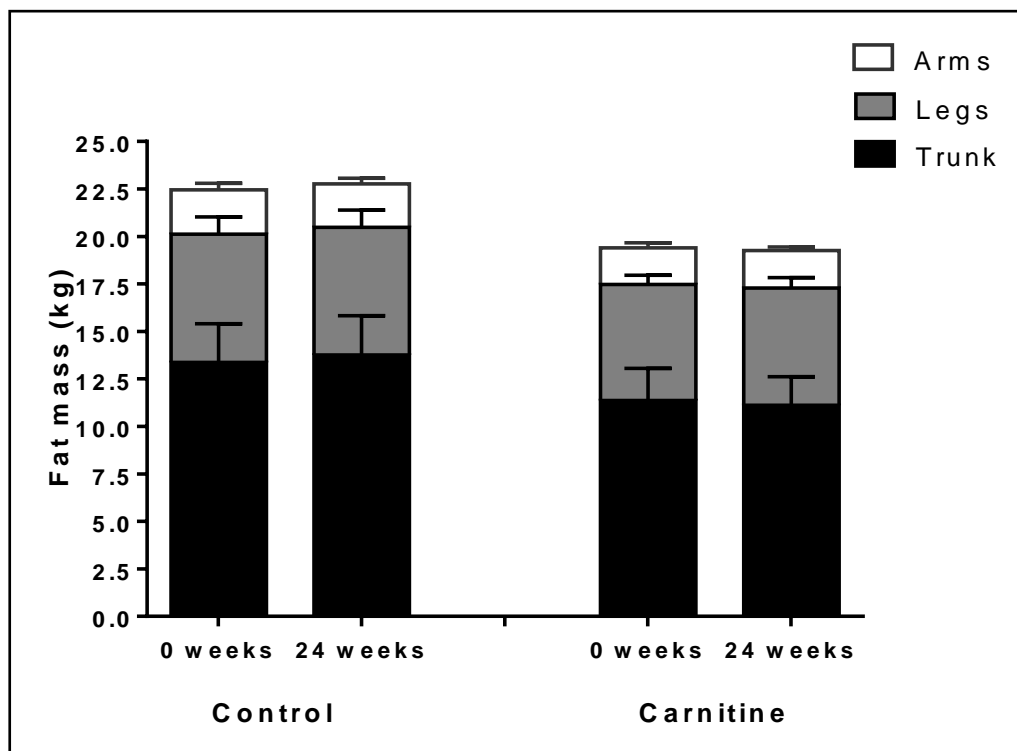
Figure 6.14: Fat mass (kg).Trunk (black bars), leg (grey bars) and arm (opened bars) fat mass before (0) and 24 weeks after once daily oral ingestion of either 4.5 grams placebo (Control)and insulinogenic beverage (n=7) or 4.5 grams l-carnitine-l-tartrate (Carnitine) and insulinogenic beverage (n=7). All values are means ±SEM.

After 24 weeks of twice weekly 1 hour cycling at 50% VO_2 max, no difference was seen in body mass, BMI, lean body mass or fat mass and fasting plasma glucose in both Carnitine and Control groups.

6.4.8 Incremental shuttle walk test

The ISWT did not differ in both groups at baseline and after 24 weeks.

6.4.9 SF36v2

Table 6.2: SF 36v2™ Health Questionnaire Scores (%) completed by participants in carnitine (n=6) and control (n=6) at week 0 and week 24. NS non-significant

Health domains	Control		Carnitine		
	Week 0	Week 24	Week 0	Week 24	p value
Physical function	95.8	94.3	90.8	95.0	NS
Physical role functioning	95.8	96.4	79.2	90.6	P<0.001
Emotional role functioning	98.6	98.8	95.8	91.7	NS
Vitality	75.0	76.8	75.0	77.1	NS
Mental Health	80.8	83.6	88.3	88.3	NS
Social functioning	97.9	98.2	97.9	97.9	NS
Bodily Pain	89.2	90.7	85.4	82.5	NS
General Health	79.2	77.9	75.8	75.8	NS

SF36v2 scores only improved in the functional physical role domain in the carnitine group after 24 weeks.

6.5 Discussion

As demonstrated in Chapter 3, older people exhibited lower fatty acid oxidation (most likely secondary to impaired IMCL utilisation) during light-intensity exercise and reduced glucose disposal rates compared to young. This study proposed that ingestion of carnitine with an insulinogenic beverage may improve fatty acid oxidation rates in older people and that this may translate to improving insulin sensitivity and body composition in this age group. A major finding from this study was that a 16% increase in muscle total carnitine improved total fatty acid oxidation, in particular IMCL oxidation. The improvement in IMCL oxidation however did not affect insulin sensitivity or body composition in older individuals. The ability of carnitine to improve fatty acid oxidation in older people during low-intensity exercise is advantageous as it demonstrates that fatty acid oxidation in older people can improve without undertaking high-intensity exercise or training many times per week that may prove unrealistic and unattainable in the majority of the older population. Previously 12 weeks of aerobic training at 75% VO_2 max 5 days per week was shown to improve IMCL and insulin sensitivity in older, obese and sedentary individuals (Pruchnic *et al.* 2004).

This study is the first to show that total muscle carnitine content can be successfully increased in older healthy men after 24 weeks of oral carnitine ingestion in combination with an insulinogenic supplement. In previous studies, an increment in total muscle carnitine was only observed following intravenous infusion of carnitine and oral carbohydrate supplementation (Stephens *et al.* 2006) and more recently via oral ingestion of L-carnitine in young healthy recreational athletes (Wall *et al.* 2011,Stepehens *et al.* 2013). It would appear that the daily insulinogenic beverage in this study was effective in elevating insulin to levels that facilitated uptake of carnitine into muscle, in concert with previous studies of carnitine supplementation (Stephens *et al.* 2006, Wall *et al.* 2011, Stephens *et al.* 2013). A total of 80 g

of carbohydrate supplement twice daily was required to increase retention and facilitate uptake of carnitine in muscle (Wall *et al.* 2011) but this may prove deleterious and cause a positive energy balance and fat mass accumulation in older individuals, as observed with the control group in the study by Stephens *et al.* 2013. Therefore, a compromise of 44.4 g of carbohydrate and addition of 13.8 g of protein, which can stimulate a modest rise in insulin secretion without increasing blood glucose levels (Krekozski *et al.* 1986), was considered and proved successful in retaining total carnitine content in muscle.

Although there is considerable research examining carnitine status in populations these tended to be measurements of blood and urine, whilst those of muscle were mostly carried out in younger individuals (Carlin *et al.* 1986, Harris *et al.* 1987, Janssen *et al.* 1989, Arenas *et al.*1991). Of the few examining muscle carnitine levels, regression data by Costell *et al.* (1989) indicated an inverse relationship between age and carnitine levels in healthy humans and mice. Whereas skeletal muscle free carnitine levels increased from adolescence to adulthood in rats, levels then tended to decline with advancing age. The underlying cause of why carnitine levels are reduced in ageing is not entirely known but low carnitine levels have been associated with insulin resistance (Noland *et al.* 2009), which in turn is associated with increasing age. Reduced free carnitine levels in muscle were found to be a feature in rat models of insulin resistance including ageing (Bernard *et al.* 2008). Previous studies reported baseline mean levels of 20-25 mmol kg dm^{-1} in young healthy individuals (Wall *et al.* 2011, Stephens *et al.* 2013) but there are limited studies examining total carnitine content in muscle of older human volunteers. In concert with this study, Starling *et al.* (1995) showed that resting free and total muscle carnitine were similar in older vs. younger people (20.3 ± 0.9 vs 21.6 ± 0.7 and 26.1 ± 0.9 vs 26.4 ± 0.6 mmol kgdm^{-1}) respectively. However the older volunteers in Starling's study had an age range lower than the present study (47.6 ± 8.8 vs.69.2 ± 0.6 years respectively).

Having demonstrated that skeletal muscle total carnitine content can be successfully increased after 24 weeks supplementation in healthy older people, another major finding from this study was that carnitine increased fat oxidation (most likely IMCL) during low-intensity exercise. The ability of carnitine loading to promote fatty acid oxidative capacity in humans has been shown before in young healthy individuals (Stephens *et al.* 2013, Wall *et al.* 2011). This may be the result of adaptations leading to diversion of carbohydrate oxidation towards storage and favouring fat oxidation, and is likely to occur because carnitine is rate-limiting to CPT-1.

Carnitine appeared to improve muscle metabolism during exercise as muscle lactate did not increase post exercise compared to baseline. Indeed, in a previous study by Stephens *et al* (2006), muscle lactate content reduced following L-carnitine infusion at rest and was associated with blunting of PDC activity. However contrary to findings from the same study and a study by Wall *et al* (2011) (whereby carnitine reduced muscle glycolysis and increased glycogen storage in muscle at rest and during exercise in young healthy people, respectively, there appeared to be greater glycogen utilisation during exercise not only in the control group but also in the carnitine-fed group.Therefore oral supplementation of carnitine for 6 months does not appear to have an effect on reducing muscle glycogen utilisation in older people.

The notion that carnitine may influence insulin sensitivity stem from observations that insulin resistant states are often associated with lower (~25%) carnitine concentrations (Tamamogullari *et al.* 1999) and carnitine supplementation improved glucose homeostasis in healthy (Galloway 2011) and insulin-resistant humans (Rahbar *et al.* 2005). Perturbations in muscle mitochondrial fuel metabolism in animal models of glucose intolerance including incomplete fatty acid oxidation as a result of carnitine insufficiency were reversed by administration of oral carnitine and further improved whole body glucose tolerance (Mingrone *et al.* 1999). Indeed low rates of fatty acid oxidation due to decreased function of

CPT-1 involved in the carnitine shuttle in mitochondria is associated with insulin resistance, possibly caused by accumulation of IMCL intermediates that interfere with insulin signalling (Dobbins *et al.* 2011). Human studies examining the effect of carnitine supplementation on glucose disposal or insulin sensitivity are limited and mostly involved acute intravenous administration of L-carnitine. These studies demonstrated that L-carnitine infusion during the hyperinsulinaemic euglycaemic clamp in insulin-resistant individuals with diabetes increased peripheral glucose disposal (Capaldo 2005), whole body glucose disposal and glucose oxidation (Mingrone *et al.* 1999). A bolus of L-carnitine was also shown to increase glucose disposal, carbohydrate oxidation and respiratory quotient in people with T2DM by means of the intravenous glucose tolerance test (analysed via the minimal model technique). Insulin sensitivity index remained unchanged (De Gaetano *et al.* 1999). It is worth pointing out that these studies involved adults aged less than 60 years of age and that carnitine was administered intravenously.

Despite an increase in the ability to utilise IMCL, increasing muscle total carnitine and fat oxidation in older healthy males in this study did not appear to affect skeletal muscle insulin sensitivity as demonstrated by unchanged glucose disposal rates and 2DG uptake in skeletal muscle at 24 weeks. Fasting plasma glucose concentrations were however reduced after 24 weeks of ingesting carnitine. Exercise at 50% VO_2 max twice weekly for an hour did not affect fat oxidation or insulin sensitivity in the control group. Whereas other studies have showed an improvement in markers of insulin sensitivity (fasting insulin, HOMA-IR) (Pruchnic *et al.*, 2004) and via the insulin-glucose clamp (Goodpaster 2003), these were most likely exercise training-induced effects of exercise up to 75% VO_2 max (Hughes *et al.* 1995,Cox *et al.* 1999) or vigorous endurance exercise training (Kirwan *et al.* 1993).

This study further showed that under insulin-stimulated conditions fat oxidation rates at rest in the carnitine group remained unaltered but carbohydrate oxidation rates increased in

addition to an increase in resting energy expenditure pre-insulin after 24 weeks of daily supplementation with carnitine. Oral supplementation of carnitine may have improved the metabolic flexibility of switching substrates in response to insulin. Besides carnitine's main involvement with mitochondrial transport and subsequent beta-oxidation of long-chain fatty acids it also functions to sequester and export inhibitory acetyl-coenzyme A units as acetyl carnitine, which favours glucose oxidation (McGarry and Brown 1997).

Taken together the findings indicate that although increasing the bioavailability of carnitine was able to improve beta-oxidation particularly during low-intensity exercise, this may not have reached levels sufficient to affect whole body glucose disposal.

There was a tendency for SSL lipid droplets to reduce post exercise at 24 weeks in the control group indicating perhaps an exercise training effect. Lipid droplets in the IMF region tended to reduce post exercise in the carnitine group. As demonstrated in study 1 (Chapter 3), lipid was seen to accumulate to a greater extent in the SSL regions of muscle of older overweight participants during light-intensity exercise and this was attributed to blunting of IMCL oxidation capacity. However despite an improvement in fatty acid oxidation in the carnitine group, and comparable body fat mass, there was less of a reduction of percentage lipid droplet accumulation within the SSL region of muscle post-exercise compared to control. The significance of this cannot be entirely reconciled without further examination of the lipid moieties involved and reasons for the difference in the degree of accumulation. However carnitine appeared to prevent the accumulation of IMF lipid during light-intensity exercise, perhaps indicating a role for carnitine on this distinct pool. Again the significance of this is unknown, and the lipid moieties involved should be examined further. No changes in body composition were seen in both the carnitine and control groups after 24 weeks of ingesting either carnitine or placebo in combination with daily supplement of 220mls of an insulinogenic beverage (44.4 grams and 13.8g protein) equating to 330kcals extra energy. In

a study involving carnitine and insulinogenic beverage in young healthy volunteers, fat mass accrual was prevented with oral supplementation of carnitine (Stephens *et al.* 2013). The investigators postulated that ingesting carnitine may have obviated the effect of increased energy intake from the carbohydrate supplement by increased fuel utilisation during exercise. Lean mass was also similar in both groups at 24 weeks. It may be that a longer period of carnitine loading and sustainability of exercise may be required to see significant changes in body composition in older individuals, for this to occur individuals may have to train for longer and at higher activity levels as the Wall *et al.* (2011) and Stephens *et al.* (2013) studies used triathletes who were regularly training at higher exercise intensities. 24 weeks of light-intensity exercise twice weekly (control group) also did not appear to improve lean nor fat mass. Nevertheless fat oxidation in volunteers in this study increased despite exercising at the same exercise intensities before and after carnitine.

No changes were seen with regards to the incremental shuttle walk test (which is an indicator of VO_2 peak) in both groups although there was a perceived improvement in the physical functioning role in the carnitine group at 24 weeks. The incremental shuttle walk test (ISWT) is a reproducible, standardised exercise test originally developed to evaluate functional capacity in patients with chronic progressive obstructive disease (Singh *et al.* 1992) and later validated for use in other chronic diseases (ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories). When compared to the few studies examining the ISWT in older healthy individuals the mean distance walked (560 ± 22 m) in this study was comparable to distance walked in a study by Jurgensen *et al.* (2011) (508 ±160m, mean age 58± 10 yrs) and Lee *et al.* (2005) (440m (range 360-520m, mean age 61 ± 10 yrs). However Dyer *et al.* (2002) reported a much lower mean distance walked in much older subjects (243±21m, age range 70-85) indicating the negative correlation of age with ISWT that may be influenced by gradual reduction in muscle mass, strength and maximal oxygen uptake in

parallel with ageing (Fleg *et al.*1998, Evans *et al.* 1993). This corresponds with the lower maximal oxygen uptake (VO_2 max) seen in older volunteers in this study compared to younger healthy controls. No difference was seen after 24 weeks in mean distance walked in both groups in this study, although participants in the carnitine group walked an average 25.7m further than at 0 weeks.

The overall SF36v2 scores did not differ between participants in the carnitine and control groups at baseline, participants in the carnitine group on average scored better in the physical role functioning health domain. The improvement was related to an increase in the ability to accomplish more and having less difficulties and limitations with work and daily activities of living (Users Manual SF36v2TM, QualityMetric, Lincoln USA).

6.6 Conclusions

In conclusion, this study has shown for the first time that oral carnitine supplementation taken with an insulinogenic beverage for 6 months can increase skeletal muscle total carnitine content in older healthy humans. The increased carnitine availability promoted fatty acid oxidation during low-intensity exercise, presumably from IMCL. Perhaps a longer period of carnitine feeding and exercise is required to appreciate an improvement in insulin sensitivity, or an underlying mechanism that resists the increased fatty acid oxidation from translating to increasing insulin sensitivity is yet unknown, and remains to be elucidated. Future studies should also control for confounding factors such as habitual activity and dietary intake in participants, as these may also impact on the overall effect on insulin sensitivity. Finally as carnitine has been shown to improve glucose tolerance in insulin-resistant states other than ageing, the role of carnitine supplementation in overweight/obese individuals and T2DM should also be explored.

Chapter 7: Final Summary and Discussion

Two major pertinent issues affecting the 21st century are the growing ageing population and overweight and obesity rates. Over 10 million people in the UK are aged 65 and above, with numbers expected to almost double by 2050. By then obesity is predicted to affect 60% of adult men, 50% of adult women and 25% of children (Foresight 2007). In line with this, the number of people with diabetes is expected to more than double worldwide (Wild *et al.* 2004) as a result of the ageing population, with the largest rise in people aged 65 and over. Clearly these numbers pose major public health concerns and hugely impact on healthcare costs. A key issue of the 2015 Parliament was once more focussed on addressing the growing social and economic impact of ageing and related chronic conditions on healthcare and social systems in the UK (www.parliament.uk / business/ publications/ research/ key-issues-parliament-2015). Over 10 billion pounds annually are spent on management of T2DM. The growing literature on studies related to insulin resistance is testament that this issue is significant and strategies to improve insulin sensitivity are warranted. Efforts to tackle these problems are clearly still hampered by outstanding issues concerning nutrition and insulin resistance.

The studies presented in this thesis set out to explore these important issues; in particular the effects of overfeeding macronutrients and of specific micronutrient supplementation on insulin sensitivity, and sought to investigate the influence of age and overweight/obesity on insulin sensitivity in healthy people. The general literature on these subjects is inconclusive on several questions that the studies have endeavoured to answer:

1) Does ageing per se cause insulin resistance, or are there other factors associated with ageing that contributes to the decline in insulin sensitivity?

2) As ageing is associated with insulin resistance and sarcopenia, is there an effect of reduced insulin sensitivity on muscle protein metabolism and what are the underlying mechanisms?

3) Is increased liver fat content and perturbations to carbohydrate and fat metabolism due to energy excess or is it macronutrient specific?

4) Can micronutrients that are specific to influencing fatty acid metabolism, ie fat oxidation, impact on improving impaired fat utilisation and insulin sensitivity particularly in the elderly?

With the emergence of increased obesity rates and the ageing population, there is growing interest as to whether it is age, physical inactivity or body composition per se that affects insulin sensitivity in older healthy people. This question was explored in the first study (Chapter 3), where fat metabolism, insulin sensitivity and skeletal muscle metabolism were compared in old and young healthy men. It was proposed that it is factors associated with ageing and not ageing per se that contributes to the decline in insulin sensitivity. Ageing and insulin resistance is also associated with sarcopenia, a significant risk factor of disability in the ageing population. It was also demonstrated in Chapter 3 that older overweight and less insulin-sensitive men have lower functional capacity as demonstrated with the Incremental shuttle walk test (ISWT), a feature of sarcopenia or inactivity. The following chapter (Chapter 4) presented results from examining the effect of insulin resistance, induced by acutely elevating lipid concentrations in young healthy men, on MPS, providing insight to the likely mechanisms involved in perturbed MPS and insulin sensitivity. Besides physical inactivity, diets are clearly a driver in the development of central obesity, visceral fat and hence insulin resistance. The third study (Chapter 5) thus investigated to what extent liver fat and hepatic insulin sensitivity were affected by 2 weeks of increased energy intake of fat, and how this compared to high carbohydrate feeding with the premise that liver fat accumulation and hepatic insulin resistance are energy, rather than macronutrient -mediated. Novel

strategies are therefore warranted in addressing the growing rates of obesity and insulin resistance. The final study (Chapter 6) aimed to investigate the effects of carnitine supplementation on skeletal muscle fat metabolism and insulin sensitivity in older healthy men. The main findings are chapter specific and were summarised within the chapters themselves. This section will amalgamate the empirical findings and discuss further research required to further increase our understanding of current issues.

Ageing is associated with metabolic perturbations, exacerbated by increasing visceral fat, skeletal muscle lipid accumulation, sarcopenia and reduced insulin sensitivity. However there is mounting evidence including findings from this thesis to suggest that it is not age per se but body composition and physical inactivity that affects insulin sensitivity and skeletal muscle metabolism. As revealed from data presented in chapter 3, older healthy men have greater predisposition to lipid accumulation, reduced fat metabolism and insulin resistance, and these appear to be largely influenced by body fat and to a certain extent, physical inactivity. The general impression of the current literature is that although ageing is often implicated in perturbations to metabolism and insulin sensitivity, it would appear that it is mostly factors associated with ageing such as increasing adiposity, reduced lipid utilisation and impaired metabolism and a sedentary state that increases insulin resistance. More often than not, studies involving older groups and investigating insulin sensitivity do not control for these factors that may influence results. The findings from this thesis assert the importance and influence of adiposity and habitual physical activity on skeletal muscle insulin sensitivity. Thus, people do not necessarily develop insulin resistance simply because they are 'growing older', but this is likely to be due to development of obesity, accumulation of IMCL and associated reduction in physical activity with ageing. Therefore strategies should be targeted at encouraging increased activity, a healthy balanced diet and therapies to prevent accumulation of excess fat; visceral or skeletal. The findings would also fit with the general

consensus that increased adiposity, liver fat deposition and IMCL accumulation all predispose to a decline in insulin sensitivity and hepatic metabolism. Along the line of physical inactivity and insulin resistance is the increasing incidence of sarcopenia, a decline in muscle mass and functional capacity. A stark difference between the ISWT of the older lean and overweight older men was observed in Chapter 3 and as Chapter 4 demonstrated, insulin resistance led to perturbations of MPS in response to amino acid ingestion under hyperinsulinaemic euglycaemic conditions, associated with an impairment of signalling located downstream of the Akt-mTOR signalling pathway at the level of translation initiation.

Closely associated with increasing overweight and obesity rates, controversy surrounding energy excess vs. macronutrient influence on fat accumulation and insulin resistance persists but provides strong impetus to research, even dominating headlines in social media in recent years. With various conflicting information on which is worse, carbohydrate, fat or energy excess the ultimate conclusions are not so clear-cut thus dividing public and indeed research community opinions alike. Newspaper headlines such as 'Butter is NOT bad for you' and 'Saturated fat is ok after all' are likely to confuse and lead to increased consumption of processed foods high in fat. On the other hand, replacing saturated fat with refined carbohydrates would also have potentially negative side-effects. The studies in this thesis were designed to examine the effects of modulating macronutrients on liver and muscle fat, metabolism and insulin sensitivity. The general consensus generated from current research appears to indicate that energy excess leading to overweight or obesity is associated with insulin resistance. Although the effect of high fat diets on insulin resistance is inconclusive, carbohydrate excess, in particular fructose has been shown to promote insulin resistance (Stanhope et al 2009). The effects of high fat vs high carbohydrate diets are scarce so no definitive conclusion have been ascertained. The study presented in this thesis showed that 2 weeks of energy excess per se caused a significant increase in liver fat content and fasting

plasma TAGs, most likely due to the process of DNL when overfed with a diet high in carbohydrate, with a (borderline) greater increase of liver fat from baseline compared to the high-fat fed group. Whilst the findings of this study support the notion that energy excess can increase liver fat content in 2 weeks the results did not show a significant differential effect between carbohydrate and fat overfeeding. Carbohydrate excess appears to increase liver fat from baseline, with a trend seen following fat overfeeding. Nevertheless, the change in metabolites normally associated with liver fat accumulation appears to indicate a greater deleterious effect of high carbohydrate intake on liver fat, whereas high fat contributed to a rise in ApoB (thought to be a contributor to cardiovascular risk) and ApoA1 (perhaps as an indicator of the liver's response to the rise in ApoB concentrations). Contrary to expectations, the increase in liver fat did not lead to a difference in hepatic or whole body insulin resistance as a consequence of energy excess or manipulation of carbohydrate or fat content. This is similar to outcomes seen in the study by Johnston et al (2013), whereby diets high in glucose and fructose increased liver fat content but did not affect hepatic insulin resistance. Several reasons could explain this outcome. It could be that 25% excess energy of fat and carbohydrate for 2 weeks is insufficient to increase insulin resistance. Stanhope et al (2009) shown that a 10 week 25% excess energy diet in the form of fructose-sweetened beverages increased DNL and visceral adiposity, resulting in dyslipidaemia and reduced insulin sensitivity in overweight/obese healthy humans. Perhaps given a longer period of time, chronic ingestion of nutrients whether in excess of carbohydrate or fat may eventually lead to hepatic and skeletal muscle insulin resistance. A greater feeding stimulus may therefore be required to appreciate the effects of a short-term moderate dietary excess that correlates with insulin resistance. The ingestion of diets with greater energy excess than that used in this study have been investigated (Westerbacka *et al.* 2005, Le *et al.* 2009, Horton *et al.*, Brons *et al.* 2011, Sevastionova *et al.* 2012). Whilst some of these studies showed significant effects

with liver fat or insulin sensitivity, it should be borne in mind that these diets may not be entirely representative of typical intakes in the general population. Diets in this study were designed to be realistic and simulate that of which might be consumed under habitual circumstances in the present population. Furthermore, the ethical concerns relating to prolonged or excessive overfeeding in humans would preclude studies involving excessive or prolonged dietary intakes.

Acknowledging the studies in the current literature and indeed from findings of studies undertaken for this thesis, the increasing prevalence of overweight, obesity and insulin resistance either from overnutrition or as a consequence of factors that accompany ageing warrant strategies to overcome these problems. This would include novel use of nutrients that may modulate metabolism, energy expenditure and insulin sensitivity. Dietary restriction, weight loss and exercise have been advocated in concerted efforts to improve insulin sensitivity in older people, and yet the number of elderly people with insulin resistance continues to rise. Realising that physical inactivity declines and adiposity increases with age, increased resources and focus have been placed in encouraging the older generation to exercise and eat healthier. Although training and exercise programmes are clearly beneficial in promoting weight loss, cardiorespiratory fitness and even insulin sensitivity in older people, in most cases, these regimens can be difficult for most to sustain. Various nutrients have been shown to improve insulin sensitivity but studies are limited and contradictory. Carnitine has been shown to increase the relative contribution of muscle fat oxidation to total fat oxidation during light intensity exercise in older people (Chapter 5), and appears to exert positive effects on skeletal muscle metabolism and metabolites. Older healthy people retained skeletal muscle total carnitine, however contrary to expectations ingestion of carnitine for 6 months did not improve skeletal muscle insulin sensitivity. There was a tendency for the accumulation of lipid to decrease post-exercise in both control and carnitine-fed groups,

indicating that exercise, even at low-intensities can reduce the accumulation of lipid in muscle, but more importantly increase fatty acid oxidation in the carnitine group. It may be that a longer period, greater amount of carnitine or other ways of increasing the bioavailability of carnitine are required to appreciate a robust effect, or that there are still underlying mechanisms involved in ageing muscle not yet explored. Nevertheless the findings show promise and future studies to extend on data are warranted, including investigation into the use of carnitine in overweight and people with pre-diabetes.
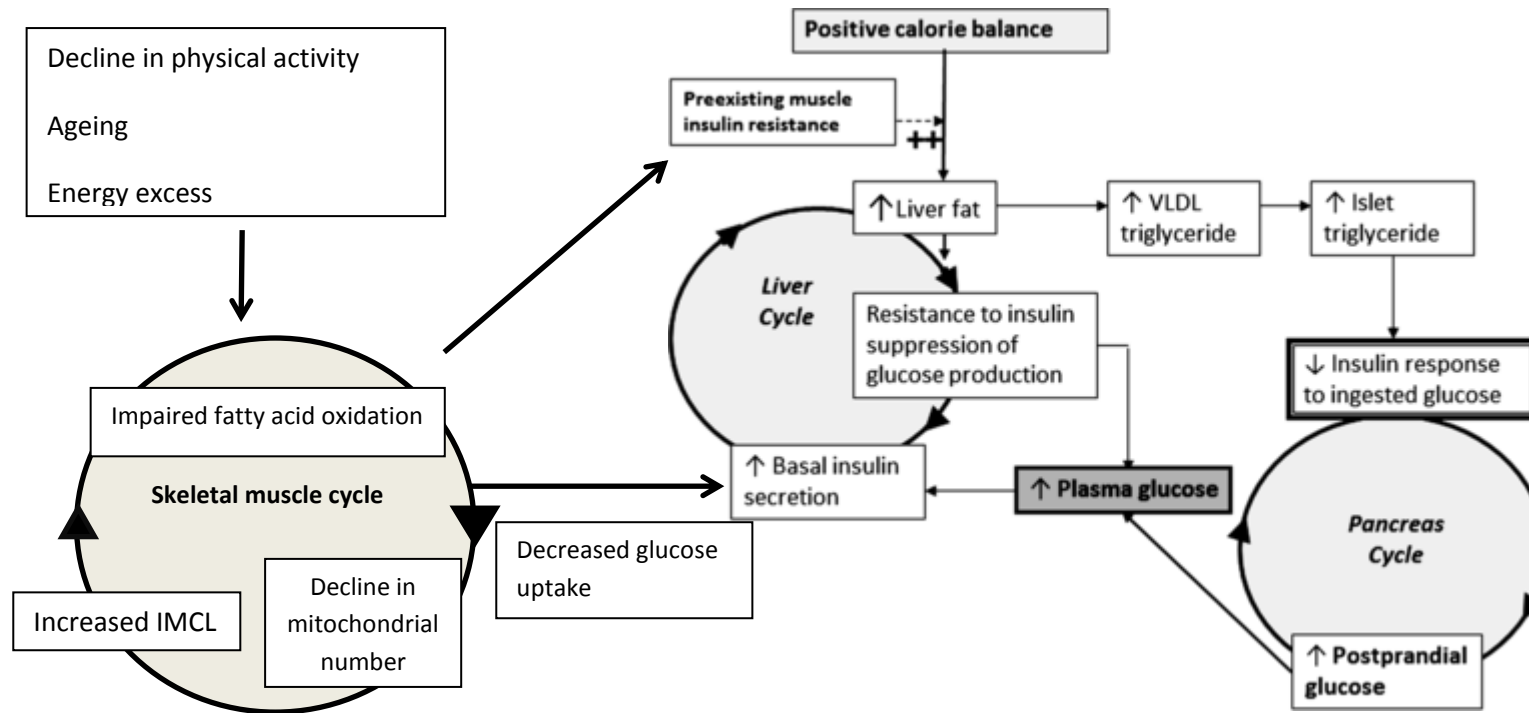
Figure 7.1: Tricycle hypothesis; amalgamation of Taylor's twin cycle hypothesis (Taylor 2008) and the skeletal muscle cycle.

Findings from the overfeeding study stress the importance of the deleterious effects of a positive energy balance on liver fat content and metabolism. Skeletal muscle lipid accumulation contributes to insulin resistance in ageing as seen in the study comparing young vs. older subjects. To reconcile the twin cycle hypothesis of Taylor (who acknowledges muscle insulin resistance as a factor implicated in development of T2DM), and De Fronzo's postulation of skeletal muscle resistance as an important contributor to T2DM, a combined three cycle (tricycle) hypothesis is proposed (Figure 7.1). In addition to the twin cycle hypothesis that chronic energy excess leads to accumulation of liver fat with eventual spill-over into the pancreas, over time, (ageing and continuous ingestion of energy excess) accumulation of skeletal muscle lipid could overwhelm the capacity of muscle to oxidise fatty acids. Ageing, reduced physical activity and decline in skeletal muscle mitochondrial content can all lead to muscle insulin resistance and contribute to a reduction in skeletal muscle glucose uptake and thus increased insulin secretion. Indeed this would presumably also occur in younger people who are in chronic positive energy balance. Considerably more studies in future should explore the effects of chronic nutrient overfeeding on hepatic and skeletal muscle insulin sensitivity, adipose tissue and pancreatic fat concurrently to further understand the interplay between these tissues and of which precedes the other. This would conceivably influence targeted strategies in the prevention of insulin resistance in at-risk groups of the population. For example strategies to reduce lipid delivery (particularly during exercise) and improve adipose tissue function, such as the use of acipimox (Daniele 2014) and pioglitazone (Bajaj *et al.* 2003), and further examination of the use of carnitine are justified. This should be in conjunction with the continued emphasis on 'eating less, exercising more'.

7.1 Study limitations

Several limitations to studies undertaken in this study are acknowledged. The major limitations of the high fat vs. high carbohydrate study was that it was not a cross-over design and did not involve an isoenergetic-fed control group per se; rather the participants presented as their own controls having completed an isoenergetic diet prior to embarking on the overfed period. On the other hand a cross-over study may pose issues with compliance and retention of participants for longer periods of overfeeding. Body composition was assessed using body impedance analysis and subcutaneous fat analyses. ^{1}H MRS of liver and skeletal muscle was also not performed at the beginning of the isoenergetic period. This would ascertain baseline liver and muscle fat, but the isoenergetic diet was not designed to provide excess energy so it has to be assumed liver fat remained the same during that period. Utilising indirect calorimetry at baseline would have been ideal for assessing energy expenditure and requirements as opposed to estimating basal metabolic rate and energy intake using diet diaries and energy equations. The study was also performed exclusively in male White European men and not under strict controlled conditions, thus activity levels were not controlled for and may be a confounding factor. The intervention was relatively short and so the possible effects of a longer dietary regimen cannot be excluded, however this will need to be balanced between practicality, adherence to dietary regimen and ethics. Power calculations were made for the primary outcome measures but not for the secondary outcomes.

Several limitations in the carnitine study are also acknowledged. Diets and day to day habitual activities of participants were not controlled for so may have influenced outcomes of the study. On the other hand, this represents the use of carnitine within a habitual setting. Compliance may have been an issue; however participants attended over 75% of their twice weekly cycling sessions at the laboratory and regular measurements of serum carnitine levels were undertaken.

7.2 Future directions

There is certainly still a lot of scope for increasing our understanding and exploration in the effects of nutrients and ageing on insulin sensitivity.

The observations and findings of studies undertaken have unraveled several questions that warrant further investigation and should be addressed in the future:

1) Explore the underlying mechanisms and insulin pathways contributing to the accumulation of lipid in specific regions of skeletal muscle in older vs. young people. Why does subsarcolemmal lipid accumulation mostly occur in muscle of overweight/obese and older people and how does it affect insulin sensitivity? Importantly, are there ways to avert or slow down this phenomenon?

2) Further investigate the role of physical activity and body composition including the contribution of subcutaneous fat/visceral fat in pre-diabetes or T2DM on skeletal muscle lipid and metabolism.

3) Explore the effects of high fat vs high carbohydrate on liver fat and skeletal muscle in people with pre-diabetes or impaired glucose tolerance (IGT).

4) To further investigate in detail the effects of the type of carbohydrate (polysaccharide vs monosaccharides) and fat (PUFA vs MUFA and saturated) on insulin sensitivity.

5) To not only investigate the effects of diet on liver fat but further determine if participants are genetically insulin sensitive or resistant. Investigating pancreatic fat via MRI/^{1}H MRS would also be beneficial to further understand the cross talk between fatty liver, pancreatic fat cells and inflammation that may accentuate beta-cell dysfunction.

6) Investigating the interplay of effects of diets on muscle, liver, pancreas and adipocytes in elucidating the pathogenesis of insulin resistance.

7) Investigate the effects of intravenous vs oral carnitine in older and younger subjects, older lean and overweight/obese, controlling for habitual physical activity and diets.

8) Exploration of gene expressions of insulin signalling pathways and fat metabolism in carnitine-fed subjects.

9) Examine skeletal muscle protein synthesis of older healthy people in response to lipid-induced insulin resistance and whether this can be improved with carnitine supplementation.

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Appendices

Breakfast	Weight (g)
Medium sliced bread	50
Skimmed milk 1 pint	293
Flora butter	20
Cereal	50
Skimmed milk powder	9
Lunch	
Large cornish pasty	227
Crisps	30
Peach	100
Dinner	
Chicken in white wine with mash	450
Garden Peas	80
Jelly	175
Snacks	
Chocolate digestive biscuits	51
Crisp biscuits	44

Figure 1 :Example food menu 2200 kcals

Table 1: Example breakdown of energy composition 2200kcal menu

Nutrient	Quantity	Energy	Percentage
Protein	90.5	362	16
Carbohydrate	286	1072.6	49
Fat	85.6	770.7	35
		2205.3	100
Composition of CHO			
Sugars	71.2	266.9	12.1
Starch	84.4	316.6	14.4
Composition of Fat			
SFA	38	342.1	15.5
MUFA			
PUFA			

Table 2: Example breakdown of energy composition of high fat diet

HF diet			
Nutrient	Quantity	Energy	Percentage
Protein	90.5	362.3	13.5
Carbohydrate	286.2	1073.1	40
Fat	138.7	1248.2	46.5
		2683.6	100
Composition of CHO		267.4	10
Sugars		316.6	11.8
Starch			
Composition of Fat			
SFA		643.5	24
MUFA			
PUFA			

Table 3: Example breakdown of energy composition of high carbohydrate diet

HC Diet			
Nutrient	Quantity	Energy	Percentage
Protein	90.5	362	13.2
Carbohydrate	430.4	1614	58.8
Fat	85.6	770.7	28
		2746.7	100
Composition of CHO			
Sugars		266.9	9.7
Starch		316.6	11.5
Composition of Fat			
SFA	38	342.1	12.5
MUFA			
PUFA			

Table 4: Example energy requirements during isoenergetic and 2 week hyperenergetic periods

	TEE (kcal)	Energy from fat(kcal /day)	Energy from fat (%)	Energy from CHO (kcal/day)	Energy from CHO (%)	Energy from protein (kcal/day)	Energy from protein (%)
Isoenergetic period	2200	800	35	1100	55	300	15
Hyper-energetic (+25%)HC	2750	800	28	1650	60	300	12
Hyper-energetic (+25%) HF	2750	1300	46	1125	41	325	13

Table 5: Dropouts from Study 4 (Chapter 6)

Participant	Age	Comments
2	Older	Died due to cholangiocarcinoma unrelated to study (Severe Adverse Event – SAE)
5	Older	Discontinued following vasovagal event post muscle biopsy
7	Older	Developed arrythmia following light-intensity exercise
9	Older	Discontinued as averse to chronic intake of nutritional drink
14	Older	Developed deceleration of heart rate during light-intensity exercise
16	Older	Developed vasovagal episode during light-intensity exercise during study visit
18	Older	Decided to discontinue with study due to inability to complete exercise during study visit
8	Young	Decided to discontinue study after painful muscle biopsy
9	Young	Did not return for follow-up study visit
10	Young	Did not return for follow-up study visit