Modulation of muscle fuel metabolism in human volunteers by increasing the availability of muscle acetyl-CoA and carnitine moieties

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CONTENTS

TITLE PAGE 1

CONTENTS 2

ABSTRACT 7

DECLARATION 9

ACKNOWLEDGEMENTS 10

ABBREVIATIONS 12

Chapter 1. GENERAL INTRODUCTION 18

1.1. Acetyl group availability 19

1.1.1. Structure, function and regulation of PDC 21

1.1.2. The concept of 'acetyl group deficit' during non-steady state exercise 24

1.1.3. Acetyl group stockpiling via sodium acetate administration 29

1.2. Carnitine 33

1.2.1. Background 33

1.2.2. Physiological functions of carnitine in skeletal muscles 34

1.2.2.1. Transfer of long chain fatty acids across the mitochondrial membrane 38

1.2.2.1.1. Cellular and sub-cellular physiology 39

1.2.2.1.2. Enzymes 39

1.2.2.1.3. Transporters 40

1.2.2.1.4. First enzymatic reaction 41

1.2.2.1.5. Second enzymatic reaction 42

1.2.2.2. Acetyl group buffering 42

1.2.3. Increasing muscle carnitine content 46

1.2.4. The role of Insulin in increasing carnitine uptake into muscle cells 51

1.3. Integration of muscle fuel use at rest and during steady-state exercise 56

1.3.1. Fatty acid and glucose uptake into muscle cells 56

1.3.2. Glucose-fatty acid cycle (Randle cycle) 57

1.3.3. Malonyl-CoA hypothesis 60

1.3.4. Free carnitine availability 64

1.4. The impact of increased muscle carnitine content on fuel metabolism and energy expenditure 68

1.5. Lipid accumulation, insulin resistance and a potential role for carnitine 70

1.5.1. Insulin signalling pathway in skeletal muscle cells 72

1.5.2. Mechanisms of insulin resistance 72

1.6. Weight loss and a potential role for carnitine 75

1.7. Aims and hypotheses 80

Chapter 2. GENERAL METHODS 82

2.1. Ethics, volunteer recruitment and screening 83

2.2. Maximum O2 consumption (VO2max) 85

2.2.1. Measurement of VO_2max in Chapter 3 and 4 86

2.2.2. Measurement of VO_2max in Chapter 5 86

2.3. Body composition 88

2.4. Blood sampling and analysis 89

2.4.1. Serum insulin concentration 90

2.4.2. Plasma total carnitine concentration 91

2.5. Muscle sampling and analysis 92

2.5.1. Muscle metabolite extraction 93

2.5.2. Muscle free creatine (Cr) content 95

2.5.3. Muscle phosphocreatine (PCr) content 97

2.5.4. Muscle lactate content 100

2.5.5. Muscle glycogen content 101

2.5.6. Muscle free carnitine content 104

2.5.7. Muscle long chain acyl-carnitine content 104

2.5.8. Muscle acetylcarnitine and acetyl-CoA content 105

2.5.9. Muscle total carnitine content 107

2.5.10. Muscle pyruvate dehydrogenase complex activity (PDCa) 107

2.5.11. Muscle 2-deoxyglucose (2-DG) content 108

2.6. Coefficients of variation for analytical measurements 109

2.7. Statistics 111

Chapter 3. THE IMPACT OF INCREASING SKELETAL MUSCLE ACETYL GROUP AVAILABILITY BY SODIUM ACETATE INFUSION ON FUEL UTILISATION DURING LOW AND HIGH INTENSITY STEADY-STATE EXERCISE IN YOUNG HEALTHY, MALE VOLUNTEERS 112

3.1. Introduction 113

3.2. Methods 119

3.3. Results 127

3.4. Discussion 144

Chapter 4. THE IMPACT OF INCREASING SKELETAL MUSCLE CARNITINE CONTENT ON RESTING FUEL METABOLISM IN YOUNG, HEALTHY, MALE VOLUNTEERS 149

4.1. Introduction 150

4.2. Methods 154

4.3. Results 163

4.4. Discussion 174

Chapter 5. THE IMPACT OF INCREASING SKELETAL MUSCLE CARNITINE CONTENT, CALORIC RESTRICTION AND EXERCISE ON WEIGHT LOSS IN HEALTHY, OVERWEIGHT FEMALE VOLUNTEERS 180

5.1. Introduction 181

5.2. Methods 186

5.3. Results 197

5.4. Discussion 214

Chapter 6. GENERAL DISCUSSION 221

6.1. Overview of studies and findings 222

6.2. The impact of stockpiling muscle acetyl group on fuel selection during steady-state low intensity exercise in healthy young male volunteers (Chapter 3) 223

6.3. The impact of stockpiling muscle acetyl group on fuel selection during steady-state high intensity exercise in healthy young male volunteers (Chapter 3) 224

6.4. Increasing muscle carnitine content via chronic supplementation of carnitine and carbohydrate in healthy young male volunteers (Chapter 4) 225

6.5. The impact of increased muscle carnitine content on body mass and percentage total body fat in healthy young male volunteers (Chapter 4) 226

6.6. The impact of increased muscle carnitine content on whole blood glucose disposal and insulin sensitivity in healthy young male volunteers (Chapter 4) 227

6.7. Increasing muscle carnitine content via a novel approach involving chronic supplementation of carnitine plus carbohydrate and protein in healthy overweight female volunteers (Chapter 5) 228

6.8. The impact of increased muscle carnitine content, caloric restriction and low intensity exercise on resting whole-body fat and carbohydrate oxidation in healthy overweight female volunteers (Chapter 5) 229

6.9. The impact of increased muscle carnitine content, caloric restriction and low intensity exercise on body mass and body fat mass in healthy overweight female volunteers (Chapter 5) 231

6.10. Summary, future work and limitations 232

REFERENCES 235

Appendix 1: Volunteers' screening and consent forms 270

Appendix 2: DEXA Scan information leaflet 274

Appendix 3: Procedure for Insulin assay (Coat-a-Count) 278

Appendix 4: Biochemical buffers and reagents 282

ABSTRACT

This thesis investigated the impact of sodium acetate infusion on muscle fuel metabolism during low and high intensity exercise and demonstrated a decrease in fat oxidation during the former and a decrease in muscle lactate production during the latter. We propose that the rate of fat oxidation decreased during low intensity exercise due to acetate being preferentially metabolised over fat and possibly reduced muscle free carnitine availability. During high intensity exercise, muscle lactate accumulation decreased due to higher acetyl-CoA supply to the TCA cycle.

This thesis also investigated the impact of chronic carnitine supplementation on overall glucose disposal and demonstrated a decrease in blood glucose and serum insulin concentrations following an OGTT in the carnitine group post-supplementation compared to baseline, coupled with a decrease in muscle 2-deoxyglucose accumulation in the carnitine group post-supplementation compared to control. We propose that carnitine-induced increased fat oxidation caused a decrease in hepatic fat deposition which in turn resulted in increasing glucose disposal by the liver. We also propose that increased hepatic glucose disposal resulted in reduced hepatic glucose release and hence diminished serum insulin concentrations.

The impact of a chronic lifestyle intervention protocol involving either oral supplementation with carnitine (carnitine group) or placebo (control group) combined with carbohydrate and protein, together with regular exercise and a prescribed diet on muscle fuel metabolism and body composition in overweight

volunteers was investigated in this thesis. This study demonstrated that increased muscle carnitine content caused an increase in fasting fat oxidation and a decrease in carbohydrate oxidation at rest. The carnitine group did not show any significant difference in body mass and body fat mass losses compared to the control group. Whether increased carnitine content, in combination with caloric restriction and exercise, has any impact on body composition seems likely but needs further investigations.

DECLARATION

Work presented in this thesis, including recruitment of volunteers, leading and running the studies, exercise tests, blood and muscle samplings, cannulations, setting up intravenous infusions and indirect calorimetry analysis has been performed by myself at the School of Life Sciences, University of Nottingham Medical School between September 2011 and 2015; with the following exceptions:

The analysis of muscle metabolites in **Chapter 3, 4 and 5** was performed by Dr Chris Shannon at the School of Life Sciences, University of Nottingham Medical School.

The analysis of serum insulin concentration in **Chapter 4** was performed by Ms Karen Swift and Ms Sally Cordon at the School of Life Sciences, University of Nottingham Medical School.

The analysis of muscle 2-DG tracer in **Chapter 5** was performed by Mr Kevin Bailey at the School of Life Sciences, University of Nottingham Medical School.

The diet reviews and diet prescriptions in **Chapter 5** were performed by Dr Moira Taylor and Miss Aisling Burns at the School of Life Sciences, University of Nottingham Medical School.

I hereby declare that this thesis has been composed by myself. No part of this thesis has been submitted in any previous application for a higher degree.

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ABBREVIATIONS

ACC: acetyl-CoA carboxylase

ACS: acyl-CoA synthetase

ADP: adenosine diphosphate

Akt: protein kinase B

AMP: adenosine monophosphate

AMPK: adenosine monophosphate activated protein kinase

aPKC: atypical protein kinase C

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

AUC: area under the curve

°C: Celsius

C: carbon atom

Ca^{+}: calcium ion

CACT: carnitine acylcarnitine translocase

CARN: carnitine

CAT: carnitine acetyl transferase

CD: cluster of differentiation

CON: control

CPT1: carnitine palmitoyltransferase 1

CPT2: carnitine palmitoyltransferase 2

CoASH: free co-enzyme A

Cr: creatine

DAG: diacyl glycerol

DCA: dichloroacetate

DEXA: dual-energy X-ray absorptiometry

2-DG: 2-deoxyglucose

dm: dry muscle

dw: dry weight

EE: energy expenditure

EEE: estimated energy expenditure

F-1-6-P: fructose-1-6-bisphosphate

F-6-P: fructose 6-phosphate

FAT: fatty acid translocase

FATP: fatty acid transport protein

FBC: full blood count

FDR: false discovery rate

FOXO: Fork head box protein O

G6P: glucose 6-phosphate

GLUT: glucose transporter

GSK3: glycogen synthase kinase 3

H^+: hydrogen ion

HK: hexokinase

IL: interleukin

IMCL: intramyocellular lipids

IMTG: intramuscular triglycerides

IR: insulin receptor

IRS: insulin receptor substrate

IU: international units

iv: intravenous

J: Joule

K^+: potassium ion

Kg: kilogram

K_m: Michaelis-Menton constant

kpm: kilopond meters

l - L: litre

LBM: Lean Body Mass

LDH: lactate dehydrogenase

LFT: liver function tests

µ: micro

m: milli

mm Hg: millimetre of mercury

mol: mole

mRNA: messenger ribonucleic acid

MRS: magnetic resonance spectroscopy

n: nano

Na^+: sodium ion

NaAc: sodium acetate

NAD^+: nicotinamide adenine dinucleotide (oxidised form)

NADH: nicotinamide adenine dinucleotide (reduced form)

O2: oxygen

OCTN2: organic cation transporter number 2

OGTT: oral glucose tolerance test

p: pico

PCA: perchloric acid

PCr: phosphocreatine

PDC: pyruvate dehydrogenase complex

PDCa: pyruvate dehydrogenase complex activation status

PDK: pyruvate dehydrogenase kinase

PDK1: phosphoinositide-dependent protein kinase 1

PDP: pyruvate dehydrogenase phosphatase

PFK: phosphofructokinase

PI3K: phosphoinositide 3 kinase

PIP3: phosphoinositol triphosphate

PPAR: peroxisome proliferator-activated receptors

RER: respiratory exchange ratio

rpm: revs per minute

RQ: respiratory quotient

Sv: sievert

TC: total carnitine

TCA: tricarboxylic acid cycle

TNF: tumour necrosis factor

TZD: Thiazolidinediones

U: units

U&E: urea and electrolytes

VE: minute ventilation

VO_2max: maximum oxygen consumption

W: watts

wm: wet muscle

ww: wet weight

Unless otherwise specified, **high intensity exercise** in this thesis refers to exercise above 75% VO_2max and **low intensity exercise** refers to exercise below 75% VO_2max.

Chapter 1

GENERAL INTRODUCTION

1.1. Acetyl group availability

The end product of glycolysis and β-oxidation is acetyl-CoA which in turn enters the tricarboxylic acid (TCA) cycle for oxidative phosphorylation and ATP production. TCA cycle is the final common oxidative pathway for lipids and carbohydrates and therefore acetyl-CoA plays a central role in linking glycolytic and β-oxidation pathways to oxidative energy production **(Fig 1.1)**. Depending on the exercise intensity, acetyl-CoA produced by fatty acid degradation and glycolysis can either enter the TCA cycle or combine with free carnitine and form acetylcarnitine. During low intensity exercise, when fat is the main fuel source utilised (Havel et al., 1963, Romijn et al., 1993, van Loon et al., 2001) and glycolytic flux is low, TCA cycle acetyl group use is matched with acetyl group supply. At high intensity exercise however, when carbohydrate is the main fuel source utilised and glycolytic and PDC flux is increased markedly (van Loon et al., 2001), acetyl group production occurs in excess of TCA cycle demand. The surplus acetyl group is buffered by carnitine to form acetylcarnitine (Harris et al., 1987, Sahlin, 1990, Constantin-Teodosiu et al., 1991a, Constantin-Teodosiu et al., 1992) which in turn guarantees that the PDC flux continues. Diminished availability of muscle free carnitine has been proposed to be a limiting factor to muscle fat oxidation during high intensity steady-state exercise in humans (van Loon et al., 2001). These will be discussed in details in due course.

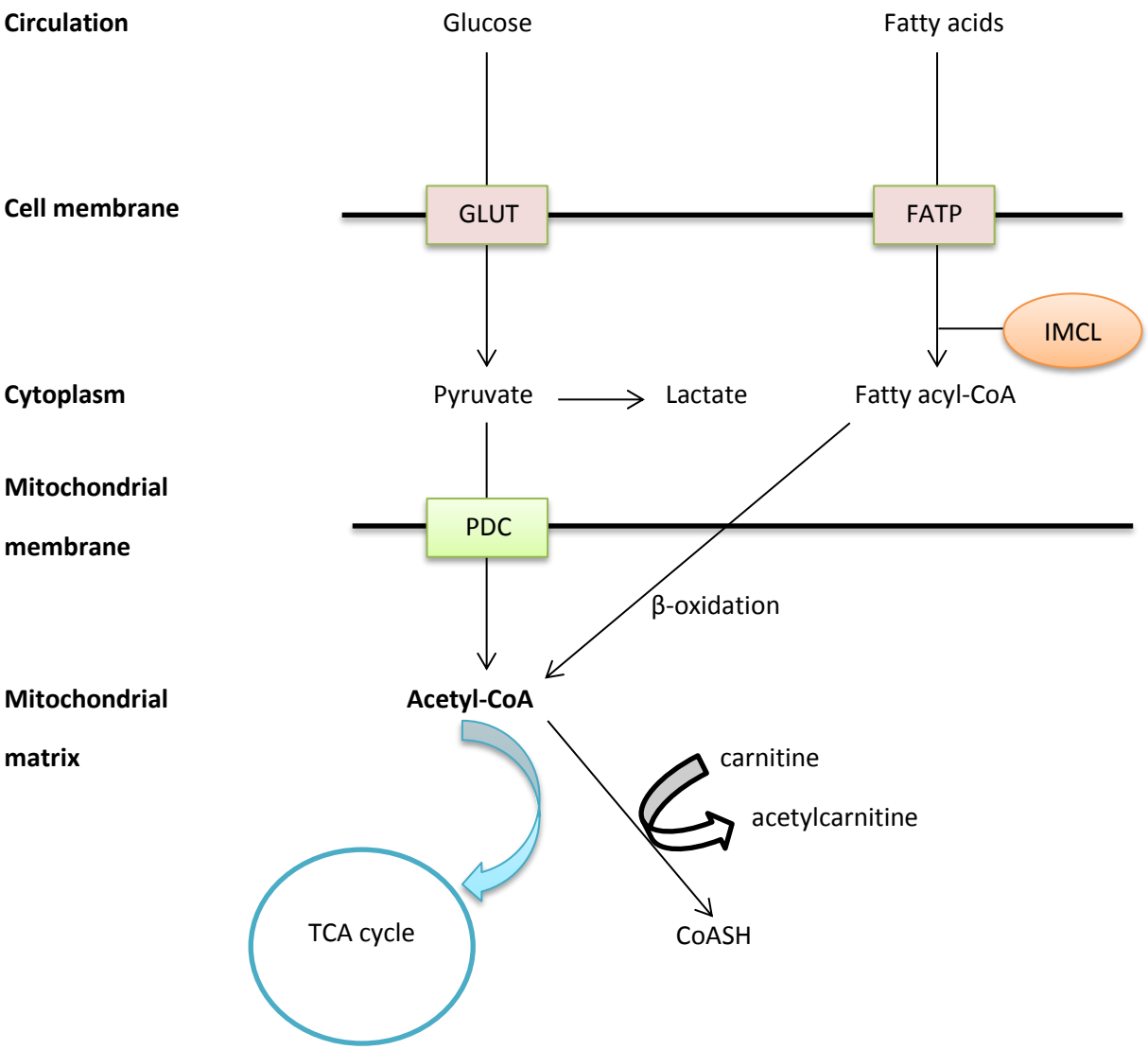


Fig 1.1. Acetyl-CoA plays a central role in linking glucose and fatty acid metabolism to the TCA cycle. Purple boxes represent transporters and green boxes represent enzymes.

1.1.1. Structure, function and regulation of PDC

Pyruvate dehydrogenase complex (PDC) is an enzyme complex consisting of three components: E1 (pyruvate dehydrogenase), a tetramer consisting of two α and two β subunits (α2β2), E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoyl dehydrogenase) (Reed and Hackert, 1990). It is located on the inner mitochondrial membrane (Schnaitman and Greenawalt, 1968, Addink et al., 1972) and converts pyruvate to acetyl-CoA in a decarboxylation reaction **(Reaction 1.1)**. It therefore functions as a link between the glycolytic pathway and the TCA cycle. During conditions of high PDC flux, pyruvate can convert to lactate in an anaerobic reaction catalysed by the enzyme lactate dehydrogenase (LDH) **(Reaction 1.2) (Fig. 1.1)**.

Reaction 1.1: This reaction is considered to be the rate-limiting step in the glycolytic pathway.

$$\text{Pyruvate} + \text{CoASH} + \text{NAD}^+ \xrightarrow{\text{PDC}} \text{acetyl-CoA} + \text{NADH} + \text{H}^+$$

Reaction 1.2: $\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$

The α subunits of E1 component contain serine residues (site 1 (serine-264), site 2 (serine-271) and site 3 (serine-203)) which can be phosphorylated and dephosphorylated by the intrinsic regulatory enzymes pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP) respectively (Hennig

et al., 1975) **(Fig. 1.2)**. Phosphorylation inactivates and dephosphorylation activates the E1 component, the main PDC enzyme component responsible for the overall decarboxylation reaction and where the rate limiting property of PDC resides (Cate et al., 1980). So far 4 isoforms of PDK and 2 isoforms of PDP have been discovered (Lawson et al., 1993, Bowker-Kinley et al., 1998, Sugden and Holness, 2006).

Site 1 is the main site out of three responsible for PDC inactivation. It is phosphorylated by all PDK isoforms but mainly by PDK2. Site 2 is mainly phosphorylated by PDK4 and site 3 is only phosphorylated by PDK1 (Sugden and Holness, 2006).

The end products of the PDC reaction **(Reaction 1.1)**, i.e. acetyl-CoA and NADH, by activating PDK exert a feedback inhibitory effect on PDC activity (Pettit et al., 1975, Cooper et al., 1975) whilst calcium ions activate PDP and act as PDC stimulators (Constantin-Teodosiu et al., 2004). Therefore the overall status of PDC activation depends on the relative activities of PDK and PDP which in turn are regulated by the concentrations of NADH, acetyl-CoA and calcium ions **(Fig. 1.2)**.

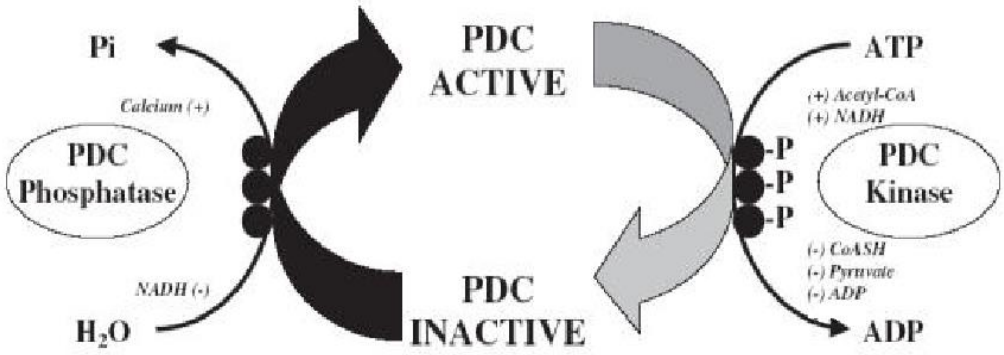


Fig. 1.2. Regulation of PDC (adapted with permission from Greenhaff et al., 2004).

1.1.2. The concept of 'acetyl group deficit' during non-steady state exercise

At the onset of exercise there is inertia in mitochondrial ATP production, which for many years was attributed to a lag in skeletal muscle blood flow and hence muscle oxygen (O_2) delivery. This phenomenon was associated with an increase in muscle energy delivery from non-mitochondrial sources, namely phosphocreatine (PCr) degradation and glycolysis, activated as a result of the accumulation of cytosolic AMP and ADP, and was termed the 'oxygen deficit' (Margaria et al., 1933, Saltin B, 1990). More recently it has been suggested that at least part of the lag in mitochondrial ATP production is attributable to a delay in PDC activation and substrate delivery to the TCA cycle (metabolic inertia) at the onset of exercise (Timmons et al., 1996, Grassi, 2001, Roberts et al., 2002, Greenhaff et al., 2002), which was coined the 'acetyl group deficit'.

Bangsbo et al. (1990) reported that during high intensity exercise in a one-legged knee-extensor exercise model in human volunteers, non-mitochondrial ATP production accounted for ~80% of total energy generated during the initial 30 seconds. During subsequent 60-90 seconds, this value decreased to ~45% and after 120 seconds to ~30%, and was associated with a simultaneous concomitant rise in mitochondrial ATP production.

Studies by Timmons et al. (1996, 1998a, 1998b) demonstrated that substrate level phosphorylation and lactate accumulation increased at the onset of exercise in both human and canine skeletal muscle, during both normal blood flow and low blood flow conditions. The authors also reported that activating the PDC via dichloroacetate (DCA) increased acetyl group delivery to the TCA cycle which in turn

augmented oxidative ATP production at the onset of exercise considerably, during both normal and reduced blood flow conditions. The team made the conclusion that at the onset of exercise, PDC flux was limiting to acetyl-CoA production, hence the increase in non-mitochondrial ATP production.

Timmons et al. (1996) used DCA to activate PDC in ischemic contractile canine gracilis muscle. By increasing PDC activity at rest by ~4-fold, the team reported less lactate accumulation (10.3 ± 3.0 vs 58.9 ± 10.5 mmol/kg dm, $p < 0.05$) and less phosphocreatine degradation (15.6 ± 6.3 vs 33.8 ± 9.0 mmol/kg dm, $p < 0.05$) during contraction in the DCA group compared to CON. After contraction for 20 minutes, the DCA group showed ~25% isometric tension reduction whilst the CON group showed a reduction by ~40% (less fatigue in the DCA group by ~15%, $p < 0.05$). The team reported that regardless of blood flow, non-mitochondrial ATP production decreased significantly in ischemic contractile canine muscle when PDC was activated by DCA. This was in favour of the PDC flux and availability of acetyl groups, and not the blood flow and O_2 delivery, being responsible for metabolic inertia.

The team also demonstrated that DCA-induced increased PDC activation by ~3-fold decreased phosphocreatine hydrolysis and lactate accumulation in human volunteers by ~50% compared to CON after 3 minutes of knee extensor exercise, with lower limb blood flow reduced by ~20% via applying 50 mmHg of positive pressure (Timmons et al., 1998b). The two study groups showed no difference in non-oxidative ATP generation after 8 minutes of exercise. The authors suggested that, similar to ischemic canine model, PDC activation and acetyl group availability decreased non-mitochondrial ATP production in ischemic human exercising

muscles; however this happened during the initial phase of skeletal muscle contraction.

The findings of the studies done by Timmons et al. (1996, 1998a, 1998b) were further supported later in a series of studies run by Roberts et al. (2002, 2005). Roberts et al. (2002) ran a study on ischemic canine gracilis muscle using DCA and managed to augment the activation of resting PDC by ~20-fold, acetyl-CoA content by ~3-fold and acetylcarnitine content by ~4-fold compared to CON ($p < 0.01$). Following 5 minutes of contraction, muscle lactate accumulation was 43 mmol/kg dm in CON and 23 mmol/kg dm in DCA group ($p < 0.01$) and there was ~40% less reliance on substrate level phosphorylation in the DCA group ($p < 0.01$).

Roberts et al. (2005) used sodium acetate administration to increase the availability of canine muscle acetyl group pool independent of PDC activation. The team reported that in canine gracilis skeletal muscle pre-treated with sodium acetate (360 mg/kg body mass), acetyl-CoA and acetylcarnitine availability increased remarkably compared to CON ($p < 0.01$), resulting in ~40% reduction in canine muscle non-mitochondrial ATP generation in the first minute of contraction. The authors concluded that muscle acetyl group stockpiling, independent of PDC activation, overcame the PDC lag and the metabolic inertia at the commencement of skeletal muscle contraction (during the 1'st minute). After the 1'st minute, PDC flux was mainly contributing to acetyl group supply to the TCA cycle and the activation of PDC was identical in both sodium acetate and CON groups.

Roberts et al. (2005) proposed that 'acetyl group deficit' existed between ~65% and ~90% VO_2max exercise intensities. This suggestion was made based on extensive

review of previous studies investigating muscle fuel metabolism at rest to exercise transition period, (Timmons et al., 1996, Timmons et al., 1998a, Timmons et al., 1998b, Howlett et al., 1999b, Evans et al., 2001, Roberts et al., 2002). At this range of workloads (between ~65% and ~90% VO_2max exercise intensities) there is a high demand for energy and therefore a delay in PDC activation at the onset of exercise (metabolic inertia) would result in a delay in acetyl-CoA production and supply to the TCA cycle (acetyl group deficit); hence increased lactate production and substrate level phosphorylation. Roberts et al. (2005) suggested that increasing acetyl group availability at the onset of exercise intensities below ~65% VO_2max and above ~90% VO_2max would not be effective in diminishing non-mitochondrial ATP production at rest to exercise transition period.

At exercise intensities below ~65% VO_2max, glycolytic and PDC flux is low and the acetyl-CoA supply can be handled by the TCA cycle. Studies run by Evans et al. (2001) and Laithwaite et al. (unpublished, 2009) demonstrated that increasing muscle acetyl group availability, by sodium acetate infusion or PDC activation, did not have any impact on non-mitochondrial ATP production at the onset of low intensity exercise, suggestive of the TCA cycle handling acetyl group supply very efficiently at low workloads.

The study run by Evans et al. (2001) involved sodium acetate infusion (4 mmol/kg over 60 minutes) to young healthy volunteers. Post-infusion the volunteers exercised for 120 seconds at 65% VO_2max and muscle biopsies were taken at 30 and 120 seconds. The team reported that non-mitochondrial ATP generation was not different between the sodium acetate and CON groups.

A study done in 2009 in our unit by Laithwaite et al. (unpublished) managed to increase resting muscle PDCa by 4-fold and resting muscle acetylcarnitine by 5.5-fold via DCA pre-treatment ($p < 0.05$). The authors reported that there were no differences in substrate level phosphorylation and muscle PCr and lactate contents between DCA and CON groups during 6 minutes of cycling exercise under normoxic conditions at 60% VO_2max.

At exercise intensities above ~90% VO_2max, PDC becomes activated very rapidly, i.e. within 5 seconds of contraction (Bangsbo et al., 2002); therefore an immediate supply of acetyl-CoA is provided to the TCA cycle. A Study run by Howlett et al. (1999b) demonstrated that increasing muscle acetyl group availability by sodium acetate infusion did not have any impact on non-mitochondrial ATP production at the onset of maximal intensity exercise in human volunteers. Howlett et al. (1999b) infused sodium acetate (4 mmol/kg over 60 minutes) to young healthy subjects and took muscle biopsies post-infusion following 10 seconds of exercise at 100% VO_2max. Although acetyl group content (acetyl-CoA and acetylcarnitine) was significantly higher in the sodium acetate group compared to CON, non-mitochondrial ATP production showed similar rises in both groups (similar changes in ATP, PCr, pyruvate and lactate levels). The team concluded that non-mitochondrial ATP generation at the onset of exercise at 100% VO_2max was not affected by acetyl group stockpiling.

1.1.3. Acetyl group stockpiling via sodium acetate administration

Increasing muscle acetyl group availability, either through pharmacological activation of PDC by DCA, or by oral or intravenous administration of sodium acetate, has been used in the past in multiple animal model and human volunteer studies. As outlined above, Timmons et al. (1996, 1998a, 1998b), Howlett et al. (1999a, 1999b), Evans et al. (2001) and Roberts et al. (2002, 2005) focused on the rest to exercise transition period when investigating the impact of increased acetyl group availability on muscle metabolism, whilst Akanji et al. (1989), Putman et al. (1995) and Smith et al. (2007, 2013) investigated the impact of increased acetyl group availability on muscle fuel metabolism at rest and/or during submaximal steady-state exercise.

Intravenous or oral sodium acetate administration has been used to create skeletal muscle acetyl group stockpiling independent of PDC activation (Akanji et al., 1989, Putman et al., 1995, Howlett et al., 1999b, Evans et al., 2001). Acetate converts to acetyl-CoA in a reaction catalysed by the enzyme acetyl-CoA synthetase (ACS) **(Reaction 1.3)**. Surplus acetyl-CoA combines with carnitine in the cytoplasm and converts to acetylcarnitine and CoASH by the enzyme cytoplasmic carnitine-acetyl-transferase (cCAT) **(Reaction 1.4)**. Acetylcarnitine could cross the mitochondrial membrane and convert back to acetyl-CoA in a reverse reaction catalysed by mitochondrial carnitine-acetyl-transferase (mCAT) **(Reaction 1.5)**.

Reaction 1.3 (in the cytoplasm):

Acetate —(Acetyl-CoA Synthetase)→ Acetyl-CoA

Reaction 1.4 (in the cytoplasm):

Acetyl-CoA + Carnitine —(cCAT)→ Acetylcarnitine + CoASH

Reaction 1.5 (in the mitochondrial matrix):

Acetylcarnitine + CoASH —(mCAT)→ Acetyl-CoA + Carnitine

Akanji et al. (1989) infused sodium acetate at a rate of 2.5 mmol/min over 60 minutes (150 mmol in total) to resting non-diabetic and diabetic subjects. It has been reported that plasma acetate levels are raised in patients with diabetes mellitus (Buckley and Williamson, 1977, Smith et al., 1986) and the aim of this study was to investigate the impact of raised plasma acetate levels on whole-body fat and carbohydrate metabolism (measured by indirect calorimetry) in diabetic patients and comparing it to a non-diabetic control group. Akanji et al. (1989) demonstrated that whole-body fat oxidation decreased from 0.05 ± 0.04 and 0.02 ± 0.02 g/min at baseline to 0.02 ± 0.03 and 0.01 ± 0.02 g/min post-infusion in non-diabetic and diabetic volunteers respectively ($p < 0.05$). No change was reported in the rate of whole-body carbohydrate oxidation post-infusion compared to baseline in either group. The team also reported significantly reduced concentrations of plasma fatty

acid and glycerol in both groups and concluded that acetate oxidation replaced fat oxidation and through an unknown mechanism reduced peripheral lipolysis too.

A study of Putman et al. (1995) reported an infusion of 400 mmol of sodium acetate (and similar dose of sodium bicarbonate as a control (CON) to match the alkalosis caused by sodium acetate) over 40 minutes to human volunteers, consisting of 20 minutes of rest followed immediately by 5 minutes of low intensity exercise (40% VO_2max) and 15 minutes of high intensity exercise (80% VO_2max). The aim of this study was to investigate the impact of increased muscle acetyl group availability on PDC activation at rest and during low and high intensity exercise. When considering PDCa and glycogen utilisation, there were no differences between sodium acetate and CON during exercise at low and high intensities. However the team reported that PDCa decreased from 0.37 ± 0.08 mmol/min/kg wm pre-rest to 0.16 ± 0.03 mmol/min/kg wm post-rest ($p < 0.05$) in sodium acetate volunteers, which was associated with a ~2.6-fold increase in acetyl-CoA/CoASH ratio. The authors concluded that increased acetyl-CoA/CoASH ratio had an inhibitory impact on PDC activation at rest in sodium acetate volunteers. As the same finding was not noticed during exercise, the authors suggested that exercise induced PDC activation overrode the inhibitory impact of increasing acetyl-CoA/CoASH.

Smith et al. (2007) investigated the impact of sodium acetate administration on fat and carbohydrate oxidation at rest in human volunteers. Examples of increased acetate availability in the body include following ethanol ingestion (Lundquist et al., 1962, Lundquist et al., 1973), during renal dialysis using acetate (Tolchin et al., 1977, Skutches et al., 1983) and following colonic fermentation of dietary fibre secondary

to increased fibre ingestion in the diet (Cummings, 1983, Pomare et al., 1985, Scheppach et al., 1991). The volunteers ingested sodium acetate at a dose of 2mmol/kg body mass with labelled [1,2-^{13}C] sodium acetate added and had their resting fat and carbohydrate oxidation measured 180 minutes later. The same volunteers took a similar dose of sodium bicarbonate one week apart to match for the sodium acetate-induced alkalosis. The authors demonstrated that post-ingestion fat oxidation increased significantly compared to baseline with sodium bicarbonate ($p < 0.01$) which the authors believed was secondary to alkalosis; however there were no changes in fat oxidation in the sodium acetate group despite a similar increase in energy expenditure. The authors reported that oxidation of the administered acetate in the sodium acetate group fully replaced the additional fat metabolised with the sodium bicarbonate.

The same team investigated the impact of sodium acetate ingestion on substrate utilisation at rest and during steady-state moderate intensity exercise (Smith et al., 2013). The subjects ingested 4 mmol/kg body mass of sodium acetate following an overnight fast. A similar dose of sodium bicarbonate was ingested by the same volunteers at least one week apart (the control visit). Each visit consisted of 2 phases: 90 minutes of post-ingestion resting followed by 120 minutes of exercise at 50% VO_2peak. The volunteers did not show any change in resting whole-body fat or carbohydrate oxidation rates during the control visit. However, post sodium acetate ingestion, the authors reported a decrease in the rate of resting whole-body fat oxidation compared to pre-ingestion ($p < 0.05$), which was not associated with any change in the rate of resting whole-body carbohydrate oxidation. The team also reported a lower rate of fat oxidation ($p < 0.05$) and a higher rate of carbohydrate

oxidation ($p < 0.05$) over the initial 15 minutes of exercise during the sodium acetate visit compared to control. No changes were reported in substrate utilisation between the 2 visits over the final 75 minutes of exercise. The authors concluded that acetate oxidation replaced fat as a substrate at rest and at the onset of steady-state moderate intensity exercise.

1.2. Carnitine

1.2.1. Background

Russian scientists Gulewitsch and Krimberg and German scientist Kutscher discovered carnitine simultaneously in muscle tissue in 1905. The name carnitine is derived from 'Carnus', the Latin term for 'flesh' as it was isolated originally from muscle extracts. The chemical structure of carnitine was established as 3-hydroxy-4-N,N,N-trimethylaminobutyric acid by Tomia and Sendju in 1927. Carnitine has two isoforms: L-carnitine and D-carnitine. L-carnitine is the active isoform whilst D-carnitine is biologically inactive (Bieber, 1988).

Carnitine is both supplied exogenously through the daily diet and biosynthesised endogenously *de novo* from the essential amino acids lysine and methionine. Vaz and Wanders (2002) suggested that around 75% of daily carnitine requirements come from dietary intake whilst the remaining 25% is produced inside the body.

Food from animal sources is the main provider of dietary carnitine, although lesser amounts are found in grains, fruits and vegetables as well (Steiber et al., 2004).

Vegetarians, compared to omnivores, have lower plasma and urine carnitine concentrations (Lombard et al., 1989) and diminished capacity to transfer carnitine into muscle tissue (Stephens et al., 2011). Human body also has the ability to produce carnitine endogenously. This happens mainly in the hepatic and renal cells from the precursor essential amino acids lysine and methionine. In a chain of reactions catalysed by the vitamins B2, B6 and C and iron (Steiber et al., 2004), the two amino acids convert initially to 6-N-trimethyllysine and then to gamma-butyrobetaine (Vaz and Wanders, 2002). Gamma-butyrobetaine in turn converts to carnitine in a reaction catalysed by gamma-butyrobetaine hydroxylase (Borum, 1983).

Skeletal muscle is where the vast majority of carnitine (more than 95%) is stored, either as free carnitine or acylcarnitine (Brass, 1995). The rest is stored in liver and kidneys with a very small amount in all other tissues (Engel and Rebouche, 1984). Considering the bulk of the skeletal muscle in the body, its remarkable energy requirements and its role in energy production during locomotion and physical activity, it is no wonder that the majority of carnitine is stored in skeletal muscle tissue. It is believed that cellular carnitine pool resides mainly in the cytoplasm (Zammit, 1999).

1.2.2. Physiological functions of carnitine in skeletal muscles

Carnitine plays two important roles in skeletal muscle cells (see review by Stephens et al., 2007a) **(Fig.1.3)**. Its first role is to facilitate the transfer of long chain fatty acids as acylcarnitine esters across the mitochondrial membrane (Fritz, 1955, Fritz

and Mc, 1959b, Bremer, 1962b, Bremer, 1962c, Fritz and Yue, 1963, Fritz and Marquis, 1965). Whilst inside the mitochondrial matrix, the fatty acyl-CoA molecules go through a series of chemical reactions called β-oxidation to produce acetyl-CoA which in turn enters the TCA cycle. This is the main function of carnitine at rest and during low intensity exercise (Stephens et al., 2006a, Wall et al., 2011).

Its second role is buffering excess acetyl-CoA in the states of high PDC flux (Friedman and Fraenkel, 1955, Childress and Sacktor, 1966, Childress et al., 1967, Constantin-Teodosiu et al., 1991a, Constantin-Teodosiu et al., 1992). This function helps to keep the balance between mitochondrial acetyl-CoA and CoASH (acetylCoA/CoASH ratio) by shuttling the acetyl groups out of the mitochondria; thereby preventing the inhibitory impact of acetyl-CoA on PDC and playing an important role in fuel-sensing and glucose metabolism when glycolytic flux in enhanced. This reaction also produces CoASH, which is needed in the formation of fatty acyl-CoA derivatives, the conversion of pyruvate to acetyl-CoA and a couple of chemical reactions in the TCA cycle which will be discussed in due course. This is the main function of carnitine during high intensity exercise (Wall et al., 2011).

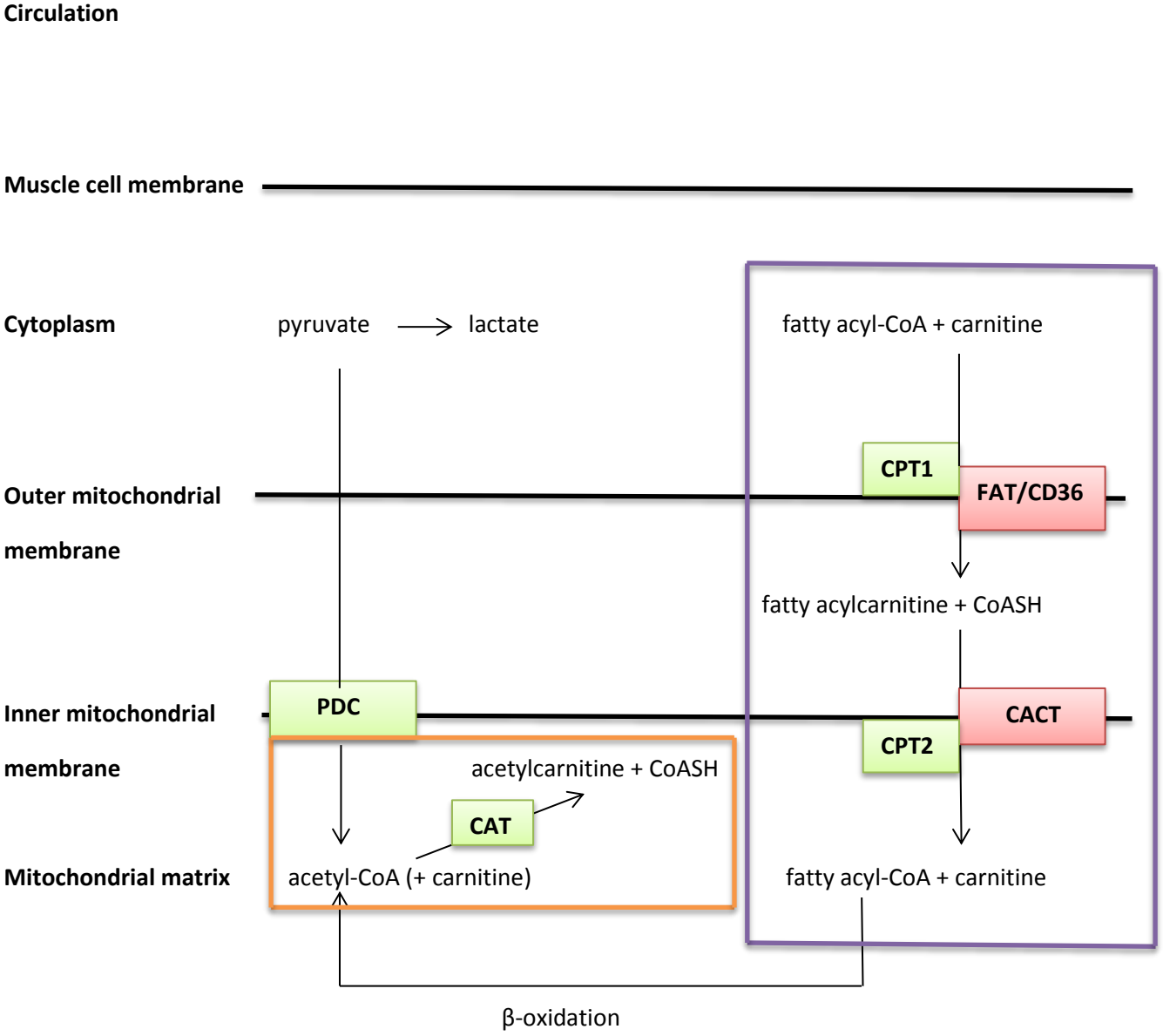

Circulation
Muscle cell membrane
Cytoplasm
pyruvate
lactate
fatty acyl-CoA + carnitine
CPT1
FAT/CD36
Outer mitochondrial
membrane
fatty acylcarnitine + CoASH
PDC
CACT
Inner mitochondrial
membrane
CPT2
acetylcarnitine + CoASH
CAT
Mitochondrial matrix
acetyl-CoA (+ carnitine)
fatty acyl-CoA + carnitine
β-oxidation

Fig.1.3. Physiological functions of carnitine in skeletal muscle cells. Red boxes represent transporters and green boxes represent enzymes. The box with purple border represents the first function of carnitine, i.e. translocation of fatty acyl-CoA derivatives across the mitochondrial membrane and the box with orange border represents the second function of carnitine, i.e. buffering excess acetyl-CoA molecules.

1.2.2.1. Transfer of long chain fatty acids across the mitochondrial membrane

Fatty acids convert to fatty acyl-CoA molecules in the cytoplasm. The oxidation of fatty acyl-CoA molecules, called β-oxidation, happens inside the mitochondria. Therefore there should be an intracellular mechanism in operation transporting fatty acyl-CoA molecules from the site of their biosynthesis in the cytoplasm, across the mitochondrial membrane into the mitochondrial matrix for β-oxidation.

The link between carnitine and fat oxidation was first discovered by the late Irving Fritz and his colleagues in rat tissues *in vitro*. Fritz I (1955) noticed that the oxidation of long chain fatty acids increased in hepatic slices of rats by adding rat muscle homogenate extracts. Fritz suggested that the carnitine available in muscle extracts was the active chemical responsible for the increase in fatty acid metabolism. In a series of studies from 1957 to 1959 Fritz and his colleagues demonstrated the same stimulatory effect of carnitine on the metabolism of long chain fatty acids *in vitro* in several other rat tissues, including heart homogenates (Fritz, 1957, Fritz, 1959a, Fritz, 1959b, Fritz and Mc, 1959a). Research done by Jon Bremer showed that synthesised fatty acyl carnitines were metabolised in the incubated mitochondria *in vitro* in different tissues of rats (liver, brain, testis, kidney and heart) and suggested that carnitine had a 'acyl-carrying function' through the mitochondrial membrane (Bremer, 1962a, Bremer, 1962b, Bremer, 1962c). Fritz and Yue (1963) suggested that carnitine was essential in long chain fatty acid translocation across the mitochondrial membrane and Fritz and Marquis (1965) reported that heart muscle mitochondria in rats were only permeable to long chain fatty acyl carnitine and impermeable to long chain fatty acyl CoA derivatives. The authors also reported

that mitochondrial-bound carnitine palmityltransferase (CPT) acted both as a transferase and a translocase of fatty acyl carnitine derivatives into the mitochondria, although the authors did not manage to locate it (Fritz and Marquis, 1965).

1.2.2.1.1. Cellular and sub-cellular physiology

Long chain acyl-CoA molecules should be transported from the cytoplasm into the mitochondria in order to become metabolised in a series of chemical reactions named β-oxidation. The translocation of long chain fatty acyl-CoA molecules across the two membranes of mitochondria, i.e. the outer and the inner membranes, is accomplished by carnitine through two enzymatic reactions (catalysed by the enzymes carnitine palmitoyltransferase 1 and 2) and two transporters (carnitine acylcarnitine translocase and fatty acid translocase FAT/CD36) (for a review see Stephens et al. 2007a).

1.2.2.1.2. Enzymes

CPT1: Carnitine palmitoyltransferase 1 is a protein enzyme located on the outer mitochondrial membrane. Murthy and Pande (1987) used malonyl-CoA, an inhibitor of CPT1, to accurately locate the activity of the enzyme in mitochondria from rat hepatic and cardiac tissues. The authors reported that malonyl-CoA bound to and inhibited CPT1 on the outer aspect of the outer mitochondrial membrane whilst the

CPT1 activity *per se* resided on the inner aspect of the outer mitochondrial membrane. The K_m of CPT1 for human skeletal muscle free carnitine has been reported to be 0.48 mM (McGarry et al., 1983, McGarry et al., 1989). It was discussed earlier that the main location of cellular carnitine pool is cytoplasm (Zammit, 1999). Considering the fact that CPT1 activity resides on the inner aspect of outer mitochondrial membrane and that the cellular carnitine pool is mainly cytoplasmic, it is believed that free carnitine availability to CPT1 is lower than free carnitine measurements in skeletal muscle biopsy samples. This has led some to hypothesise the concept of compartmentalization of skeletal muscle carnitine inside the cells (Stephens et al., 2006a).

CPT2: Carnitine palmitoyltransferase 2 is a protein enzyme located on the inner mitochondrial membrane. Using detergents, antibodies generated to purified carnitine palmitoyltransferase, western blotting and immunodetection of proteins in rat hepatic tissue, Woeltje et al. (1987) reported that CPT2 was an immunologically distinct protein compared to CPT1 and that it resided on the inner aspect of the inner mitochondrial membrane.

1.2.2.1.3. *Transporters*

FAT/CD36: Fatty acid translocase FAT/CD36A is a transport protein located on the outer mitochondrial membrane translocating long chain fatty acylcarnitine

molecules across the outer mitochondrial membrane. Bezaire et al. (2006) used an inhibitor of FAT/CD36 (sulfo-N-succimidyl-oleate) on mitochondria from human skeletal muscle samples and reported that the oxidation of long chain fatty acids (palmitate) declined significantly, whilst the oxidation of medium chain fatty acids (oleate) was unaffected. The authors did not notice any changes in the activity of CPT1 whilst FAT/CD36 was inhibited and concluded that FAT/CD36 was functioning downstream of CPT1 and was likely involved in the transfer of long chain fatty acids from CPT1 to CACT across the outer mitochondrial membrane.

CACT: Carnitine acylcarnitine translocase is a transport protein located on the inner mitochondrial membrane (Ramsay and Tubbs, 1975, Indiveri et al., 1990, Indiveri and Palmieri, 1990) translocating fatty acylcarnitine into the mitochondrial matrix in exchange for free carnitine moving out of the mitochondrial matrix into the cytoplasm (Pande, 1975).

1.2.2.1.4 First enzymatic reaction

Long chain fatty acyl-CoA converts to long chain fatty acylcarnitine and CoASH in a reversible esterification reaction with carnitine, catalysed by CPT1 **(Reaction 1.6)**. It is long chain fatty acylcarnitine that can get transferred across the mitochondrial membrane into the mitochondrial matrix (Fritz and Marquis, 1965). The mitochondrial membrane is impermeable to fatty acyl-CoA molecules.

Reaction 1.6 (In cytoplasm):

carnitine + long chain fatty acyl-CoA $\xrightarrow{\text{CPT1}}$ long chain fatty acylcarnitine + CoASH

1.2.2.1.5. Second enzymatic reaction

Inside the mitochondrial matrix, long chain fatty acylcarnitine combines with CoASH and converts back to long chain fatty acyl-CoA and free carnitine **(Reaction 1.7)**, catalysed by carnitine-palmitoyl-transferase 2 (CPT2).

Reaction 1.7 (In mitochondrial matrix):

long chain fatty acylcarnitine + CoASH $\xrightarrow{\text{CPT2}}$ long chain fatty acyl-CoA + carnitine

1.2.2.2. Acetyl group buffering

Friedman and Fraenkel (1955) discovered the enzyme carnitine acetyltransferase (CAT) in the hepatic extracts from pigeon and sheep and noticed that it catalysed the reversible transfer of acyl groups between carnitine and coenzyme A. Childress and Sacktor (1966) noticed that at the onset of flight, acetylcarnitine content increased by 4-fold in the flight muscles of blowflies (Phormia Regina) which was associated with a sharp and significant increase in PDC activity. This was despite the fact that blowflies did not oxidise fat during flight.

Harris et al. (1987) reported that free carnitine decreased and acetylcarnitine increased significantly in human skeletal muscle tissue after 4 minutes of high intensity exercise at 90% VO_2max. Total carnitine content at baseline was 20 mmol/kg dm of which 19% was acetylcarnitine and 77% was free carnitine. Following 4 minutes of high intensity exercise, there was a 6.2 mmol/kg dm increase in acetylcarnitine content associated with a 7.9 mmol/kg dm decline in free carnitine content. Total carnitine content was unaffected though. The authors reported that carnitine buffered excess acetyl groups whilst PDC activity was accelerated.

A study by Sahlin et al. (1990) involved exercising human volunteers for 10 minutes at 40% and 75% VO_2max followed by exercise to exhaustion at 100% VO_2max for about 4 minutes. The team reported that skeletal muscle pyruvate, free carnitine and acetylcarnitine concentrations did not change during low intensity exercise; however at higher intensities muscle free carnitine concentration decreased significantly whilst pyruvate and acetylcarnitine concentrations increased significantly. The simultaneous increase in muscle pyruvate and acetylcarnitine concentrations at high intensity exercise suggested that carnitine functioned as a sink for acetyl-CoA; however the fact that no changes were noticed at low intensity exercise, suggested that TCA cycle could handle the acetyl-CoA supply in a state of low PDC flux.

The role of carnitine in buffering excess acetyl groups at high intensity exercise was further supported by multiple metabolic studies done by Constantin-Teodosiu (1991a, 1992, 1996). A study by Constantin-Teodosiu (1991a) involved exercising

human volunteers on a bike at 30%, 60% and 90% VO_2max, each period lasting 3 to 4 minutes. There was a significant increase in PDC activity, acetyl-CoA and acetylcarnitine contents with increasing exercise workload associated with a remarkable decrease in free carnitine and CoASH in human skeletal muscle samples. The team reported that for 1 accumulated mole of acetyl-CoA, there were roughly 500 moles of acetyl groups binding to carnitine. The authors also proposed that carnitine buffered excess acetyl groups in the event of upgraded PDC activity and for the first time calculated the amount of acetylcarnitine produced in relation to the amount of acetyl-CoA accumulated.

A year later in 1992, Constantin-Teodosiu et al. cycled human volunteers to exhaustion at 75% VO_2max. The team reported that PDC was fully active after 3 minutes of exercise and remained active during the exercise period. Acetyl-CoA content increased significantly from 8.4 ± 1.0 at baseline to 31.6 ± 3.3 µmol/kg dm at exhaustion with a parallel significant increase in acetylcarnitine content from 2.9 ± 0.7 at baseline to 15.6 ± 1.6 mmol/kg dm at exhaustion. At the same time CoASH decreased significantly from 45.3 ± 3.1 to 25.9 ± 3.1 µmol/kg dm paralleled with a significant decline in free carnitine from 18.8 ± 0.7 to 5.7 ± 0.5 mmol/kg dm. The increased acetyl-CoA/CoASH ratio did not impact the PDC activation status, although the glycogen content declined by 90%. The authors attributed the changes to the buffering role of carnitine which guaranteed that PDC flux continued.

Constantin-Teodosiu et al. (1996) reported that the role of carnitine in buffering excess acetyl groups was more pronounced in type 1 than type 2 skeletal muscle fibres. Free carnitine content was significantly lower ($p < 0.001$) and acetylcarnitine

content significantly higher ($p < 0.005$) in type 1 fibres compared to type 2 following exhaustive exercise at 75% VO_2max. The authors proposed that the discrepancy in carnitine and acetylcarnitine contents between type 1 and type 2 muscle fibres following high intensity exercise was due to higher number of mitochondria in type 1 fibres.

Therefore during a state of high PDC flux, surplus acetyl-CoA combines with carnitine to form acetyl-carnitine and CoASH **(Reaction 1.8) (Fig. 1.1** and **1.3)**. This reaction is catalysed by the enzyme CAT (carnitine acetyl-transferase) and ensures that the PDC flux continues and that there is enough CoASH available for pyruvate decarboxylation by PDC **(Reaction 1.1)**, the synthesis of fatty acyl-CoA molecules **(Reaction 1.9)**, and a couple of reactions in the TCA cycle **(Reactions 1.10 and 1.11)**.

Reaction 1.8 (In the mitochondrial matrix):

Acetyl CoA + carnitine $\xrightarrow{\text{CAT}}$ acetylcarnitine + CoASH

CoASH is needed in the following reactions:

Reaction 1.9: The conversion of fatty acids to fatty acyl-CoA in the cytoplasm:

Fatty acid + CoASH $\xrightarrow{\text{Fatty acyl-CoA synthase}}$ fatty acyl-CoA

Reaction 1.10: The conversion of α-ketogluterate to succinyl-CoA in the TCA cycle inside the mitochondria:

α-ketogluterate + NAD^+ + CoASH $\xrightarrow{\text{α-ketogluterate dehydrogenase}}$ succinyl-CoA + NADH + H^+

Reaction 1.11: The conversion of β-ketoacyl-CoA to acyl-CoA in the TCA cycle inside the mitochondria:

β-ketoacyl-CoA + CoASH $\xrightarrow{\text{β-ketoacyl-CoA thiolase}}$ acyl-CoA + acetyl-CoA

1.2.3. Increasing muscle carnitine content

Increasing carnitine content in the body and investigating its impact on fuel metabolism has been of interest to scientists in the past few decades. Part of this interest stems from the fact that carnitine facilitates the translocation of fatty acids across the mitochondrial membrane for β-oxidation (Fritz, 1955, Fritz and McEwen, 1959, Bremer, 1962a, Bremer, 1962b, Fritz and Yue, 1963, Fritz and Marquis, 1965) and therefore increased carnitine content in the body could potentially enhance fat oxidation. In fact commercial carnitine supplements have been sold worldwide for a long time as an aide to weight loss and improved exercise performance.

Barnett et al. (1994) fed 4 g/day carnitine to human volunteers for 14 days and noticed no change in skeletal muscle carnitine content post-feed compared to pre-feed. Vukovich et al. (1994) used a higher dose (6 g/day) for 14 days and similarly

the muscle carnitine content remained unaffected after the 2 week supplementation period. Brass et al. (1994) infused carnitine at a dose of 185 mμmol/kg over 2 hours to human volunteers and noticed no differences in the muscle carnitine content post-infusion compared to baseline. As there was a dramatic rise in plasma carnitine level, the authors suggested that the carnitine pool in human skeletal muscle tissue was segregated from the carnitine pool in plasma. Wächter et al. (2002) fed human volunteers 2 g of carnitine twice a day for 3 months and noticed no change in muscle carnitine content immediately after and two months following the 3 month supplementation period.

It was demonstrated by Steenge et al. (1998) that insulin had a stimulatory effect on skeletal muscle creatine (Cr) accumulation in human volunteers. This study also showed that this stimulatory effect happened only when serum insulin concentration was increased to ~100 mU/l and above. In this study, 12.4 g of creatine was administered to human volunteers alongside a 300-min euglycaemic insulin clamp on four separate occasions involving four different insulin infusion rates: 5, 30, 55, 105 mU/m^2/min. The authors reported a significant increase in skeletal muscle creatine content ($p < 0.05$), which only happened at the insulin infusion rates of 55 and 105 mU/m^2/min. (i.e. no significant muscle creatine accumulation was observed at insulin infusion rates of 5 and 30 mU/m^2/min). The authors concluded that insulin stimulated muscle creatine accumulation only when present at high (~100 mU/l) or supra-physiological (~200 mU/l) concentrations. Skeletal muscle creatine transport happens through a Na^+-dependent membrane transporter protein against a concentration gradient (Loike et al., 1986, Wallimann et al., 1992, Sora et al., 1994, Odoom et al., 1996). It is believed that the stimulatory

impact of insulin on Na^+-dependent muscle creatine accumulation occurs via Na^+/K^+ ATPase pump activation (Clausen and Kohn, 1977, Hundal et al., 1992, Marette et al., 1993, Odoom et al., 1996). This study highlighted the role of insulin and its interaction with the Na^+/K^+ ATPase pump in facilitating trans-cellular transport of molecules via Na^+-dependent membrane protein channels.

Stephens et al. (2006b) demonstrated for the first time that a state of hyperinsulinaemia was needed alongside a state of hypercarnitinaemia to facilitate carnitine uptake into the skeletal muscle cells, resulting in acutely elevated skeletal muscle carnitine pool. The authors reported that increased plasma carnitine concentration (~500 μmol/L) following 5 hours of iv carnitine infusion in human volunteers increased muscle total carnitine content from 22.0 ± 0.9 at baseline to 24.7 ± 1.4 mmol/kg dm post-infusion ($p < 0.05$) only when plasma insulin concentration was increased as well (to 149.2 ± 6.9 mIU/L), using an insulin clamp technique. The authors also reported a significant 2.3-fold increase in the mRNA expression of OCTN2, responsible for carnitine transport into the skeletal muscle cells. No difference in muscle carnitine content was noted when plasma insulin remained at physiological level. The team reported that insulin played a role in carnitine uptake into the cells and that this was achieved by via OCTN2 receptor. This is consistent with the hypothesis that insulin stimulates Na^+/K^+ ATPase pump activity which in turn activates OCTN2, causing carnitine and Na^+ uptake into muscle cells. This will be discussed in details in due course.

A subsequent study by the same group (Stephens et al., 2007b) reported that there was a threshold for the stimulatory impact of insulin on carnitine uptake by muscle

cells. The team created a state of hypercarnitinaemia by infusing 15 mg/kg bolus intravenous carnitine, followed by 10 mg/kg/h over 5 hours. By using simultaneous insulin clamps of 5, 30, 55 and 105 mU/m^2/min, the team reported that plasma total carnitine concentration was significantly lower at the end of 55 and 105 mU/m^2/min clamps compared to 5 and 30 mU/m^2/min clamps. The authors proposed that high serum insulin concentration is needed to stimulate carnitine uptake into skeletal muscle cells and to increase muscle total carnitine content. The team calculated this serum insulin concentration threshold to be ≥ 90 mU/L.

Increasing muscle carnitine content acutely following iv administration of carnitine and insulin would not be possible in the everyday setting. Although infusion studies were successful in acutely increasing muscle carnitine content (Stephens et al., 2006b, 2007b), there was no evidence available in literature on whether oral feeding strategy could work and whether it could be effective more chronically than the infusion studies. Following successful parenteral attempts in our unit in increasing carnitine retention in human volunteers, Stephens et al. (2007c) investigated the impact of oral carbohydrate intake (in order to stimulate insulin secretion) combined with carnitine ingestion on skeletal muscle carnitine pool. The team fed 3 g/day of carnitine plus either 4 × 500 ml/day solutions, each containing 94 g CHO (to the CHO group) or 4 × 500 ml/day solutions of flavoured water (in control group). Following 2 weeks of feed, the CHO group showed a significant increase in the area under the curve (AUC) of serum insulin ($p < 0.001$), significant decreases in total carnitine concentration in plasma ($p < 0.05$) and 24 hour total carnitine excretion in urine ($p < 0.05$) compared to the control group. The same group of volunteers took part in another study when they were fed the same dose

of carnitine plus either 2 × 500 ml/day solutions, each containing 94 g CHO (to the CHO group) or 2 × 500 ml/day solutions of flavoured water (in control group) for 2 weeks. The urinary total carnitine concentration was lower in the CHO group, compared to the control group ($p < 0.001$), following the 2 week period of the study. This was the first study to show that oral ingestion of carnitine combined with carbohydrate could increase the retention of carnitine in the skeletal muscles. This again pointed to the fact that hyperinsulinaemia was required in addition to hypercarnitinaemia in order to increase skeletal muscle carnitine accumulation. Based on the findings of this study, the authors reported that skeletal muscle total carnitine content could be increased by chronic oral intake of carnitine and carbohydrate (to stimulate insulin secretion). The team calculated that combined carnitine and carbohydrate oral feed would increase muscle carnitine content by a further 0.1 % per day compared to carnitine only feed. This would be equivalent to 10% increase in muscle carnitine content following 100 days of combined carnitine and carbohydrate oral feed, which the authors believed would have a significant impact on muscle fuel selection and metabolism. In line with this hypothesis, Wall et al. (2011) demonstrated that ingesting 80 g of carbohydrate plus 2 g of L-carnitine L-tartrate twice a day for 24 weeks managed to increase muscle total carnitine content by 21% compared to baseline ($p < 0.05$) and 30% compared to control ($p < 0.05$) (Wall et al., 2011). Elevated skeletal muscle carnitine content had a significant impact on muscle fuel selection and metabolism which will be discussed later in this chapter.

1.2.4. The role of Insulin in increasing carnitine uptake into muscle cells

It was outlined earlier that muscle cells store the vast majority of carnitine in the body (Brass, 1995) but are not able to biosynthesise it. It can therefore be deciphered that there is a very efficient system in place in charge of transporting carnitine molecules actively across the muscle cell membrane against a high gradient from the extracellular fluid to the cytoplasm.

Rebouche CJ (1977) was the first to suggest that an active carrier system existed in the extensor digitorum muscle in rats responsible for transferring carnitine into the muscle cells against a significant gradient. He also suggested that the transport system was only responsible for transferring carnitine into the muscle cells and not out of the cells, i.e. it is a unidirectional active transport system. This carrier transport system was identified by Tamai et al. (1998) to be organic cation transporter 2 (OCTN2) **(Fig. 1.4)**. It is a protein located on the skeletal muscle cell membrane and functions as a 1:1 co-transporter of carnitine and Na^+ ions into the muscle cells. The OCTN2 gene (SLC22A5) was later found to be located on human chromosome 5q31 (Lamhonwah and Tein, 1999).

The function of OCTN2 is closely linked with Na^+/K^+ ATPase pump (**Fig. 1.4**), discovered in 1957 by Nobel Prize winning scientist Skou. This pump consists of two α (catalytic) and two β (glycoprotein) subunits and hydrolyses one molecule of ATP to provide the required energy for transporting three Na^+ ions outside in exchange for two K^+ ions inside the cell; hence providing an electro-chemical gradient for Na^+ and K^+ ions across the cell membrane. The Na^+ electro-chemical gradient is needed

for the function of OCTN2 in co-transporting Na^+ and carnitine into the cells (Ohashi et al., 1999).

Mutations in the OCTN2 gene, causing dysfunction of the protein carrier, result in a disease called 'primary systemic carnitine deficiency' (Nezu et al., 1999). People suffering with this autosomal recessive disorder have a remarkable 85% reduction in muscle carnitine content (Engel and Angelini, 1973) and develop cardiomyopathy, skeletal myopathy and hypoglycaemia (Nezu et al., 1999, Tein, 2003).

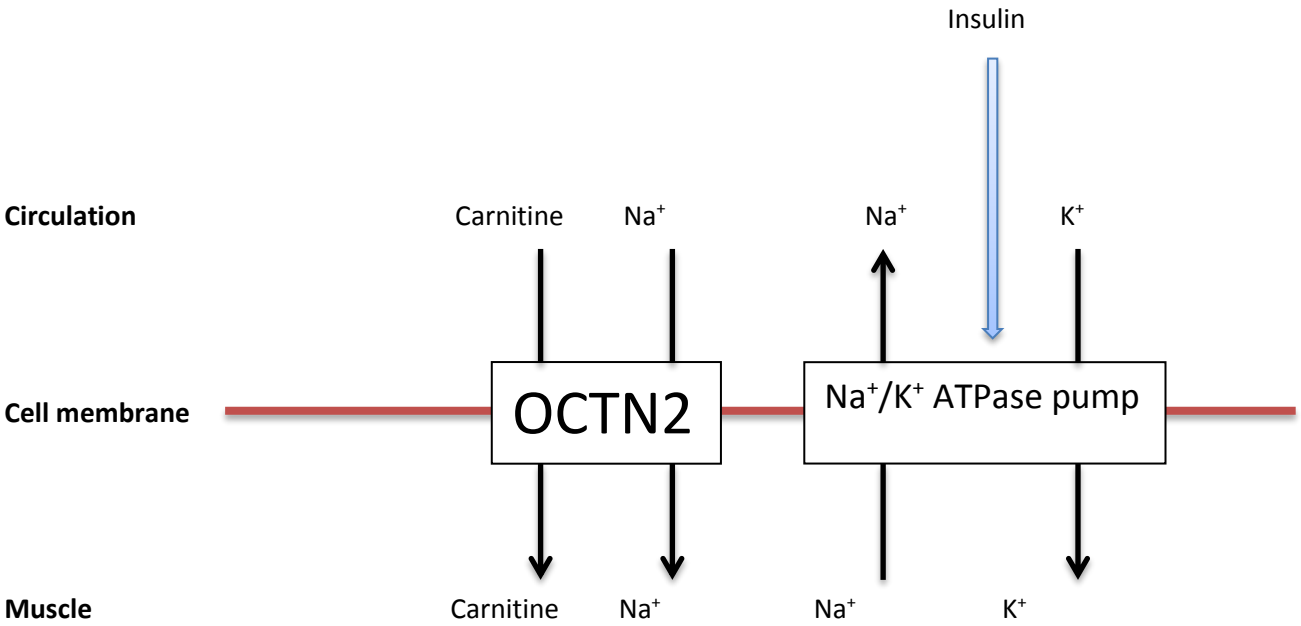


Fig. 1.4. Carnitine transport into the muscle cells and the interaction between OCTN2 and Na^+/K^+ pump. Insulin has a stimulatory effect on the Na^+/K^+ pump.

Muscle contraction has been shown to exert a stimulatory effect on carnitine uptake into the muscle cells in animal models (Furuichi et al., 2012). Muscle contraction causes translocation of GLUT4 and FAT/CD36 to the muscle cell membrane, resulting in increased uptake of glucose and fatty acids respectively (Bonen et al., 2000). Furuichi et al. (2012) hypothesised that a similar mechanism, i.e. translocation of carnitine transporter to the muscle cell membrane secondary to muscle contraction would increase muscle carnitine uptake. The team demonstrated that electrical contraction of the rat hind limb muscles *in vivo* caused a ~50% increase in skeletal muscle uptake clearance of l-[(3)H]carnitine compared to contralateral resting hind limb muscles ($p < 0.05$). Using immunostaining and western blotting techniques, the team demonstrated an associated increase in OCTN2 signal in the muscle cell membrane following electrical contraction compared to the contralateral non-exercising muscles ($p < 0.05$). The team concluded that, at least in animal models, muscle contraction/exercise has a positive stimulatory effect on carnitine uptake into the muscle cells which is exerted through enhanced OCTN2 translocation to the cell membrane.

Insulin too has a stimulatory effect on carnitine uptake into the muscle cells (Ewart and Klip, 1995, Sweeney and Klip, 1998, Stephens et al., 2006b). As outlined above, enhanced Na^+/K^+ pump activity brings about an increase in Na^+ gradient across the cell membrane (with higher concentration outside the cell), facilitating the co-transport of Na^+ and carnitine into the cells via OCTN2. Elevation of intracellular Na^+, translocation of the pump subunits to the plasma membrane and enhanced sensitivity of the pump to Na^+ have been proposed as possible mechanisms underlying the stimulatory impact of insulin on the Na^+/K^+ ATPase pump:

A. Some studies have suggested that an insulin-induced increase in intracellular Na^+, via hormonal activation of Na^+/H^+ exchanger pumping Na^+ in and H^+ out, could be responsible for augmented Na^+/K^+ pump activity (Fehlmann et al., 1981a, Fehlmann et al., 1981b, Fehlmann and Freychet, 1981, Rosic et al., 1985). Although Brodsky J.L. (1990) suggested that insulin-induced increased intracellular Na^+ level happened through the action of an insulin-sensitive Na^+ conductive channel.

B. Insulin-induced translocation of Na^+/K^+ ATPase pump subunits to the plasma membrane has been reported as another mechanism responsible for hormonal activation of the pump (Grinstein and Erlij, 1974, Gavryck et al., 1975, Lytton, 1985a, Lytton, 1985b, Lytton et al., 1985). As discussed earlier, the Na^+/K^+ ATPase pump consists of two α and two β subunits. The translocation of the subunits α_2 and β_1 from the cytoplasm to the cell membrane in rat muscle tissue is enhanced by insulin, increasing in turn the activity of the pump (Hundal et al., 1992).

C. There are also studies suggesting that the Na^+/K^+ ATPase pump sensitivity to intracellular Na^+ ions increases by insulin, in turn upgrading the activity of the pump (Clausen and Everts, 1989, Clausen and Nielsen, 1994, Clausen, 1996). McGill et al. (1991) demonstrated that the increased insulin-induced sensitivity of the pump in the rat adipocyte tissue was due to an enhanced affinity of α_1 and α_2 subunits to Na^+ (McGill and Guidotti, 1991, McGill, 1991).

It can be concluded that the underlying mechanisms for the excitatory effect of insulin on the Na^+/K^+ pump are varied and complex and applied at different levels.

At molecular level, OCTN2 mRNA expression upgrades following chronic carnitine and CHO supplementation. Stephens et al. (2013) demonstrated increased abundance of skeletal muscle OCTN2 mRNA expression after 12 weeks of twice daily supplementation of 2 g of L-carnitine L-tartrate and 80 g of carbohydrate. The authors reported that 20% increase in skeletal muscle carnitine content ($p < 0.05$ post-supplementation compared to baseline) was associated with enhanced expression of OCTN2 (SLC22A5) mRNA. Whilst relative OCTN2 (SLC22A5) mRNA abundance in the control group was 0.88 ± 0.08 post-supplementation compared to baseline, it was 1.36 ± 0.17 in the carnitine group (< 5% FDR CARN vs. CON).

1.3. Integration of muscle fuel use at rest and during steady–state exercise

In order to understand the impact of skeletal muscle carnitine on fuel selection and oxidation, we need first to fully comprehend the integration of muscle fuel metabolism and selection at rest and during exercise.

1.3.1. Fatty acid and glucose uptake into muscle cells

Fatty acid transport proteins (FATPs) are responsible for facilitated transport of lipids into the cells. In muscle cells this is mainly done by FATP1 (Schaffer and Lodish, 1994, Abumrad et al., 1999, Bonen et al., 1999). Whilst inside the cell the lipids either get stored in lipid droplets called IMCL (intramyocellular lipid); or

convert to signalling and structural molecules (sphingolipids, eicosanoids, phospholipids, ceramide, DAG, etc); or convert to fatty acyl-CoA and get transferred into the mitochondria for β-oxidation. Research by Stahl et al. (2002) and Fisher RM et al. (2005) reported that insulin regulates fatty acid uptake by stimulating FATP1 translocation from cytoplasm to plasma membrane.

Using a euglycaemic insulin clamp technique, hepatic and femoral venous blood sampling and endogenous glucose production measurement, DeFronzo et al. (1985) calculated that 90% of total body glucose uptake was accomplished by the muscles. Glucose crosses the muscle cell membrane via GLUT4. Inside the cell it gets phosphorylated by hexokinase (HK) to glucose-6-phosphate (G6P). It then either converts to glycogen and gets stored in the cell (via glycogen synthase) or enters the glycolytic pathway. Research by Woerle et al. (2003), using direct ^{13}C MRS measurements demonstrated that after a standard meal normal healthy volunteers had nearly 30% of the ingested glucose stored in the muscle cells as glycogen.

1.3.2. Glucose-fatty acid cycle (Randle cycle)

The idea of a 'glucose-fatty acid cycle' was proposed by Philip Randle (1963) and his colleagues; to be named later in his honour, the 'Randle cycle'. Early studies by Randle et al. (1964) on rat cardiac and diaphragmatic muscle tissues demonstrated that increased fatty acid oxidation caused a direct decrease in glycolysis, independent of any hormones. Randle suggested that glucose and fatty acids competed for their oxidation in muscle tissue and that the oxidation of one nutrient had a direct inhibitory impact on the other. Randle suggested that increased fat

oxidation caused increased levels of acetyl-CoA which in turn converted to and increased citrate level **(Reaction 1.12)**. Acetyl-CoA has a direct inhibitory effect on PDC activity (discussed earlier in this chapter) whilst citrate crosses the mitochondrial membrane, enters the cytoplasm and inhibits PFK **(Reaction 1.13)** resulting in increased levels of G6P. This in turn inhibits HK **(Reaction 1.14)** and decreases glucose entry into the cell. The inhibitory impact of increased fatty acid oxidation on glycolysis as suggested originally by Randle et al. (1963, 1964) is depicted in **Fig. 1.5**.

Reaction 1.12:

Acetyl-CoA + oxaloacetate + H2O $\xrightarrow{\text{citrate synthase}}$ citrate + CoASH

Reaction 1.13: The conversion of F-6-P to F-1-6-P by PFK

Fructose-6-phosphate + ATP $\xrightarrow{\text{PFK}}$ fructose-1-6-bisphosphate + ADP

↑ Inhibited by citrate

Reaction 1.14: Glucose transport into the cells and its phosphorylation by HK

Glucose + ATP $\xrightarrow{\text{HK}}$ Glucose-6-phosphate + ADP

↑ Inhibited by increased G-6-P level

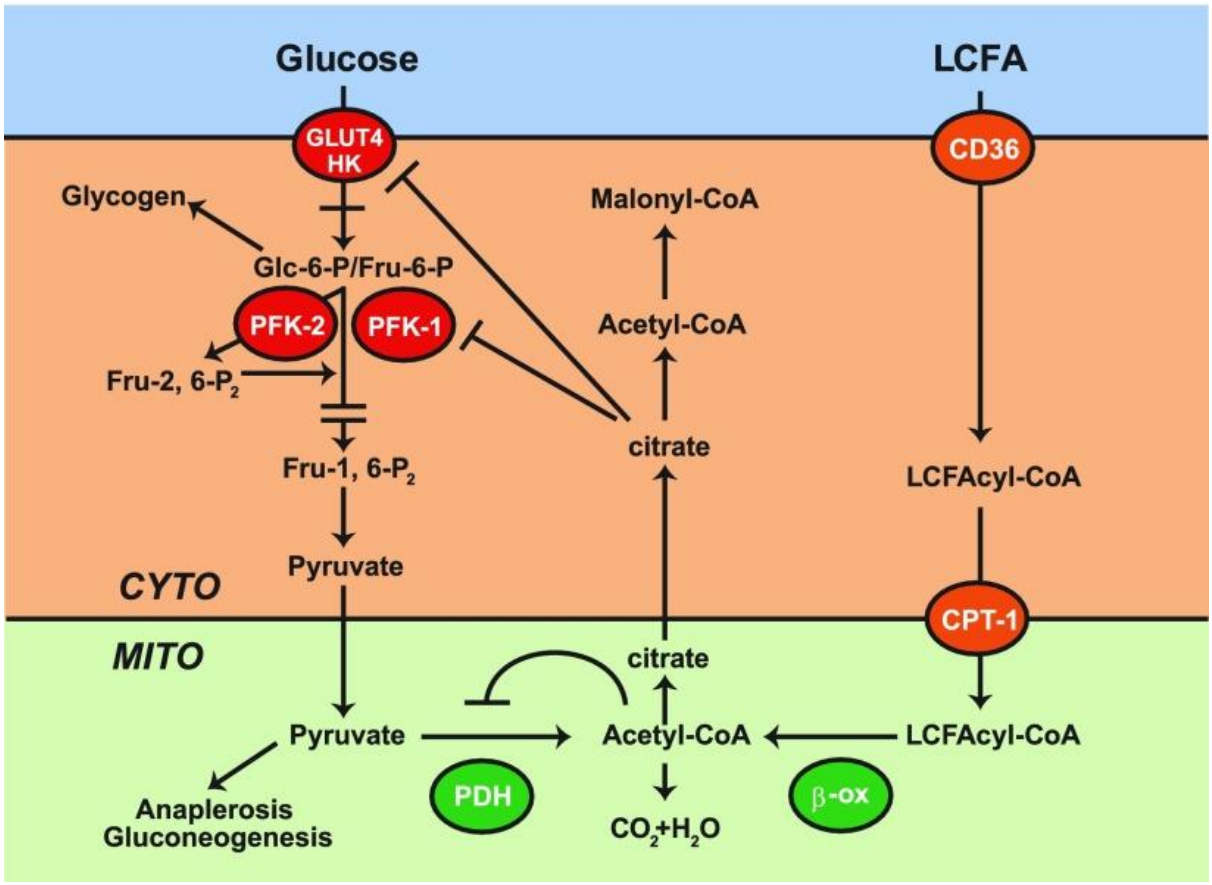
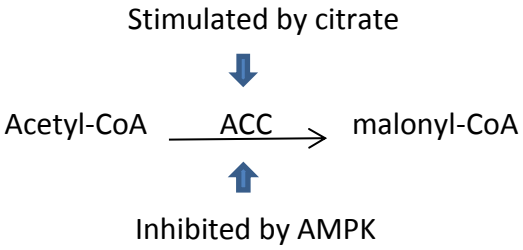


Fig. 1.5. The Randle cycle (adapted with permission from Hue and Taegtmeyer, 2009)

1.3.3. Malonyl-CoA hypothesis

Randle et al. did not describe the molecular events responsible for the inhibitory effect of increased glucose metabolism on fat oxidation; however McGarry et al. (1977) reported that malonyl-CoA had an inhibitory impact on hepatic oleic acid oxidation and ketogenesis in rats *in vitro* and suggested that this inhibitory effect was exerted via CPT1 suppression. Malonyl-CoA is produced from acetyl-CoA catalysed by the enzyme acetyl-CoA carboxylase (ACC), itself under the regulatory control of citrate (Saha et al., 1999, Saha et al., 1997) and AMP-activated protein kinase (AMPK) (Saha and Ruderman, 2003, Saha et al., 2000), which in turn stimulate and inhibit it **(Reaction 1.15)(Fig. 1.6)**.

Reaction 1.15: The conversion of acetyl-CoA to malonyl-CoA by ACC



The authors proposed that increased CHO oxidation results in increased acetyl-CoA concentration which in turn causes a raise in malonyl-CoA content; culminating in depressed CPT1 activity and reduced fat oxidation at rest. Two studies run by Båvenholm et al. (2000) and Rasmussen et al. (2002) were supportive of this finding.

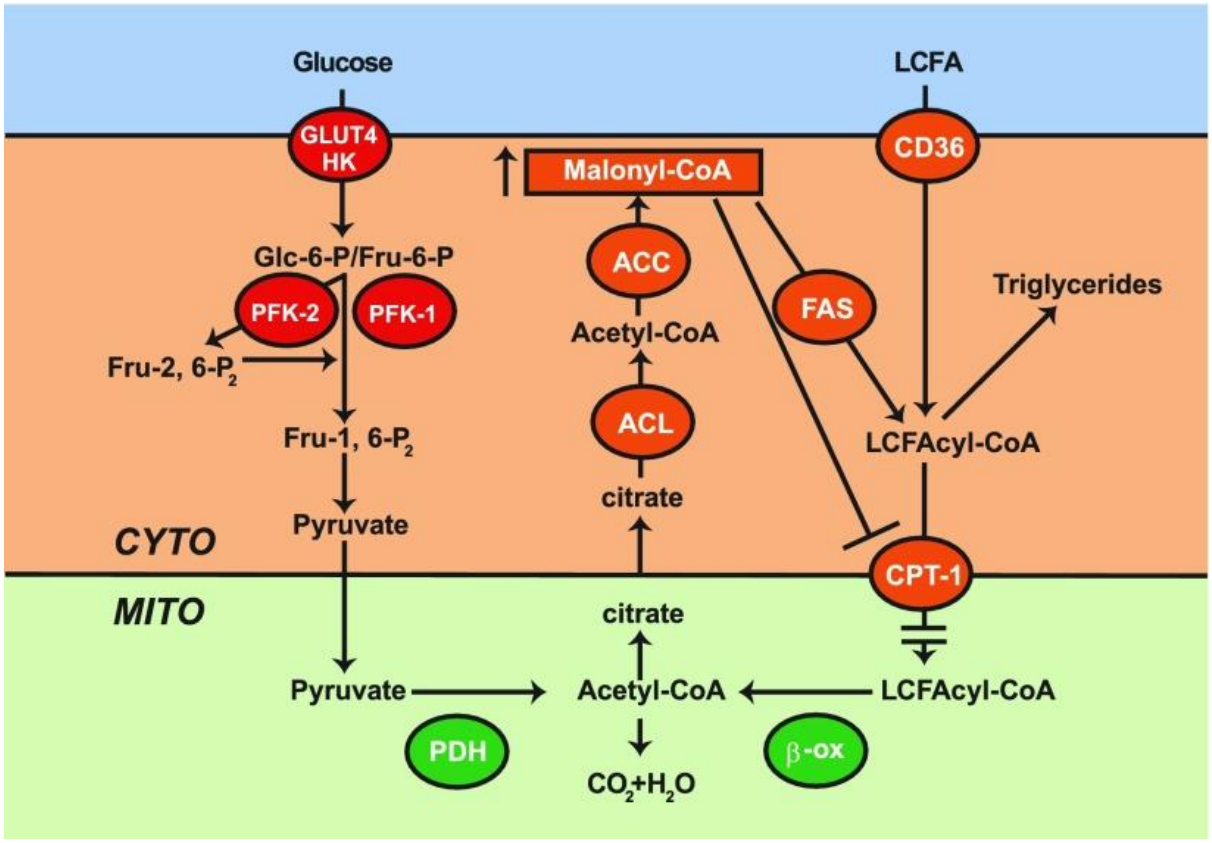


Fig. 1.6. Malonyl-CoA hypothesis (adapted with permission from Hue and Taegtmeyer, 2009).

The study by Båvenholm et al. (2000) involved a low dose vs a high dose euglycaemic hyperinsulinaemic clamp. During the low dose clamp (Insulin's infusion rate: 0.25 mU/kg/min and glucose infusion rate: 2.0 ± 0.2 mg/kg/min), there was a 22% decrease in whole-body fat oxidation measured by indirect calorimetry ($p < 0.01$ compared to baseline) but no change in muscle malonyl-CoA concentration. However during the high dose clamp (the rate of insulin infusion: 1.0 mU/kg/min and the rate of glucose infusion: 8.1 ± 0.7mg/kg/min), there was a further 41% decrease in whole-body fat oxidation ($p < 0.001$) which was associated with a significant increase in muscle malonyl-CoA and acetyl-CoA carboxylase contents (muscle malonyl-CoA increased from 0.20 ± 0.01 at baseline to 0.24 ± 0.01 nmol/g during the clamp; $P < 0.001$). The authors reported that high dose glucose and insulin infusion reduced whole-body fat oxidation through the inhibitory impact of malonyl-CoA on fatty acid metabolism.

Rasmussen et al. (2002) used a tracer study with a 5 hourly hyperglycaemic (156 ± 4 mg/dl) hyperinsulinaemic (43 ± 7 μU/ml) clamp. Using isotope methodology associated with femoral arterial and venous blood sampling, the team reported a significant decrease in muscle long chain fatty acid oxidation combined with a significant increase in muscle malonyl-CoA content (from 0.13 ± 0.01 at baseline to 0.35 ± 0.07 nmol/g during the clamp; $p < 0.05$). The team came to the same conclusion that hyperglycaemia associated with hyperinsulinaemia caused an increase in malonyl-CoA concentration which in turn suppressed fat oxidation.

However the results of some other studies cast doubt on the physiological importance of malonyl-CoA in suppressing the enzyme CPT1 *in vivo*. Odland et al.

(1996) cycled healthy human volunteers for 70 minutes, including 10 minutes at 40% VO_2max and 60 minutes at 65% VO_2max, and took muscle biopsies at minutes 10, 20 and 70. The authors did not report any significant changes in muscle malonyl-CoA concentration at any time during exercise compared to baseline and suggested that muscle malonyl-CoA content did not have any impact on muscle fuel metabolism. A study done by Dean et al. (2000) investigated skeletal muscle ACC and malonyl-CoA content at baseline and following one-legged knee extensor exercise at 60%, 85% and 100% knee extensor VO_2max and their association with whole-body fat oxidation (measured by indirect calorimetry). The authors reported a decrease in muscle ACC and malonyl-CoA concentrations with increasing exercise intensity which was associated with increasing whole-body fat oxidation at 60% and 85% VO_2max and declining fat oxidation at 100% VO_2max (whole-body fat oxidation increased from 121 mg/min at baseline to 258 ± 35 mg/min at 60%, 264 ± 63 mg/min at 85% and then declined to 174 ± 76 mg/min at 100% VO_2max). The authors suggested that reduced muscle malonyl-CoA content at low intensity exercise could have a stimulatory effect on whole-body fat oxidation; however at higher intensities whole-body fat oxidation was not affected by muscle malonyl-CoA content.

Therefore, considering all the results available so far, it can be concluded that although there might be a role for muscle malonyl-CoA in regulating fat oxidation at rest, further investigations are needed to clarify its impact on fatty acid metabolism during exercise. Although Dean et al. (2000) attributed increased fat oxidation during low intensity exercise to decreasing malonyl-CoA content; van Loon et al. (2001) put more emphasis on carnitine as playing a major role in muscle fuel

selection and metabolism during steady-state exercise. This will be discussed in details in due course.

1.3.4. Free carnitine availability

Krogh and Lindhard (1920) suggested for the first time that less fat was metabolised during heavy exercise. This was based on substrate metabolism measurements on air samples collected from a Jaquet respiration chamber whilst the volunteers cycled on an ergometer bike in 20 minute periods.

Later studies on blood lipids during exercise suggested that free fatty acid efflux from blood increased during exercise (Carlson and Pernow, 1959, Friedberg et al., 1960, Carlson and Pernow, 1961, Havel et al., 1963). The assumption was that blood flow increased to the exercising muscles, resulting in more fatty acids' uptake into and oxidation inside the muscle cells.

Tracer studies done by Havel et al. (1963) and Jones et al. (1980) shed more light on muscle fuel metabolism during low and high intensity exercise. A study by Havel et al. (1963) involved infusing intravenous palmitate-1-C^{14} over 4 hours to healthy human volunteers. During hours 2 and 3, the volunteers walked on a treadmill at the speed of 3 to 4 miles/hour (low intensity exercise). The team noticed that there was a significant raise in the contribution of free fatty acids towards metabolism from rest to low intensity exercise. Whilst fasting subjects achieved 25 to 26% of energy metabolism at rest from oxidation of fatty acids, the corresponding values increased to 41 to 49% during low intensity exercise.

A study by Jones et al. (1980) demonstrated that plasma palmitate (14C) turnover rate decreased significantly from 104 ± 16 μnml/min at baseline to 63 ± 8 μnml/min during 40 minutes of exercise at 70% VO_2max. At the same time there was a remarkable fall in the rate of appearance of free fatty acids during exercise at 70% VO_2max compared to exercise at 36% VO_2max (the two exercise sessions were run on separate days) suggestive of reduced peripheral adipose tissue lipolysis. The findings of the two tracer studies run by Havel et al. (1963) and Jones et al. (1980) suggested that exercise intensity had an impact on fat oxidation with fat oxidation increasing at low intensity and decreasing at higher intensity, which the authors attributed to decreased peripheral lipolysis.

Using [6,6-$^2H_2$]glucose and [$^2H_2$]palmitate tracers, Romijn et al. (1993) reported that with increasing exercise intensity from 25% to 85% VO_2max, the rate of appearance of free fatty acids decreased (from 25.8 ± 2.6 to 17.0 ± 3.4 μmol/kg/min, $p < 0.05$), associated with a simultaneous significant raise in the rate of appearance of glucose ($p < 0.05$). At the same time, maximum peripheral adipose tissue lipolysis occurred during low intensity exercise. The authors concluded that increasing exercise intensity shifted the overall body metabolism from fat to carbohydrates.

A comprehensive study on the impact of exercise intensity upon total body fat and carbohydrate metabolism was undertaken by van Loon et al. in 2001. His study involved [6,6-$^2H_2$] glucose and [U-^{13}C] palmitate tracer administration to volunteers cycling at 40%, 55% and 75% *W*max. Each exercise period lasted 30 minutes and muscle biopsy samples were taken at baseline and immediately following each exercise period. The authors noticed that total fat and carbohydrate metabolism

rates increased in parallel from rest to steady-state low and medium intensity exercise. However, at steady-state high intensity exercise, total fat oxidation rate declined whilst total carbohydrate oxidation rate continued rising up. There was a steady increase in total carbohydrate oxidation rate from 0.15 ± 0.04 g/min at baseline to 1.44 ± 0.35 g/min at 40% *W*max exercise ($p < 0.05$), 2.04 ± 0.41 g/min at 55% *W*max exercise ($p < 0.05$) and 3.91 ± 0.32 g/min at 75% *W*max exercise ($p < 0.05$). This was associated with a proportional significant steady increase in muscle PDC activation from 0.57 ± 0.08 mmol/min/kg wm at baseline to 1.18 ± 0.010, 1.23 ± 0.17 and 1.67 ± 0.32 mmol/kg/min wm at low, medium and high intensity exercise periods respectively. On the other hand total fat oxidation rate followed a different trend. Whilst total fat oxidation increased from 0.08 ± 0.02 g/min at rest to 0.68 ± 0.11 g/min at 40% *W*max exercise ($p < 0.05$) and 0.79 ± 0.15 g/min at 55% *W*max exercise ($p < 0.05$), there was a decline to 0.51 ± 0.14 g/min at 75% *W*max exercise ($p < 0.05$ compared to other exercise intensities). Skeletal muscle free carnitine content decreased from 15.87 ± 1.17 mmol/kg dm at rest to 11.25 ± 1.13 mmol/kg dm at low intensity ($p < 0.05$), 10.29 ± 0.84 mmol/kg dm at medium intensity ($p < 0.05$) and 5.61 ± 0.76 mmol/kg dm at high intensity exercise ($p < 0.05$). The team proposed that a reduction in muscle free carnitine availability to CPT1, due to high PDC flux resulting in increased acetylation of free carnitine, was responsible for the reduction in total fat oxidation rate at steady-state high intensity exercise. The authors suggested that at a value of less than 6 mmol/kg dm, muscle free carnitine availability becomes limiting to the enzyme CPT1.

The impact of high muscle glycolytic flux on whole-body fat oxidation during high intensity exercise was investigated by pre-treating the volunteers with a high

carbohydrate diet and comparing the results with pre-treatment with low carbohydrate diet (Putman et al., 1993). The team demonstrated that exercise to exhaustion at 75% VO_2max following 3 days of high carbohydrate diet, was associated with significantly higher muscle PDC activity, acetyl-CoA/CoASH ratio, acetyl-CoA and acetylcarnitine contents and concomitantly suppressed whole-body fat oxidation compared to low carbohydrate diet. A study run by Roepstorff et al. (2005) delivered similar results. This study involved cycling at 65% VO_2max for 60 minutes. The day before the experimental visit, the subjects undertook one bout of cycling to deplete skeletal muscle glycogen store and then had either a low-CHO (primarily fat) or a high-CHO diet (primarily carbohydrate). The team noticed that the low-CHO group had significantly lower levels of acetyl-CoA and acetylcarnitine ($p < 0.001$) and significantly higher level of free carnitine ($p < 0.01$) during exercise compared to the high-CHO group. This was associated with a significantly higher leg fat oxidation in the low-CHO group compared to the high-CHO group. The team suggested that higher glycolytic flux in the high-CHO group resulted in reduced free carnitine availability which in turn caused a decrease in fat oxidation.

The above-mentioned studies suggest that the inhibitory impact of high glycolytic flux on fatty acid oxidation could be due to decreased muscle free carnitine content (secondary to enhanced acetyl-CoA production and acetylcarnitine concentration) and not the impact of malonyl-CoA as previously thought.

1.4. The impact of increased muscle carnitine content on fuel metabolism and energy expenditure

Stephens et al. (2006a) investigated the impact of increased skeletal muscle carnitine content on muscle fuel metabolism. The team managed to increase skeletal muscle total carnitine content by 15% ($p < 0.01$) following intravenous carnitine infusion (normal saline in CON group) during a euglycaemic hyperinsulinaemic clamp for 5 hours (plasma carnitine level of 600 µmol/L and plasma insulin level of 160 mU/L). The team reported that by increasing skeletal muscle carnitine content, muscle PDC activity decreased by 30% ($p < 0.05$) and muscle lactate content reduced by 40% ($p < 0.05$) in the CARN group compared to CON. On the other hand, muscle glycogen content increased by 30% ($p < 0.01$) and long chain acyl-CoA content raised by 40% ($p < 0.05$) in the CARN group compared to CON. The authors suggested that acute muscle carnitine retention increased the oxidation of long chain fatty acids which in turn resulted in increased muscle acetyl-CoA content. This in turn had an inhibitory effect on PDC activity resulting in reduced CHO oxidation and increased glycogen storage.

Research in our laboratory (Wall et al., 2011) showed for the first time that increased skeletal muscle carnitine content by 21% ($p < 0.05$), by taking 80 g CHO combined with 2 g L-carnitine L-tartrate twice daily over 24 weeks, decreased muscle glycogen utilisation by 55% ($p < 0.05$) compared to CON and declined PDC activation by 31% ($p < 0.05$) compared to baseline at steady-state low intensity exercise (50% VO_2max) consistent with increased fat and decreased CHO oxidation.

The team also reported increased PDC activation by 38% ($p < 0.05$) and acetyl-carnitine content by 16% ($p < 0.10$), decreased lactate content by 44% ($p < 0.05$) and a better matching of PCr/ATP ratio ($p < 0.05$) in skeletal muscle cells at steady-state high intensity exercise (80% VO_2max) indicating a more efficient PDC flux and reduced anaerobic energy production due to the buffering effect of carnitine on acetyl-CoA.

The authors reported that whilst at steady-state low intensity exercise carnitine functioned mainly as a translocator of fatty acids into the mitochondria resulting in enhanced fat oxidation; at higher intensities its function switched mainly to buffering excess acetyl groups produced by accelerated glycolysis culminating in decreased anaerobic energy production. Therefore the dual roles of carnitine during exercise follow its two originally described functions (see '*1.2.2. Physiological functions of carnitine in skeletal muscles'*) and depend on the exercise intensity.

Stephens et al. (2013) demonstrated that increasing muscle total carnitine content by 20% ($p < 0.05$), following 12 weeks of twice daily oral supplementation of 2 g of L-carnitine L-tartrate and 80 g of carbohydrate, was associated with 200% increase in muscle long chain acyl-CoA and 6% increase in whole-body energy expenditure during steady-state low intensity exercise (20 min of cycling at 50% VO_2max) ($p < 0.05$). The control group volunteers, who ingested 80 g of carbohydrate twice daily over the same period of time, did not show any change in whole-body energy expenditure post-supplementation compared to baseline during the same bout of exercise. In the carnitine supplemented volunteers, there was a trend for whole-body fat oxidation to increase by 10% ($p = 0.09$) following 12 weeks of

supplementation and the rate of whole-body fat oxidation post-supplementation was significantly different between the two groups. The authors suggested that increased whole-body energy expenditure following increased muscle carnitine content was secondary to enhanced whole-body fat oxidation and was in line with the role of carnitine in translocating long chain fatty acyl-CoA molecules across the mitochondrial membrane via the enzyme CPT1.

1.5. Lipid accumulation, insulin resistance and a potential role for carnitine

The excess calorie intake associated with ingesting calorie rich food sources, becomes stored primarily as TAG in the adipose tissues, resulting in turn in weight gain and obesity (Frayn et al., 2006). However some of the excess energy 'overflows' to ectopic sites in liver, skeletal muscle, kidneys and possibly β cells of pancreas (Danforth, 2000, Shulman, 2000, Unger, 2003). Research by Phillips et al. (1996) and Pan et al. (1997) reports that there is a direct relationship between insulin resistance and lipid content in the skeletal muscle cells (IMCL: Intramyocellular lipid). This has made some researchers to suggest that the pathophysiology of insulin resistance and diabetes mellitus type 2 has a 'lipocentric' basis (Savage et al., 2007). In order to understand the interaction between skeletal muscle lipid accumulation and insulin resistance, we need first to comprehend the insulin signalling pathway (**Fig. 1.7**).

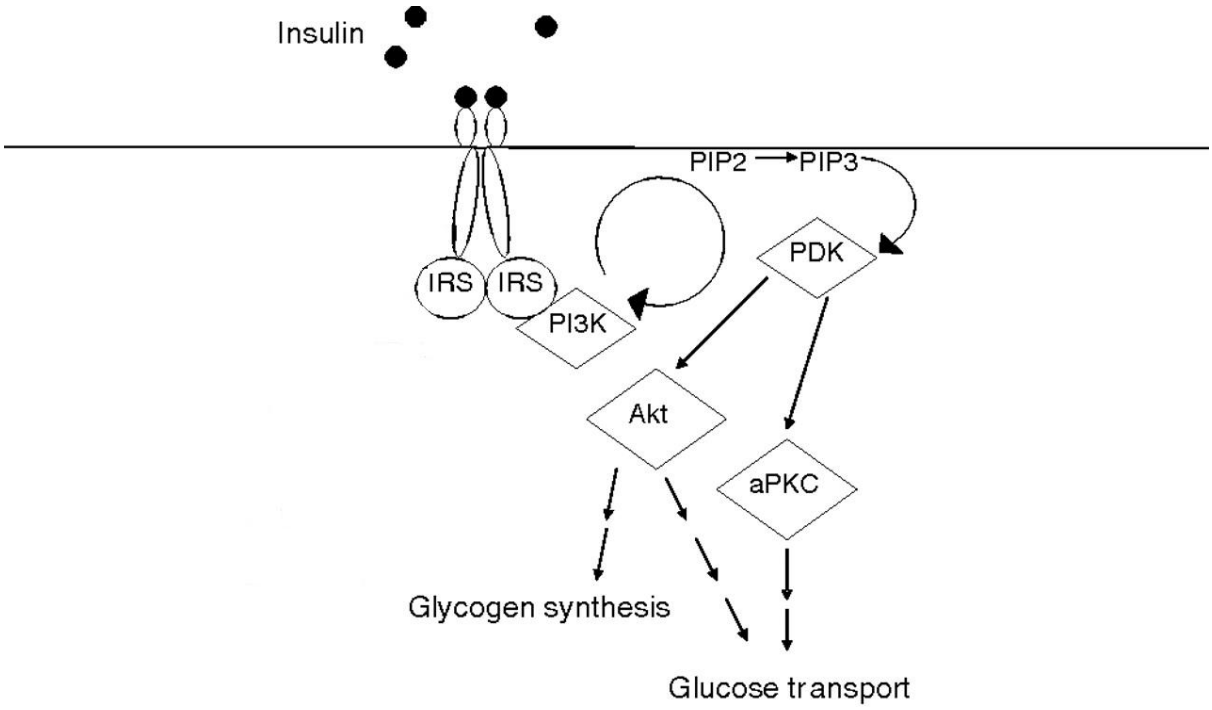


Fig. 1.7. Insulin signalling pathway (adapted with permission from Corcoran et al., 2007).

1.5.1. Insulin signalling pathway in skeletal muscle cells

Insulin receptor (IR), a tyrosine kinase heterotetramer, consists of two α and two β subunits (Rosen, 1986). On binding to insulin, a conformational change happens to IR causing its auto-phosphorylation (Van Obberghen et al., 2001). This in turn causes the attachment of insulin receptor substrates (IRSs) to the receptor (Rosen, 1987) resulting in tyrosine phosphorylation of some components of IRSs which in turn leads to PI3K (phosphoinositide 3 kinase) activation (White et al., 2002). Activated PI3K catalyses a reaction ending in the formation of PIP3 (phosphoinositol triphosphate), which in turn activates PDK1 (phosphoinositide-dependent protein kinase 1). PDK1 in turn activates aPKC (atypical protein kinase C) and Akt (protein kinase B). aPKC and Akt bring about the translocation of GLUT4 from the cytoplasm to the cell membrane. Akt inactivates GSK3 (glycogen synthase kinase 3), promoting synthesis of glycogen from glucose (Corcoran et al., 2007).

1.5.2. Mechanisms of insulin resistance

It has been suggested that increased IMCL, via increasing the content of DAG and ceramides, decreases insulin sensitivity in muscle tissues (Samuel et al., 2010, Samuel and Shulman, 2012). The content of these two metabolites has been found to be elevated in skeletal muscle cells of insulin resistant people (Heydrick et al., 1991, Chen et al., 1991, Itani et al., 2002). It has been reported that this inhibitory effect is exerted at the level of insulin signalling pathway. DAG and ceramides activate cPKC and nPKC (Bronfman et al., 1988, Nishizuka, 1995, Nishizuka and Nakamura, 1995) which cause serine phosphorylation of IRS-1, rather than tyrosine

phosphorylation, in turn resulting in inhibition of PI3K binding to IRS-1 (Savage et al., 2007).

PPARs and FOXO1 have also been reported as potential mediators involved in insulin resistance. PPARs (peroxisome proliferator-activated receptors) are nuclear receptors which exert regulatory control over some genes associated with oxidation of lipids and carbohydrates (Corcoran et al., 2007). It has been suggested that raised IMTG can cause down-regulation of PPARs resulting in insulin resistance (Ferre, 2004, Guerre-Millo et al., 2000, Verma et al., 2004, Wang and Tafuri, 2003). This is the reason why PPARs have been a target for the management of diabetes mellitus. TZDs (Thiazolidinediones) are medications commonly used in medical practice for the management of diabetes mellitus type 2 (Boden et al., 2003) which act by activating PPARs.

In a study run by Constantin-Teodosiu (2012), healthy human volunteers cycled for 60 minutes at 75% VO_2max following an iv infusion of normal saline after consuming a control diet (CD). Same volunteers repeated the same bout of exercise on 2 separate occasions following iv infusion of normal saline or DCA after consuming a high fat diet (HFD and HFD + DCA respectively). Muscle biopsies were taken at baseline and minutes 10 and 60 and were analysed for PDK, FOXO1, PPARα mRNA and PDCa. The team reported that HFD increased PDK2, PDK4, FOXO1 and PPARα mRNA and decreased PDCa at rest compared to CD. PDCa and whole-body carbohydrate oxidation increased following exercise in both HFD and CD but to a lesser extent in the former, whilst PDK4 and FOXO1 mRNA remained elevated in the HFD. HFD + DCA increased PDC activity and whole-body carbohydrate oxidation

during exercise. The authors reported that HFD had an inhibitory impact on PDC activity and carbohydrate oxidation which was exerted via up-regulation of PDK4 and FOXO1. The authors also reported that PDC activation by DCA improved the inhibitory impact of HFD on carbohydrate oxidation during exercise.

And finally inflammatory mediators, especially TNF-α and IL-6 have been put forward as potential mediators involved in insulin resistance (Kanety et al., 1996, Guo and Donner, 1996, Kim et al., 2004, Bastard et al., 2002, Pickup et al., 1997).

Studies involving overexpression of CPT1, either directly via adenovirus or electro-poration or indirectly via blocking the enzyme ACC-2 (acetyl-CoA carboxylase-2), managed to increase fat oxidation and improve insulin sensitivity *in vitro*. Perdomo et al. (2004) reported that adenovirus-induced CPT1 over-expression in L6 myotubes was associated with a significant increase in the oxidation of long chain fatty acids associated with a 60% increase in insulin-induced [^{14}C] glucose incorporation into glycogen in the cell cultures. Running a similar study, Sebastian et al. (2007) came to the same conclusion. Using electro-transfer to over-express CPT1 in hindlimb muscle of rats and a hyperinsulinaemic euglycaemic clamp, the team reported a decline in the accumulation of lipid metabolites (TAG, DAG and ceramides) and improved insulin stimulated glucose metabolism in L6E9 myotubes.

Although the impact of acute hypercarnitinaemia on glucose disposal has been investigated in a few metabolic studies (Ferrannini et al., 1988, De Gaetano et al., 1999, Galloway et al., 2011), we are not aware of any studies investigating the impact of chronic carnitine supplementation on overall glucose metabolism and insulin resistance in human volunteers. Considering the role of carnitine in

transporting fatty acids across the mitochondrial membrane, it would be plausible to speculate that carnitine could potentially improve insulin resistance and glucose disposal by increasing fatty acid oxidation and decreasing IMCL in muscle cells.

1.6. Weight loss and a potential role for carnitine

Obesity is associated with increased risk of hypertension, cardiovascular disease, stroke, diabetes mellitus type 2, mental health issues and reduced self-esteem (James, 2008a, James, 2008b, James, 2008c). According to '2010 to 2015 government policy: obesity and healthy eating' published by the Department of Health (DoH), 61.9% of adults living in England are overweight or obese. The numbers for children are of great concern as there has been a significant rise in the prevalence of childhood obesity in the last decade in England with 28% of children aged between 2 and 15 currently either overweight or obese. £5 billion is spent annually in the UK National Health System (NHS) to deal with the costs of medical, surgical and mental health issues related to being overweight or obese. This obviously puts a great burden on the already busy hospitals and the government policy makers are constantly coming up with new ideas and plans to encourage people lose weight (2010 to 2015 government policy: obesity and healthy eating published by DoH).

Unlike carbohydrate stores in the body which are regulated very tightly by adjusting metabolism to dietary intake, fat balance is not as tightly regulated and total body fat mass can increase markedly by increasing dietary fat intake (Abbott et al., 1988). Whilst dietary carbohydrate can be absorbed by the cells and oxidised quite rapidly

via the action of insulin hormone secreted from the pancreas (Blaak and Saris, 1996), dietary fat does not metabolise as rapidly and there is a tendency for fat to become accumulated in the adipose tissues (Frayn et al., 2006). Researches performed by Flatt et al. (1985), Schutz et al. (1989) and Schrauwen et al. (1997a, 1997b) demonstrate that unlike carbohydrates, an increase in dietary fat brings about only a slow adaptive rise in fat metabolism, suggesting that body's limited capacity to adjust fat oxidation in proportion to dietary fat could result in the excess fat becoming accumulated in the adipose tissues, causing obesity. Although it is widely believed that obesity happens when the balance between dietary fat intake and fat oxidation changes in favour of the former, there is evidence that some people may be, possibly genetically, low fat oxidisers and predisposed to the development of obesity (Blaak, 2005).

Gaster and Beck-Nielsen (2004) and Gaster et al. (2004) compared fatty acid uptake and palmitate oxidation in cultured human myotubes from subjects with diabetes mellitus type 2 and compared it with myotubes from normal control subjects. The teams reported that although fatty acid uptake was not different between the two groups, diabetic subjects demonstrated a significantly reduced palmitate oxidation in cultured myotubes compared to control and suggested that, at least to some extent, decreased *in vivo* muscle fat oxidation in type 2 diabetic patients was of primary genetic origin.

Zurlo et al. (1990) reported that reduced fat to carbohydrate oxidation ratio could be used as a predictor of weight gain. In his study on 152 Pima Indians, 24 hour RQ was measured whilst the subjects were fed a standard weight-maintenance diet.

The subjects were then followed up for a total period of 3 years. It was recorded that the baseline 24 hour RQ was associated with later body mass ($p < 0.01$) and body fat mass ($p < 0.05$) changes in 111 subjects whom follow-up data were available for. The mean follow up period was 25 ± 11 months. Compared to subjects with lower 24 hour RQ (10'th percentile), there was a 2.5 times higher risk of gaining more than 5 kg body mass in subjects with higher 24 hour RQ (90'th percentile). The authors concluded that, independent of EE, RQ was an independent factor predicting subsequent body mass gain and body fat mass gain. In other words, Pima Indian subjects who were low fat oxidisers with higher RQ were more prone to develop weight gain.

Astrup et al. (1994) demonstrated that increasing fat content in the diet of post-obese women was associated with a failure to increase fat oxidation proportionately. In this study a group of post-obese women consumed a high fat diet (50% fat energy) for 72 hours before and during a 24 hour stay in a respiratory chamber. Compared to the CON group who ingested a normal standard diet, the high fat diet group failed to increase fat to carbohydrate oxidation ratio proportionately ($p < 0.01$).

In the NUGENOB study (Nutrient-Gene interactions in human obesity), a major cohort study run by Blaak and his colleagues on (2007) 701 obese individuals in 8 European countries including the UK, it was reported that independent of body composition, sex, age, research centre and REE, post-prandial EE following a high fat meal showed a blunted increase in the obese individuals compared to the lean control group. In this study the subjects had a meal challenge consisting of 95% fat

with an energy content equivalent to 50% of estimated REE. REE was 4.29 ± 0.13 kJ/min in the control group (BMI less than 25 kg/m^2), 5.13 ± 0.08 kJ/min in subjects with a BMI 30 to 35 kg/m^2, 5.46 ± 0.11 kJ/min in subjects with a BMI 35 to 40 kg/m^2 and 5.99 ± 0.18 kJ/min in subjects with a BMI above 40 kg/m^2. Following 180 minutes of the high fat meal challenge, the post-prandial EE, adjusted for REE and energy content of the meal, increased to 1018 ± 12 kJ/min in the control group (BMI less than 25 kg/m^2), 1005 ± 6 kJ/min in subjects with a BMI 30 to 35 kg/m^2, 1018 ± 8 kJ/min in subjects with a BMI 35 to 40 kg/m^2 and 1016 ± 11 kJ/min in subjects with a BMI above 40 kg/m^2. The authors concluded that obesity caused a decrease in fat-induced thermogenesis but could not say whether this decrease was a primary factor contributing to obesity or whether it was a secondary adaptation to obesity (Blaak et al., 2007). The same team also reported that with increasing BMI, post-feed fat oxidation showed a significantly blunted increase in those obese subjects who had a relatively low pre-feed fat oxidation ($p < 0.01$) (Blaak et al., 2006). The team categorised the subjects into 8 sub-categories based on 4 BMI categories (<25, 30-35, 35-40, >40 kg/m^2) and 4 quartiles of fasting fat oxidation. As can be seen in **Fig. 1.8**, obese subjects with a lower pre-meal fat oxidation demonstrated a reduced increase in post-meal fat oxidation as well. The authors concluded that, at least some obese people might have a lower capacity to oxidise fat during fasting and post-prandially (Blaak et al., 2006).

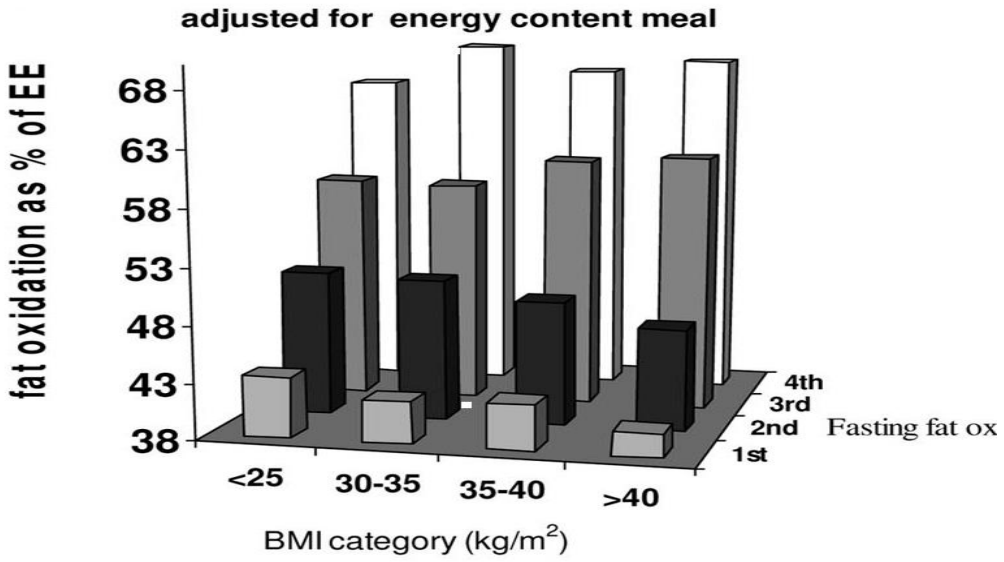


Fig. 1.8. Post-meal fat oxidation, as percentage of EE, following a high fat load according to BMI category and quartiles of fasting fat oxidation (adapted with permission from Blaak et al., 2006).

The above-mentioned studies suggest that some people with high BMI might be, perhaps genetically, low fat oxidisers and therefore predisposed to obesity (Zurlo et al., 1990, Gaster et al., 2004, Blaak et al., 2006). However weight loss via caloric restriction alone does not always restore the ability to increase fat oxidation (Astrup et al., 1994). Thus, individuals with obesity face a serious challenge if they are to maintain even modest weight loss in today's obesogenic environment and therefore investigations into the mechanisms underlying reduced fasting fatty acid oxidation in obesity, as well as nutritional strategies to aid weight loss are warranted.

We are not aware of any study which has investigated the impact of chronic carnitine supplementation on weight loss. Considering the role of carnitine in transporting fatty acids across the mitochondrial membrane for β-oxidation, it would be plausible to speculate that in the context of a weight loss strategy involving a diet and/or exercise regime, carnitine could also play a role by enhancing fatty acid oxidation in people with high BMI who might be low fat oxidisers.

1.7. Aims and hypotheses

The aim of this thesis was to investigate the impact of increased skeletal muscle acetyl-CoA and carnitine contents on muscle fuel metabolism in healthy volunteers. Sodium acetate infusion was used in order to increase muscle acetyl-CoA content (Chapter 3) and chronic oral carnitine supplementation, in combination with carbohydrate (Chapter 4) or carbohydrate and protein (Chapter 5), was used in order to increase muscle carnitine content.

Firstly, we hypothesised that increasing muscle acetyl group availability by sodium acetate infusion would decrease whole-body fat oxidation during steady-state low intensity exercise as a result of either decreased free carnitine availability or due to administered acetate oxidation substituting fat utilisation, and would decrease the contribution of non-mitochondrial ATP production during steady-state high intensity exercise due to increased availability of acetyl groups to the TCA cycle.

Secondly, we hypothesised that increasing skeletal muscle carnitine content by chronic oral carnitine and carbohydrate supplementation would result in increased whole-body and muscle fat oxidation and by doing so would improve whole-body glucose disposal and serum insulin concentration following an oral glucose tolerance test compared with a control group.

Thirdly, we hypothesised that a novel feeding approach of chronic oral carnitine, carbohydrate and protein supplementation, would increase skeletal muscle carnitine content and that this in combination with caloric restriction and low intensity exercise training would lead to an increase in whole-body fat oxidation and by doing so would result in reduced body fat mass and greater lean mass compared to control.

CHAPTER 2

GENERAL METHODS

2.1. Ethics, volunteer recruitment and screening

Healthy recreationally active (running, swimming or cycling 2 - 5 times per week) male volunteers aged 18 to 30 were recruited into the study described in Chapter 3. For the study described in Chapter 4 healthy sedentary male volunteers aged 18 to 30 were recruited. Healthy female volunteers aged 18 to 45 with a body mass index of 27 to 35 kg/m^2 were recruited into the study described in Chapter 5. All volunteers taking part in these studies were non-smokers and non-vegetarians. As per the University of Nottingham Medical School Ethics Committee guidelines, subjects who had taken part in any other study in the preceding 3 months to the commencement of studies described in this thesis were not recruited. All studies were approved by the University of Nottingham Medical School Ethics Committee and were performed in accordance with the declaration of Helsinki (1964).

Volunteers were recruited by poster advertisements from students and staff of the University of Nottingham and Queen's Medical Centre (Chapters 3 and 4). For Chapter 5, volunteers were recruited from the general public of Nottingham city and the surrounding areas by local press advertisements.

Potential participants expressing enthusiasm in taking part in research received the relevant study information sheet, including a DEXA leaflet (Chapters 4 and 5) before screening and were encouraged to read it carefully. All received a verbal explanation of the study in question, either on the phone or in person, and were informed that they could withdraw from the study at any point and were not required to provide an explanation to do so. Volunteers were informed that they

could not donate blood before or during the study. Additionally, potential participants interested in the study described in chapter 5 were informed that their involvement would stop immediately if they became pregnant.

Subjects deciding to take part were invited to the David Greenfield Human Physiology Unit, University of Nottingham Medical School, for screening. A letter was also sent to the General Practitioner of each volunteer informing them of the volunteer's decision to take part in the study. All volunteers signed an informed consent prior to screening.

Each screening lasted approximately 30 minutes and involved a medical history with emphasis on past medical history, drug history and allergies, which was followed by measurement of height and weight (body mass index), heart rate and blood pressure and 12 lead ECG. A venous blood sample was also obtained for Full Blood Counts (FBC), Urea and Electrolytes (U&Es), Liver Function Tests (LFTs) and Coagulation screen (Pathology, Queen's Medical Centre).

Blood results and the ECG recordings were confirmed as normal by the clinician in charge before the subjects were enrolled into any study. In case of any abnormalities the subject was dropped from the study and a letter was given to the subject to take to his/her GP for consideration of further investigations and follow up. See the screening and consent forms in the **Appendix 1**.

2.2. Maximum O_2 consumption (VO_2max)

All studies involved determination of VO_2max in each volunteer at the outset of each study (Vmax, SensorMedics corporation, California, USA). Volunteers taking part in Chapter 4 also had VO_2max determined again at the end of the study (i.e. baseline and 24 weeks). VO_2max was determined with an exhaustive, incremental and continuous test involving volunteers exercising on an electrically braked cycle ergometer (Lode Excalibur, Lode, Groningen, Netherlands; Chapters 3 and 4**)** or during running on a motorised treadmill (Woodway ELG 70, Weil am Rhein, Germany; Chapter 5**)**. Volunteers received verbal encouragement to continue exercising as long as possible during each VO_2max test. Volunteers wore a mouth-piece and a nose clip throughout each test to collect expired gases and determine VO_2, VCO_2 and VE using an on-line gas analysis system (Vmax, SensorMedics corporation, California, USA). A plateau in oxygen consumption or oxygen consumption at the highest workload sustainable for 3 minutes (Chapters 3 and 4) or for 1 minute (Chapter 5) (in the absence of a plateau) and with an RER > 1.2 were considered to represent VO_2max. Cardiac rate and rhythm were monitored via a 3-lead heart monitor (GE Medical Systems Technology Inc. Buckinghamshire, UK) and perceived exertion was recorded at the end of each incremental exercise workload using the Borg scale (Borg, 1982a, Borg, 1982b). Specifics of each VO_2max test are outlined below.

2.2.1. Measurement of VO_2max in Chapter 3 and 4

Volunteers started cycling at a workload of 1 W/kg body mass on an electronically braked cycle ergometer (Lode Excalibur, Lode, Groningen, Netherlands) which was increased every 3 minutes in 30W increments to the point of volitional exhaustion. All were asked to cycle at a cadence of 70-80 rpm. A confirmatory VO_2max test was performed 7 days later. This test was shorter involving the workload being increased to the intensity achieved at exhaustion in the previous test following a 5 minute low intensity warm-up.

2.2.2. Measurement of VO_2max in Chapter 5

Volunteers ran to the point of volitional exhaustion on a motor driven treadmill (Woodway ELG 70, Weil am Rhein, Germany). The following exercise protocol was used:

		Speed (km/hour)	Gradient (%)
-	Min 0-5	3.5	0
-	Min 5-8	4.5	0
-	Min 8-11	5.5	0
-	Min11-14	6.5	0
-	Min14-15	6.5	2
-	Min15-16	6.5	4
-	Min16-17	6.5	6
-	Min17-18	6.5	8
-	Min18-19	6.5	10
-	Min19-20	6.5	12
-	Min 20-21	6.5	14
-	Min 21-22	6.5	16
-	Min 22-23	6.5	18
-	Min 23-24	6.5	20
-	Min 24-25	6.5	22

Based on the VO_2max value achieved, exercise workloads equivalent to 50% and 80% VO_2max were calculated for the volunteers taking part in Chapter 3 and 50% VO_2max for volunteers participating in Chapter 5 in the following way:

A. The mean VO_2 for the last minute of each steady-state exercise workload was calculated. For Chapter 5 this would apply to the first 4 exercise cycles (min 0 – 14).

B. The calculated mean VO_2 values were plotted on an Excel graph (Microsoft Excel 2010, CA, USA) with the X axis representing the workload (watts) for Chapter 3 and speed (km/h) for Chapter 5 and the Y axis representing the VO_2 in L/min.

C. The linear trend line for the calculated mean VO_2 values was plotted on the graph and the corresponding equation was used to calculate the 50% and 80% VO_2max for Chapter 3 and the 50% VO_2max for Chapter 5.

2.3. Body composition

Volunteers taking part in studies described in Chapter 4 and 5 had body composition quantified using DEXA (Lunar Prodigy DEXA, GE Medical Systems, Bedford, UK). Information on the DEXA scan was provided to the volunteers in a 'DEXA leaflet' (See **Appendix 2**). All the volunteers filled a safety questionnaire before the scan, changed into scrubs and were asked to remove all the metallic accessories. The female volunteers in Chapter 5 had a urine β-hCG test done to rule pregnancy out.

The DEXA scan was used to determine body composition and involved a very low dose of radiation (less than 1 μSv), nearly 100 times less than a chest X-ray radiation

dose. Relevant to our studies were the scan reports on fat mass and its distribution in the body i.e. total body, arms, legs, trunk, android and gynoid fat percentages. No volunteers were recruited that had an abnormally low lean body mass.

2.4. Blood sampling and analysis

Volunteers in Chapter 3 and 4 had a retrograde cannula (Venflon, Becton Dickinson, New Jersey, USA) inserted in a distal forearm or dorsal hand vein at the onset of each experimental visit for blood sample collection. Cannulation was undertaken following a subcutaneous injection of local anaesthetic (1% lignocaine, B. Braun, Melsungen, Germany) whilst volunteers rested semi supine on a bed. In order to arterialise the venous drainage of the hand, a hand was inserted in a heating device (Trent Thermal Technology, Science Park, Nottingham, UK) at an air temperature of 55 °C at least 20 minutes prior to blood sample collection. Blood samples were obtained via a 3 way tap (BD Connecta, Becton Dickinson, Helsingborg, Sweden), and a slow continuous infusion of normal saline (0.9% isotonic sodium chloride, Maco Pharma Ltd, London, UK) was used for the duration of the study to keep the cannula patent.

At each sampling time-point, 1 ml blood sample was initially taken and discarded to avoid blood sample dilution with saline. After which a 1 ml venous blood sample was taken in Chapter 3 for immediate pH (ABL80 Blood Gas Analyser, Copenhagen, Denmark) at the bedside. 8 ml arterialised-venous blood samples were taken in Chapter 4 at each time-point and were immediately analysed for blood glucose concentration (YSI 2300 STATplus, Yellow Springs Instruments, Yellow Springs, Ohio,

USA) at the bedside. Then 5 ml of the main blood sample decanted in a heparin tube, containing 75 µl EGTA, and 3 ml into a non-preservative tube for serum collection. The tubes were centrifuged at 3000 rpm at 4 °C for 10 min (the green heparin/EGTA tubes were centrifuged immediately and the serum tubes were allowed to stand for 30 minutes before centrifugation). Plasma and serum was then stored in labelled eppendorf tubes and kept at -80 °C until analysed.

2.4.1. Serum insulin concentration

A radioimmunoassay kit (Coat-a-Count Insulin, Diagnostics Products Corporation, Los Angeles, CA, USA) was used to measure serum insulin concentration in Chapter 4. Serum samples were combined with 1 ml of ^{125}Iodine labelled insulin. The endogenous insulin contained within the samples competes with the exogenous labelled insulin for insulin-specific antibody sites located on the wall of a polypropylene test tube. The samples were then vortexed and incubated at room temperature in the lab. After 24 hours of incubation, the competitive binding of the exogenous and endogenous insulins was terminated by decanting. Subsequently a gamma counter (Cobra Ⅱ Auto Gamma, Canberra-Packard, Melbourne, Australia) was used to measure the magnitude of radioactivity, which was then converted via a calibration curve into an insulin concentration for each sample. The regression coefficients were usually > 0.9995. For the further details see **Appendix 3**: procedure for insulin assay.

2.4.2. Plasma total carnitine concentration

Plasma total carnitine concentration was determined in Chapter 4. 1.2 ml of chloroform/methanol (3:2) was added on 50 µL of plasma from the samplers in order to extract free, short-chain, medium-chain and long-chain acyl carnitine moieties, together making up total carnitine. The samples were then centrifuged and the supernatant was decanted and evaporated to leave a powder. Following this, samples were incubated at 50 °C in 0.1 M KOH for 2 hours in order to hydrolyse the acyl-carnitine bonds and then neutralised with 0.5 M HCl. In this way, free carnitine represented the total carnitine concentration.

A modified version of the Cederblad and Lindstedt radioenzymatic method (Cederblad and Lindstedt, 1972) was used to measure free carnitine in standards (0-3, 600 pmol) and samples (prepared with H_2O to a volume of 120 µL). Samples were first incubated in a 1 M phosphate buffer consisting of 300 µM acetyl-CoA, 4 µM [^{14}C]acetyl-CoA, 40 µM N-ethylmalemide (NEM) and carnitine acetyltransferase (CAT) enzyme for 30 min. The reaction was catalysed by CAT causing the endogenous carnitine to become labelled with [^{14}C]acetyl-CoA as [^{14}C]acetylcarnitine **(reaction 2.1)**. The role of NEM was to sequester CoASH, making sure that the reaction carried on and ensuring that the endogenous carnitine became radioactively labelled as [^{14}C]acetylcarnitine.

Reaction 2.1:

$$\text{Free carnitine} + [^{14}\text{C}]\text{acetyl-CoA} \xleftrightarrow{\text{CAT}} [^{14}\text{C}]\text{acetylcarnitine} + \text{CoASH}$$

Then the samples were passed through 150 mm glass pipettes containing positive ion-exchange resin (Dowex 1-X8, 100-200 mesh). The resin contained chloride as a counter ion and caused the labelled positively charged [^{14}C] acetylcarnitine to separate from any excess labelled negatively charged [^{14}C]acetyl-CoA. Consequently, the negatively charged [^{14}C]acetyl-CoA bound to the resin whilst the positively charged [^{14}C]acetylcarnitnine washed through the glass pipettes. It was collected and quantified in a β-radioactivity scintillation counter (Tri-Carb 2100TR, Canberra-Packard, Melbourne, Australia). The slope of the regression line from the standard curve was used to calculate the concentration of free (total) carnitine in the samples in μmol/L. The regression coefficients were usually > 0.9995.

2.5. Muscle sampling and analysis

Muscle biopsy samples were obtained in studies described in Chapter 3, 4, and 5 using the Bergstrom percutaneous needle biopsy technique (Bergstrom, 1975). The biopsy area of the quadriceps was shaved and an iodine solution was used to clean the skin in the antero-lateral side of the thigh. Using an aseptic technique and following subcutaneous and deep administration of 5 ml of lignocaine as local anaesthetic (1% lignocaine, B. Braun, Melsungen, Germany) muscle samples were taken from Vastus Lateralis muscle with the subject lying prone on a bed. An incision was made in the skin and muscle fascia using a scalpel and a 5 or 6 mm Bergstrom biopsy needle (Bignell Surgical Instruments Ltd, West Sussex, UK) was inserted through the incision site into the muscle to obtain 50 – 200 mg wet muscle sample. All samples were immediately frozen in liquid nitrogen and stored in

eppendorfs in liquid nitrogen to be analysed at a later date (see individual study chapters for details of measurements). For biopsies taken during exercise (Chapter 3), the biopsy sites were prepared in the same way before exercise commenced. At the immediate cessation of the exercise, each volunteer lay back onto a bed and had the biopsy taken within 10 seconds. After each biopsy, hand pressure was immediately applied to the site for several minutes after which a standard dressing was applied on muscle biopsy incision area including Steri-Strip (3M Deutschland GmbH, Neuss, Germany), tegaderm (3M Deutschland GmbH, Neuss, Germany) and coban compression bandage (3M Deutschland GmbH, Neuss, Germany). Before leaving the laboratory, a leaflet on how to look after the muscle biopsy areas was provided to each volunteer, which included the contact details of the clinician in charge should the volunteer wish to make contact.

2.5.1. Muscle metabolite extraction

All muscle biopsy samples were stored under liquid nitrogen until analysed. In order to extract muscle metabolites, approximately 6-10 mg of each muscle biopsy sample was freeze-dried overnight (Edwards Freeze Dryer Modulyo) and powdered. Using a pestle and mortar, the powdering was performed in the lab under a non-heating lamp and magnifying glass for 20 minutes (per sample). All visible blood and connective tissue was removed. 1 ml of ice-cold 0.5M PCA, containing 1 mmol/L EDTA, was then added to each aliquot of powdered muscle. This method of extracting the metabolites contained within the powder was originally described by Harris et al. (1974). Samples were then kept on ice over a period of 10 minutes with

each one, in turn, being subjected to vigorous intermittent vortexing. Extracts were then centrifuged for 3 minutes at 4°C at 10000 rpm. The resulting supernatants were removed and neutralised by adding 2.2 mol/L $KHCO_3$. The extracts were then re-centrifuged for 3 minutes at 4°C at 10000 rpm, followed by the removal and storage of their supernatants at -80 °C for later analysis of metabolites. The extraction dilution factor (F) for each sample was calculated as follows **(calculation 2.1)**. See **Appendix 4** for biochemical buffers and reagents.

Calculation 2.1:

$$EF = \frac{VolPCA \times (VolSUP + VolKHCO3)}{(VolSUP \times WtMUS)}$$

Where:

EF = Extraction Factor

VolPCA = Volume of PCA added (µl)

VolSUP = Volume of PCA supernatant (µl)

VolKHCO3 = Volume of KHCO3 added to the PCA extract (µl)

WtMUS = Weight of the muscle powder (mg)

The assays for phosphocreatine, lactate and glycogen are all NAD^+ linked reactions. This means that when measured at a wavelength of 340 nm, light absorbance of 6.22 OD changes for every mmol of NAD^+ being reduced to NADH. Consequently, by using a spectrophotometer (Spectra Max 190, Molecular Devices Ltd., Wokingham, UK), the change in light absorbance was measured in order to calculate the metabolite content of each sample.

2.5.2. Muscle free creatine (Cr) content

Free creatine was calculated using a modification of the method of Harris et al. (1974). Twenty µL of PCA extract or an H_2O blank was added to a plate and incubated with a glycine buffer containing PK, LDH, 150 µM NADH, 1mM PEP and 1.5 mM ATP for 5 minutes and set to pH 9. Then the light absorbance was read spectrophotometrically at a wavelength of 340 nm. By adding CPK, ATP was hydrolysed to ADP (by the endogenous free creatine) **(reaction 2.2)**. Then PK catalysed the phosphorylation of ADP by PEP to form ATP and pyruvate **(reaction 2.3)**. In the final reaction pyruvate was reduced by NADH to form NAD^+ and lactate (catalysed by LDH) **(reaction 2.4)**.

Reaction 2.2: ATP + Cr $\xrightarrow{\text{CPK}}$ ADP + PCr

Reaction 2.3: ADP + PEP $\xrightarrow{\text{PK}}$ ATP + pyruvate

Reaction 2.4: Pyeuvate + NADH $\xrightarrow{\text{LDH}}$ lactate + NAD^+

After an incubation period of 30 minutes, the absorbance was read again at 340 nm. The change in absorbance was used to calculate the free creatine content in mmol/kg dm **(calculation 2.2)**.

Calculation 2.2:

$$Cr\ (mmol/kg\ dm) = \frac{(Vol2 \times (Abs2 - Bla2) - Vol1 \times (Abs1 - Bla1)) \times EF}{(6.22 \times SVol)}$$

Vol1: initial reaction volume (µL)

Vol2: reaction volume after adding CPK (µL)

Abs1: absorbance of the sample before the addition of CPK

Abs2: absorbance of the sample after the addition of CPK

6.22: mM absorption coefficient for NADH at a wavelength of 340 nm

SVol: sample volume

EF: PCA extraction factor (L/kg)

2.5.3. Muscle Phosphocreatine (PCr) content

Muscle PCr content was determined spectrophotometrically in NADPH/NADH linked reactions (Harris et al., 1974) as outlined below:

20 µL of the PCA extract or an H2O blank was added to a 96 well plate and incubated for 5 min with a triethanolamine buffer set to pH 7.5 containing 40 µM ADP, 5 mM glucose and 1mM NADP. The light absorbance was read in the plate spectrophotometer at a wavelength of 340 nm. CPK (dissolved in 0.5% $NaHCO_3$ + 0.05% BSA) was then added to the reaction mixture to catalyse the phosphorylation of ADP by endogenous PCr to form ATP and creatine **(reaction 2.5)**.

Reaction 2.5:

$$\text{Phosphocreatine} + \text{ADP} + H^+ \xrightarrow{\text{CPK}} \text{Creatine} + \text{ATP}$$

The resultant ATP was then hydrolysed by glucose to form ADP and Glu-6-P **(reaction 2.6)** which subsequently reduced NADP+ to NADPH and 6-P-gluconolactone in a reaction catalysed by Glu-6-PDH **(reaction 2.7)**.

Reaction 2.6: $\text{ATP} + \text{glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{Glu-6-P}$

Reaction 2.7:

$$\text{Glu-6-P} + \text{NADP}^+ \xrightarrow{\text{Glu-6-PDH}} \text{6-P-gluconolactone} + \text{NADPH} + \text{H}^+$$

The samples were incubated for 15 minutes after the addition of CPK to have the reaction completed, after which the absorbance was read again at 340 nm. The reduction of $NADP^+$ to NADPH caused a change in absorbance which was used to calculate the PCr concentration, measured in mmol/kg dm, present in the original sample **(calculations 2.3, 2.4 and 2.5)**.

Calculation 2.3:

$$[\text{G-6-P}]\ \text{mmol/kg dry muscle} = \frac{(V2\,(A2 - Bl2) - V1\,(A1 - Bl1)) \times F}{6.22 \times SV}$$

Calculation 2.4:

$$[\text{ATP}]\ \text{mmol/kg dry muscle} = \frac{(V3\,(A3 - Bl3) - V2\,(A2 - Bl2)) \times EF}{6.22 \times SV}$$

Calculation 2.5:

$$[PCr]\ mmol/kg\ dry\ muscle = \frac{(V4\ (A4 - Bl4) - V3\ (A3 - Bl3))\ x\ EF}{6.22\ x\ SV}$$

Where:

V1 = initial reaction volume (µl)

V2 = reaction volume after the addition of Glu-6-PDH (µl)

V3 = reaction volume after the addition of HK (µl)

V4 = reaction volume after the addition of CPK (µl)

A1 (or Bl1) = absorbance of the sample before the addition of Glu-6-PDH, HK or CPK (or blank)

A2 (or Bl2) = Absorbance of the sample following the addition of Glu-6-PDH (or blank)

A3 (or Bl3) = Absorbance of the sample following addition of HK (or blank)

A4 (or Bl4) = Absorbance of the sample following addition of CPK (or blank)

EF = PCA extraction factor (L/kg)

6.22 = mMr absorption coefficient of NADPH at 340 nm (cm^2/µmol)

SV = sample volume

2.5.4. Muscle lactate content

Muscle lactate content was determined using a modification of the enzymatic assay developed by Harris et al. (1974). 20 µL of the PCA extract or an H_2O blank was added to a 96 well plate and incubated with a glycine buffer set to pH 9 and fresh NAD^+ for 5 minutes. The light absorbance was then read in the plate spectrophotometer at a wavelength of 340 nm. The assay began by adding lactate dehydrogenase (LDH) catalysing the oxidation of the endogenous lactate by NAD^+ to form pyruvate and NADH **(reaction 2.8)**.

Reaction 2.8: $\text{Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{pyruvate} + NADH + H^+$

The reduction of NAD^+ to NADH caused a change in absorbance which was used to calculate the lactate concentration measured in mmol/kg dm, present in the original sample **(calculation 2.6)**.

Calculation 2.6:

$$[\text{Lactate}]\ \text{mmol/kg dry muscle} = \frac{(V2\ (A2 - Bl2) - V1\ (A1 - Bl1)) \times F}{(6.22 \times SV)}$$

Where:

V1 = initial reaction volume (µl)

V2 = reaction volume after the addition of LDH (µl)

A1 (or Bl1) = absorbance of the sample before the addition of LDH (or blank)

A2 (or Bl2) = absorbance of the sample after the addition of LDH (or blank)

EF = PCA extraction factor (L/kg)

6.22 = mM absorption coefficient of NADPH at 340 nm (cm^2/µmol)

SV = sample volume

2.5.5. Muscle glycogen content

Muscle glycogen content was determined using a modification of the method of Harris et al. (1974) and utilized 1 - 3 mg of freeze dried muscle powder. Muscle glycogen was first hydrolysed by heating at 80 °C for 10 minutes in 100 – 160 µl of 0.1 mol/L NaOH. This was followed by neutralization with 400 – 640 µl of buffered HCl (3:1 ratio of 0.2 mol/L citric acid/0.2 mol/L Na2HPO4 buffer: 0.1 mol/L HCl). The resultant mixture was then incubated for 1 hour in 15 – 25 µl aminoglucosidase and the supernatant stored at - 80 °C for the subsequent measurement of glucose. The extraction factor of the sample was calculated by the following equation **(calculation 2.7)**.

Calculation 2.7:

$$EF = \frac{Vol_{NaOH} + Vol_{buffer} + Vol_{AGDase}}{Wt_{mp}}$$

Where:

EF = extraction factor

Vol_{NaOH} = volume of sodium hydroxide added (µL)

Vol_{buffer} = volume of citrate and hydrochloric acid buffer added (µL)

Vol_{AGDase} = volume of sodium AGDase added (µL)

Wt_{mp} = weight of muscle powder (µg)

Muscle glycogen was determined by 25 µL of the muscle extract or an H_2O blank being added to a 96 well plate and incubated with a triethanolamine (TEA) buffer (set to pH 8.2 and containing 0.75 mM ATP, 1 mM NAD^+ and glu-6-PDH) for 5 minutes. The light absorbance was then read in the plate spectrophotometer at a wavelength of 366 nm. The assay started by adding HK which initiated a 2 step reaction. First the endogenous glucose was phosphorylated by ATP to form ADP and Glu-6-P, in a reaction catalysed by HK. Then the Glu-6-P reduced NAD^+ to NADH and produced 6-P-gluconolactone, catalysed by the Glu-6-PDH added in the initial reagent. Following 20 minutes of incubation at room temperature the absorbance

was read again at 340 nm. The reduction of NAD^+ to NADH caused a change in absorbance which was used to calculate the glucose concentration (equal to muscle glycogen content), present in the original sample and measured in glycosyl units mmol/kg dm **(calculation 2.8)**.

Calculation 2.8:

$$\text{Glycogen (glucosyl units mmol/kg dm)} = \frac{(V2 \times (A2 - Bl2) - V1 \times (A1 - Bl1)) \times EF}{(3.4 \times SV)}$$

Where:

V1 = initial reaction volume (µl)

V2 = reaction volume after the addition of HK (µl)

A1 (or Bl1) = absorbance of the sample before the addition of HK (or blank)

A2 (or Bl2) = absorbance of the sample after the addition of HK (or blank)

EF = PCA extraction factor (L/kg)

3.4 = mM absorption coefficient of NAD^+ at 340 nm ($cm^2/\mu mol$)

SV = sample volume

2.5.6. Muscle free carnitine content

A modification of the method described by Cederblad and Lindstedt assay (1972) was used to determine muscle free carnitine content. In summary, 10 µL of the PCA extract, made up to 120 µL with H_2O, was used to determine muscle free carnitine content in a manner similar to that described earlier for plasma free carnitine concentration. Muscle free carnitine content was calculated based on the slope of the regression line from a standard curve and conversion to mmol/kg dm. The regression coefficient was usually > 0.9995.

2.5.7. Muscle long chain acyl-carnitine content

PCA metabolite extraction left an acid insoluble pellet, which was used to measure muscle long chain acyl-carnitine content. First, the pellet was re-suspended in 0.5 ml of KOH (0.2 M), containing DTT (10 mM), and dissolved by vortexing. Then samples were incubated at 55 °C for 2 hours to achieve acyl-carnitine bond hydrolysis. Following this samples were cooled on ice, neutralised by adding 20 µL PCA (5M), vortexed and centrifuged at 4 °C for 3 minutes at 10000 rpm. The supernatant was extracted and 30 µL was added to 90 µL H_2O and analysed for free carnitine as described for plasma free carnitine concentration (Cederblad and Lindstedt, 1972). The content of free carnitine was calculated by using the slope of the regression line from a standard curve and conversion to mmol/kg dm. The regression coefficient was usually > 0.9995.

2.5.8. Muscle acetylcarnitine and acetyl-CoA content

Cederblad et al. (1990) method was used to determine muscle acetylcarnitine content in PCA muscle extracts. First the samples and standards were incubated in a HEPES buffer, containing 11 mM of EDTA, 0.166 mM of CoA and CAT, for 30 minutes. This facilitated the acetyl group transfer from acetylcarnitine to CoASH, in a reaction catalysed by carnitine acetyltransferase, as outlined below **(reaction 2.9)**.

Reaction 2.9:

Acetylcarnitine + CoASH $\xleftrightarrow{\text{CAT}}$ acetyl-CoA + carnitine

Then, by heating the samples at 95 °C for 3 minutes, the CAT enzyme became dysfunctional and the reaction stopped. Afterwards [^{14}C]-oxaloacetate was added to acetyl-CoA to produce citrate and CoASH, catalysed by the enzyme citrate synthase **(reaction 2.10)**.

Reaction 2.10:

[^{14}C]-oxaloacetate + acetyl-CoA $\xleftrightarrow{\text{CS}}$ [^{14}C]-citrate + CoASH

Fresh [^{14}C]-oxaloacetate was in turn produced from [^{14}C]-aspartate (Cooper et al., 1986), catalysed by the enzyme glutamic oxalacetic transaminase (GOT) **(reaction 2.11)**.

Reaction 2.11:

[^{14}C]-aspartate + α-ketoglutarate $\xleftrightarrow{\text{GOT}}$ [^{14}C]-oxaloacetate + L-glutamate

Any remaining [^{14}C]-oxaloacetate was transaminated back to [^{14}C]-aspartate enabling the [^{14}C]-citrate to be recovered, by the addition of 1 ml of Dowex (H^+) resin (18 g/30 ml reagent grade water) removing the negatively charged [^{14}C]-aspartate from the supernatant. Sample radioactivity was determined within a 0.5 ml aliquot of the supernatant, to which 5 ml of scintillation fluid was added, with each sample being counted over 5 minutes (1214 Rackbetar, β-liquid scintillation counter, LKB Wallac). An acetyl-CoA standard curve (0 – 125 pmol; $r > 0.9999$) was run in duplicate within each batch of analysis.

The supernatant was also quantified in a β-radioactivity scintillation counter (Tri-Carb 2100TR, Canberra-Packard, Melbourne, Australia). Acteylcarnitine concentration was calculated by using the slope of the regression line from the standard curve.

2.5.9. Muscle total carnitine content

Muscle total carnitine consists of free carnitine, acetylcarnitine and long chain acyl-carnitine; therefore muscle total carnitine analysis involved the summation of these carnitine moieties. None of the carnitine moieties can cross the muscle cell membrane. Consequently, in order to compensate for any errors arising from non-muscular components in the muscle biopsy samples, the muscle total carnitine content was corrected for the muscle total creatine content (combination of PCr and free creatine content).

2.5.10. Muscle pyruvate dehydrogenase complex activity (PDCa)

Muscle PDC activation status was assessed using the method of Constantin-Teodosiu et al. (1991b). Approximately 5 mg of wet muscle was homogenised on ice with a glass tissue homogeniser for 50 seconds in an ice cold sucrose buffer. Subsequently the amount of PDC in its active state was conformationally locked by the addition of DCA and NaF to the buffer, to inhibit the intrinsic kinase (PDK) and phosphatase (PDP) respectively. 30 µL of each homogenised sample was added to 720 µL aliquots of a pre-warmed (37 °C) incubation buffer containing NAD^+ and CoASH. This was followed by the addition of 26 mM pyruvate. This started the conversion of pyruvate to acetyl-CoA, with the rate of conversion dependent on the amount of endogenous PDCa **(reaction 2.12)**.

Reaction 2.12:

$$\text{Pyruvate} + NAD^{+} + \text{CoASH} \xrightarrow{\text{PDCa}} \text{acetyl-CoA} + \text{NADH}$$

Every minute for 3 minutes, 210 µL of the incubation mixture was removed and added to 0.5 M PCA to arrest the reaction, in order to measure the rate of pyruvate conversion to acetyl-CoA. Following centrifugation for 2 minutes at 14000 rpm, the supernatant was removed and stored at -80 °C for the analysis of acetyl-CoA (Cederblad et al., 1990) at a later date. The slope of the regression line from the acetyl-CoA produced at minutes 1, 2 and 3 was used to calculate the PDCa in mmol acetyl-CoA/kg wm. The regression coefficient was invariably > 0.9995.

2.5.11. Muscle 2-deoxyglucose (2-DG) content

Thirty to 40 mg of wet muscle was ground to powder under liquid nitrogen. The muscle powder was transferred to 70% ethanol, vortex mixed and centrifuged at 5000 gravity for 10 minutes. Then the supernatant of the sample was dried down under nitrogen and reformed in doubly distilled water at a pH 9. Afterwards the sample was divided into two equal fractions. Ten µL of FDG (fluorodeoxyglucose) was added to the first fraction for determining free 2-DG concentration. Twenty µL 100 mmol/L of $MgCl_2$ and 1 U of alkaline phosphatase were added to the second fraction. The sample was vortex mixed and heated at 37° C for 50 minutes. Ten µL FDG was then added for determining total 2-DG. Both fractions were dried under nitrogen. Then the samples were derivatized and concentrations were determined

spectrophotometrically with reference to a standard curve of known 2-DG concentration (Cuthbertson et al., 2007, Babraj et al., 2009).

2.6. Coefficients of variation for analytical measurements

The coefficients of variation (CV) for the measurements of substrate and metabolite concentrations in blood and contents in muscle are presented in **Table 2.1**. This was used to demonstrate the relative variability of the analyses and was calculated as follows **(calculation 2.9)**.

Calculation 2.9:

$$CV\ \% = \frac{\text{Standard deviation of repeated measurement}}{\text{Mean of repeated measurement}} \times 100$$

Table 2.1: coefficients of variance

Tissue/fluid	Measurement	n	CV (%)
Blood	Glucose	10	2.7
Plasma	Carnitine	10	3.4
Serum	Insulin	10	3.9
Muscle	Free carnitine	10	3.0
Muscle	Acetylcarnitine	10	2.2
Muscle	PCr	10	3.4
Muscle	Glycogen	10	2.2
Muscle	Lactate	10	3.6
Muscle	PDCa	10	5.6

2.7. Statistics

All the values within this thesis are reported as means ± SEM, calculated using Microsoft Excel 2010 (Microsoft Corporation, CA, USA). Any impact of time or treatment, on any variables measured in the studies presented in this thesis (metabolite concentrations, expired gases, body composition and exercise performance), were statistically analysed using a repeated measures two-way (time and treatment) analysis of variance (ANOVA) on GraphPad Prism 6 software (GraphPad Software Inc, CA, USA). On finding a significant time or treatment effect, a Bonferroni's post-hoc test or Student's t test were used to detect individual differences (GraphPad Software Inc, CA, USA). Statistical significance was declared at $p < 0.05$, and a trend for difference at $0.05 < p < 0.1$. All the graphs presented in this thesis were made by the GraphPad Prism 6 software too.

Chapter 3

THE IMPACT OF INCREASING SKELETAL MUSCLE ACETYL GROUP AVAILABILITY BY SODIUM ACETATE INFUSION ON FUEL UTILISATION DURING LOW AND HIGH INTENSITY STEADY-STATE EXERCISE IN YOUNG HEALTHY VOLUNTEERS

3.1. Introduction

Increasing muscle acetyl group availability has been tried in multiple animal model (Timmons et al., 1996, Roberts et al., 2002, Roberts et al., 2005) and human volunteer studies (Timmons et al., 1998a, Timmons et al., 1998b, Howlett et al., 1999a, Howlett et al., 1999b, Evans et al., 2001). This has been achieved either through oral or intravenous sodium acetate administration (Howlett et al., 1999b, Evans et al., 2001, Roberts et al., 2005) or via pharmacological activation of PDC (Timmons et al., 1996, Timmons et al., 1998a, Roberts et al., 2002). Some studies investigated the impact of increased muscle acetyl group availability on muscle metabolism in the rest to exercise transition period (Timmons et al., 1996, Howlett et al., 1999a and 1999b, Evans et al., 2001, Roberts et al., 2002) when it was believed that there was a state of PDC lag and acetyl group deficit (Greenhaff et al., 2002), whilst others focused on the effect of acetyl group stockpiling on muscle fuel metabolism during rest and sub-maximal steady-state exercise (Akanji et al., 1989, Putman et al., 1995, Smith et al., 2007, Smith et al., 2013). Relevant to our research would be the studies which administered sodium acetate to human volunteers and investigated its impact on fuel metabolism at rest and/or during sub-maximal steady-state exercise.

Akanji et al. (1989) infused sodium acetate at a rate of 2.5 mmol/min over 60 minutes to non-diabetic and diabetic subjects at rest. The team aimed to investigate the impact of increased plasma acetate concentration, as reported to happen in patients suffering with diabetes mellitus (Buckley and Williamson, 1977, Smith et al., 1986), on substrate utilisation in patients with diabetes mellitus and a non-

diabetic control group. The authors reported significantly lower post-infusion whole-body fat oxidation in both groups compared to corresponding baselines ($p < 0.05$) despite no changes in energy expenditure, and suggested that administered acetate oxidation replaced fat metabolism in both groups.

Randle cycle was originally reported in studies performed *in vitro* in rat heart muscle preparations (Garland and Randle, 1964, Garland et al., 1964, Kerbey et al., 1977, Kerbey et al., 1976, Wieland et al., 1971a, Wieland et al., 1971b), rat skeletal muscle preparations (Kruszynska et al., 1990) and rat muscle mitochondrial preparations (Ashour and Hansford, 1983). Randle et al. (1963) suggested that increasing fatty acid oxidation resulted in increased acetyl-CoA concentration which in turn exerted an inhibitory impact on PDC activation. In order to investigate Randle cycle in human skeletal muscle *in vivo*, Putman et al. (1995) infused 400 mmol of sodium acetate to human volunteers over 40 minutes: 20 minutes of rest followed immediately by 5 minutes of exercise at 40% VO_2max and 15 minutes of exercise at 80% VO_2max. The same dose of sodium bicarbonate was used as a control in the same volunteers two weeks apart to match the metabolic alkalosis induced by sodium acetate. The team hypothesised that increasing muscle acetyl-CoA content secondary to sodium acetate infusion would decrease skeletal muscle PDC activity at rest and during low and high intensity exercise, consistent with the classic Randle cycle hypothesis. The team reported that muscle PDCa declined significantly following 20 minutes of rest in the sodium acetate group ($p < 0.05$, compared to baseline) which was associated with a ~2.6-fold raise in acetyl Co-A/ CoASH ratio ($p < 0.05$, compared to baseline). However there were no differences between the sodium acetate and the CON groups in muscle PDCa, acetyl

CoA/CoASH ratio and glycogen utilisation during exercise at low and high intensities. The team reported that acetyl CoA/CoASH ratio had an inhibitory impact on PDCa at rest as originally reported by Randle et al. (1963). However this was overcome during exercise at low and high intensities and the authors concluded that the classic Randle cycle was functional in humans only at rest and not during exercise.

Smith et al. (2007) investigated resting muscle fuel metabolism 180 minutes following oral ingestion of sodium acetate (2 mmol/kg body mass) with labelled [1,2-^{13}C] sodium acetate added and demonstrated the blocking of alkalosis-induced increased fat oxidation associated with sodium bicarbonate (CON) ingestion at the same dose. The authors reported that post-ingestion fat oxidation increased significantly in sodium bicarbonate (CON) group ($p < 0.01$, compared to pre-ingestion) but observed no change in fat oxidation in the sodium acetate group. The authors concluded that this was due to the administered acetate replacing nearly fully the additional fat oxidation observed during the sodium bicarbonate study. A later study by the same team (Smith et al., 2013) reported a decrease in the rate of whole-body fat oxidation at rest 90 minutes after ingestion of sodium acetate (4 mmol/kg body mass; $p < 0.05$ compared to pre-ingestion). This was not reciprocated by any change in resting carbohydrate oxidation. Furthermore, no changes were reported in the rates of fat or carbohydrate oxidation at rest during a control visit (a similar dose of sodium bicarbonate ingested by the same volunteers one week apart). The resting phase of the study was followed by 120 minutes of exercise at 50% VO_2peak and the authors reported a lower rate of fat oxidation ($p < 0.05$) and a higher rate of carbohydrate oxidation ($p < 0.05$) over the initial 15

minutes of exercise following sodium acetate ingestion compared to control. The authors concluded that the administered acetate replaced the fat contribution to fuel oxidation at rest and at the onset of moderate intensity exercise. There were no differences in substrate utilisation over the final 75 minutes of exercise between the sodium acetate and control visits.

In summary, the above-mentioned human studies suggested that administered acetate replaced fat as an oxidative fuel at rest (Akanji et al., 1989, Smith et al., 2007) and at the onset of moderate intensity exercise (Smith et al., 2013) and that increased muscle acetyl group availability had an inhibitory impact on PDC activity at rest, and not during exercise (Putman et al., 1995).

Muscle lactate accumulation during rest to exercise transition

A study by Karlsson and Saltin (1970) demonstrated that the majority of lactate accumulation in the exercising muscle happened during the rest to exercise transition, with a lesser accumulation during the steady-state exercise. In this study the volunteers performed maximal bicycle exercise at a mean of 1630 kpm/min for 16 minutes and had muscle biopsies taken during exercise at minutes 2, 6 and 16. Whilst muscle lactate increased sharply to 8.5 mmol/kg ww at minute 2, it only rose by 1 to 9.5 mmol/kg ww at minute 6 and by a further 2.5 to 12 mmol/kg ww at minute 16, i.e. 70% of the lactate accumulated in the initial 2 minute rest to steady-state transition. The authors suggested that skeletal muscle lactate content measured at the end of the steady-state exercise reflected the initial rest to exercise transition which coincides with acetyl group deficit and PDC inertia.

Acetate metabolism

Numbers between 80% to 100% have been put forward for the percentage of administered sodium acetate metabolising in human body (Burnier et al., 1992, Chiolero et al., 1993, Akanji et al., 1989, Smith et al., 2007).

Akanji et al. (1989) infused 150 mmol of sodium acetate over 60 minutes to non-diabetic and diabetic subjects and made a simplifying assumption that 90% of administered acetate was oxidised by the subjects. This assumption was made based on a comprehensive review of previous studies investigating acetate metabolism in human volunteers and animal models (Vreman et al., 1980, Richards et al., 1982, Skutches et al., 1979, Harper et al., 1953, Davidson et al., 1978, Shelmet et al., 1988). Assuming that 90% of administered acetate was metabolised, the team calculated the 'non-acetate' VO_2 and VCO_2 values (oxidation of 1 mmol of acetate utilises 44.8 ml O2 and produces 22.4 ml CO2) and from that, using Frayn formulae (Frayn, 1983), calculated fat and carbohydrate oxidation rates. The team also reported that changing the assumption for the percentage of administered acetate metabolised from 90% to 75% had no impact on the magnitude of difference between values for pre and post-infusion fat and carbohydrate oxidation rates.

Burnier et al. (1992) and Chiolero et al. (1993) reported that 100% of administered acetate was metabolised in their studies. Burnier et al. (1992) infused sodium acetate at a rate of 2.5 mmol/min over 120 minutes and Chiolero et al. (1993) infused sodium acetate at a rate of 20 µmol/kg/min over 180 minutes to human volunteers. This assumption was made based on the fact that the body cannot store

acetate and acetate excretion in the urine is negligible; and also noticing that in their studies the net rate of acetate oxidation equalled its infusion rate.

Using a stable isotope technique, Smith et al. (2007) calculated that 80% of the administered acetate had been oxidised at rest 180 minutes following 2 mmol/kg sodium acetate ingestion.

Bearing in mind that fat is the principal fuel utilised during rest and steady-state low intensity exercise and carbohydrate the main fuel source during steady-state high intensity exercise (Van Loon et al. 2001), we designed a study to determine the impact of stockpiling muscle acetyl groups independent of PDC activation on skeletal muscle fuel metabolism during steady-state exercise at 50% and 80% VO_2max. We hypothesised that increasing muscle acetyl group availability by sodium acetate infusion would decrease fat oxidation during steady-state low intensity exercise as a result of either decreased free carnitine availability (secondary to free carnitine being acetylated to acetylcarnitine) limiting mitochondrial fatty acid translocation or due to increased muscle acetate oxidation substituting fat utilisation and would decrease the contribution of non-mitochondrial ATP production during steady-state high intensity exercise due to increased availability of acetyl groups to the TCA cycle.

3.2. Methods

Subjects

Seven healthy young male volunteers (age 22.3 ± 0.6 yr, body mass 71.9 ± 3.8 kg, body mass index 23.5 ± 1.2 kg/m^2) took part in this study.

Study protocol

All the volunteers were non-vegetarians and recreationally active and screened before being recruited into the study. The screening consisted of taking a medical history, a 12 lead ECG, measurement of height and weight and a blood sample which was sent to Queens Medical Centre pathology laboratory to have FBC, U&E, LFT and clotting screen checked. All volunteers signed a consent form and were informed that they could leave the study at any time and were under no obligation to provide any explanations.

This study consisted of 4 visits to the David Greenfield Human Physiology Unit in the Medical School, Queens Medical Centre, Nottingham **(Fig. 3.1)**. The first visit consisted of a physical exercise test to determine VO_2max where volunteers cycled to exhaustion at 3 minute 30W workload increments on an electronically braked cycle ergometer (Lode Excalibur, Lode, Groningen, Netherlands). VO_2max measurement was needed to calculate the exercise intensities for volunteers during subsequent laboratory visits, i.e. 50% and 80% VO_2max (Vmax, Sensor Medics corporation, California, USA).

The second laboratory visit was a repeat VO_2max test, which was done to verify that maximal oxygen consumption had been reached in the previous exercise test. This was shorter than the first visit and involved increasing the exercise workload to the highest intensity reached in the previous test following 5 minutes of low intensity warm up exercise.

On the third and fourth (experimental) visits, subjects reported to the laboratory after an overnight fast and were randomised to either a sodium bicarbonate (control) or sodium acetate (NaAc) visit **(Fig 3.1)**. The two visits were one week apart.

The subjects were asked to abstain from alcohol and strenuous physical activity for 48 hours before and to fast from 10 pm the night before each experimental visit. On the day of an experimental visit, volunteers reported to the laboratory at 09:00 am and were asked to rest semi-supine on a bed while a cannula (Venflon, Becton Dickinson, New Jersey, USA) was inserted into a dorsal hand vein of one arm for blood sampling, through a 3 way tap attached to the cannula (BD Connecta, Becton Dickinson, Helsingborg, Sweden), and into an ante-cubital vein of the contralateral arm for the infusion of either sodium bicarbonate or sodium acetate. A continuous infusion of either sodium bicarbonate or sodium acetate (500 mmol in a 1 L solution), was commenced and continued for 80 minutes. The same dose and volume of sodium bicarbonate was used as a control intervention to match the metabolic alkalosis and the volume effect created by sodium acetate infusion. A normal saline drip (0.9% sodium chloride, Maco Pharma Ltd, London, UK) was

attached to the retrograde cannula to keep it patent throughout the experimental visit.

The sodium acetate solution was quality controlled and made each day of investigation (Queen's Medical Centre pharmacy, Nottingham). Sodium bicarbonate polyfusors, 250 mmol in 500 ml solution (Fresenius Kabi ltd, Runcorn, UK) were also supplied by Queen's Medical Centre pharmacy. Two sodium bicarbonate polyfusor bags were used in each visit to make it total 500 mmol in 1 L solution equivalent in dose and volume to the sodium acetate infusion. After 30 minutes of rest on bed, the subjects were asked to cycle on an electronically braked cycle ergometer (Lode Excalibur, Lode, Groningen, Netherlands) for 30 minutes at a predetermined workload equivalent to 50% VO_2 max followed immediately by 20 minutes at 80% VO_2 max.

The cardiac rate and rhythm of the volunteers was monitored continuously by a three lead heart monitor (GE Medical Systems Technology Inc. Buckinghamshire, UK) throughout the study.

The flow of the volunteers along the study is depicted in a diagram **(Diagram 3.1)** based on CONSORT statement guidelines (Begg et al., 1996).

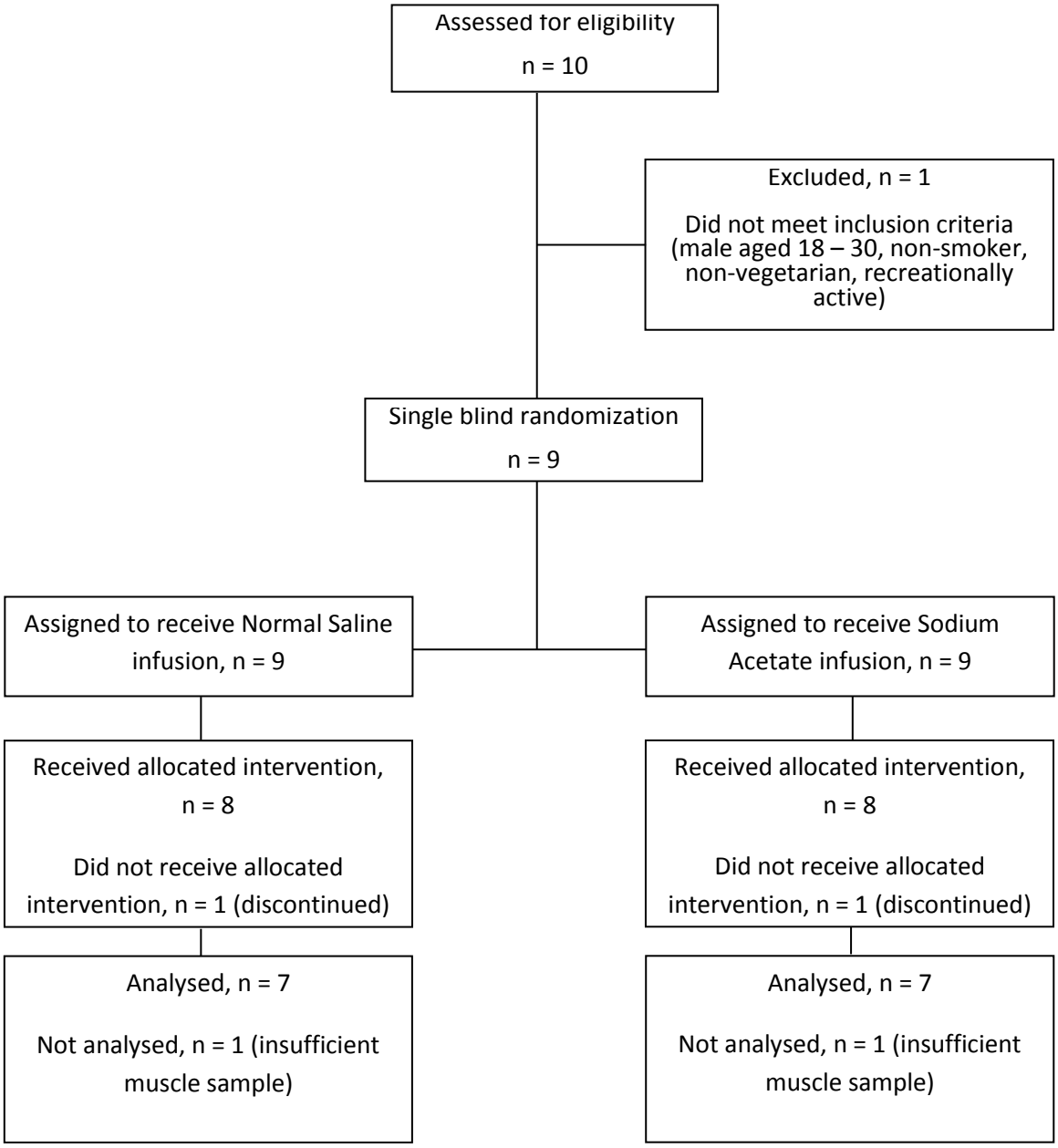


Diagram 3.1. The flow of the volunteers along the study depicted on the basis of CONSORT statement guidelines.

Sample collection and analysis

Indirect calorimetry

Expired gases were collected online for VO_2 and VCO_2 determination for 10 minutes at the end of rest and exercise periods (i.e. min 20-30, min 50-60 and min 70-80), to calculate whole-body fat and carbohydrate oxidation rates by indirect calorimetry (Vmax, SensorMedics corporation, California, USA).

The values for fat and carbohydrate oxidation during the sodium acetate visit were corrected for acetate metabolism, as previously described by Akanji et al (1989), in the following way: Oxidation of 1 mmol of acetate utilises 44.8 ml O_2 and produces 22.4 ml CO_2. We assumed that, in our study, 80% of acetate was oxidised at rest and 100% during exercise. This simplifying assumption was based on extensive literature review and the findings of multiple previous studies on acetate metabolism (See '*Acetate metabolism*' in the Chapter Introduction). As our infusion rate was 6.25 mmol/min (500 mmol over 80 min), we calculated that in our study 224 ml/min O_2 consumed at rest (6.25 × 44.8 × 0.8) and 280 ml/min O_2 during exercise (6.25 × 44.8 × 1.0) were attributable to acetate metabolism, whilst 112 ml/min CO_2 at rest (6.25 × 22.4 × 0.8) and 140 ml/min CO_2 at exercise (6.25 × 22.4 × 1.0) were liberated. These values were subtracted from the VO_2 and VCO_2 values at the corresponding time points ('uncorrected' values) to give the non-acetate VO_2 and VCO_2 values ('corrected' values). From the 'corrected' values, using Frayn formulae (Frayn, 1983), fat and carbohydrate (CHO) oxidation rates were calculated for the sodium acetate visit:

Fat oxidation (g/min): [1.67 × (VO_2 – VCO_2)]

Fat oxidation (kJ/min): [39.4 × Fat oxidation (g/min)]

CHO oxidation (g/min): [(4.55 × VCO_2) – (3.21 × VO_2)]

CHO oxidation (kJ/min): [15.6 × CHO oxidation (g/min)]

(VO_2 and VCO_2 are in L/min)

Muscle biopsy

Muscle biopsy samples were obtained from the Vastus Lateralis muscle immediately following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max (i.e. min 30, 60 and 80). Muscle biopsies were obtained using the Bergstrom percutaneous needle biopsy technique under sterile conditions after injection of Lignocaine 2% (B. Braun, Melsungen, Germany) as local anaesthetic. All muscle biopsy sites were prepared immediately before the experimental study started to make sure that biopsy sampling was undertaken in the shortest possible time. Muscle biopsy samples were immediately frozen in liquid nitrogen and then stored 'wet' in eppendorfs kept in liquid nitrogen. The samples were later freeze dried, powdered and analysed for PDCa, free carnitine, acetylcarnitine, acetyl CoA, PCr, glycogen and lactate. For details of analyses see CHAPTER 2: GENERAL METHODS.

Blood samples

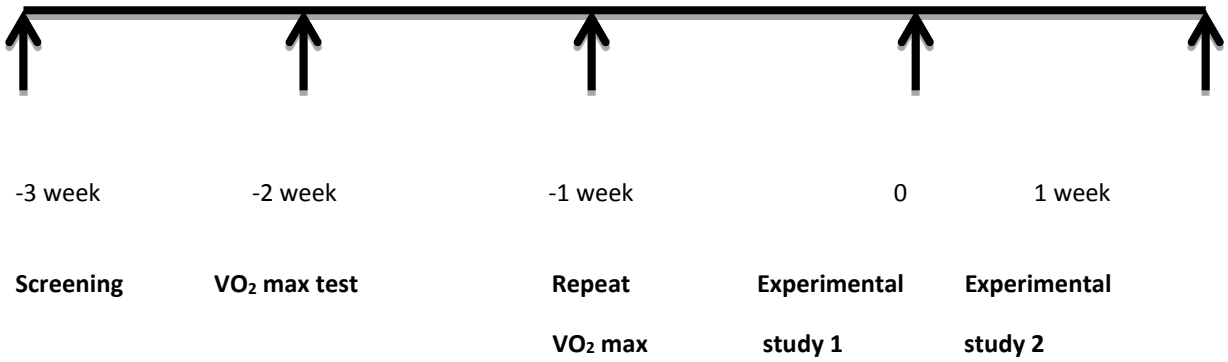
Venous blood samples were taken from a 3 way tap attached to a retrograde cannula at baseline and then every 15 minutes during rest and exercise at 50% VO_2max and every 10 minutes during exercise at 80% VO_2max for the bed side measurement of pH (i.e. min 0, 15, 30, 45, 60, 70 and 80).

At the end of the study all the lines were removed, the volunteers were fed and were allowed to leave the unit only after their venous blood pH measured at the bedside was back in the normal range (7.35 – 7.45). A standard dressing was applied on muscle biopsy incision areas including Steri-Strip (3M Deutschland GmbH, Neuss, Germany), tegaderm (3M Deutschland GmbH, Neuss, Germany) and coban compression bandage (3M Deutschland GmbH, Neuss, Germany) and a leaflet on how to look after the muscle biopsy areas was provided to them as well.

Statistical analysis and calculations

A repeated measures (RM) two-way ANOVA for interaction and exercise intensity effects with a Bonferroni multiple comparison test (GraphPad Prism 6, GraphPad Software Inc, California, USA) was performed to locate differences in muscle PDCa, carnitine, acetylcarnitine, glycogen, lactate and PCr contents and fat and carbohydrate oxidation rates. Statistical significance was declared at $p < 0.05$, and a trend for difference at $0.05 < p < 0.1$. All the values presented in text, tables, and figures are means ± standard error of the mean (SEM). All the graphs too were made by the GraphPad Prism 6 software.

Protocol:



Experimental visits:

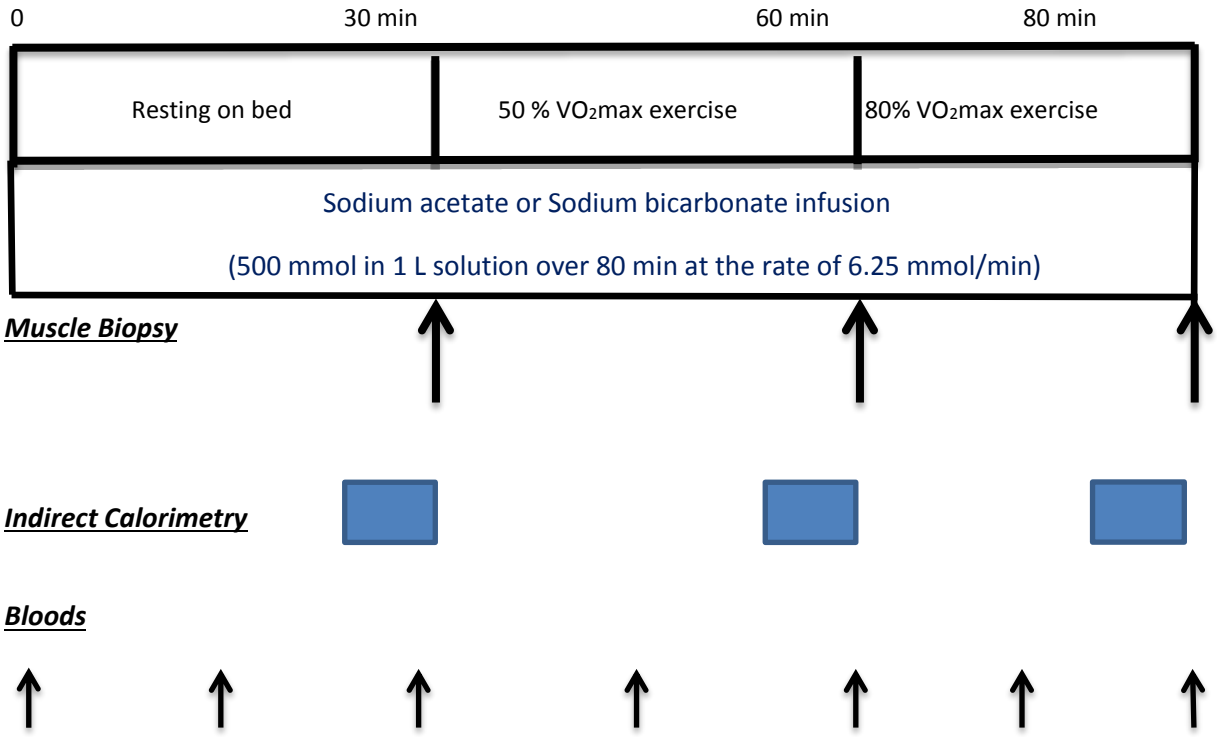


Figure 3.1. Study protocol and experimental visits

3.3. Results

Calorimetry data

Fat oxidation

Whole-body fat oxidation rate increased from 1.63 ± 0.60 and 0.38 ± 0.18 kJ/min following 30 minutes of rest to 11.40 ± 2.10 and 7.21 ± 1.38 kJ/min following 30 minutes of exercise at 50% VO_2max during sodium bicarbonate (CON) and sodium acetate (NaAc) infusions, respectively. Whole-body fat oxidation rate then decreased to 1.02 ± 0.69 and 0.16 ± 0.12 kJ/min following 20 minutes of exercise at 80% VO_2max during sodium bicarbonate (CON) and sodium acetate (NaAc) infusions, respectively **(Fig. 3.2)**. At 50% VO_2max the rate of whole-body fat oxidation was 37% lower in NaAc compared to CON ($p < 0.05$). There was no difference in whole-body fat oxidation between NaAc and CON at rest or during exercise at 80% VO_2max.

Carbohydrate oxidation

Whole-body carbohydrate oxidation rate increased from 4.86 ± 1.21 and 6.05 ± 1.70 kJ/min following 30 minutes of rest to 19.21 ± 2.34 and 18.33 ± 1.82 kJ/min following 30 minutes of exercise at 50% VO_2max during CON and NaAc administration, respectively. Whole-body carbohydrate oxidation rate increased

further to 56.66 ± 2.96 and 59.31 ± 4.69 kJ/min following 20 minutes of exercise at 80% VO_2max during CON and NaAc administration, respectively **(Fig. 3.3)**. The rate of whole-body carbohydrate oxidation was not different between CON and NaAc at any time-point.

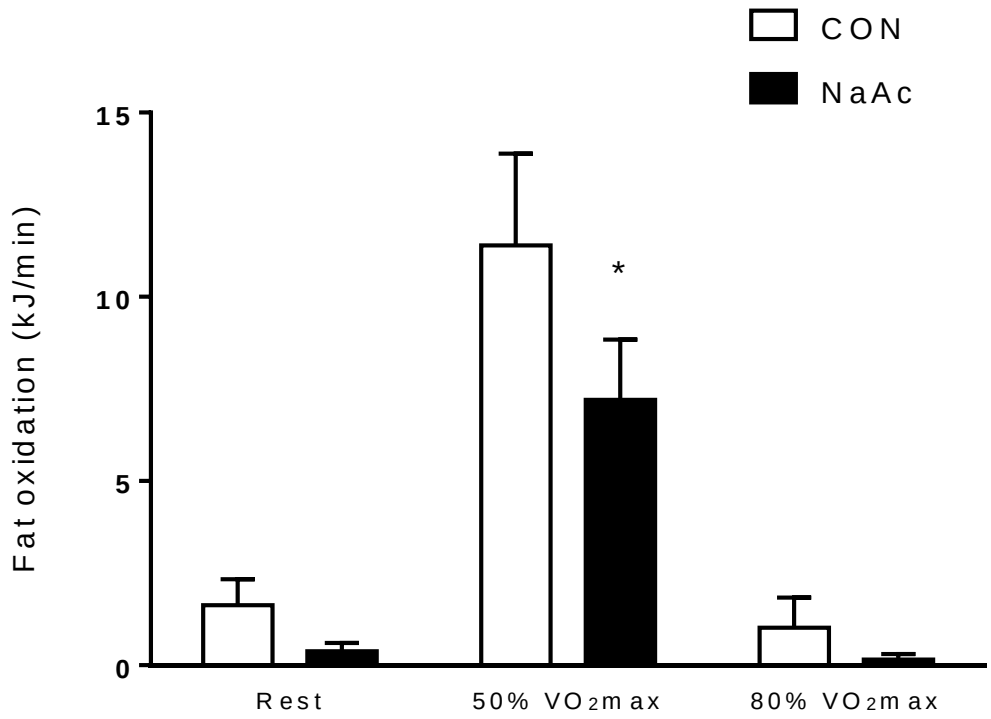


Figure 3.2. Whole-body fat oxidation following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as kJ/min.

Significantly different from the corresponding CON value: * $p < 0.05$.

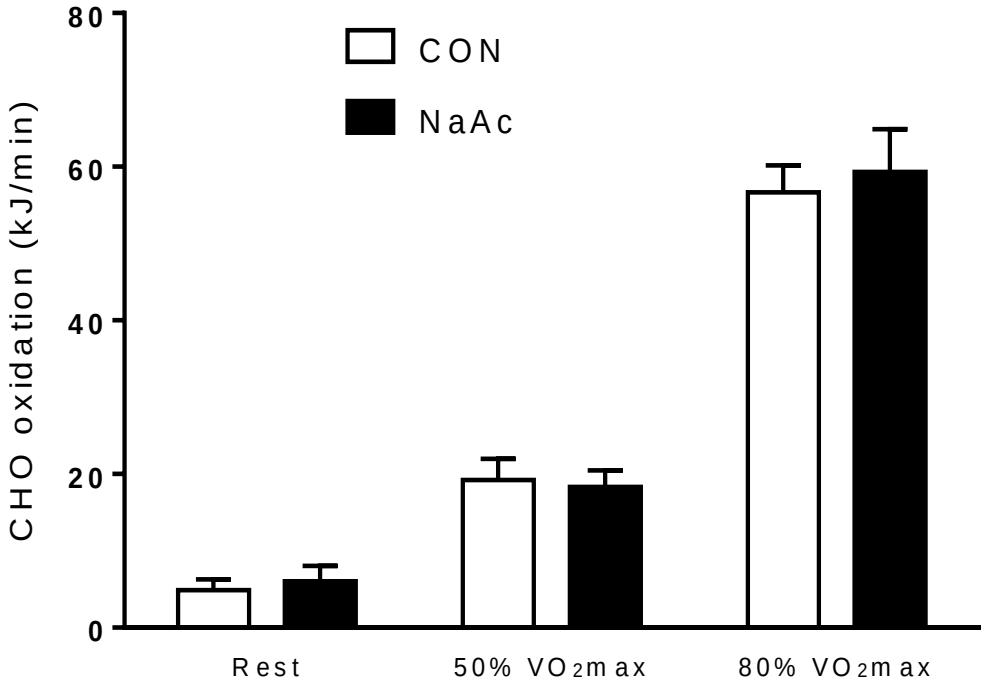


Figure 3.3. Whole-body carbohydrate oxidation following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as kJ/min.

Muscle carnitine moieties

Muscle free carnitine content decreased from 19.66 ± 0.98 and 11.71 ± 1.81 mmol/kg dm following 30 minutes of rest during CON and NaAc infusion, to 15.85 ± 1.29 and 11.28 ± 1.81 mmol/kg dm following 30 minutes of exercise at 50% VO_2max, respectively. Muscle free carnitine content declined further to 8.94 ± 1.91 and 5.98 ± 0.69 mmol/kg dm following 20 minutes of exercise at 80% VO_2max during CON and NaAc infusion, respectively **(Fig. 3.4)**.

Muscle acetylcarnitine content increased from 3.56 ± 0.70 and 7.38 ± 0.94 mmol/kg dm following 30 minutes of rest during CON and NaAc infusion, to 5.13 ± 1.27 and 8.21 ± 1.30 mmol/kg dm following 30 minutes of exercise at 50% VO_2max, respectively. Muscle acetylcarnitine content increased further to 11.81 ± 1.80 and 11.33 ± 1.11 mmol/kg dm following 20 minutes of exercise at 80% VO_2max during CON and NaAc infusion, respectively **(Fig. 3.5)**.

Muscle free carnitine content was 40% lower in NaAc compared to CON ($p < 0.05$) and muscle acetylcarnitine content was 2 times higher ($p < 0.05$) following 30 minutes of infusion at rest. Following 30 minutes of exercise at 50% VO_2max, muscle free carnitine content was 29% lower in NaAc compared to CON ($p = 0.06$), but there was no difference in acetylcarnitine content. Similarly, neither muscle free carnitine content nor muscle acetylcarnitine content were different when comparing groups at 80% VO_2max.

The contribution of acetylcarnitine (AC), free carnitine (FC) and long chain acylcarnitine (LCAC) to muscle total carnitine (TC) content is depicted in **Fig. 3.6**.

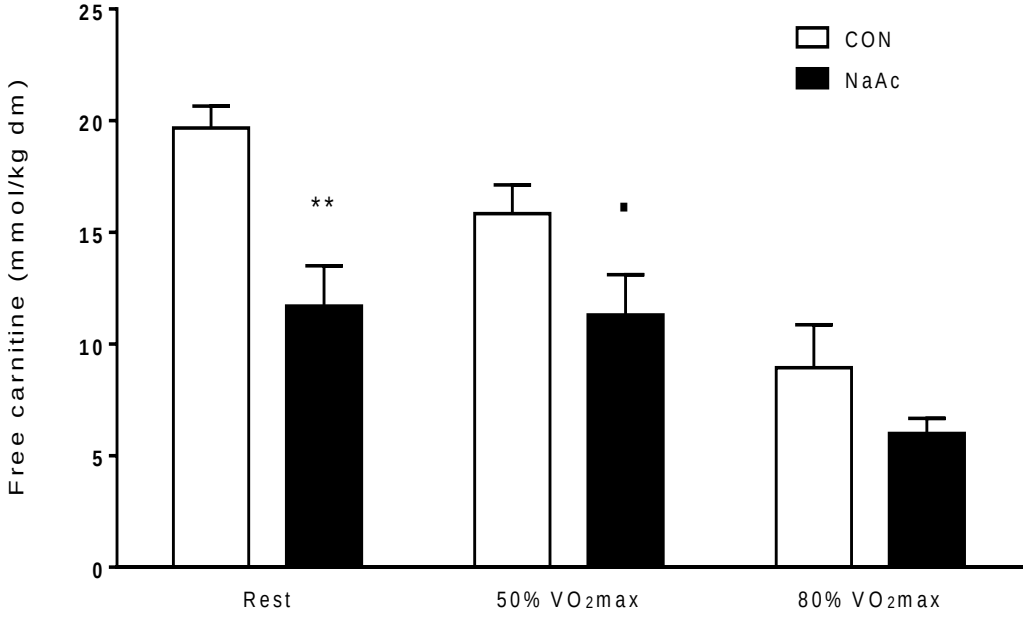


Figure 3.4. Skeletal muscle free carnitine content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Significantly different from the corresponding CON value: * $p \leq 0.05$.

Trend for difference from the corresponding CON value: ▪ $p = 0.06$.

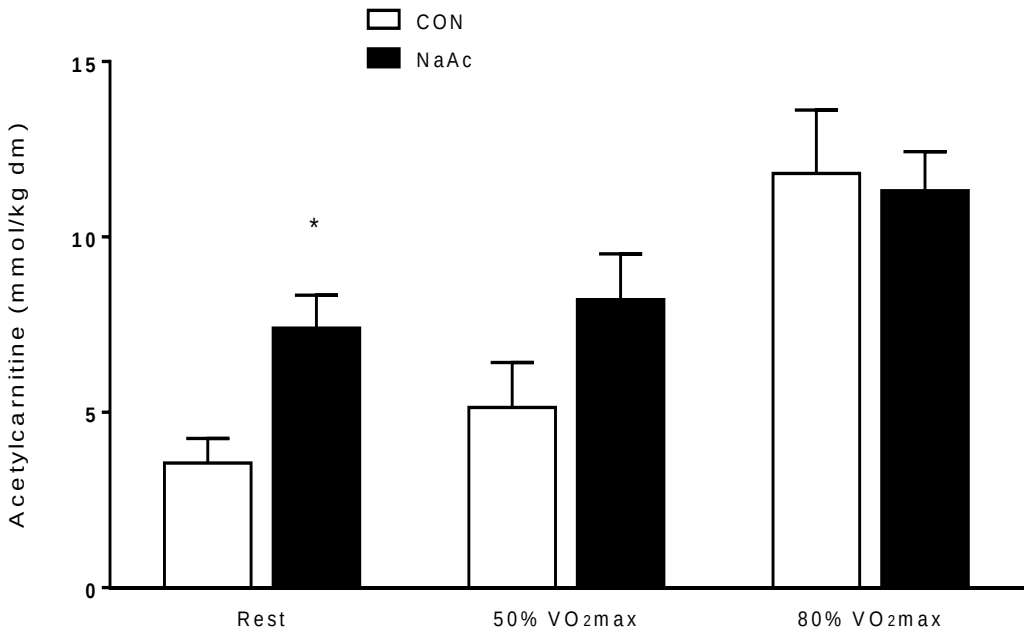


Figure 3.5. Skeletal muscle acetylcarnitine content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 min. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Significantly different from the corresponding CON value: * $p \leq 0.05$.

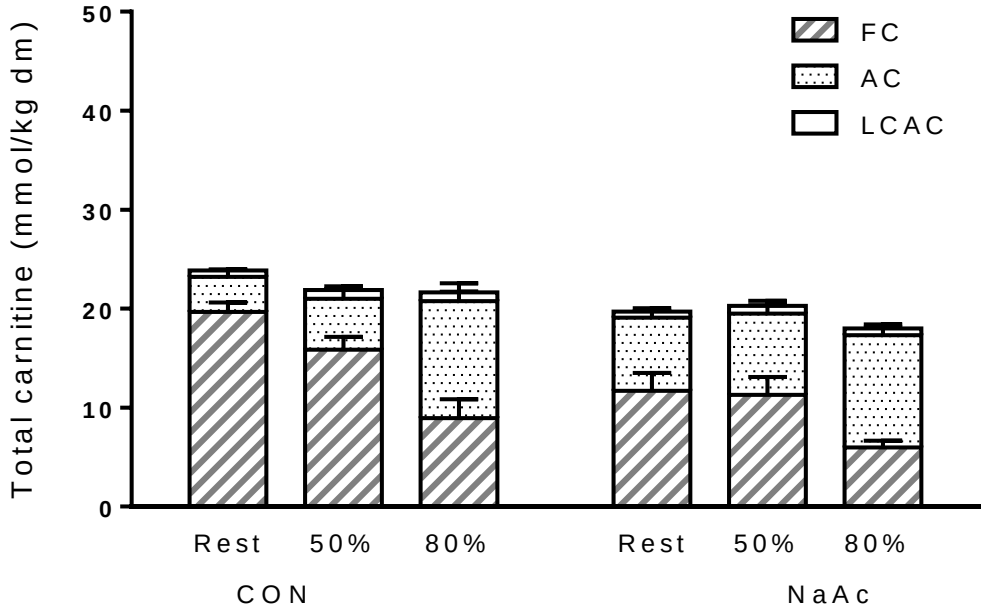


Figure 3.6. Skeletal muscle total carnitine (TC) content, consisting of long chain acylcarnitine (LCAC), acetylcarnitine (AC) and free carnitine (FC), following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, n = 7) or sodium acetate (NaAc, n = 7) was infused over 80 min. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Muscle metabolites

Muscle acetyl-CoA content decreased from 15.67 ± 3.00 and 20.88 ± 4.64 µmol/kg dm following 30 minutes of rest, to 9.69 ± 1.36 and 19.49 ± 3.28 µmol/kg dm following 30 minutes of exercise at 50% VO_2max during CON and NaAc infusion, respectively. Muscle acetyl-CoA content increased to 24.51 ± 4.04 and 23.28 ± 2.29 µmol/kg dm following 20 minutes of exercise at 80% VO_2max during CON and NaAc infusion, respectively **(Fig. 3.7)**. Muscle acetyl-CoA content was 2 times greater in NaAc compared to CON following 30 minutes of exercise at 50% VO_2max ($p < 0.05$). There was no difference in muscle acetyl-CoA content at rest and during exercise at 80% VO_2max when comparing CON and NaAc interventions.

Muscle lactate content increased from 2.38 ± 0.71 and 3.64 ± 0.79 mmol/kg dm following 30 minutes of rest, to 11.88 ± 4.80 and 7.32 ± 2.40 mmol/kg dm following 30 minutes of exercise at 50% VO_2max during CON and NaAc infusion, respectively. Muscle lactate content increased further to 72.01 ± 10.69 and 50.91 ± 8.00 mmol/kg dm following 20 minutes of exercise at 80% VO_2max in CON and NaAc interventions, respectively **(Fig. 3.8)**. There were no differences in muscle lactate content at rest and during exercise at 50% VO_2max between the two experimental visits; but muscle lactate content was 29% lower in NaAc compared to CON following 20 minutes of exercise at 80% VO_2max ($p < 0.05$).

Muscle PCr content decreased from 83.27 ± 3.16 and 73.75 ± 3.56 mmol/kg dm following 30 minutes of rest, to 72.06 ± 7.43 and 65.54 ± 6.10 mmol/kg dm following 30 minutes of exercise at 50% VO_2max during CON and NaAc infusion, respectively. Muscle PCr content declined further to 30.04 ± 8.33 and 35.63 ± 7.37

mmol/kg dm following 20 minutes of exercise at 80% VO_2max in CON and NaAc, respectively **(Fig. 3.9)**.

Muscle glycogen content decreased from 323.7 ± 20.8 and 267.7 ± 29.9 mmol/kg dm following 30 minutes of rest to 281.6 ± 32.0 and 235.9 ± 31.4 mmol/kg dm following 30 minutes of exercise at 50% VO_2max in CON and NaAc respectively. Muscle glycogen content declined further to 160.1 ± 31.3 and 128.7 ± 28.6 mmol/kg dm following 20 minutes of exercise at 80% VO_2max in CON and NaAc, respectively **(Fig. 3.10)**.

Muscle PDCa increased from 2.51 ± 1.19 and 2.03 ± 0.51 mmol/kg wm following 30 minutes of rest, to 6.81 ± 2.13 and 5.32 ± 2.12 mmol/kg wm following 30 minutes of exercise at 50% VO_2max in CON and NaAc, respectively and then increased further to 13.19 ± 2.70 and 12.02 ± 3.47 mmol/kg wm following 20 minutes of exercise at 80% VO_2max, respectively **(Fig. 3.11)**.

Muscle PCr, glycogen and PDCa values were not different between NaAc and CON groups at any time-point.

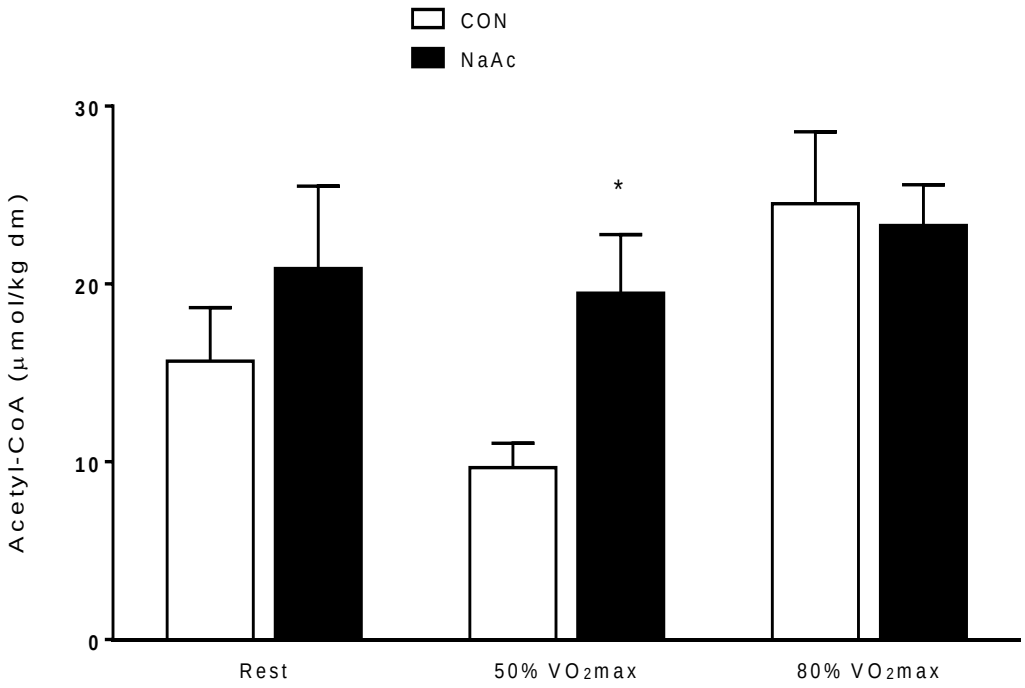


Figure 3.7. Skeletal muscle acetyl-CoA content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as µmol/kg dm.

Significantly different from the corresponding CON value: * $p < 0.05$.

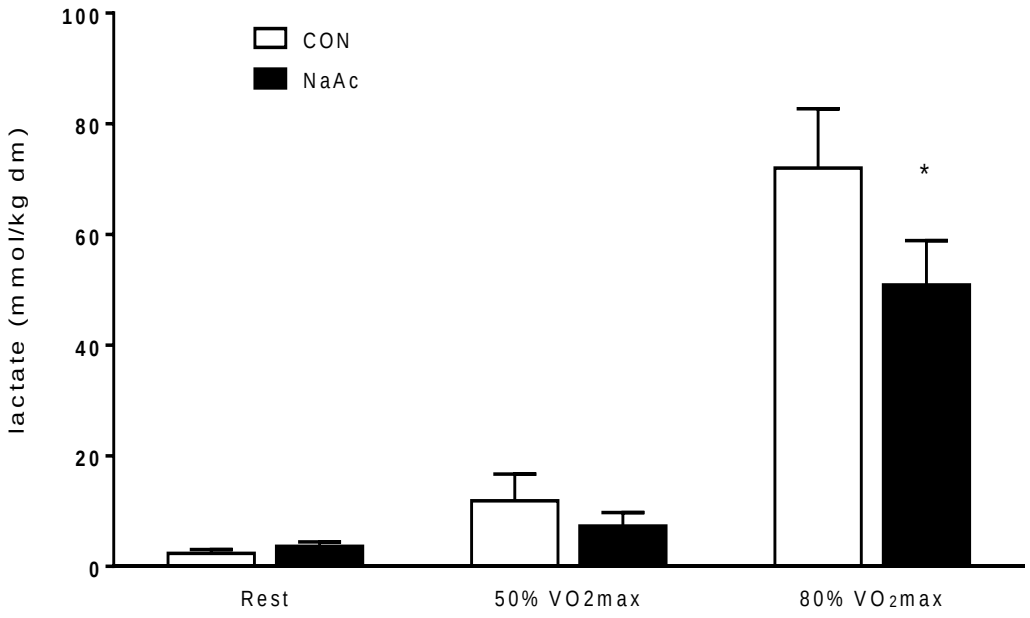


Figure 3.8. Skeletal muscle lactate content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Significantly different from the corresponding CON value: * $p < 0.05$.

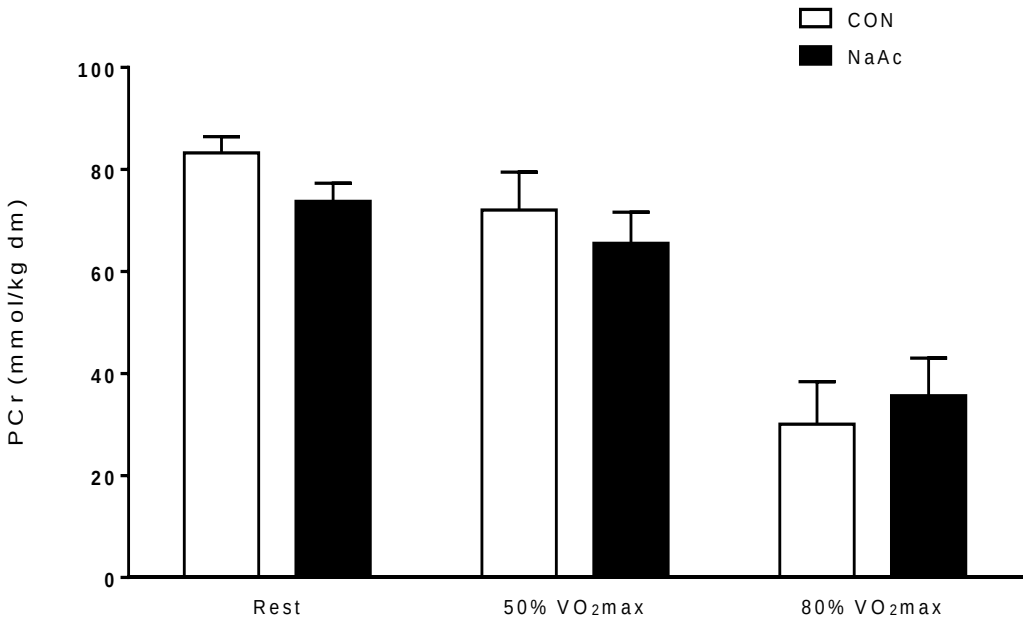


Figure 3.9. Skeletal muscle PCr content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

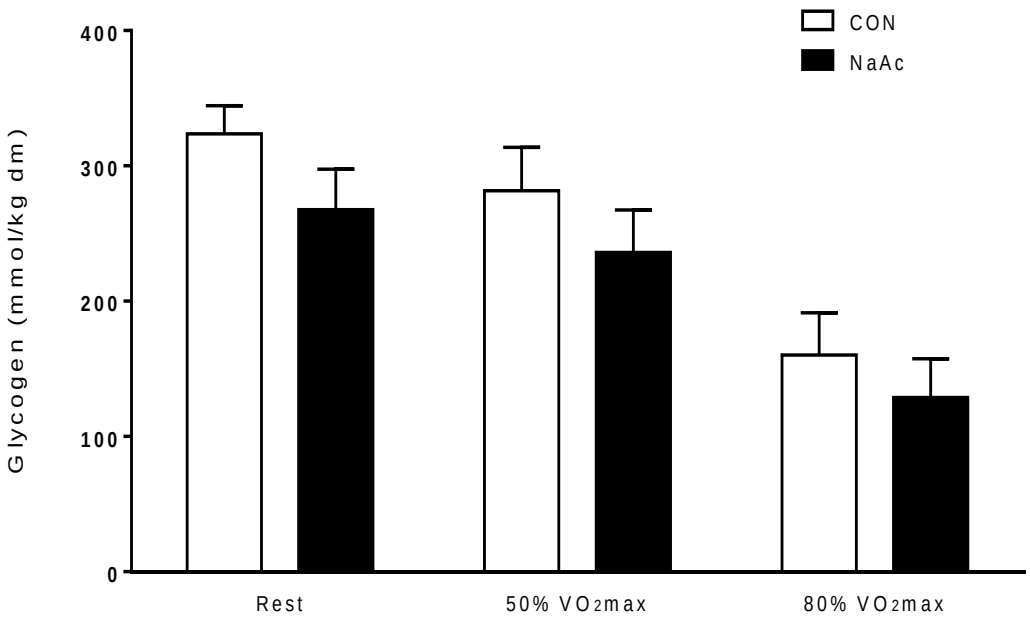


Figure 3.10. Skeletal muscle glycogen content following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, grey blocks; n = 7) or sodium acetate (NaAc, black blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

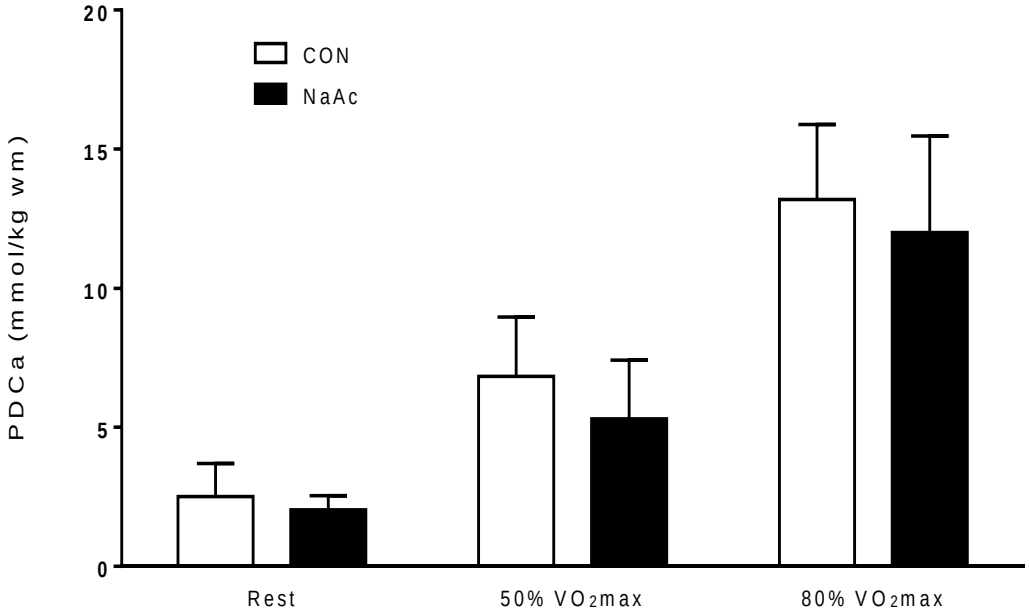


Figure 3.11. Skeletal muscle PDCa following 30 minutes of rest, 30 minutes of exercise at 50% VO_2max and 20 minutes of exercise at 80% VO_2max, during which 1 L of either 500 mmol of sodium bicarbonate (CON, open blocks; n = 7) or sodium acetate (NaAc, filled blocks; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg wm.

Venous blood pH

Venous blood pH was not different between NaAc and CON at any time-point **(Table 3.1)**.

Table 3.1. Venous blood pH during 30 minutes of rest (min 0, 15 and 30), 30 minutes of exercise at 50% VO_2max (min 45 and 60) and 20 minutes of exercise at 80% VO_2max (min 70 and 80), during which a 1 L solution of either 500 mmol of sodium bicarbonate (CON; n = 7) or sodium acetate (NaAc; n = 7) was infused over 80 minutes. All values are means ± standard error of the mean (SEM).

	NaAc	**CON**
0 min	7.38 ± 0.00	7.38 ± 0.01
15 min	7.47 ± 0.01	7.46 ± 0.01
30 min	7.48 ± 0.01	7.46 ± 0.01
45 min	7.48 ± 0.01	7.47 ± 0.01
60 min	7.52 ± 0.01	7.52 ± 0.01
70 min	7.49 ± 0.01	7.48 ± 0.01
80 min	7.50 ± 0.01	7.48 ± 0.01

3.4. Discussion

The aim of the present study was to investigate the impact of increasing muscle acetyl group availability independent of PDC activation, on muscle fuel metabolism during steady-state low intensity (50% VO_2max) and steady-state high intensity (80% VO_2max) exercise. We used sodium acetate infusion, 500 mmol over 80 minutes (6.25 mmol/min), to increase acetyl group availability and used the same dose and infusion rate of sodium bicarbonate as a control (CON) to match the metabolic alkalosis and the volume effect created by sodium acetate infusion. We demonstrated that augmenting skeletal muscle acetyl group content reduced whole-body fat oxidation during steady-state exercise at 50% VO_2max ($p < 0.05$ compared to the corresponding CON value) and reduced muscle lactate accumulation during steady-state exercise at 80% VO_2max ($p < 0.05$ compared to corresponding CON value). Given these metabolic changes occurred under conditions of very similar blood acid-base status conditions, we conclude they did not happen secondary to metabolic alkalosis.

The rate of whole-body carbohydrate oxidation in the present study followed the pattern of change described by van Loon et al. (2001), i.e. carbohydrate oxidation rate increased incrementally in both interventions from rest to steady-state low intensity exercise and then from low to steady-state high intensity exercise. The rate of whole-body carbohydrate oxidation was no different between NaAc and CON at rest or during exercise at both workloads. Similarly the pattern of change in the rate of whole-body fat oxidation followed that reported by van Loon et al. (2001), i.e. the rate of fat oxidation increased from rest to low intensity exercise but

then proceeded to decline as exercise intensity increased further. However, in the present study the rate of whole-body fat oxidation was 37% lower in the NaAc treatment group following 30 minutes of exercise at 50% VO_2max compared to the corresponding value in the CON group ($p < 0.05$). The fact that the rate of whole-body carbohydrate oxidation during low intensity exercise was no different between NaAc and CON, would suggest that the lower rate of fat oxidation in NaAc volunteers could be due to administered acetyl groups preferentially oxidised over fat. A similar hypothesis was proposed by Akanji et al. (1989) when it was suggested that oxidation of exogenous acetate replaced fat and thereby resulted in a decrease in whole-body fat oxidation at rest whilst CHO oxidation was not affected. Considering that fat is the main fuel source utilised at rest (Romijn et al., 1993) and during steady-state low intensity exercise (van Loon et al., 2001), it would be plausible to speculate that the administered acetate-induced reduction in fat oxidation at rest (Akanji et al., 1989) could as well happen during steady-state low intensity exercise. A study by Smith et al. (2013) reported a suppression of the rate of whole-body fat oxidation at rest and during the initial 15 minutes of 120-min exercise at 50% VO_2peak following sodium acetate administration compared to control (sodium bicarbonate ingestion). The authors also reported that over the final 75 minutes of exercise, the rate of fat oxidation was not different between the sodium acetate and control visits. However in the present study we found a decrease in the rate of whole-body fat oxidation following 30 minutes of exercise at 50% VO_2max after sodium acetate administration. There are 3 main differences between the two studies which can possibly explain this difference. Firstly, a continuous iv infusion of sodium acetate was used in the present study in contrast

to a single dose of oral sodium acetate in Smith et al. (2013). Indeed, sodium acetate infusion was still ongoing in the present study at the time the indirect calorimetry was performed during the low intensity exercise. Secondly, exercise at 50% VO_2max in the present study was preceded by a much shorter resting period (30 minutes) compared to Smith et al. (2013; 90 minutes). The indirect calorimetry performed during low intensity exercise in the present study was undertaken at minute 60 of study, which was earlier compared to Smith et al. (2013). Finally, the volunteers were exposed to a higher dose of sodium acetate when the indirect calorimetry was performed during the low intensity exercise in the present study (375 mmol of sodium acetate over 60 minutes), compared to Smith et al. (2013; 295.7 mmol).

The present study also reports a 2-fold greater muscle acetylcarnitine content ($p < 0.05$) and a 40% lower muscle free carnitine content ($p < 0.05$) in NaAc group relative to CON immediately prior to the onset of exercise at 50% VO_2max (i.e. the end of the rest period). A principal physiological function of carnitine known since 1950s is the translocation of fatty acids into mitochondrial matrix for β-oxidation (Fritz, 1955; Fritz and Mc, 1959; Fritz and Yue, 1963; Fritz and Marquis, 1965). In line with this function of carnitine, it is also possible that the decrease in muscle free carnitine availability may have limited mitochondrial fatty acid translocation, thereby contributing to diminished fatty acid oxidation during low intensity exercise. Muscle free carnitine content at the end of low intensity exercise during the NaAc visit in the present study was reported to be 11.28 ± 1.81 mmol/kg dm. It was discussed earlier that muscle free carnitine availability becomes limiting to the enzyme CPT1 at values less than 6.0 mmol/kg dm (van Loon et al., 2001). It is

obvious that the reported muscle free carnitine content in the present study is higher than the threshold value suggested by van Loon et al. (2001). However due the compartmentalization of cellular carnitine pool (intracellular carnitine is mainly cytoplasmic whilst the activity of CPT1 resides mainly on the inner aspect of outer mitochondrial membrane), free carnitine content measured in muscle biopsy samples does not reflect the intracellular free carnitine available to the catalytic site of the enzyme CPT1 (Stephens et al., 2006a). Therefore it remains a possibility that sodium acetate infusion acetylated cellular carnitine pool to an extent which diminished the intracellular carnitine available to the active site of CPT1.

We also reported that muscle lactate content was 29% lower in NaAc compared to CON at the end of steady-state high intensity exercise ($p < 0.05$). This was associated with a 2-fold higher acetyl-CoA content at the end of steady-state low intensity exercise (i.e. at the immediate onset of high intensity exercise) in the NaAc compared to CON ($p < 0.05$). There was no statistically significant difference in muscle glycogen and PCr content and PDCa, when comparing NaAc and CON during exercise at 80% VO_2max.

Karlsson and Saltin (1970) demonstrated that the majority of lactate accumulation in the exercising muscle happened during rest to exercise transition period, with a lesser accumulation during the steady-state exercise. Therefore muscle lactate content measured at the end of steady-state exercise reflects mainly the lactate accumulated during the initial transition phase. We propose therefore that in the present study the lower muscle lactate accumulation reported during high intensity

exercise in NaAc is secondary to greater acetyl-CoA supply to the TCA cycle and reflects less lactate accumulated during the initial transition period.

In summary stockpiling of muscle acetyl groups via sodium acetate infusion decreases the rate of whole-body fat oxidation during steady-state low intensity exercise due to acetate being preferentially metabolised over fat and possibly reduced muscle free carnitine content. At steady-state high intensity exercise, muscle lactate accumulation decreases due to higher acetyl-CoA supply to the TCA cycle.

CHAPTER 4

THE IMPACT OF INCREASING SKELETAL MUSCLE CARNITINE CONTENT ON RESTING FUEL METABOLISM IN YOUNG, HEALTHY, MALE VOLUNTEERS

4.1. Introduction

The two main physiological functions of carnitine include the translocation of long chain fatty acids from the cytoplasm into the mitochondria for β-oxidation (Fritz, 1955; Fritz and Mc, 1959; Fritz and Yue, 1963; Fritz and Marquis, 1965) and the buffering of excess acetyl-CoA produced during the states of high glycolytic and PDC flux (Childress et al. 1966; Harris et al. 1987; Sahlin et al. 1990; Constantin-Teodosiu et al. 1991).

Stephens et al. (2006b) investigated the impact of insulin on carnitine uptake by the muscle cells and demonstrated for the first time that in addition to a state of hypercarnitinaemia, a state of hyperinsulinaemia was required in order to increase skeletal muscle carnitine uptake. The authors reported a significant acute increase in skeletal muscle carnitine content ($p < 0.05$ post-infusion compared to pre-infusion) following an insulin clamp and a 5-hourly iv carnitine infusion to create a state of hyperinsulinaemia (149.2 ± 6.9 mU/L) and hypercarnitinaemia (~500 µmol/L). Hypercarnitinaemia (~500 µmol/L) associated with a fasting insulin concentration did not change muscle carnitine content in the control group volunteers. Later research in our lab (Stephens et al., 2007b) demonstrated that there was a threshold above which insulin exerted its stimulatory effect on carnitine uptake by the muscle cells. The authors calculated this threshold to be ~90 mU/L. The role of insulin in promoting carnitine uptake by skeletal muscle cells is believed to be exerted via the stimulatory effect of insulin on Na^+/K^+ ATPase pump (Ewart and Klip, 1995; Sweeney and Klip, 1998) which is very closely linked to organic

cation transporter 2 (OCTN2), located on the cell membrane and responsible for the transport of carnitine into the muscle cells (Tamai et al. 1998).

We also investigated the impact of acutely increased skeletal muscle carnitine content on muscle fuel metabolism for the first time in our lab (Stephens et al., 2006a). The authors reported that intravenous carnitine infusion for 5 hours (plasma carnitine level of 600 μmol/L) during a euglycaemic hyperinsulinaemic clamp (plasma insulin level of 160 mU/L) caused a 15% increase in skeletal muscle total carnitine content ($p < 0.01$). Infusing normal saline to the control group volunteers associated with hyperinsulinaemia had no impact on muscle total carnitine content. The authors also reported that by increasing skeletal muscle carnitine content, muscle PDC activity decreased by 30% ($p < 0.05$) and muscle lactate content reduced by 40% ($p < 0.05$) in the carnitine group compared to control. On the other hand, muscle glycogen content increased by 30% ($p < 0.01$) and long chain acyl-CoA content raised by 40% ($p < 0.05$) in the carnitine group compared to control. The authors suggested that acute muscle carnitine retention increased the oxidation of long chain fatty acids which in turn resulted in increased muscle acetyl-CoA content. This in turn had an inhibitory effect on PDC activity resulting in reduced CHO oxidation and increased glycogen storage.

Wall et al. (2011) was the first to demonstrate that muscle carnitine content could be increased by chronic oral ingestion of carnitine and carbohydrate (to stimulate insulin secretion). The authors reported an increase in muscle total carnitine content by 21% following twice daily oral ingestion of 80 g of carbohydrate plus 2 g of L-carnitine L-tartrate for 24 weeks ($p < 0.05$ compared to baseline). The authors

also reported an increase in body mass and body fat mass in control group volunteers following 24 weeks of twice daily supplementation of 80 g of carbohydrate ($p < 0.05$ compared to baseline) which the team believed was secondary to the extra calories taken in the daily carbohydrate supplements. No change in body mass or body fat mass was reported in the carnitine group volunteers following 24 weeks of supplementation which the authors attributed to the impact of carnitine on enhancing fatty acid oxidation.

Stephens et al. (2013) demonstrated that enhanced fatty acid oxidation secondary to increased skeletal muscle carnitine content was associated with upgraded expression of muscle genes involved with fat metabolism. The authors reported that twice daily supplementation of 80 g of CHO and 2 g of L-carnitine L-tartrate over 12 weeks increased muscle total carnitine content by 20% compared to baseline ($p < 0.05$) and that this was associated with an upgrade in the expression of 73 out of 187 genes regulating skeletal muscle fuel metabolism, especially 'fatty acid metabolism', 'PPAR signalling' and insulin signalling' genes. The authors also reported that carnitine prevented the gain in body mass (1.9 kg; $p < 0.05$ compared to baseline) and body fat mass (1.8 kg; $p < 0.05$ compared to baseline) noticed in the control group volunteers who ingested isoenergetic carbohydrate over 12 weeks.

It has been reported that lipid content in the skeletal muscle cells (IMCL) exerts an inhibitory impact on insulin sensitivity (Phillips et al. 1996; Pan et al. 1997). Whilst some researchers suggested that this negative effect is implemented via increasing concentrations of DAG and ceramides in muscle cells (Heydrick et al., 1991; Itani et

al., 2002), others put more emphasis on down-regulation of PPARs and FOXO1 (Ferre, 2004, Guerre-Millo et al., 2000, Verma et al., 2004, Wang and Tafuri, 2003) and inflammatory mediators, mainly TNF-α and IL-6 (Kanety et al., 1996, Guo and Donner, 1996, Kim et al., 2004, Bastard et al., 2002, Pickup et al., 1997).

Bearing the above information in mind, it is plausible to speculate that increasing muscle total carnitine content could improve muscle insulin sensitivity by augmenting muscle fatty acid oxidation and decreasing IMCL. The aim of the present study was to investigate whole blood glucose disposal during an OGTT in the muscle carnitine-loaded state in healthy, young, male volunteers. We hypothesised that chronic carnitine supplementation (2 g L-carnitine L-tartrate plus 80 g CHO twice a day for 24 weeks) would increase muscle total carnitine content and by doing so would result in enhanced muscle lipid oxidation and muscle insulin sensitivity which would in turn cause an improvement in whole blood glucose disposal during an OGTT.

4.2. Methods

Subjects

Twelve healthy, young, sedentary male volunteers took part in this study. All were students in the University of Nottingham and were randomised into control (CON, n = 6) and carnitine (CARN, n = 6) intervention groups (**Table 4.1**).

Study protocol

Following informed consent, all volunteers were screened in David Greenfield Human Physiology Unit in the Medical School, Queen's Medical Centre, Nottingham before being recruited into the study. The screening consisted of taking a medical history, a 12 lead ECG, measurement of height and weight and a venous blood sample which was sent to Queen's Medical Centre pathology laboratory to have FBC, U&E, LFT and clotting screen checked. All volunteers were healthy non-vegetarians, non-smokers and pursued a sedentary lifestyle. Information on the DEXA scan was provided to the volunteers in a 'DEXA leaflet' (See **Appendix 2**). All provided informed consent to take part in the study and were aware they could withdraw at any time with no obligation to provide an explanation of why they wished to do so.

The study consisted of six visits to the David Greenfield Human Physiology Unit at the Medical School, Queen's Medical Centre, Nottingham (**Fig. 4.1**). The first visit consisted of a physical exercise test to determine VO_2max, which involved

volunteers cycling to exhaustion on an electronically braked cycle ergometer (Lode Excalibur, Lode, Groningen, Netherlands) whilst the workload was increased every 3 min in 30W increments. At least two days later, the VO_2max test was repeated to verify that maximal oxygen consumption had been reached in the first test. This was shorter than the first visit and involved increasing the workload to the highest workload achieved in the previous test after low intensity warm up exercise of 5 min duration.

The third laboratory visit consisted of a DEXA scan followed by a 4 hour experimental study divided into fasting and OGTT phases, each lasting 2 hours (**Fig 4.2**). The subjects were asked to abstain from alcohol and strenuous physical activity for 48 hours before and to fast from 10 pm the night before the study visit. The volunteers reported to the unit at 8:30 am and after having a DEXA scan performed were asked to rest semi-supine on a bed while a cannula (Venflon, Becton Dickinson, New Jersey, USA) was inserted retrogradely into a superficial hand vein for arterialized-venous blood sampling (through a 3 way tap attached to the cannula; BD Connecta, Becton Dickinson, Helsingborg, Sweden). A normal saline infusion drip (0.9% sodium chloride, Maco Pharma Ltd, London, UK) was attached to the retrograde cannula to keep it patent throughout the experimental visit. A second cannula was inserted into a contralateral forearm vein for the infusion of 2-deoxyglucose tracer. A primed continuous infusion of 2-DG was commenced at a rate of 6 mg/kg/h over 4 hours in order to directly assess muscle glucose uptake. 2-DG is a glucose analogue that closely resembles glucose in the characteristics of its transport. Thus, 2-DG is effectively trapped within skeletal muscle where its content can be determined as a surrogate of glucose uptake. After 2 hours the subjects

were asked to ingest a 500 ml beverage containing 80 g of carbohydrate (Vitargo; Swecarb AB, Stockholm, Sweden) in order to test oral glucose tolerance and insulin response. The 2-DG infusion was quality controlled and made each day of investigation (Queen's Medical Centre pharmacy, Nottingham).

The flow of the volunteers along the study is depicted in a diagram **(Diagram 4.1)** based on CONSORT statement guidelines (Begg et al., 1996).

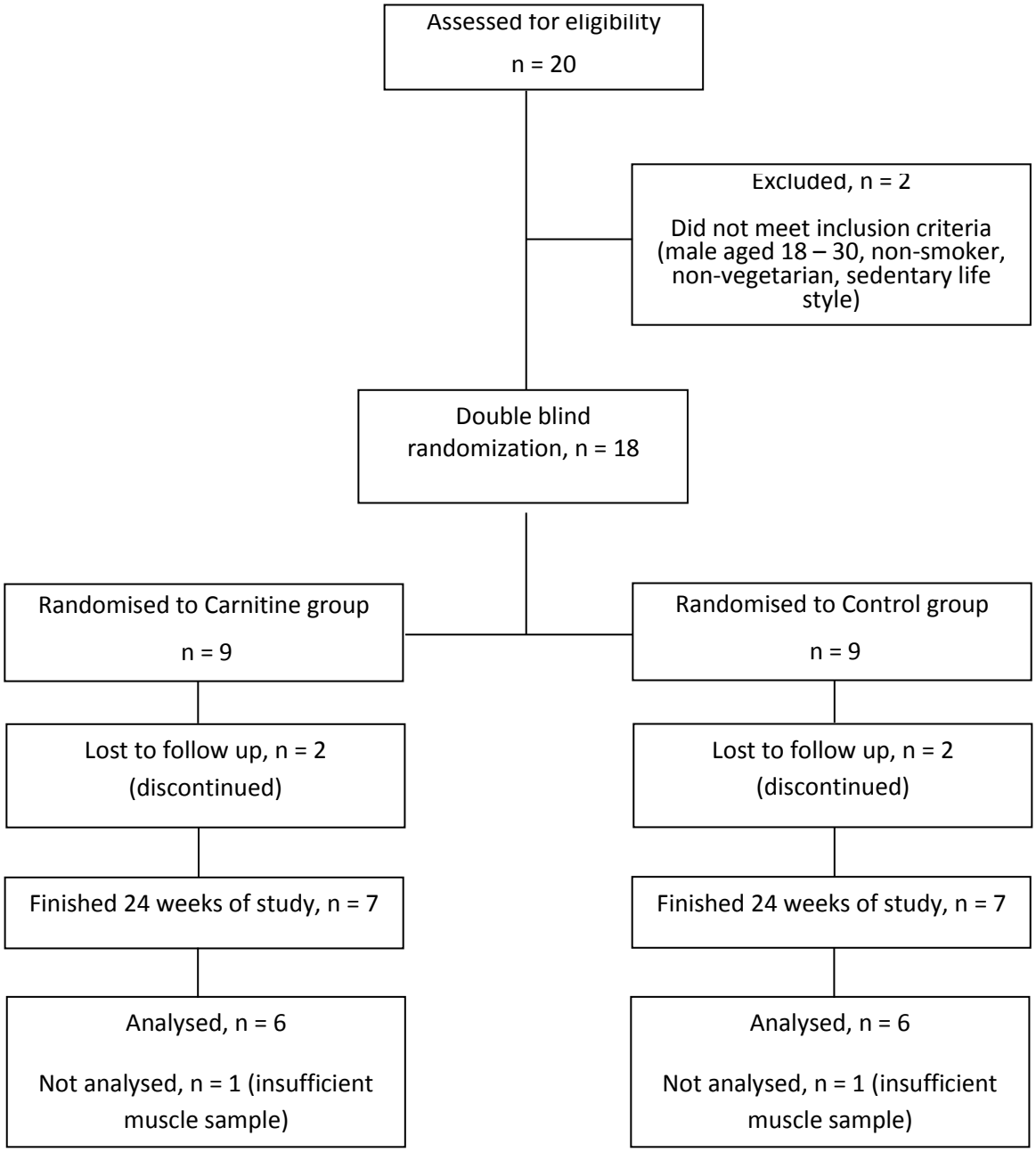


Diagram 4.1. The flow of the volunteers along the study depicted on the basis of CONSORT statement guidelines.

Sample collection and analysis

Blood samples

Arterialised-venous blood samples were taken at baseline and then every 30 minutes during the fasting period and every 15 minutes during the OGTT period for the measurement of whole blood glucose and serum insulin concentrations (**Fig. 4.2**). Whole blood glucose concentration was measured at the bedside using a glucose analyser (YSI 2300 STATplus, Yellow Springs Instruments, Yellow Springs, Ohio, USA). For details of analyses see CHAPTER 2: GENERAL METHODS.

Muscle biopsy

Muscle biopsy samples were obtained from the vastus lateralis muscle at baseline and at the end of fasting and OGTT periods (i.e. 0, 2, and 4 h) (**Fig. 4.2**). Muscle biopsies were obtained using the Bergstrom percutaneous needle biopsy technique under sterile conditions after injection of Lignocaine 2% (B. Braun, Melsungen, Germany) as local anaesthetic.

Muscle biopsy samples were snap frozen in liquid nitrogen. The samples were later freeze dried, powdered and analysed for muscle carnitine and 2-DG concentration. For details of analyses see CHAPTER 2: GENERAL METHODS.

At the end of the study all the lines were removed and the volunteers were fed. A standard dressing was applied on muscle biopsy incision areas including Steri-Strip

(3M Deutschland GmbH, Neuss, Germany), tegaderm (3M Deutschland GmbH, Neuss, Germany) and coban compression bandage (3M Deutschland GmbH, Neuss, Germany) and a leaflet on how to look after the muscle biopsy wounds was provided to them as well.

Randomisation and supplementation protocol

The volunteers taking part in the present study were asked to ingest a 500 ml beverage containing 80 g of carbohydrate (Vitargo; Swecarb AB, Stockholm, Sweden) (CON; n=6) or 80 g of CHO combined with 2 g of L-carnitine L-tartrate (containing 1.36 g of L-carnitine; Lonza Ltd., Switzerland) (CARN; n=6) twice a day for 24 weeks; with the first supplement in the morning and the second one 4 hours later. This supplementation strategy was tried in our unit by Wall et al. (2011) and managed to increase muscle total carnitine content by 21% compared to baseline ($p < 0.05$).

Similar VO_2max and repeat VO_2max tests and an experimental metabolic trial were repeated after 24 weeks of supplementation (visits 4, 5 and 6) (**Fig. 4.1**).

Statistical analysis

A repeated measures (RM) two-way ANOVA for interaction and time effects, with a Bonferroni multiple comparison test (GraphPad Prism 6, GraphPad Software Inc, California, USA), was performed to locate differences in muscle total carnitine and 2-DG contents, serum insulin level, blood glucose level, DEXA scan body fat

percentage and VO_2max. Statistical significance was declared at $p < 0.05$, and a trend for difference at $0.05 < p < 0.1$. All the values presented in text, tables, and figures are means ± standard error of the mean (SEM). All the graphs too were made by the GraphPad Prism 6 software.

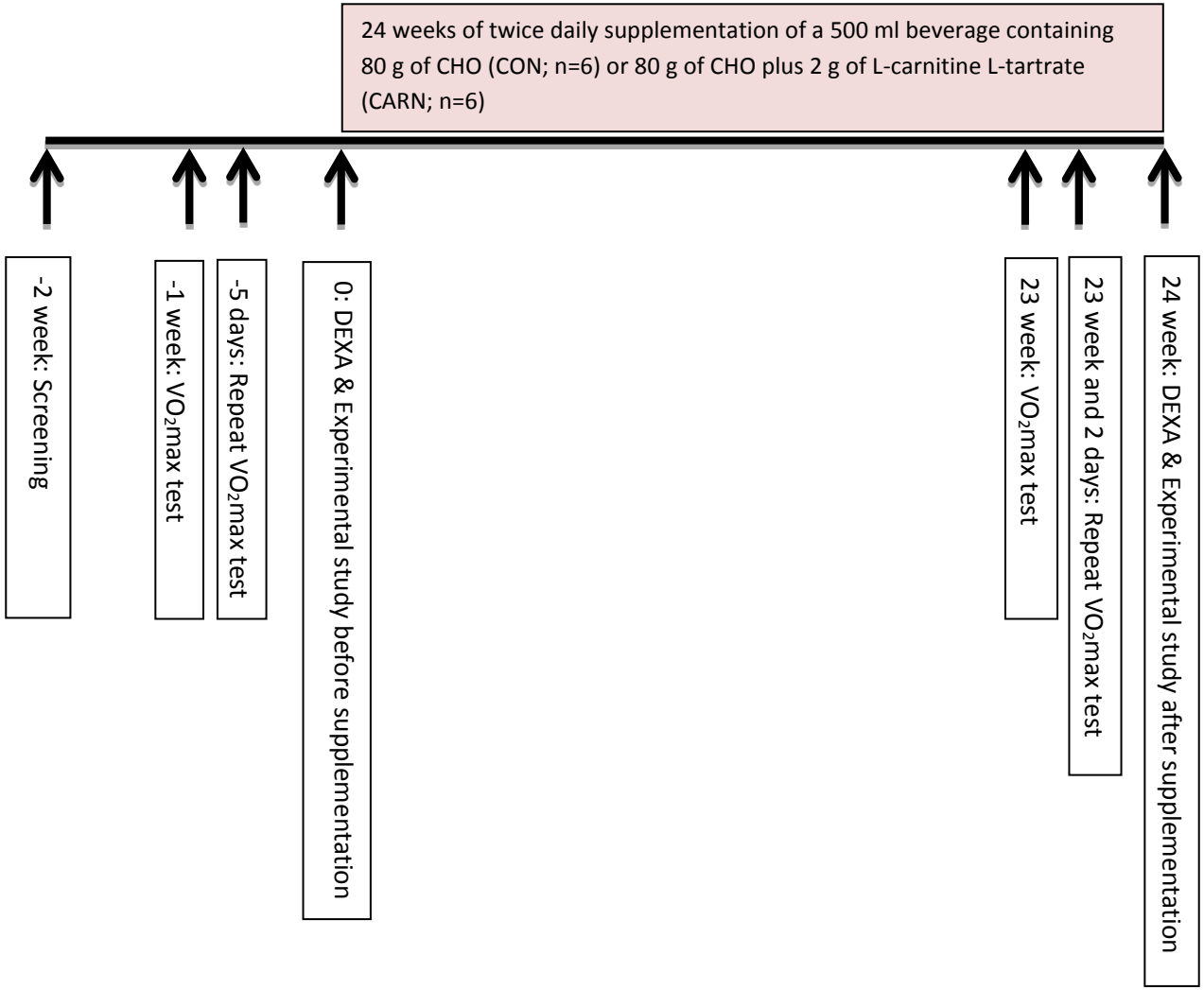


Fig.4.1. Study protocol

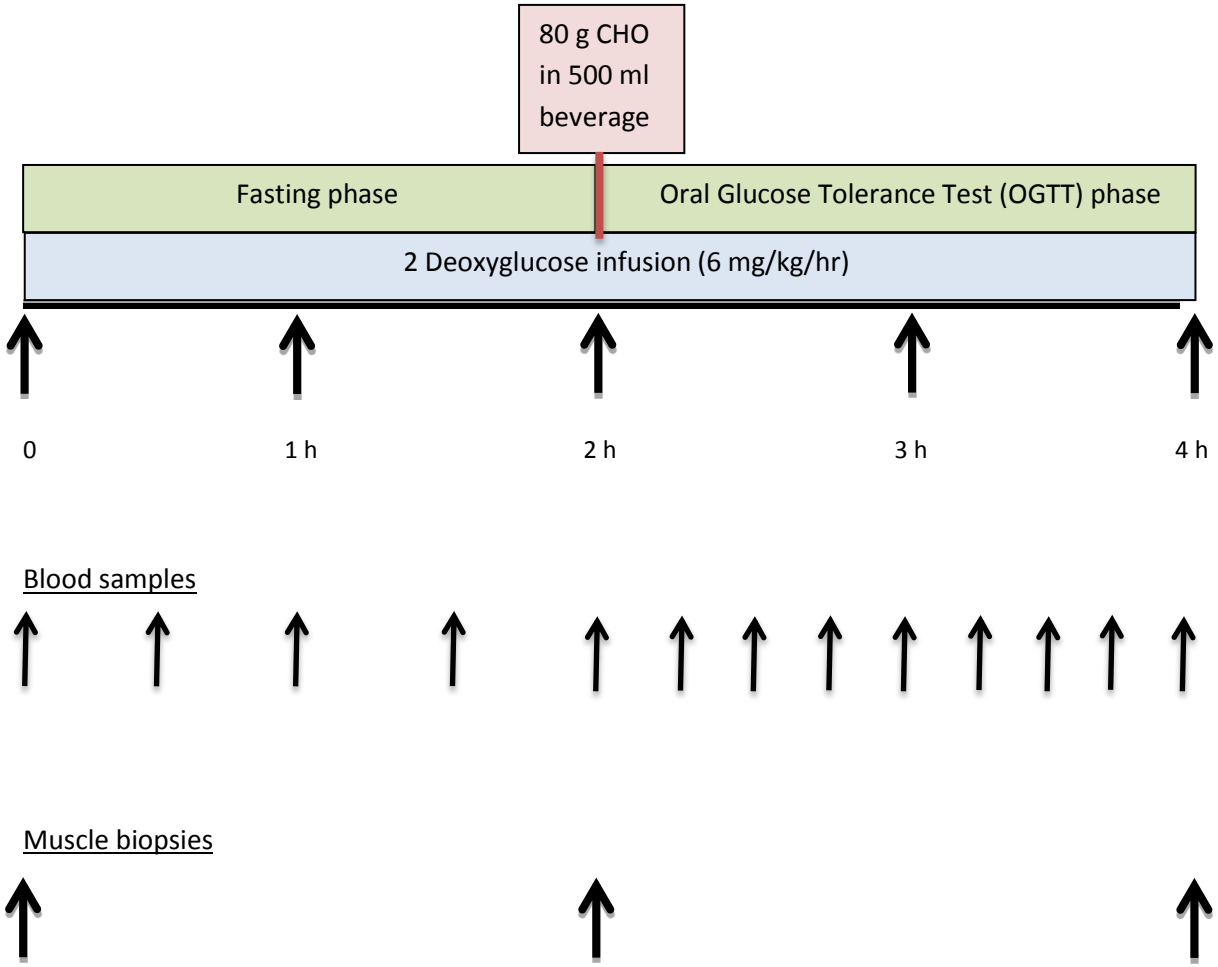


Fig.4.2. Experimental metabolic visits (24 weeks apart; i.e. before and after supplementation).

4.3. Results

Subject characteristics

Subject characteristics are displayed in **Table 4.1**. There was no difference in body mass or total body fat percentage between CON and CARN groups before supplementation. However the CON group demonstrated 2.3 kg increase in body mass ($p < 0.05$) and 2.7% increase in total body fat percentage ($p < 0.05$) following 24 weeks of twice daily oral ingestion of 80 g of CHO. There was no statistically significant changes in body mass or total body fat percentage in the CARN group following 24 weeks of twice daily oral ingestion of 80 g of CHO and 2 g of L-carnitine L- tartrate. VO_2max did not change following 24 weeks of supplementation in either CON or CARN groups.

Table 4.1. Subject characteristics before (0) and 24 weeks after twice daily oral ingestion of either 80 g of CHO (CON; n=6) or 80 g of CHO containing 2 g L-carnitine L- tartrate (CARN; n=6). All values are means ± standard error of the mean (SEM).

Significantly different from before supplementation (0): ¶ $p < 0.05$.

	0		24	
	CON	CARN	CON	CARN
Age (years)	24.3 ± 1.2	23.3 ± 1.8	-	-
Height (cm)	176.6 ± 1.6	179.3 ± 1.4	-	-
Body mass (kg)	76.6 ± 2.7	79.3 ± 1.4	78.9 ± 3.0 ¶	80.2 ± 1.7
BMI (kg/m^2)	24.6 ± 1.1	24.7 ± 0.5	25.3 ± 1.3	25.0 ± 0.7
VO_2max (ml/kg/min)	45.8 ± 1.3	45.8 ± 1.6	45.8 ± 1.7	45.9 ± 1.3
Total body fat (%)	20.0 ± 1.2	21.7 ± 1.3	22.7 ± 1.4 ¶	22.0 ± 1.4

Blood glucose

In the CON group there was no significant difference in whole blood glucose concentration following 24 weeks of twice daily ingestion of 80 g of CHO at any time-point during fasting or OGTT phases (**Fig. 4.3).**

In the CARN group whole blood glucose concentration decreased significantly at minutes 75, 90 and 105 during the OGTT phase following 24 weeks of twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (from 10.0 ± 1.0 to 8.2 ± 0.5 mmol/L at min 75 ($p < 0.001$); from 9.7 ± 1.0 to 7.5 ± 0.7 mmol/L at min 90 ($p < 0.0001$) and from 8.6 ± 1.1 to 7.1 ± 0.7 mmol/L at min 105 ($p < 0.05$)). There was no significant difference after supplementation compared to baseline at any time-point during the fasting phase and at minutes 15, 30, 45, 60 and 120 during the OGTT phase (**Fig 4.4)**.

OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO and lasts 120 minutes.

Serum insulin

In the CON group there was no significant difference in the serum insulin concentration following 24 weeks of twice daily ingestion of 80 g of CHO at any time-point during fasting or OGTT phases (**Fig. 4.5).**

In the CARN group serum insulin concentration declined at minutes 60 and 90, and there was a trend for a decrease at minute 120, during the OGTT phase following 24 weeks of twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (from 182.2 ± 23.0 to 136.9 ± 22.5 mIU/L at min 60 ($p < 0.05$), from 140.5 ± 23.3 to 78.9 ± 17.2 mIU/L at min 90 ($p < 0.001$) and from 100.2 ± 31.6 to 61.3 ± 19.2 mIU/L at min 120 ($p = 0.06$)). There was no significant difference after supplementation compared to baseline at any time-point during the fasting phase and at minute 30 during the OGTT phase (**Fig. 4.6).**

OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO and lasts 120 minutes.

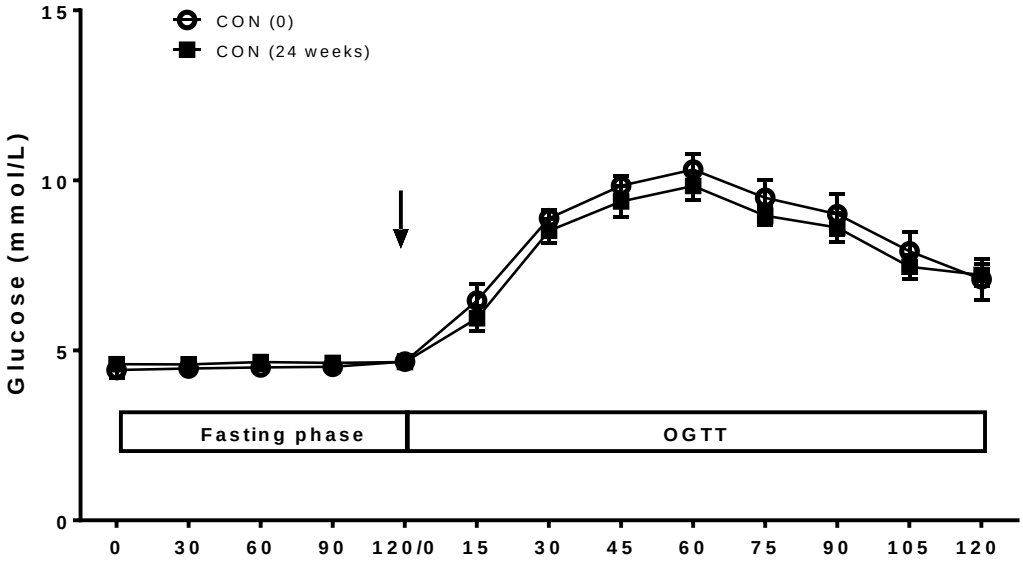


Fig. 4.3. Whole blood glucose concentration during fasting and OGTT phases before and after 24 weeks of twice daily ingestion of 80 g of CHO (unfilled circles and filled squares respectively) (CON n=6). All values are means ± standard error of the mean (SEM) and expressed as mmol/L. OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO (the arrow). Each phase lasts 120 minutes.

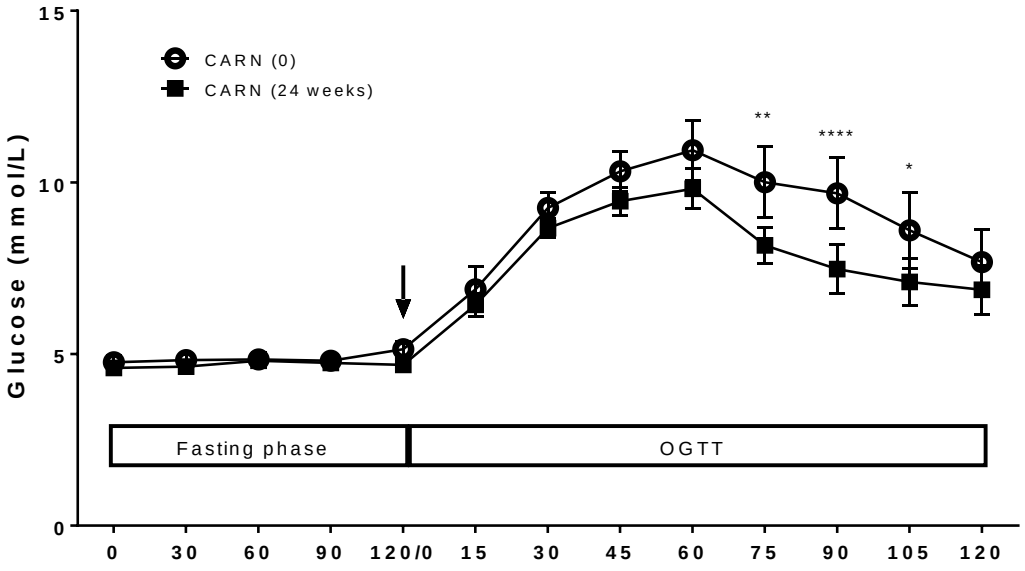


Fig. 4.4. Whole blood glucose concentration during fasting and OGTT phases before and 24 weeks after twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (unfilled circles and filled squares respectively) (CARN, n = 6). All values are means ± standard error of the mean (SEM) and expressed as mmol/L. OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO (the arrow). Each phase lasts 120 minutes.

Significantly different from the corresponding time-point before supplementation: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

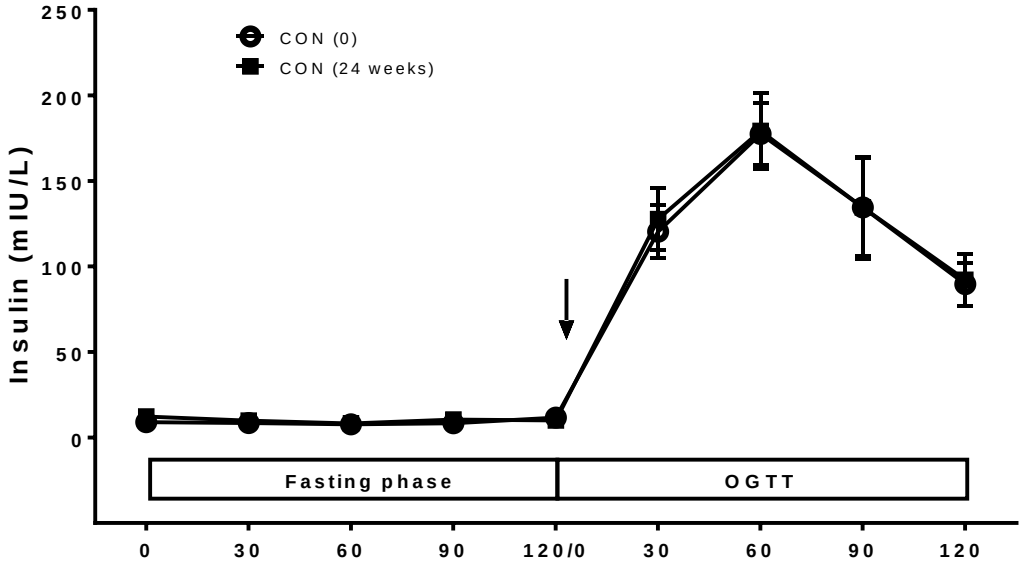


Fig. 4.5. Serum insulin concentration during fasting and OGTT phases before and 24 weeks after twice daily ingestion of 80 g of CHO (unfilled circles and filled squares respectively) (CON, n = 6). All values are means ± standard error of the mean (SEM) and expressed as mIU/L. OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO (the arrow). Each phase lasts 120 minutes.

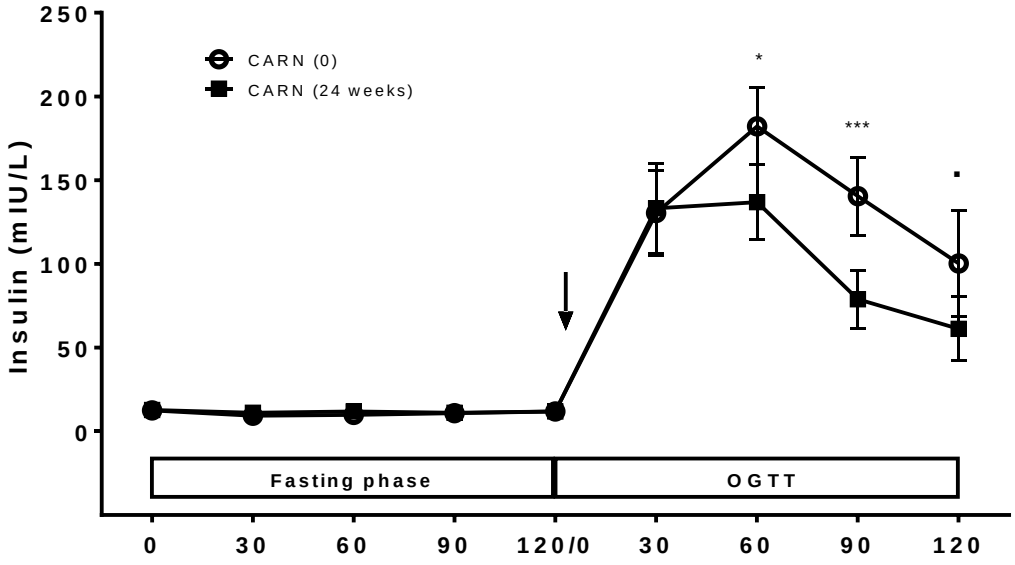


Fig. 4.6. Serum insulin concentration during fasting and OGTT phases before and 24 weeks after twice daily supplementation of 80 g of CHO and 2 g of L-carnitine L-tartrate (unfilled circles and filled squares respectively)(CARN, n = 6). All values are means ± standard error of the mean (SEM) and expressed as mIU/L. OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO (the arrow). Each phase lasts 120 minutes.

Significantly different from the corresponding time-point before supplementation: * $p < 0.05$; *** $p < 0.001$

Trend for difference from the corresponding time-point before supplementation: ▪ $p = 0.06$.

Skeletal muscle total carnitine content

Muscle total carnitine content increased by 18% in the CARN group following 24 weeks of twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (from 21.68 ± 2.09 to 25.52 ± 2.69 mmol/kg dm; $p < 0.05$). There was no change in muscle total carnitine content in the CON group following 24 weeks of twice daily ingestion of 80 g of CHO (from 20.20 ± 0.43 to 20.57 ± 1.77 mmol/kg dm) (**Fig. 4.7**).

Skeletal muscle 2-Deoxyglucose tracer accumulation

Muscle 2-DG accumulation was significantly lower during the OGTT phase in the CARN group compared to CON following 24 weeks of supplementation ($p < 0.05$). Muscle 2-DG uptake post-supplementation was 0.39 ± 0.18 and -0.25 ± 0.23 µmol/kg dm/min in the CON and CARN groups respectively. Muscle 2-DG uptake pre-supplementation was not different during the OGTT phase between the two groups (**Fig. 4.8**).

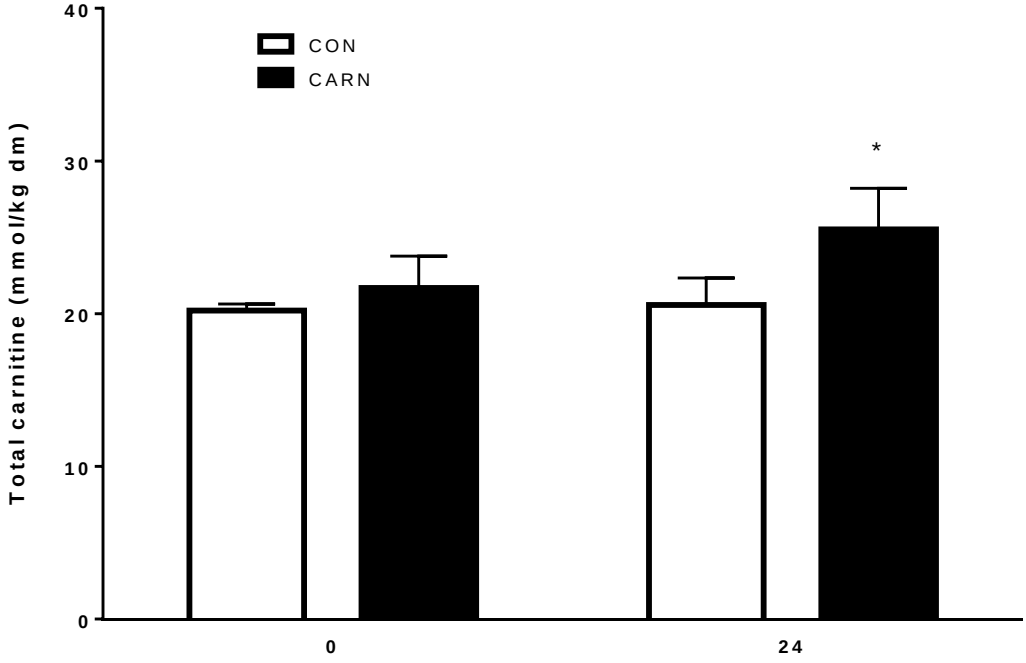


Fig. 4.7. Skeletal muscle total carnitine content before and 24 weeks after twice daily ingestion of 80 g of CHO (CON, open blocks; n = 6) or twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (CARN, filled blocks; n = 6). All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Significantly different from before supplementation: * $p < 0.05$.

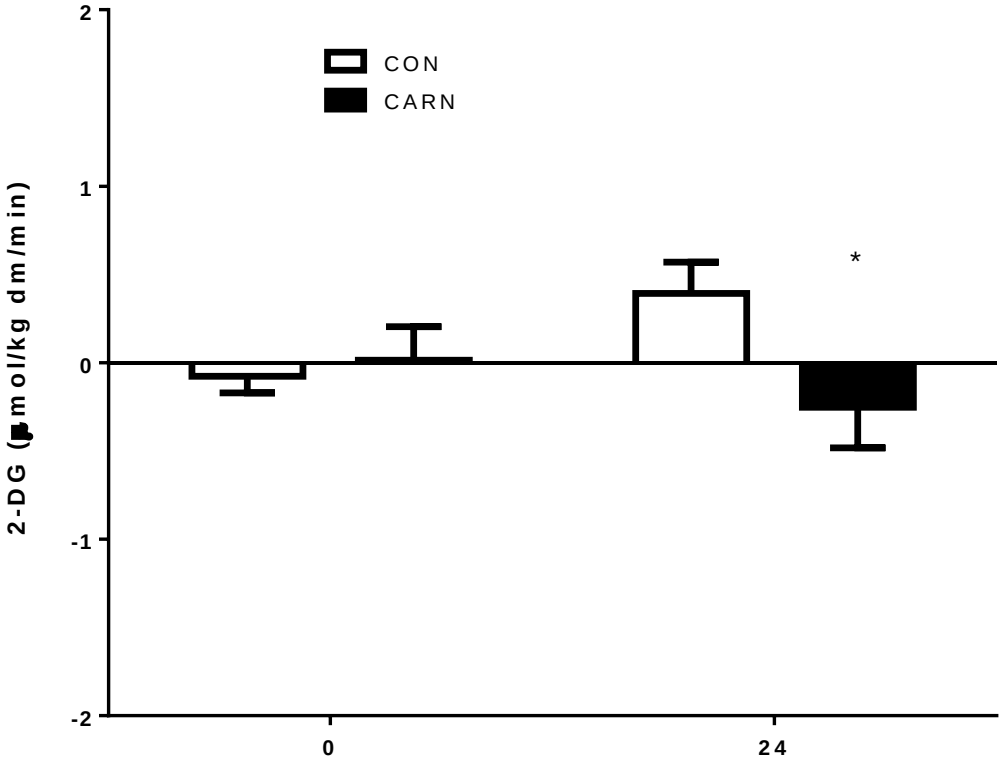


Fig. 4.8. Skeletal muscle 2-deoxyglucose tracer accumulation during the OGTT phase before and 24 weeks after twice daily ingestion of 80 g of CHO (CON, open blocks; n = 6) or twice daily ingestion of 80 g of CHO and 2 g of L-carnitine L-tartrate (CARN, filled blocks; n = 6). All values are means ± standard error of the mean (SEM) and expressed as µmol/kg dm/min. OGTT phase starts after drinking a 500 ml beverage containing 80 g of CHO and lasts 120 minutes.

Significantly different from the corresponding CON value: * $p < 0.05$.

4.4. Discussion

The aim of the present study was to investigate blood glucose disposal during an OGTT in the muscle carnitine-loaded state in healthy, young, sedentary volunteers. We hypothesised in the present study that chronic L-carnitine and CHO ingestion in sedentary individuals would increase muscle total carnitine content and lipid oxidation and by doing so would improve whole-body insulin response to an oral glucose tolerance test compared to control. Major findings of the present study were that augmented skeletal muscle carnitine content prevented an increase in body mass and total body fat percentage observed in the CON group ($p < 0.05$ post-supplementation compared to baseline) and resulted in a reduction in whole blood glucose concentration during an OGTT compared to before supplementation, which was associated with a parallel reduction in serum insulin concentration, neither of which was observed in the CON group. Concomitant with this, muscle 2-DG accumulation during an OGTT was lower in the CARN group compared to CON following 24 weeks of supplementation ($p < 0.05$).

Previous research done by Wall et al. (2011) in our unit on healthy recreationally active individuals, demonstrated that skeletal muscle carnitine content could be increased by chronic oral carnitine and CHO supplementation ($p < 0.05$). Following the same supplementation regime (twice daily ingestion of 2 g of L-carnitine L-tartrate and 80 g of CHO over 24 weeks), we managed to augment skeletal muscle total carnitine content by 18% in the present study ($p < 0.05$ post-supplementation compared to baseline).

As discussed in the GENERAL INTRODUCTION and Chapter INTRODUCTION in details, it is known since 1950s that carnitine is essential for the translocation of long chain fatty acids across the mitochondrial membrane for intra-mitochondrial β-oxidation (Fritz et al. 1955, Fritz & McEwen 1959, Fritz & Yue 1963). It is also known that increasing muscle carnitine content, either acutely or chronically, enhances fat oxidation at rest and during steady-state low intensity exercise (Stephens et al., 2006b & 2013, Wall et al., 2011). At a molecular level, chronic carnitine supplementation over 12 weeks is reported to cause an upgraded expression of skeletal muscle genes related to 'fatty acid metabolism' (Stephens et al., 2013). In the present study, the increased muscle total carnitine content was associated with prevention in increased body mass and total body fat percentage reported in the CON group. Whilst there was 2.7% increase in total body fat percentage ($p < 0.05$ post-supplementation compared to baseline) and 2.3 kg gain in body mass ($p < 0.05$ post-supplementation compared to baseline) in the CON group following 24 weeks of twice daily ingestion of 80 g of CHO, there was no significant change in body mass or body fat percentage in the CARN group (following 24 weeks of twice daily ingestion of 80 g CHO and 2 g L-carnitine L-tartrate). These findings are in line with similar findings from previous studies in our lab (Wall et al., 2011, Stephens et al., 2013). The two intervention groups in the present study were pursuing a sedentary life style (as evidenced by VO_2max findings) and were following an *ad libitum* diet for the duration of the study. It is therefore plausible to speculate that the extra calories taken by the CON group (80 g of CHO twice a day, equivalent to 640 kcal per day, for 24 weeks) caused the increase in body mass and body fat percentage. As these changes were not reported in the CARN group who ingested isoenergetic

carbohydrates over the same period of time, it would therefore be plausible as well to speculate that carnitine-induced augmented fat oxidation prevented the increase in body mass and body fat percentage.

It was also discussed in details in the GENERAL INTRODUCTION and Chapter INTRODUCTION that increased accumulation of IMCL has a negative impact on muscle insulin sensitivity (Phillips et al., 1996, Pan et al., 1997) and that carnitine-induced increased fat oxidation is associated with increased expression of 'insulin signalling' gene network (Stephens et al., 2013). We therefore hypothesised that carnitine-induced augmented fat oxidation in the present study would improve whole-body insulin response to an oral glucose tolerance test. In line with this hypothesis, increased muscle total carnitine content in the present study in the CARN group was linked to a reduction of whole-blood glucose concentration during an OGTT compared to baseline (18% reduction in blood glucose at minute 75 ($p < 0.001$); 23% reduction at minute 90 ($p < 0.0001$); and 18% reduction at minute 105 ($p < 0.05$)) which was associated with a parallel reduction in serum insulin concentration compared to baseline (25% reduction at minute 60 ($p < 0.05$); 44% reduction at minute 90 ($p < 0.001$); and 39% reduction at minute 120; ($p = 0.06$)). These findings suggest that increased muscle carnitine content in the present study was linked with an improvement in body glucose disposal during an OGTT which was associated with decreased insulin release. However, there was 164% lower 2-DG accumulation during the OGTT in the CARN group compared to CON ($p < 0.05$) following 24 weeks of supplementation which suggests that the above-mentioned decline in whole blood glucose disposal during an OGTT did not happen at the level of muscle. This could be explained by either a decrease in the absorption of glucose

from the intestinal tract or a first pass effect of liver on the glucose delivered to the hepatic cells. Glucose is delivered to the liver by the portal vein and is transported into the hepatic cells via glucose transporter 2 (GLUT2), a transporter protein located on the hepatic cell membrane. Whilst inside the hepatic cells, glucose becomes phosphorylated and converts into glucose-6-phosphate (G6P), a reaction catalysed by the enzyme hexokinase (HK). Glucose-6-phosphate then either converts to glycogen (glycogenesis) and becomes stored in the hepatic cells or enters the glycolytic pathway. The glycogen stored in the hepatic cells can be catalysed back into glucose in a biochemical process called glycogenolysis. Intra-hepatic glycogen storage therefore functions as a reserve of glucose for extra-hepatic organs and tissues (Bollen et al., 1998). Liver can also convert proteins and lipids into glucose through a metabolic pathway called gluconeogenesis. By balancing the uptake of glucose and its metabolism (glycolysis) or storage as glycogen (glycogenesis) on one hand and the release of glucose via gluconeogenesis and glycogenolysis on the other hand, the liver plays a key role in maintaining blood glucose level at a very narrow range (fasting blood glucose level range of 3.5 – 5.5 mmol/L and random blood glucose level ≤ 7.8 mmol/L) (Nordlie et al., 1999). Glucose stimulates the secretion of the hormone insulin from the pancreatic β cells. Insulin in turn binds to insulin receptor (IR) on plasma cell membranes and affects the glucose uptake by the cells (Insulin signalling pathway is depicted in **Fig. 1.7**). Low blood glucose levels are associated with decreased insulin secretion, resulting in diminished cellular glucose uptake.

Considering the important role liver plays in human metabolism and metabolic regulation and the close metabolic links between skeletal muscle and liver (Frayn

and Elliott, 1986, Kotronen et al., 2009), it is plausible to speculate that decreased glucose release into the systemic circulation following chronic carnitine supplementation happened at the level of the liver. We therefore propose that carnitine-induced increased fat oxidation in the present study resulted in less hepatic fat deposition over time, thereby increasing glucose disposal by the liver. This would in turn reduce the systemic blood glucose concentration and would therefore result in diminished insulin release.

The reported reduction in whole-blood glucose concentration and insulin concentration following an OGTT in muscle carnitine-loaded state in the present study could potentially have significant implications in the management of impaired glucose tolerance or diabetes mellitus type 2. So far all the physiological functions of carnitine have been discovered and published in animal and healthy human models. The present study suggests very strongly that there could be a role for carnitine in the management of impaired glucose tolerance and diabetes mellitus and that this is exerted through enhanced glucose disposal via liver. Designing a similar carnitine supplementation study in patients suffering with impaired glucose tolerance or diabetes mellitus type 2, focusing on the role of liver and the interaction between skeletal muscle and liver brought about by increased muscle carnitine content, would be the next step forward to shed more light on carnitine and its physiological metabolic functions.

In summary, this is the first study to demonstrate that chronic carnitine supplementation improves overall glucose disposal and insulin response in body during an oral glucose tolerance test. We propose that this improvement happens

at the level of liver and not at the level of skeletal muscle (as evidenced by decreased skeletal muscle 2-DG uptake). We also propose that decreased hepatic fat deposition as a result of increase in muscle fat oxidation, results in enhanced hepatic glucose oxidation which in turn causes an improvement in overall glycaemia.

CHAPTER 5

THE IMPACT OF INCREASING SKELETAL MUSCLE CARNITINE CONTENT, CALORIC RESTRICTION AND EXERCISE ON WEIGHT LOSS IN HEALTHY, OVERWEIGHT FEMALE VOLUNTEERS

5.1. Introduction

It was demonstrated in Chapter 4 that increased muscle carnitine content was associated with an improvement in body glucose disposal and insulin release during an oral glucose tolerance test. It was also demonstrated that chronic carnitine and carbohydrate supplementation prevented an increase in body mass and body fat percentage observed in the control group volunteers who ingested isoenergetic carbohydrate. This was speculated to be secondary to the role of carnitine in augmenting fatty acid oxidation in skeletal muscle tissue (Stephens et al., 2006a, Stephens et al., 2013). The impact of increased muscle carnitine content on preventing a gain in body mass and body fat mass has also been reported in previous studies in our lab (Wall et al., 2011, Stephens et al., 2013). Therefore, in the present study, we aimed to investigate the impact of chronic carnitine supplementation, in conjunction with low intensity exercise and diet, on weight loss in young overweight volunteers. As mentioned above all previous studies (Wall et al., 2011, Stephens et al., 2013) and the study described in Chapter 4 used carbohydrate as an adjunct to carnitine supplementation in order to increase muscle carnitine content. However chronic carbohydrate supplementation caused an increase in body mass and body fat mass in the CON group as a result of the extra calories taken daily in addition to an *ad libitum* diet (80 g of CHO twice a day equivalent to 640 kcal per day) over 12 weeks (Stephens et al., 2013) or 24 weeks (Wall et al., 2011, Chapter 4). Taking high calorie carbohydrates as an adjunct to carnitine supplementation does not seem to be advisable in the context of a weight loss study. We therefore designed a novel lower calorie oral supplementation

protocol to increase skeletal muscle carnitine content in the present study which will be discussed in details in due course.

Increasing muscle total carnitine content over a 24 week period in young, recreationally active subjects by ingesting an L-carnitine/CHO beverage on a twice daily basis prevented an increase in body fat mass observed in volunteers who ingested an isoenergetic CHO supplementation alone (Wall et al., 2011, Chapter 4). Specifically, a control group that ingested 80 g of CHO twice daily for 24 weeks, gained 2.4 kg in body mass over 12 weeks ($p < 0.01$) which increased to 2.7 kg after 24 weeks ($p < 0.05$) (Wall et al., 2011). These changes were paralleled by a corresponding increase in total body fat mass ($p < 0.5$). On the other hand, the CARN intervention group that ingested 80 g of CHO in combination with 2 g of L-carnitine L-tartrate twice a day for the same period of time demonstrated no net gain in either body mass or body fat mass following 12 and 24 weeks of supplementation. The authors proposed the increase in muscle carnitine content increased muscle fat oxidation, which in turn prevented the augmented adiposity and weight gain observed in the control group. Similar findings were reported by Stephens et al. (2013) using the same supplementation protocol over 12 weeks. In addition, the authors were able to show that increasing muscle carnitine content was associated with a 6% increase in resting energy expenditure, which was unchanged in the control group (Stephens et al., 2013). This was attributed to a carnitine mediated increase in muscle fat oxidation, evidenced by a marked increase in muscle long-chain acyl-CoA content and the up-regulation of 73 genes relating to fuel metabolism, with 'insulin signalling', 'peroxisome proliferator-activated receptor signalling' and 'fatty acid metabolism' as the three most enriched

pathways. These findings strongly suggest that molecular adaptation occurred secondary to increasing muscle carnitine content that functioned to increase capacity for muscle fat oxidation.

Collectively these findings suggest that by increasing fat oxidation and energy expenditure, increasing muscle carnitine content could potentially contribute to total body fat loss and in turn weight loss, particularly during periods of reduced energy intake and/or increased energy expenditure. This becomes of important health and medical relevance if we consider the current global obesity epidemic. Obesity increases the risk of morbidity and mortality and reduces quality of life independent of age, sex or ethnicity. There is strong evidence in medical literature that obesity increases the risk of cardio-vascular disease, hypertension, diabetes mellitus type 2 and stroke. There is accumulating evidence that there may be a primary, possibly genetically, determined component of fat oxidation, which may result in a rate of low fat oxidation that predisposes people to obesity development. For example, a study of 701 obese individuals (NUGENOB) demonstrated that the contribution of postprandial fat oxidation to total energy expenditure decreased with increasing BMI, but only in the obese subjects with a relatively low fasting fat oxidation (Blaak et al., 2006). Also, longitudinal studies in Pima Indians have shown that low fat-oxidisers show a greater risk of body mass gain over time, compared with high-fat oxidisers (Zurlo et al. 1990). This is consistent with data showing that skeletal muscle of insulin resistant or type 2 diabetic subjects is characterised by a low fasting fat oxidative capacity (Gaster et al. 2004).

Leading health authorities recommend weight loss as a primary treatment strategy for obesity reduction and intentional weight loss in most adults is associated with a reduction in many of the health complications of obesity. However, weight loss via caloric restriction alone does not always restore the ability to increase fat oxidation. For example, post-obese women have lower post-prandial and 24-h fat oxidation as compared with never-obese women (Astrup et al. 1994). Thus, individuals with obesity face a serious challenge if they are to maintain even modest weight loss in today's obesogenic environment and, therefore, investigations into the mechanisms underlying reduced fasting fatty acid oxidation in obesity, as well as strategies to increase rates of fatty acid oxidation and aid weight loss are warranted.

The aim of this study was to determine whether 16 weeks of twice daily L-carnitine feeding in combination with a meal replacement supplement (to stimulate insulin mediated muscle carnitine accumulation), caloric restriction and low intensity exercise training increased muscle total carnitine content in young, obese women, and if so whether it was associated with changes in fuel oxidation and increased fat mass loss relative to a control group.

Specifically, we aimed to determine whether:

a) Muscle total carnitine content was increased in obese, young females following 16 weeks of twice daily L-carnitine feeding (2 g of L-carnitine L-tartrate twice a day) in combination with a meal replacement supplement (25 g of CHO and 18 g of protein to stimulate insulin secretion and muscle carnitine accumulation), and caloric restriction (minus 500 kcal/day requirements) and low intensity exercise training (two exercise sessions per week, each lasting one hour at 50% VO_2max).

b) Any change in muscle total carnitine content during 16 weeks of caloric restriction and low intensity exercise training was associated with an increase in whole-body fat oxidation and increased loss of whole-body and regional fat masses relative to an appropriate control group.

5.2. Methods

Subjects

Ten healthy, sedentary, overweight female volunteers took part in this study. They were recruited by advertising the study on boards at the University of Nottingham Medical School and the Queen's Medical Centre and by advertising in the local Metro newspaper. Volunteers were randomised into intervention control (CON, n = 5) and carnitine (CARN, n = 5) groups and all were non-vegetarians and non-smokers. **(Table 5.1)**.

Study protocol

All subjects were screened in the David Greenfield Human Physiology Unit at the University of Nottingham Medical School before being recruited into the study. The screening consisted of taking a medical history, a 12 lead ECG, measurement of height, weight, blood pressure and heart rate and collecting a venous blood sample which was sent to Queen's Medical Centre pathology laboratory for FBC, U&E, LFT and clotting screen. Information on the DEXA scan was provided to the subjects in a 'DEXA leaflet' (**Appendix 2**). All volunteers signed a consent form and were informed that they could leave the study at any time and were under no obligation to provide an explanation for this. Volunteers were also informed that in the case of pregnancy, they would be immediately withdrawn from the study.

This study consisted of the following laboratory visits (**Fig. 5.1**):

- A VO_2max test
- Two resting metabolic study visits 16 weeks apart, each lasting 5 hours. A DEXA scan was performed immediately before each resting metabolic study.
- Eight diet visits at baseline, 1, 2, 4, 6, 8, 12 and 16 weeks, each lasting approximately 30 minutes. At baseline, 4, 8, 12 and 16 weeks, the volunteers submitted a 4 day diet diary and activity monitoring to the study dietician.

The first visit consisted of a physical exercise test to determine VO_2max where volunteers ran continuously to voluntary exhaustion on a motorised treadmill (Woodway ELG 70, Weil am Rhein, Germany) whilst the treadmill speed and incline was adjusted (see below). Volunteers received verbal encouragement to continue running throughout the test whilst wearing a mouth-piece and nose clip to allow monitoring of expired gas volume and composition using a calibrated online gas analysis system (Vmax, SensorMedics corporation, California, USA). Heart rate and rhythm were monitored via a three-lead cardiac monitor (GE Medical Systems Technology Inc. Buckinghamshire, UK) and perceived exertion was recorded at the end of each exercise cycle using the Borg scale (Borg G.A. 1982). The details of each exercise test are as follows:

		Speed (km/hour)	Gradient (%)
-	Min 0-5	3.5	0
-	Min 5-8	4.5	0
-	Min 8-11	5.5	0
-	Min11-14	6.5	0
-	Min14-15	6.5	2
-	Min15-16	6.5	4
-	Min16-17	6.5	6
-	Min17-18	6.5	8
-	Min18-19	6.5	10
-	Min19-20	6.5	12
-	Min 20-21	6.5	14
-	Min 21-22	6.5	16
-	Min 22-23	6.5	18
-	Min 23-24	6.5	20
-	Min 24-25	6.5	22

Based on each individual's maximum O_2 consumption value, 50% VO_2max was calculated for each individual volunteer (See the CHAPTER 2: GENERAL METHODS for the calculations).

The experimental metabolic studies were divided into 3 phases: an initial 1 hour period followed by a 2 hour pre-meal phase and a 2 hour post-meal phase (**Fig 5.2**).

Subjects were asked to abstain from alcohol and strenuous physical activity for 48 hours before and to fast from 10 pm the night before each experimental study visit. Volunteers reported to the unit at 8:30 am and had a DEXA scan performed to assess body composition (all underwent a urine β-hCG test before the DEXA scan to rule out pregnancy). Subjects were then asked to rest semi-supine on a bed for the next 5 hours. After 3 hours subjects consumed a "milkshake" containing 44 g of 'Slim Fast' (Slim Fast powder, Unilever Ltd., London, UK) in 300 ml of skimmed milk containing approximately 25 g of CHO and 18 g of protein.

The flow of the volunteers along the study is depicted in a diagram **(Diagram 5.1)** based on CONSORT statement guidelines (Begg et al., 1996).

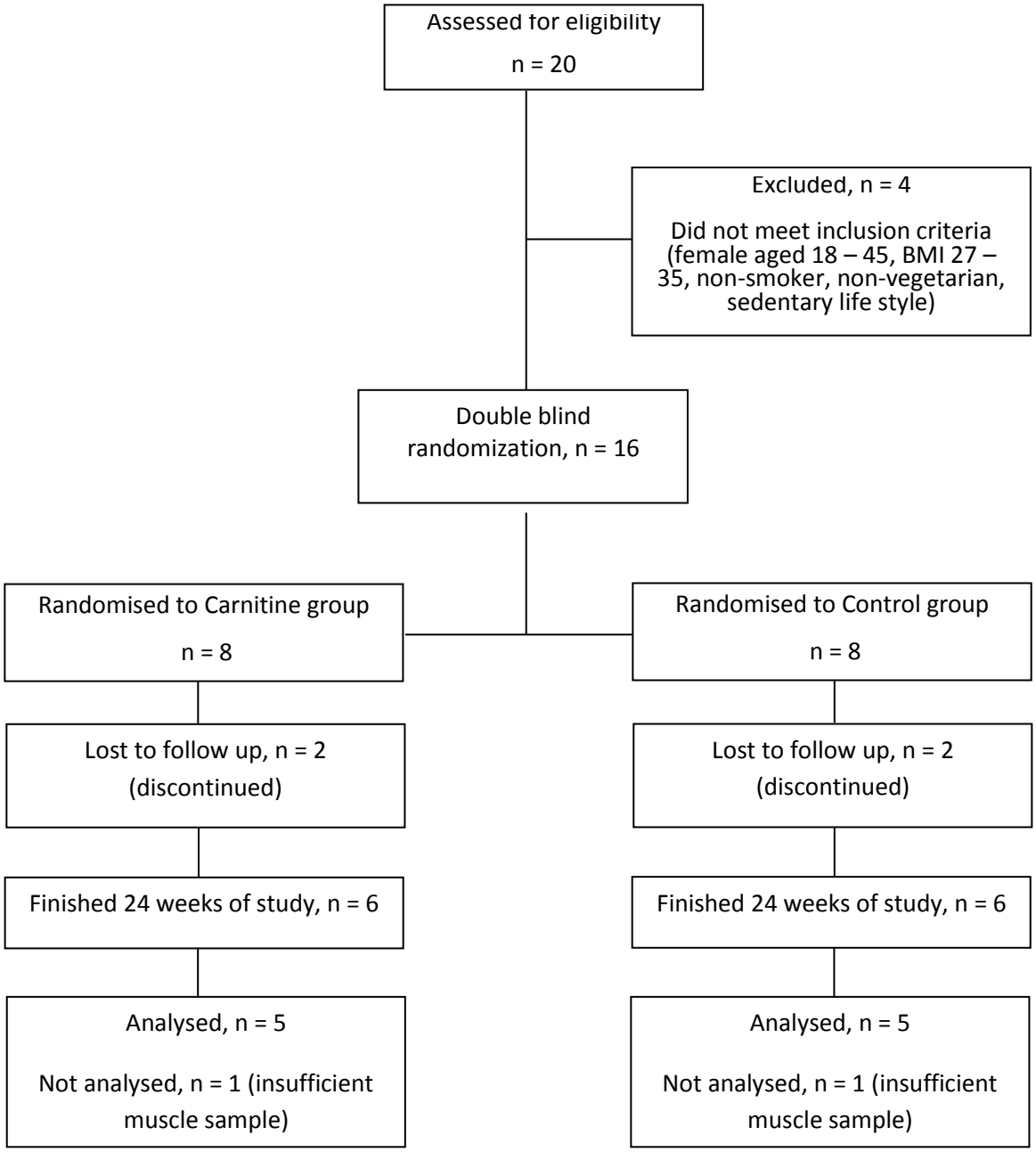


Diagram 5.1. The flow of the volunteers along the study depicted on the basis of CONSORT statement guidelines.

Sample collection and analysis

Indirect calorimetry

Expired gases were collected online for 10 minutes at hours 0, 1, 2, 3 and 4 **(Fig. 5.2)** for VO_2 and VCO_2 determination in order to calculate whole-body fat and carbohydrate oxidation rates by indirect calorimetry (Vmax, SensorMedics corporation, California, USA).

Fat and carbohydrate oxidation rates and energy expenditure (EE) were calculated based on Frayn formulae (Frayn K.N. 1983):

Fat oxidation (g/min): $[1.67 \times (VO_2 - VCO_2)]$

Fat oxidation (kJ/min): [39.4 × Fat oxidation (g/min)]

CHO oxidation (g/min): $[(4.55 \times VCO_2) - (3.21 \times VO_2)]$

CHO oxidation (kJ/min): [15.6 × CHO oxidation (g/min)]

EE (kJ/min): $(15.9 \times VO_2) + (5.2 \times VCO_2)$

(VO_2 and VCO_2 are in L/min)

Muscle biopsy

Muscle biopsy samples were obtained from the vastus lateralis muscle at hours 0, 2 and 4 **(Fig. 5.2)**. The samples were obtained using the Bergstrom percutaneous needle biopsy technique under sterile conditions after subcutaneous and deep injection of Lignocaine 2% (B. Braun, Melsungen, Germany) to anaesthetise the local area. Muscle biopsy samples were stored in liquid nitrogen and were later dried, powdered and analysed for muscle total carnitine content. For details of analyses see CHAPTER 2: General Methods.

At the end of the study all the lines were removed and the volunteers were fed. A standard dressing was applied on muscle biopsy incision areas including Steri-Strip (3M Deutschland GmbH, Neuss, Germany), tegaderm (3M Deutschland GmbH, Neuss, Germany) and coban compression bandage (3M Deutschland GmbH, Neuss, Germany).

Randomisation and supplementation protocol

Volunteers were randomly allocated in to 1 of 2 intervention groups (CON and CARN) and were asked to drink a meal replacement milkshake containing 25 g of CHO and 18 g of protein (Slim Fast powder, Unilever Ltd., London, UK), prescribed by a registered dietician/nutritionist, and either placebo (2.25 g of maltodextrin) or 2 g of L-carnitine L-tartrate (Lonza Ltd., Switzerland) twice a day for 16 weeks **(Fig. 5.1)**. The volunteers were provided with an electronic spoon to take the exact amount prescribed by the dietician. They were asked to mix the 'Slim Fast' powder

in 300 ml of skimmed milk, and take it twice a day, once in the morning replacing breakfast and once in the early afternoon replacing midday meal. The dieting plan aimed to reduce the calorie intake by 500 kcal per day for each subject. To calculate the daily calorie intake, the volunteers filled a 4 day diet diary and an International Physical Activity Questionnaire (IPAQ) and submitted it to the dietician before the first metabolic experimental visit. Basal metabolic rate (BMR) was calculated for each subject using Henry's equations (Henry, 2005):

BMR (Kcal/24 h) for volunteers aged 18-30 = [13.1 × Body Mass (kg)] + 558

BMR (Kcal/24 h) for volunteers aged 30-60 = [9.74 × Body Mass (kg)] + 694

Daily energy requirement was then calculated by BMR multiplied by the physical activity level factor calculated from IPAQ, which was then reduced by 500 kcal per day. Diet diaries at 4, 8, 12 and 16 weeks **(Fig. 5.1)** were used to estimate compliance to the diet regime.

Each subject had 7 further diet reviews with the dietician at 1, 2, 4, 6, 8, 12 and 16 weeks **(Fig. 5.1)**. They were requested to fill a 4 day diet diary and the International Physical Activity Questionnaire (IPAQ) form at 4, 8, 12 and 16 weeks as well. The 'reported energy intake' was calculated as the mean energy intake from week 4, 8, 12 and 16 food diaries.

All volunteers were also requested to exercise twice a week for the 16 week supplementation period in order to increase energy expenditure. This consisted of

exercising at 50% VO_2max for one hour in their personal time either on a treadmill at the local gym, at home or in our unit or on a convenient route calculated on the Google Maps.

Statistical analysis

A repeated measures (RM) two-way ANOVA for interaction and time effects, with a Bonferroni multiple comparison test (GraphPad Prism 6, GraphPad Software Inc, California, USA), was performed to locate differences in body mass, muscle total carnitine content, DEXA scan findings (body fat percentage and android fat mass) and indirect calorimetry findings (fat and carbohydrate oxidation and RER). Statistical significance was declared at $p < 0.05$, and a trend for difference at $0.05 < p < 0.1$. All the values presented in text, tables, and figures are means ± standard error of the mean (SEM). All the graphs too were made by the GraphPad Prism 6 software.

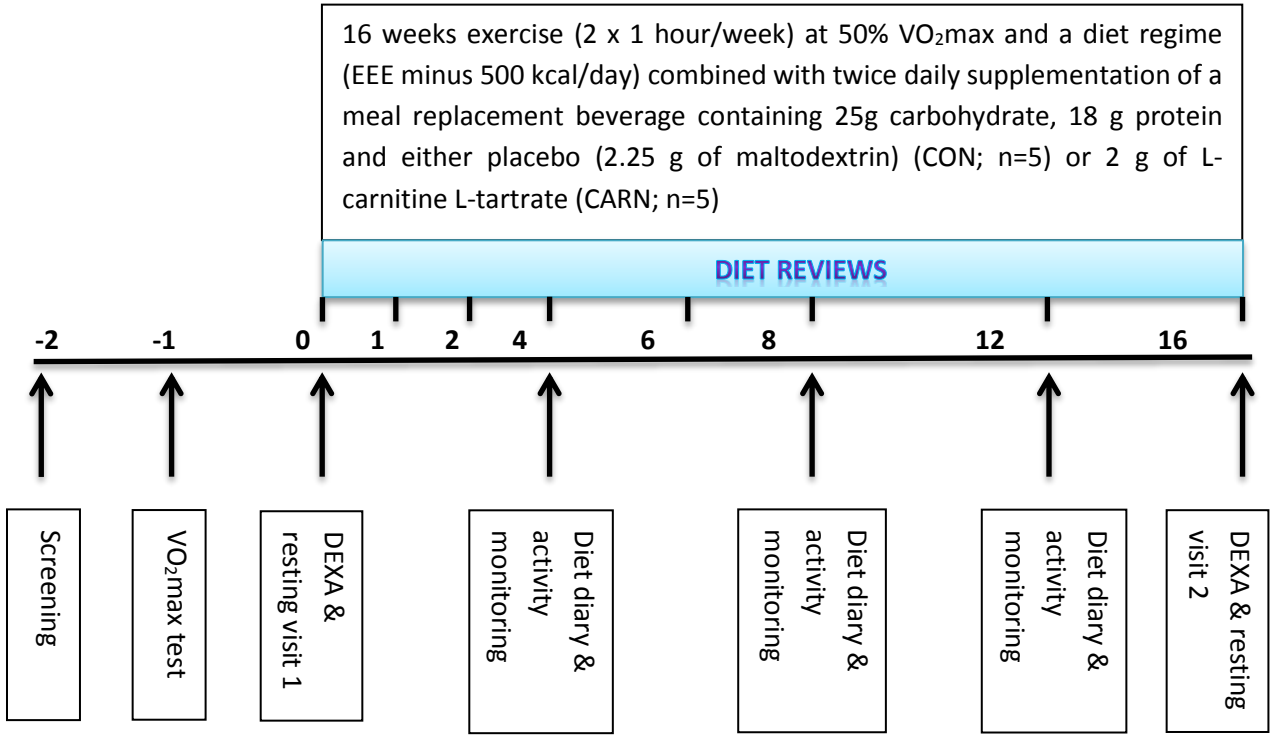


Fig 5.1. Study protocol

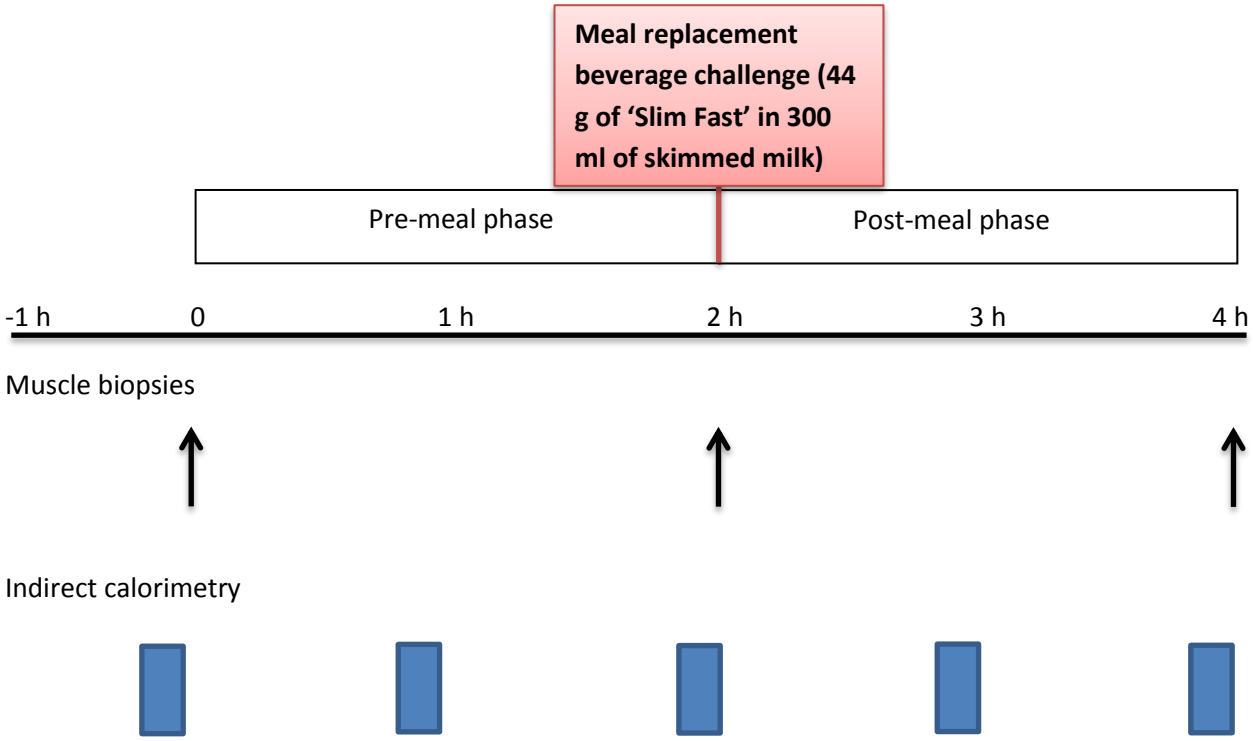


Fig 5.2. Metabolic study visit

5.3. Results

Subject physical characteristics, estimated energy expenditure, prescribed diet, reported energy intake and DEXA measurements

Subject physical characteristics, estimated energy expenditure (EEE), prescribed diet, reported energy intake and DEXA measurements at baseline are displayed in **Table 5.1**. See the Chapter Methods for EEE calculations. 'Prescribed diet' is EEE minus 500 kcal/day and 'Reported energy intake' is the mean of energy intakes based on food diaries from 4, 8, 12 and 16 weeks. There were no differences in body mass, BMI, EEE or DEXA measurements between CON and CARN groups at baseline.

Table 5.1. Subject physical characteristics, estimated energy expenditure (EEE), prescribed diet, reported energy intake and DEXA measurements at baseline in control (CON, n=5) and carnitine (CARN, n=5) groups. All values are means ± standard error of the mean (SEM).

	CON	CARN
Age (years)	31.9 ± 4.1	33.7 ± 5.3
Height (m)	1.65 ± 0.03	1.68 ± 0.03
Body mass (kg)	82.5 ± 3.4	85.3 ± 5.6
BMI (kg/m^2)	30.3 ± 1.0	30.1 ± 1.3
EEE (kcal/day)	2281 ± 129	2335 ± 171
Prescribed diet (kcal/day)	1780 ± 129	1835 ± 171
Reported energy intake (kcal/day)	1611 ± 59	1619 ± 89
Percentage total body fat mass (%)	45.82 ± 1.89	44.64 ± 2.01
Android fat mass (kg)	3.20 ± 0.18	3.40 ± 0.23

Calorimetry data

Fat oxidation

Whole-body fat oxidation rate decreased from 108.1 ± 7.3 to 77.6 ± 17.6 J/min/kg LBM in the CON group and from 100.7 ± 6.1 to 83.6 ± 16.7 J/min/kg LBM in the CARN group at baseline following a meal replacement beverage challenge (44 g 'Slim Fast' in 300 ml skimmed milk). Following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max, whole-body fat oxidation rate declined from 120.7 ± 10.9 to 102.5 ± 16.6 J/min/kg LBM in the CON group and from 131.2 ± 4.7 to 106.7 ± 13.2 J/min/kg LBM in the CARN group following a similar meal replacement beverage challenge. There was a statistically significant difference between whole-body fat oxidation rate during the pre-meal phase at baseline and after 16 weeks in the CARN group ($p < 0.05$) (**Fig. 5.3**).

Carbohydrate oxidation

Whole-body carbohydrate oxidation rate increased from 10.6 ± 4.2 to 56.7 ± 16.2 J/min/kg LBM in the CON group and from 14.8 ± 6.3 to 46.4 ± 17.3 J/min/kg LBM in the CARN group at baseline following a meal replacement beverage challenge (44 g 'Slim Fast' in 300 ml skimmed milk). Following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day)

and twice weekly exercise for 1 hour at 50% VO_2max, whole-body carbohydrate oxidation rate increased from 9.2 ± 6.7 to 36.8 ± 13.4 J/min/kg LBM in the CON group and from 0.0 ± 0.0 to 31.1 ± 11.2 J/min/kg LBM in the CARN group following a similar meal replacement beverage challenge. There was a statistically significant difference between whole-body carbohydrate oxidation rate during the pre-meal phase at baseline and after 16 weeks in the CARN group ($p < 0.05$) (**Fig. 5.4**).

Respiratory exchange ratio (RER)

RER increased from 0.73 ± 0.01 to 0.82 ± 0.04 in the CON group and from 0.74 ± 0.01 to 0.81 ± 0.04 in the CARN group at baseline following a meal replacement beverage challenge (44 g 'Slim Fast' in 300 ml skimmed milk). Following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max, RER increased from 0.71 ± 0.02 to 0.78 ± 0.03 in the CON group and from 0.68 ± 0.01 to 0.76 ± 0.02 in CARN the group following a similar meal replacement beverage challenge. There was a statistically significant difference between RER during the pre-meal phase at baseline and after 16 weeks in the CARN group ($p < 0.05$) (**Fig. 5.5**).

Energy expenditure (EE)

Whole-body energy expenditure increased from 118.7 ± 6.3 to 134.4 ± 3.1 J/min/kg LBM in the CON group and from 115.6 ± 2.4 to 129.9 ± 6.0 J/min/kg LBM in the

CARN group at baseline following a meal replacement beverage challenge (44 g 'Slim Fast' in 300 ml skimmed milk). Following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max, whole-body energy expenditure increased from 123.9 ± 2.7 to 139.3 ± 4.2 J/min/kg LBM in the CON group and from 120.8 ± 4.4 to 136.0 ± 5.9 J/min/kg LBM in the CARN group following a similar meal replacement beverage challenge (**Fig. 5.6**).

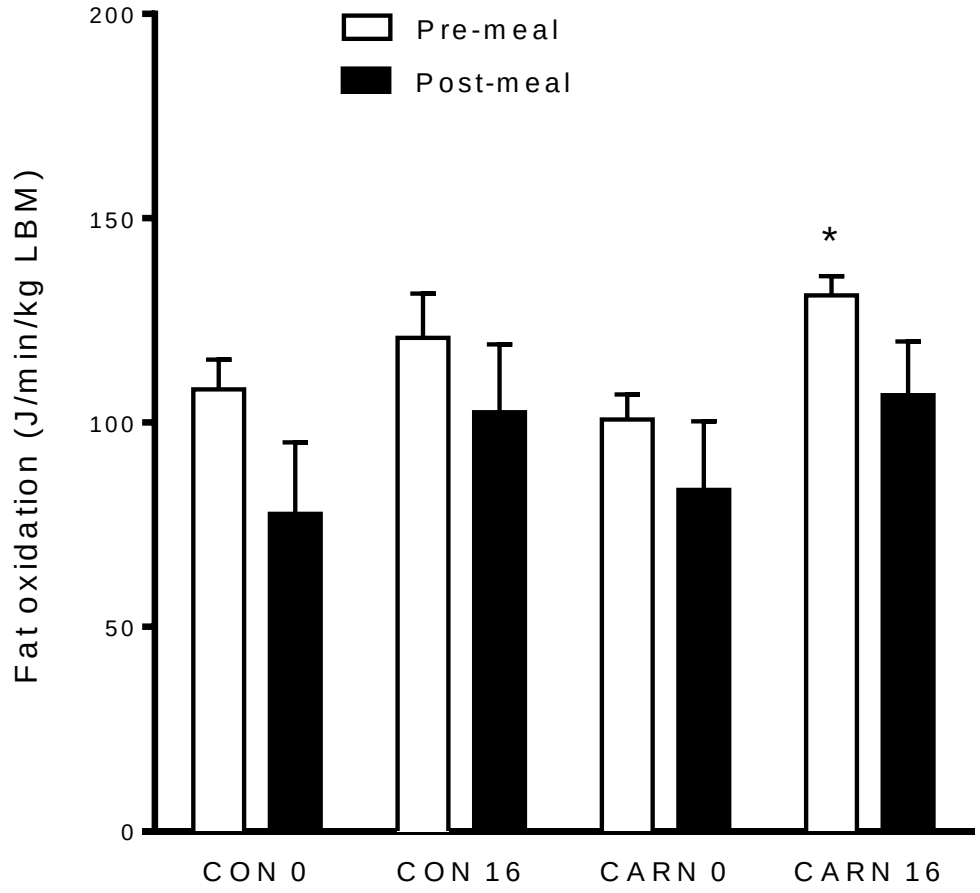


Figure 5.3. Whole-body fat oxidation rate during pre-meal (unfilled blocks) and post-meal (filled blocks) phases at baseline (0) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (n = 5) and CARN (n = 5) groups. Post-meal phase starts after drinking a meal replacement beverage (44 g of 'Slim Fast' in 300 ml of skimmed milk). Each phase lasts 120 minutes. All values are means ± standard error of the mean (SEM) and expressed as J/min/kg LBM. Significantly different from the corresponding value at baseline: * $p < 0.05$.

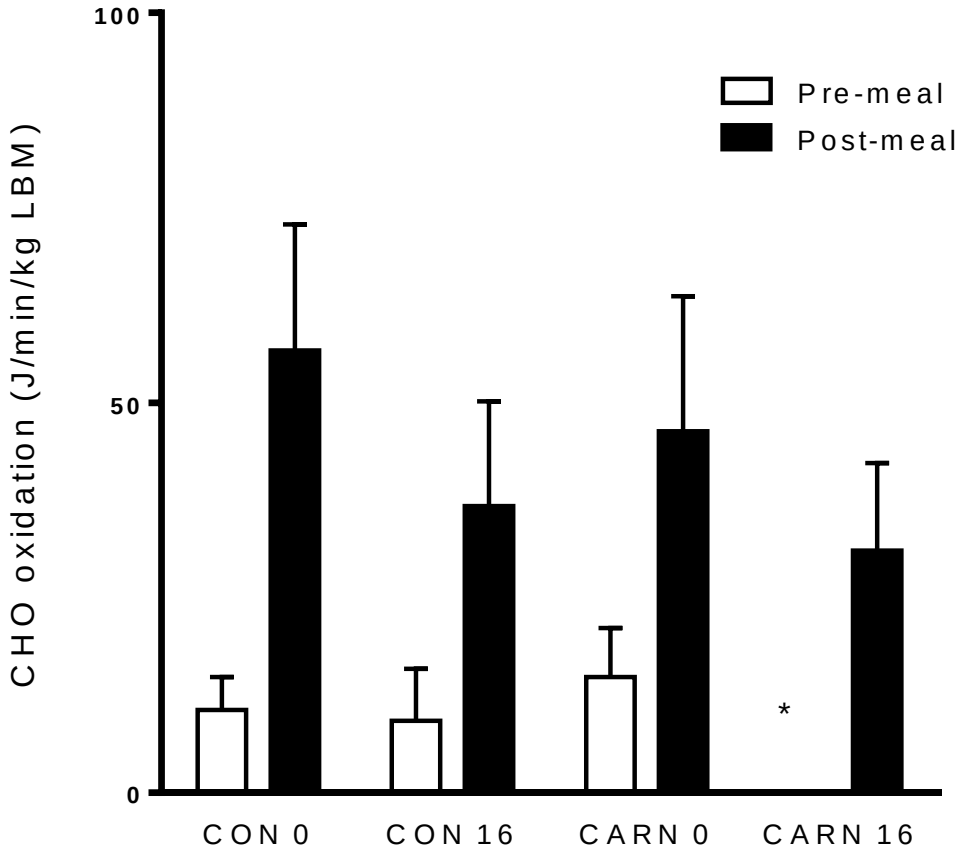


Figure 5.4. Whole-body carbohydrate oxidation rate during pre-meal (unfilled blocks) and post-meal (filled blocks) phases at baseline (0) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (n = 5) and CARN (n = 5) groups. Post-meal phase starts after drinking a meal replacement beverage (44 g of 'Slim Fast' in 300 ml of skimmed milk). Each phase lasts 120 minutes. All values are means ± standard error of the mean (SEM) and expressed as J/min/kg LBM.

Significantly different from the corresponding value at baseline: * $p < 0.05$.

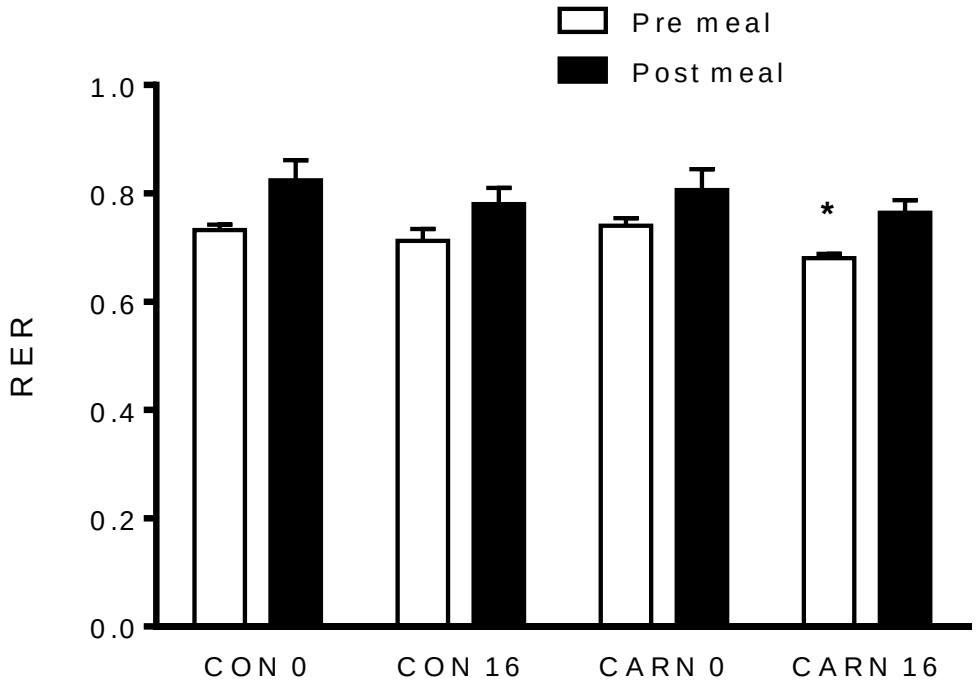


Figure 5.5. Respiratory exchange ratio (RER) during pre-meal (unfilled blocks) and post-meal (filled blocks) phases at baseline (0) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (n = 5) and CARN (n = 5) groups. All values are means ± standard error of the mean (SEM). Post-meal phase starts after drinking a meal replacement beverage (44 g of 'Slim Fast' in 300 ml of skimmed milk). Each phase lasts 120 minutes.

Significantly different from the corresponding value at baseline: * $p < 0.05$.

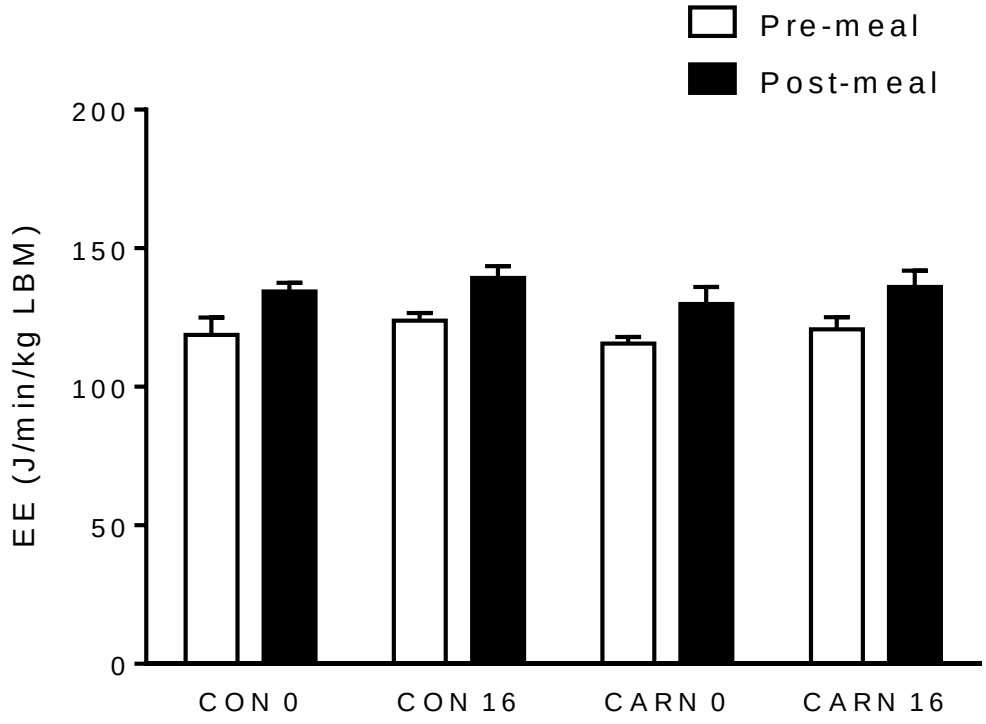


Figure 5.6. Energy expenditure during pre-meal (unfilled blocks) and post-meal (filled blocks) phases at baseline (0) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (n = 5) and CARN (n = 5) groups. Post-meal phase starts after drinking a meal replacement beverage (44 g of 'Slim Fast' in 300 ml of skimmed milk). Each phase lasts 120 minutes. All values are means ± standard error of the mean (SEM) and expressed as J/min/kg LBM.

Muscle total carnitine content

There was no change in muscle total carnitine content in the CON group following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus placebo (2.25 g of maltodextrin), a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max. Muscle total carnitine content increased from 16.66 ± 1.56 to 20.94 ± 1.37 mmol/kg dm in the CARN group following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max ($p < 0.01$) (**Fig. 5.7**).

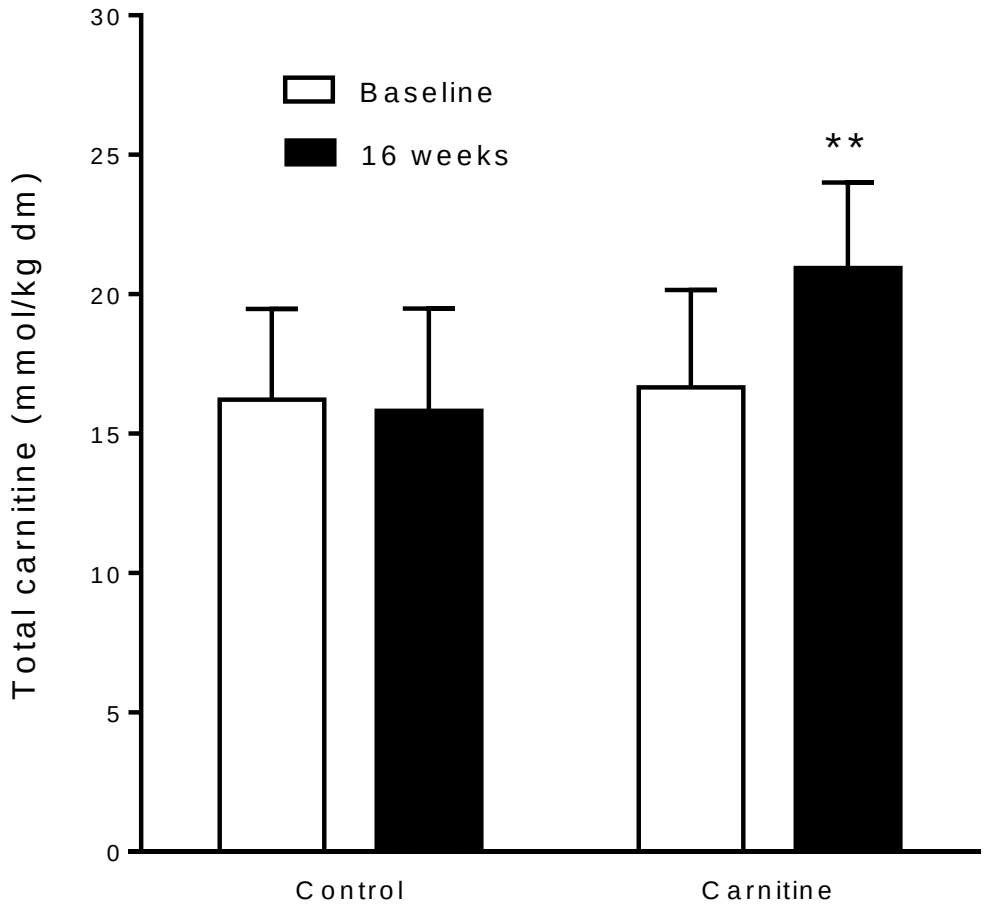


Fig. 5.7. Muscle total carnitine content at baseline (unfilled blocks) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max (filled blocks) in CON (n = 5) and CARN (n = 5) groups. All values are means ± standard error of the mean (SEM) and expressed as mmol/kg dm.

Significantly different from baseline: ** $p < 0.01$.

Body mass and body fat mass

Body mass and percentage weight loss

There was a gradual decrease in body mass (kg) from baseline in both CON and CARN groups over the course of the 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max, which was significantly different from baseline in both groups from 4 weeks onwards **(Fig. 5.8)**. The total body mass loss in CON and CARN amounted to 6.0 ± 0.5 kg and 7.5 ± 1.4 kg, respectively, which was not different between groups.

For the sake of clarity, percentage body mass loss (%) over the course of the study is depicted in **Fig. 5.9**. At 16 weeks percentage body mass loss from baseline was 7.26 ± 0.47 % in CON ($p < 0.0001$) and 8.70 ± 1.25 % in CARN ($p < 0.0001$).

Total body fat percentage and android fat mass

As might be expected from the body mass changes described above, there was a significant decline in percentage total body fat mass (%) following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in both CON and CARN groups (**Fig. 5.10**). Values declined from 45.8 ± 1.9 to 42.5 ± 2.1 % in CON ($p < 0.01$) and

from 44.6 ± 2.0 to 40.6 ± 1.7 % in CARN ($p < 0.01$), but there was no difference between treatment groups.

Android fat mass is the adipose tissue mass around shoulders, thorax and abdomen and therefore is a marker of central (apple-shaped) obesity. In the present study, android fat mass loss (kg) followed a similar pattern and declined from 3.20 ± 0.18 to 2.66 ± 0.20 kg in CON ($p < 0.001$) and from 3.40 ± 0.23 to 2.54 ± 0.26 kg in CARN ($p < 0.0001$), but again there was no difference between treatment groups (**Fig. 5.11**).

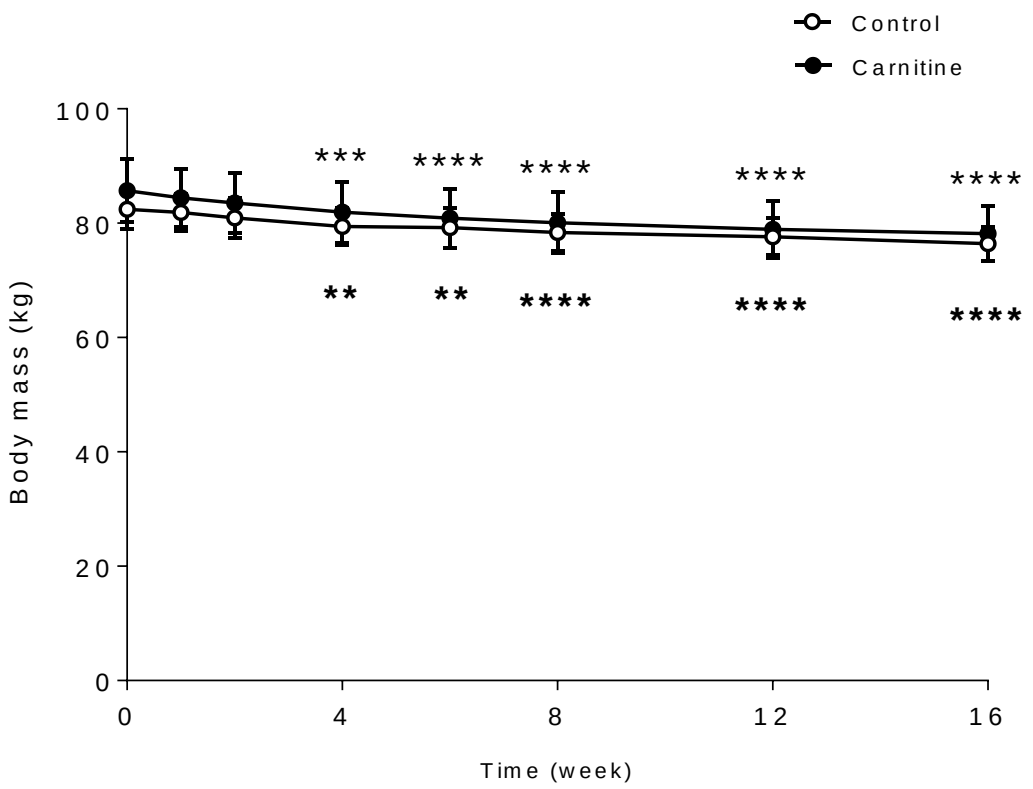


Fig. 5.8. Body mass following 1, 2, 4, 6, 8, 12 and 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (unfilled circles; n = 5) and CARN (filled circles; n = 5) groups. All values are means ± standard error of the mean (SEM) and expressed as kg.

Significantly different from baseline: ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

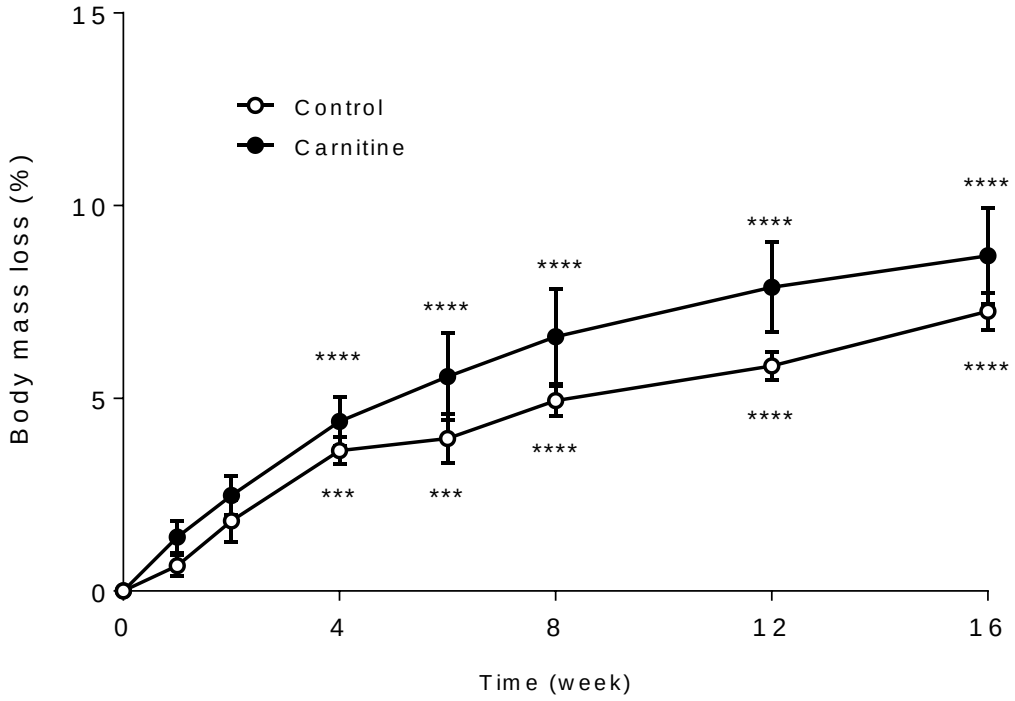


Fig. 5.9. Percentage body mass loss from baseline following 1, 2, 4, 6, 8, 12 and 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max in CON (unfilled circles; n = 5) and CARN (filled circles; n = 5) groups. All values are means ± standard error of the mean (SEM) and expressed as percentage (%).

Significantly different from baseline: *** $p < 0.001$ and **** $p < 0.0001$.

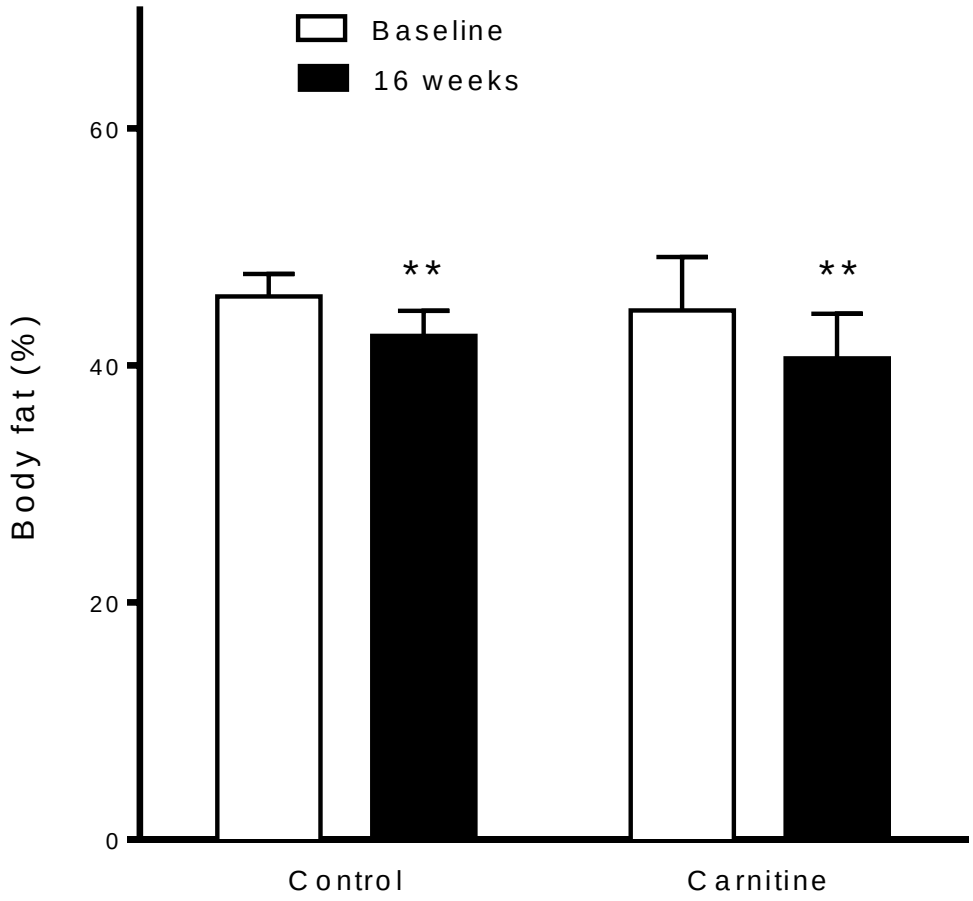


Fig. 5.10. Percentage total body fat mass at baseline (unfilled blocks) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max (filled blocks) in CON (n = 5) and CARN (n = 5) groups. All values are means ± standard error of the mean (SEM) and expressed as percentage (%).

Significantly different from baseline: ** $p < 0.01$.

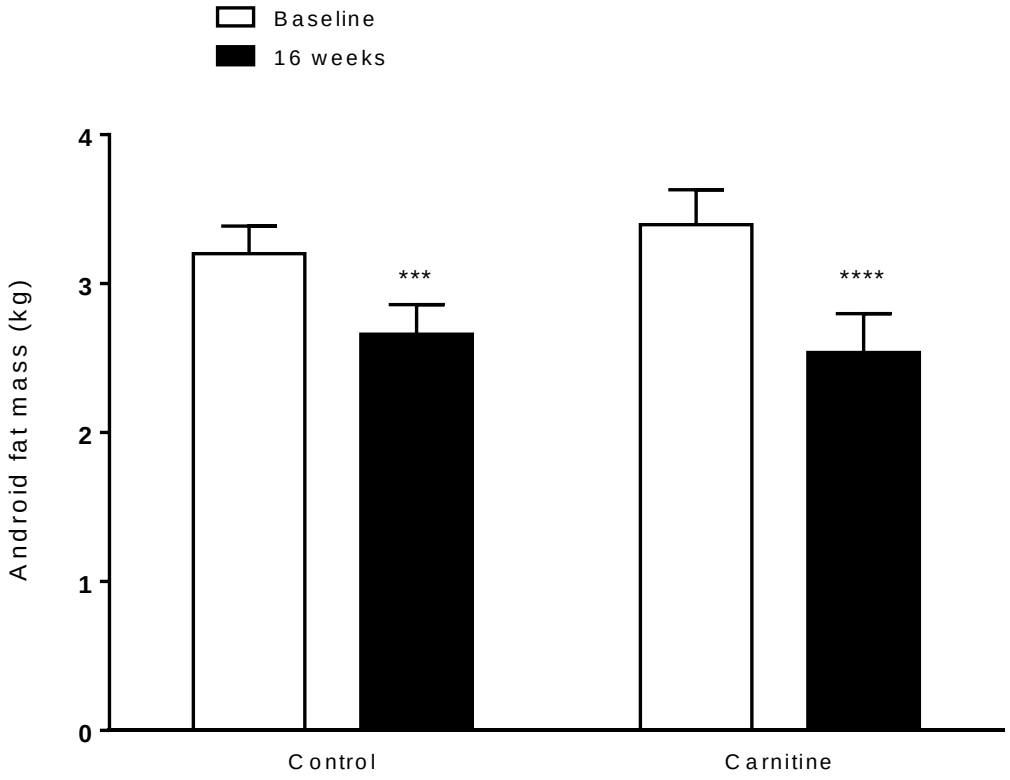


Fig. 5.11. Android fat mass at baseline (unfilled blocks) and following 16 weeks of twice daily supplementation of 25 g CHO and 18 g protein plus either placebo (2.25 g of maltodextrin) or 2 g L-carnitine L-tartrate, a diet regime (EEE minus 500 kcal/day) and twice weekly exercise for 1 hour at 50% VO_2max (filled blocks) in CON (n = 5) and CARN (n = 5) groups. All values are means ± standard error of the mean (SEM) and expressed as kg.

Significantly different from before supplementation: *** $p < 0.001$ and **** $p < 0.0001$.

5.4. Discussion

The aim of the present study was to investigate whether 16 weeks of twice daily L-carnitine feeding in combination with a meal replacement supplement (to stimulate insulin mediated muscle carnitine accumulation), caloric restriction and low intensity exercise could increase muscle total carnitine content in young, obese women, and if so whether it was associated with changes in fuel oxidation and increased fat mass loss relative to a control group. We therefore hypothesised that muscle total carnitine content would increase in obese, young female subjects following 16 weeks of twice daily L-carnitine feeding (2 g of L-carnitine L-tartrate twice a day) in combination with a meal replacement supplement (25 g of CHO and 18 g of protein to stimulate insulin mediated muscle carnitine accumulation), caloric restriction (minus 500 kcal/day requirements) and low intensity exercise training (two exercise sessions per week, each lasting one hour at 50% VO_2max) and that increased muscle total carnitine content during 16 weeks of caloric restriction and low intensity exercise training would be associated with an increase in whole-body fat oxidation and increased loss of whole-body and regional fat masses relative to an appropriate control (CON) group.

Previous research in our lab used a combination of oral carbohydrate and carnitine supplementation in order to increase muscle total carnitine content, i.e. 80 g of CHO plus 2 g of L-carnitine L-tartrate twice daily over 12 weeks (Stephens et al., 2013) or 24 weeks (Wall et al., 2011, Chapter 4). These studies reported an increase in body mass and body fat mass in the control group volunteers. In the present study, we for the first time used a novel approach to attempt to increase muscle total carnitine content. Instead of daily high calorie carbohydrate ingestion (80 g of

CHO twice a day; equivalent to 640 kcal energy intake in combination with carnitine) in addition to an *ad libitum* diet over 12 or 24 weeks, the volunteers took carnitine combined with a lower calorie meal replacement milkshake (Slim Fast, Unilever Ltd., London, UK) twice a day replacing breakfast and midday meal over 16 weeks. The milkshake contained 25 g of CHO and 18 g of protein providing 344 kcal of daily energy intake. The volunteers also were prescribed a diet (EEE minus 500 kcal/day) and underwent low intensity exercise training (two exercise sessions per week, each lasting one hour at 50% VO_2max). Stephens et al. (2007b) demonstrated that elevating serum insulin above a certain threshold (90 mU/L) in the presence of elevated plasma carnitine was sufficient to acutely increase muscle carnitine content. In the present study we hypothesised that reducing the carbohydrate content and increasing the protein content would be sufficient to elevate insulin above the 90 mU/L threshold value and would therefore increase muscle carnitine content, even in obese individuals under conditions of reduced energy intake. Steenge et al. (1998) demonstrated that a similar protocol increased muscle creatine (Cr) accumulation in human volunteers, whose muscle transport is achieved via a Na^+-dependent transporter protein located on the muscle cell membrane. It is believed that the stimulatory effect of insulin on muscle creatine accumulation happens via Na^+/K^+ pump activation (Haugland and Chang, 1975, Odoom et al., 1996). Steenge et al. (2000) demonstrated that serum insulin concentration and body creatine retention were greater when oral creatine supplementation was combined with either protein-CHO or high-CHO dietary supplements in comparison with low-CHO treatment (both $p < 0.05$). The authors reported that ingesting 50 g of protein and 50 g of CHO (protein-CHO treatment) in

combination with a creatine supplementation had the same stimulatory impact on insulin release and body creatine retention as ingesting 100 g of CHO alone (high-CHO treatment). In line with this hypothesis, a major finding of the present study is that, compared to previous successful carnitine supplementation regimes (Wall et al., 2011, Stephens et al., 2013, Chapter 4), muscle carnitine content can be elevated significantly ($p < 0.05$) by taking a lower calorie supplementation (344 kcal daily instead of 640) over 16 weeks. Whilst there was no increase in muscle carnitine content in the CON group following 16 weeks of supplementation in the present study, the CARN group demonstrated 26% rise in skeletal muscle carnitine content ($p < 0.05$). Using a lower calorie adjunct to carnitine supplement would be more appropriate in the context of a weight loss strategy where chronic ingestion of high quantities of CHO is not likely to be seen as advisable.

As discussed in the GENERAL INTRODUCTION and Chapter INTRODUCTION in details, it is known since 1950s that carnitine is essential for the translocation of long chain fatty acids across the mitochondrial membrane for intra-mitochondrial β-oxidation (Fritz, 1955, Fritz and Mc, 1959, Fritz and Yue, 1963). It is also reported that increased muscle carnitine content augments fat oxidation at rest (Stephens et al., 2006a, Stephens et al., 2013) and during low intensity steady-state exercise (Wall et al. 2011) and is associated with an upgraded expression of skeletal muscle genes related to fatty acid metabolism (Stephens et al. 2013). In line with this, another finding of the present study is that the increase in muscle carnitine content in the CARN group following 16 weeks of carnitine supplementation, a diet regime and low intensity exercise, was associated with a 30% increase in fasting (pre-meal phase) whole-body fat oxidation ($p < 0.05$ compared to baseline; **Fig. 5.3**). This

increase in fasting whole-body fat oxidation occurred concomitantly with a significant decline in the rate of fasting (pre-meal phase) whole-body carbohydrate oxidation ($p < 0.05$ compared to baseline; **Fig. 5.4**). No change in fasting whole-body fat or carbohydrate oxidation rate was noticed in the CON group after 16 weeks of oral supplementation and similar diet and exercise regimes.

Chapter 4 demonstrated that the improvement in blood glucose disposal following an OGTT happened secondary to changes in liver lipid deposition, i.e. muscle fat oxidation increased so there was less deposition of lipid in the liver which as a result improved liver insulin sensitivity. Therefore in Chapter 5, as well as an effect of carnitine supplementation on fuel selection and oxidation in muscles, there could also be a metabolic effect at the level of the liver as demonstrated in Chapter 4. This could particularly be the case in the overweight women where liver insulin resistance tends to be higher than lean subjects.

It was also discussed extensively in GENERAL INTRODUCTION and Chapter INTRODUCTION that carnitine prevents an increase in body mass and body fat mass when supplied chronically with carbohydrate. The control group volunteers who ingested carbohydrate only over the same period of time demonstrated a significant increase in body mass and body fat mass (Wall et al., 2011, Stephen et al., 2013). The findings of Chapter 4 in the present thesis are also consistent with these findings. In summary we reported in Chapter 4 that ingesting 80 g of CHO over 24 weeks caused 2.3 kg increase in body mass ($p < 0.05$) and 2.7% increase in percentage total body fat ($p < 0.05$). We reported no change in body mass or percentage total body fat in carnitine group volunteers who ingested 80 g of CHO

combined with 2 g of L-carnitine L-tartrate over the same period of time. In fact carnitine has been used commercially as an aid to weight loss and carnitine supplements are sold extensively worldwide for this purpose and as promoter of enhanced exercise performance. To our knowledge, the present study is the first which has used a comprehensive novel approach to weight loss, consisting of caloric restriction and regular exercise in addition to carnitine supplementation, in sedentary young healthy overweight volunteers. We believe this is an important new approach as obesity is on the rise in younger people in the developed countries and weight loss by caloric restriction and physical exercise does not always improve fat oxidation on its own in obese people (Astrup et al. 1994). Theoretically there could be a role for carnitine to play by increasing fat oxidation and consequently enhancing weight loss in obese people who are more likely to be low fat oxidisers.

Both CON and CARN groups lost body mass remarkably in the present study by following a similar diet regime (minus 500 kcal/day requirements) and exercise training programme (twice weekly, one hour sessions at 50% VO_2max). Although there was significant body mass loss from week 4 onwards in both groups, there was no difference between the two groups at any time-point (**Fig. 5.8** and **5.9**). Accordingly, this was associated with significant total body fat loss and android fat loss in both groups following 16 weeks of supplementation, diet regime and exercise training (**Fig. 5.10** and **5.11**). Although there were no statistically significant differences between CON and CARN groups at any time-point, the mean total body fat loss and android fat loss were greater in the CARN group after 16 weeks of supplementation, diet regime and exercise training. Specifically, the CON group lost on average 7.3% of total body fat mass and 16.9% of the android fat

mass, whilst the CARN group lost 9.1 and 25.3% respectively after 16 weeks compared to baseline. Of note, the gap in percentage weight loss widened between groups as the study progressed (**Fig. 5.9**), suggesting that if the study continued beyond 16 weeks a statistical difference in weight loss between the two groups may have become apparent. It is also possible that the present study was under-powered in terms of the number of volunteers recruited. Chronic metabolic human experiments involve low numbers of volunteers because of the nature of the measurements being made and commitment required from volunteers. Indeed, inclusion of more volunteers may have resulted in statistical difference in weight and fat mass loss between groups within the duration of the study protocol, i.e. 16 weeks.

The mean baseline muscle carnitine content in the volunteers taking part in Chapter 5 study is lower than the mean baseline muscle carnitine content in Chapter 4 study volunteers (16.4 ± 1.0 mmol/kg dm in Chapter 5 vs 20.9 ± 1.0 mmol/kg dm in Chapter 4). We are not aware of any studies in the literature comparing baseline muscle carnitine content between overweight and lean subjects; however it is likely that higher fat content of the biopsy samples in the overweight volunteers resulted in an apparent dilution. Another possible explanation would be that a degree of insulin resistance associated with high BMI in overweight subjects in Chapter 5 affected carnitine uptake by muscle cells. Another point to bear in mind is that Chapter 5 study volunteers were generally less physically active than volunteers participating Chapter 4 study. As discussed in GENERAL INTRODUCTION, muscle contraction is believed to increase muscle carnitine uptake in animal models through enhanced OCTN2 translocation to muscle cell membrane (Furuichi et al.,

2012). It remains to be answered whether a similar mechanism functions in humans and whether reduced muscle contraction/exercise is associated with diminished muscle carnitine uptake.

In summary this is the first study to demonstrate that muscle carnitine content can be increased in humans by ingesting 2 g of L-carnitine L-tartrate in combination with a shake containing 25 g of CHO and 16 g of protein twice a day for 16 weeks. We propose that the increased muscle carnitine content resulted in increased fasting whole-body fat oxidation and decreased fasting whole-body CHO oxidation post-supplementation compared to baseline, which is compatible with the role of carnitine in transferring long chain fatty acids across the mitochondrial membrane and upgrading the expression of muscle genes associated with fatty acid metabolism. Whether increased carnitine content, in combination with caloric restriction and exercise, has any impact on body mass and body fat mass seems to be likely but needs further investigations.

CHAPTER 6

GENERAL DISCUSSION

6.1. Overview of studies and findings

The overall aim of the present thesis was to investigate the modulation of fuel metabolism in healthy volunteers by changing muscle carnitine availability. In **Chapter 3** it was hypothesised that stockpiling of muscle acetyl groups would decrease total body fat oxidation rates during steady-state low intensity exercise due to either decreased muscle free carnitine availability and/or as a result of increased muscle acetate oxidation, and would thereby decrease non-mitochondrial ATP production during steady-state high intensity exercise due to increased availability of acetyl groups to the TCA cycle. **Chapter 4** hypothesised that increased muscle carnitine content would improve muscle insulin sensitivity which would in turn cause an improvement in whole blood glucose disposal during an oral glucose tolerance test. In **Chapter 5** it was hypothesised that increased muscle carnitine content, in combination with caloric restriction and low intensity exercise, would increase loss of whole-body and regional fat masses in overweight volunteers. In line with these hypotheses, the major findings of the present thesis are outlines below.

In **Chapter 3** it was demonstrated that the stockpiling of muscle acetyl group following sodium acetate infusion resulted in reduction in whole-body fat oxidation during steady-state low intensity exercise compared to control and also reduced muscle lactate content during steady-state high intensity exercise relative to control. In **Chapter 4** study it was found that increased muscle total carnitine content following 24 weeks of twice daily supplementation of 2 g of L-carnitine L-tartrate and 80 g of CHO, resulted in a decrease in whole blood glucose

concentration and serum insulin concentration following an oral glucose tolerance test relative to baseline, which was associated with reduced muscle glucose uptake compared with control. **Chapter 5** demonstrated that increased muscle carnitine content following 16 weeks of twice daily supplementation of 2 g of L-carnitine L-tartrate, 18 g of protein and 25 g of CHO, combined with caloric restriction and low intensity exercise increased whole-body fat oxidation rate and decreased whole-body CHO oxidation rate in the fasting state in overweight volunteers compared to control, but the extent of body mass loss and total body fat loss was similar between groups.

6.2. The impact of stockpiling muscle acetyl group on fuel selection during steady-state low intensity exercise in healthy, young, male volunteers (Chapter 3)

In **Chapter 3**, sodium acetate infusion was used to acutely increase skeletal muscle acetyl group content. Young, healthy male volunteers were infused with sodium acetate (NaAc) throughout each experimental visit (6.25 mmol/min over 80 minutes, 500 mmol in total) which involved 30 minutes of rest, volunteers cycling for 30 minutes at 50% VO_2max, followed immediately by 20 minutes at 80% VO_2max. On a separate occasion (one week apart) the same volunteers took part in a similar experimental protocol (CON visit) in which they were infused with sodium bicarbonate (the same volume and infusion rate as sodium acetate over the same time duration) to match the metabolic alkalosis induced by sodium acetate. The order of sodium acetate and sodium bicarbonate visit was randomised. No difference was noticed in whole-body carbohydrate oxidation during steady-state

low intensity exercise between CON and NaAc visits; however whole-body fat oxidation was 37% lower in the NaAc visit compared to CON ($p < 0.05$). Bearing in mind that the rate of whole-body carbohydrate oxidation was similar in both visits, we speculated that the lower rate of fat oxidation in the NaAc visit was due to excess acetyl groups being preferentially oxidised over fat. This is in line with findings from Akanji et al. (1989) who reported a decreased whole-body fat oxidation following 60 minutes of NaAc infusion (at the rate of 2.5 mmol/min, total dose 150 mmol) at rest in both non-diabetic and diabetic subjects.

We also demonstrated 2-fold greater muscle acetylcarnitine content ($p < 0.05$) and 40% lower muscle free carnitine content ($p < 0.05$) immediately prior to the onset of low intensity exercise in the NaAc visit compared to CON. The translocation of long chain fatty acids across the mitochondrial membrane is a principal function of carnitine known since 1950s (Fritz 1955; Fritz & McEwen 1959; Bremer 1962a; Bremer 1962b; Fritz & Yue 1963; Fritz & Marquis 1965) and therefore it is also plausible to speculate that the decrease in free carnitine availability may have limited mitochondrial long chain fatty acid translocation, thereby reducing fatty acid-derived acetyl-CoA supply to the TCA cycle, thereby also contributing to reduced fat oxidation.

6.3. The impact of stockpiling muscle acetyl group on fuel selection during steady-state high intensity exercise in healthy, young, male volunteers (Chapter 3)

Chapter 3 also demonstrated greater muscle acetyl-CoA availability immediately prior to the onset of high intensity exercise in the NaAc visit compared to CON ($p <$

0.05), which was accompanied by 29% less muscle lactate accumulation during steady-state high intensity exercise in NaAc compared to CON ($p < 0.05$).

Karlsson and Saltin (1970) demonstrated that the major part of muscle lactate accumulation during steady-state exercise occurred during the rest to exercise transition period. It was proposed therefore that the lower muscle lactate accumulation observed during high intensity exercise in NaAc was attributable to greater acetyl-CoA supply to the TCA cycle.

6.4. Increasing muscle carnitine content via chronic supplementation of carnitine and carbohydrate in healthy, young, male volunteers (Chapter 4)

Muscle total carnitine content increased by 18% in **Chapter 4** study following 24 weeks of twice daily supplementation of 80 g of CHO and 2 g of L-carnitine L-tartrate (containing 1.36 g of L-carnitine) in healthy, young male volunteers ($p < 0.05$). This is in line with findings from Wall et al. (2011) when a ~20% increase in muscle carnitine content was observed following the same supplementation protocol over the same period of time. This is also in line with the hypothesis of Stephens et al. (2007c) that carnitine accumulation in skeletal muscle follows a sustainable and cumulative pattern and can be achieved at a rate of ~0.1% increase per day via chronic oral supplementation of L-carnitine and carbohydrate and in line with multiple previous studies in our unit reporting that carnitine supplementation *per se* would not be sufficient to increase muscle carnitine content and that insulin (or glucose to stimulate insulin secretion) is needed, in combination with carnitine, to facilitate carnitine transport into the skeletal muscle cells (Stephens et al. 2006a;

Stephens et al. 2006b; Stephens et al. 2007b; Stephens et al. 2007c; Stephens et al. 2013). Stephens et al. (2007b) reported that serum insulin concentration needed to be above 90mU/l to stimulate carnitine uptake into muscle cells. This stimulatory effect of insulin is implemented via activating sarcolemmal Na^+/K^+ ATPase pump, which in turn stimulates OCTN2 transporter, facilitating the transport of carnitine and Na^+ into the cells (Ewart & Klip 1995, Sweeney & Klip 1998).

6.5. The impact of increased muscle carnitine content on body mass and percentage total body fat in healthy, young, male volunteers (Chapter 4)

In Chapter 4 the CON group gained 2.3 kg in body mass ($p < 0.05$) and 2.7% increase in percentage total body fat mass ($p < 0.05$) following 24 weeks of carbohydrate only supplementation. In contrast to this finding, there was no change in body mass and percentage total body fat mass following 24 weeks of daily L-carnitine and carbohydrate supplementation, which is in line with the findings of Wall et al. (2011). It has been speculated these responses occurred secondary to a carnitine mediated increase in resting muscle fat oxidation (Stephens et al. 2006b; Stephens et al. 2013 and **Chapter 4 and 5** this thesis) and fat oxidation during low intensity exercise (Wall et al. 2011), as a result of carnitine mediated up-regulation of fatty acid translocation across the mitochondrial membrane for β-oxidation (Fritz 1955; Fritz & McEwen 1959; Bremer 1962a; Bremer 1962b; Fritz & Yue 1963; Fritz & Marquis 1965). In keeping with this, Stephens et al. (2013) reported that 12 weeks of daily L-carnitine and carbohydrate supplementation was associated with an increase in resting energy expenditure and the mRNA expression of a number genes

linked directly to ‘fatty acid metabolism’ in muscle of healthy, young, male volunteers.

6.6. The impact of increased muscle carnitine content on whole blood glucose disposal and insulin sensitivity in healthy, young, male volunteers (Chapter 4)

Chapter 4 reported that the increase in muscle carnitine content following 24 weeks of daily L-carnitine and carbohydrate supplementation was associated with a decrease in whole blood glucose concentration and serum insulin concentration following an oral glucose tolerance test (80 g in 500 ml solution) when compared to baseline. No change in blood glucose and serum insulin concentrations was noted following 24 weeks of supplementation in the CON group. Stephens et al. (2013) reported that 12 weeks of carbohydrate and carnitine supplementation was associated with increased mRNA expression of ‘insulin signalling’ genes in muscle biopsy samples from healthy, young, male volunteers. Given it has been reported that increased IMCL (for example secondary to obesity) can have a negative impact on insulin sensitivity (Phillips et al. 1996; Pan et al. 1997) it has therefore been hypothesised that a chronic elevation of carnitine content could potentially improve muscle insulin sensitivity by increasing muscle fat oxidation and reducing IMCL. However, contrary to this proposal, Chapter 4 reported that muscle 2-DG accumulation during the OGTT was lower following 24 weeks of daily L-carnitine and carbohydrate supplementation compared to carbohydrate supplementation alone. It is therefore concluded that this improvement in whole blood glucose disposal observed following 24 weeks of daily L-carnitine and carbohydrate

supplementation could not have occurred at the muscle level. Rather, it is proposed that increased muscle fatty acid oxidation, secondary to augmented muscle carnitine content, was associated with reduced hepatic fat deposition which over time increased hepatic glucose disposal and resulted in the improved blood glucose disposal and insulin profiles observed following the OGTT after 24 weeks of daily L-carnitine and carbohydrate supplementation.

This is the first study to report an improvement in blood glucose and insulin profiles during an OGTT as a result of 24 weeks of daily L-carnitine and carbohydrate supplementation and/or an increase in muscle carnitine content.

6.7. Increasing muscle carnitine content via a novel approach involving chronic supplementation of carnitine plus carbohydrate and protein in healthy overweight female volunteers (Chapter 5)

Chapter 5 reports the first attempt to increase muscle carnitine content following 16 weeks of twice daily supplementation of 2 g of L-carnitine L-tartrate, 18 g of protein and 25 g of CHO in human volunteers. As outlined above, previous successful approaches to elevating muscle carnitine content involved combining carnitine with 80 g of glucose twice a day for 12 to 24 weeks, equivalent to 640 kcal excess energy intake per day (Wall et al. 2011, Stephens et al. 2013, **Chapter 4**). Stephens et al. (2007b) demonstrated that elevating serum insulin concentration above 90 mU/l in the presence of elevated plasma carnitine was sufficient to acutely increase muscle carnitine content. Chapter 5 therefore hypothesized that reducing the carbohydrate content to 25 g twice a day and increasing the protein

content to 18 g twice a day (the latter having a high insulinaemic effect) would be sufficient to elevate serum insulin above the 90 mU/l threshold value and would therefore increase muscle carnitine content, even in overweight individuals under conditions of reduced energy intake. This approach would increase the excess energy intake by 344 kcal per day which is lower than the previously tried supplementation regimes outlined above. This new approach would be more appropriate in the context of a weight loss strategy where ingestion of high quantities of CHO is not likely to be seen as advisable.

Bearing the above in mind we devised a supplementation protocol whereby young, healthy, overweight female volunteers took 2 g of L-carnitine L-tartrate (containing 1.36 g of L-carnitine) combined with a milkshake containing 25 g of CHO and 18 g of protein twice a day, a prescribed diet and low intensity exercise over 16 weeks. A 26% increase in muscle total carnitine content was observed post-supplementation compared to baseline ($p < 0.05$), showing for the first time that muscle carnitine content can be increased via a lower calorie adjunct.

6.8. The impact of increased muscle carnitine content, caloric restriction and low intensity exercise on resting whole-body fat and carbohydrate oxidation in healthy overweight female volunteers (Chapter 5)

Chapter 5 reported that the rate of whole-body fat oxidation at rest increased by 30% ($p < 0.05$) following 16 weeks of twice daily L-carnitine feeding (2 g of L-carnitine L-tartrate twice a day) in combination with a meal replacement supplement (25 g of CHO and 18 g of protein), caloric restriction (EEE minus 500

kcal/day) and low intensity exercise training (two exercise sessions per week, each lasting one hour at 50% VO_2max). This was also associated with a significant concomitant decline in resting whole-body carbohydrate oxidation ($p < 0.05$). These changes were not observed in control volunteers who followed the same dietary supplementation (minus the carnitine feed) reduced daily energy intake and exercise training protocols. It can be proposed therefore that the observed changes in whole-body fuel metabolism occurred secondary to carnitine mediated effects on skeletal muscle fuel selection, i.e. an increase in fat oxidation associated with a decline in carbohydrate oxidation. This is consistent with the impact of carnitine on facilitating fatty acid translocation across the mitochondrial membrane (Fritz, 1955, Fritz and Mc, 1959, Fritz and Yue, 1963) and is in line with previous research demonstrating increased fat oxidation at rest (Stephens et al. 2006a & 2013, Chapter 4) and during low intensity exercise (Wall et al. 2011) secondary to augmented muscle total carnitine content.

Chapter 4 demonstrated that the improvement in blood glucose disposal following an OGTT occurred secondary to the changes in liver lipid deposition, i.e. muscle fat oxidation increased so there was less deposition of lipid in the liver which as a result improved liver insulin sensitivity. Therefore in Chapter 5, as well as an effect of carnitine supplementation at the muscle level on fuel oxidation there could also be a metabolic effect at the level of the liver as demonstrated in Chapter 4. This could particularly be the case in the overweight women where liver insulin resistance tends to be higher than lean.

6.9. The impact of increased muscle carnitine content, caloric restriction and low intensity exercise on body mass and body fat mass in healthy overweight female volunteers (Chapter 5)

Chapter 5 is the first study to use a comprehensive approach to weight loss; consisting of a novel carnitine supplementation protocol, caloric restriction and exercise training. Stephens et al. (2013) demonstrated that chronic carnitine supplementation was associated with up-regulation of genes involved with 'fatty acid metabolism'. Therefore it was hypothesised that carnitine supplementation, in addition to caloric restriction and exercise training, would accelerate weight loss and body fat loss in overweight subjects compared to a control group who followed the same protocol (minus carnitine supplementation). There was a decrease in body mass and total body fat mass in both CON and CARN groups following 16 weeks of supplementation, caloric restriction and exercise training. Indeed both groups demonstrated significant weight loss from 4 weeks onwards, but there was no difference between groups at any time point during the 16 weeks. The weight loss was associated with significant total body fat mass loss and android fat mass loss in both groups post-supplementation compared to baseline ($p < 0.05$). Although there were no statistically significant differences between CON and CARN groups at any time point, the mean percentage total body fat loss and percentage android fat loss were greater in the CARN group after 16 weeks of supplementation. Specifically, the CON group lost on average 7.3% of total body fat mass and 16.9% of the android fat mass, whilst the CARN group lost 9.1 and 25.3% respectively after 16 weeks compared to baseline. Therefore the gap in percentage weight loss widened between groups as the study progressed, suggesting that if the study continued

beyond 16 weeks a statistical difference in weight loss between studies may have become apparent.

6.10. Summary, future work and limitations

In summary this thesis has demonstrated in Chapter 3 that stockpiling acetyl groups decreases whole-body fat oxidation at low intensity exercise due to acetate metabolism replacing fat oxidation and acetylation of the carnitine pool decreasing muscle free carnitine availability. At higher intensities increased acetyl-CoA supply to the TCA cycle decreases muscle lactate production. Administration of a higher dose of sodium acetate can be considered in future studies in order to achieve a greater acetylation of muscle carnitine pool; however the metabolic alkalosis associated with sodium acetate infusion and its potential unwanted impact on the safety of the studies could be a possible limitation.

It has also been demonstrated in Chapter 4 that increasing muscle carnitine content decreases whole blood glucose and serum insulin concentrations following an OGTT in healthy, young, male volunteers. As no increase in muscle 2-DG accumulation during the OGTT was observed, it is speculated that this effect on glycaemia is attributable to carnitine mediated effects on hepatic glucose disposal potentially due to decreased hepatic fat deposition as a result of an increase in muscle fat oxidation.

These findings could have important implications in the management of type 2 diabetes mellitus and age and obesity related insulin resistance. However there are

two main challenges for any future studies aiming at investigating the impact of increased muscle carnitine content on glucose/insulin interaction in people suffering with insulin resistance or diabetes mellitus. Firstly, carnitine retention by skeletal muscle following oral supplementation happens in an incremental and cumulative way and takes several weeks for any impact on fuel metabolism to become apparent (24 weeks in Wall et al. (2011) and Chapter 4 of this thesis, 12 weeks in Stephens et al. (2013) study and 16 weeks in Chapter 5 of this thesis). Secondly, glucose needs to be taken with carnitine in order to stimulate insulin secretion and facilitate carnitine transport into muscle cells. The ethics of feeding carbohydrates to patients with diabetes and the gain in body mass due to increased daily calorie input would be challenging to justify.

The findings of Chapter 4 clearly show that future studies aiming to investigate the impact of L-carnitine supplementation and increased muscle carnitine content on glucose disposal and insulin release in healthy volunteers or patients suffering with impaired glucose tolerance/ diabetes mellitus should focus on liver and use methods such as liver MRI scanning, to investigate hepatic function. Future studies can also investigate the possible role of carnitine in the management of steatosis (fatty liver).

Chapter 5 demonstrated that increased muscle carnitine content, combined with a prescribed diet and low intensity exercise training, increased whole-body fat oxidation and decreased whole-body CHO oxidation at rest in overweight healthy, female volunteers, which although it did not accelerate weight or fat mass loss compared with control, warrants further investigations. Detailed human volunteer

studies invariably involve low numbers of volunteers because of the nature of the measurements being made and commitment required from volunteers. It is likely therefore that Chapter 5, which included 5 volunteers in each study, was underpowered. Future studies could aim to recruit more volunteers into the study to increase the power of the study or increase the duration of the study to notice the impact of carnitine on weight loss and body fat loss.

The impact of muscle contraction/exercise on muscle carnitine uptake can also be the subject of future human studies. As discussed earlier, it has been proposed that muscle contraction increases carnitine uptake *in vivo* in animal models via OCTN2 translocation to cell membrane (Furuichi et al., 2012); however we are not aware of any studies investigating the effect of skeletal muscle exercise on carnitine accumulation in human volunteers.

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APPENDIX 1

Volunteers' screening and consent forms

Example of a volunteer's consent form:

University of Nottingham, School of Biomedical Sciences, The Medical School



Title of Project:

Name of Investigators: Dr Reza Ghasemi
Dr Francis Stephens
Professor Paul Greenhaff

Healthy Volunteer's Consent Form

Please read this form and sign it once the above named or their designated representative, has explained fully the aims and procedures of the study to you

- I voluntarily agree to take part in this study.
- I confirm that I have been given a full explanation by the above named and that I have read and understand the information sheet given to me which is attached.
- I have been given the opportunity to ask questions and discuss the study with one of the above investigators or their deputies on all aspects of the study and have understood the advice and information given as a result.
- I agree to the above investigators contacting my general practitioner [and teaching or university authority if appropriate] to make known my participation in the study where relevant.
- I agree to comply with the reasonable instructions of the supervising investigator and will notify him immediately of any unexpected unusual symptoms or deterioration of health.
- I authorise the investigators to disclose the results of my participation in the study but not my name.
- I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published.
- I authorise the investigators to disclose to me any abnormal test results.
- I understand that I can ask for further instructions or explanations at any time.
- I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing.

- I confirm that I have disclosed relevant medical information before the study.
- I shall receive an inconvenience allowance of £... If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation, but if I withdraw for any other reason, the payment to be made, if any, shall be at the discretion of the supervising investigator. (delete if not applicable)
- I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance**;** having an invasive procedure (example: venepuncture >50ml, endoscopy) or exposure to ionising radiation.
- I confirm that I have not been exposed to more than 5 mSv of ionising radiation in the last 12 months. (delete this if not applicable)

Name: ……

Address: ……..

Telephone number: ……..

Signature: ……. **Date:** ……..

I confirm that I have fully explained the purpose of the study and what is involved to:

……..

I have given the above named a copy of this form together with the information sheet.

Investigators Signature: …….. **Date**: ……

Investigators Name:……

Study Volunteer Number: ……..

Example of a volunteer's screening form:

Screening visit

Title of study:

Date:

Name:

DOB:

Age:

Address:

Mobile number:

Job:

Diet:

Physical activity:

PMH:

History of smoking:

History of alcohol drink:

History of taking nutritional supplement:

Drug history:

History of allergy:

Heart rate:

BP:

Height:

Weight:

BMI:

Bloods: ☐

ECG: ☐

Consent form: ☐

Sign and date by volunteer:

Sign and date by doctor:

APPENDIX 2

DEXA Scan information leaflet

DEXA Scan information leaflet

What is a DEXA scan and what does it measure?
DEXA stands for 'Dual Energy X-ray Absorptiometry' and it is a test used in hospitals to measure the density of bone. Density means how much of something there is in a certain amount of space and this test is usually used to help diagnose those who have brittle bones (osteoporosis). In Physiology and Nutrition research, we use the DEXA machine to measure the amount of fat and lean tissue you have in your body, as well as the density of your bones.



How does a DEXA scan work?
A DEXA scan sends X-rays of two different strengths through the body. Bone blocks a certain amount of the X-rays, whereas muscle and fat block the X-rays to a lesser extent. The denser the tissue is, the fewer X-rays get through to the detector.

What does the scan involve?
This is a simple and painless procedure, and requires very little preparation.

However metal and other objects, such as zips, buckles, bra clips / under wires and body piercing, can affect the result of the DEXA Scan. It is advisable to wear loose clothing which has no zips, metal buttons etc. If you attend wearing clothing that is not suitable, you will be asked to change into surgical tops and/or trousers prior to the scan. Lockers are provided. If you wear any kind of metal jewellery, you will need to remove it before the test. If you are unable to remove any items, please inform the DEXA operator.

On the day of the study you will be asked to have nothing to eat or drink, other than water from midnight the night before

You will also;

- Have your height and weight measured
- Complete a safety questionnaire; this questionnaire will ask you about any procedures or treatments that you have had in the past year which involved X-rays and any metal that you might have in your body.
- Go to the toilet to empty your bladder before the scan

The scan itself involves you lying on your back on a table whilst an X-ray arm passes over you. The X-ray arm does not touch you and you will not be in a “tunnel” (see picture).

You will need to remain completely still and not talk while the scan is being carried out.

If you have any hip, back or neck injury, please tell the DEXA operator before the scan.

The actual scanning time is about 10 minutes but you should allow 30 minutes for the entire procedure.



What are the risks?
The DEXA scan involves you receiving a low dose X-ray, which is a form of radiation.

We're constantly being exposed to natural radiation from the environment around us - from the earth, through rays from outer space, even from the food we eat. The radiation dose that you receive is less than 1µSv per scan. This is equivalent to the amount of radiation that you would be exposed to if you spent 15min on a transatlantic flight or the amount of radiation contained in 10 brazil nuts.

Although the dose of radiation you would be exposed to during the DEXA scan is minimal, there is a risk that even small doses of radiation can cause cancer.

The Health Protection Agency estimate that the risk of developing cancer following a dose **10 times** that of a DEXA scan is approximately 1 in 20,000. This is the same as the chance of being killed in a road traffic accident in the next year.

Is there anyone who should not have this test?
It is not advisable for you to have this scan if you are pregnant. Pregnancy test kits (requiring a urine sample) are available in the toilets. You will be asked to empty your bladder before having the DEXA scan. Please feel free to use the pregnancy test at this time if you think there might be a chance that you are pregnant. You do not need to tell anyone that you are doing so, but you can talk to the DEXA operator in confidence if you wish to.

If you have had another X-ray, or if you have had a nuclear scan (such as a bone or thyroid scan) in the last 7 days, you should not have this test

If for any reason you do not wish to proceed with the scan, please let the DEXA operator know, but you do not need to disclose the reason why.

What are the benefits?

A DEXA scan is the most accurate way of measuring your total body composition and it is a quick and simple measurement to make.

Osteoporosis, is a serious medical problem which can affect both men and women as they get older. However, we often don't know that our bones are getting weaker until we break a bone. Although the researchers are interested in your body composition, the scan will estimate the bone density of your skeleton. If the results show that the density of your bones is too low, then you will be informed and the study doctor will discuss the results with you. Therefore, having the DEXA scan could identify a problem with your bones and allow you to receive treatment at an earlier stage.

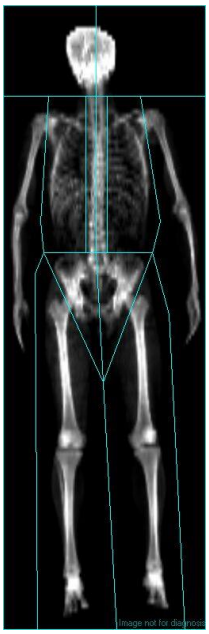
For more information please contact;

Liz Simpson: tel: 0115 8230128
liz.simpson@nottingham.ac.uk

The University of Nottingham

David Greenfield Human Physiology Laboratories

Having a DEXA Scan



APPENDIX 3

Procedure for Insulin assay (Coat-a-Count)

Principle: Insulin is a polypeptide hormone secreted from the beta cells of the pancreas that facilitates the storage and production of glucose. Presence of insulin in the blood plasma is a direct response to the circulating blood glucose which will decline in parallel with decreasing blood glucose concentration. The plasma concentration of insulin is determined using an immunoassay where ^{125}I-labelled insulin competes with insulin within a sample for sites on an insulin-specific antibody. The antibody is bound to the walls of a polypropylene tube therefore decanting the tube terminates the competition and isolates the antibody-bound fraction of the radio-labelled insulin. The tube is counted in a gamma counter to create a number that can be converted via a calibration curve to an insulin concentration.

Assay procedure: The assay takes place over two days

<u>Preparing the Calibrators (standards)</u>

- Remove 1 set of calibrators A to G from the fridge and uncap the vials
- Label one set of eppendorfs A to G (6 per letter), include one labelled AB*
- Add 6 ml of deionised water to vial A and 3 ml to vials B to G then recap and mix the vials
- Leave the vials to stand for 30 minutes before storing in a bag and freezing at -20°C.

These standards can be used for up to 30 days

*If some of the samples are from fasting subjects, insulin will not be detected unless the lowest standard is approximately 3 mIU/L. An additional point on the standard curve can be created by mixing 1 ml each of vials A and B to produce AB.

Day 1

- Take 1 set of calibrators, a high and low quality control and the samples out of the fridge, put in a rack and leave to thaw

- Make a note of the samples in the order in which they will be analysed
- Remove the green antibody-coated tubes out of the fridge (1 per sample plus 20-22 additional for the calibrators and QC's). Label the tubes as follows;

• 2 plain tubes TC (total control)

• 2 plain tubes NSB (non-specific binding)

• 2 green tubes each for each calibrator (A to G)

• 1 green tube for low and high QC (beginning of assay)

• 1 green tube for each sample

• 1 green tube for low and high QC (end of assay)

- Prepare the radioactive label. Take the vial of 125-I Insulin out of the fridge (1 vial is sufficient for 108 assay tubes). Measure out 100 ml of deionised water, add to the gel concentrate and swirl gently
- Transfer the radioactive label to a beaker for pipetting
- When the samples have thawed, mix each one by vortexing. Mix the calibrators and QC's by gentle inversion
- Using a 200 µl pipette, pipette the following;

• Nothing in the TC tubes

• 200 µl of calibrator A into the NSB tubes and each A tube (A measures zero binding- total binding of labelled insulin)

• 200 µl of each calibrator AB to G, QC's and samples into their corresponding tubes

- Using an automatic pipette, add 1 ml of the radioactive label from the beaker to each tube
- Cap the TC tubes and mix all the other tubes using a vortex

- Cover the entire rack with cling film and leave at room temperature for 18-24 hours

Day 2

- Take out the TC tubes and put them in a spare rack
- Lay out blue bench roll at several sheets thickness in a radioactive area and keep the tap running
- Decant the rack over the sink then leave it to stand upside down for 2 minutes
- Blot against the blue paper and then tap the rack firmly upside down repeatedly until no more moisture is visible on the paper. Turn the rack back up and blot the tubes
- Put the TC's back into the rack and count the tubes on a gamma counter using programme 24. Check the standards are correct and edit if not

The gamma counter automatically plots the standard curve from the calibrators and calculates the insulin concentrations from the samples. If the samples are below the lowest standards the concentration can be calculated using a spreadsheet by assuming linearity continues at lower concentrations.

APPENDIX 4

Biochemical buffers and reagents

Reagents	Conc. mmol/l	Volume / 100 µl	x 30 (µl)	x 60 (µl)	x 90 (µl)
Phosphate buffer pH 6.5	1000	25	750	1500	2250
Acetyl CoA	0.3	25	750	1500	2250
[^{14}C]Acetyl CoA	0.004	40	1200	2400	3600
NEM	0.04	10	300	600	900

CAT is ordered as 5 mg/ml and diluted 1 part enzyme with 9 parts H_20.

MUSCLE G-6-P, ATP, AND PCr

Reagents	Conc. mmol/l	Volume / 225 µl	x20 (µl)	x40 (µl)	Stock mmol/l	Mass / Volume
Triethanolamine pH 7.5-7.6	100	25	500	1000	1000	18.6 g· $100ml^{-1}$
$Mg(Ac)_2 \cdot 4\ H_20$	10				100	2.2 g· $100ml^{-1}$
$EDTA \cdot Na_2 \cdot 2\ H_20$	1				10	0.4 g· $100ml^{-1}$
DTT	1	5	100	200	50	7.8 $mg{\cdot}ml^{-1}$
$NADP \cdot Na_2 \cdot 4\ H_20$	1	10	200	400	25	20.9 $mg{\cdot}ml^{-1}$
$ADP \cdot Na \cdot 2\ H_20$	0.04	1	20	40	10	5.1 $mg{\cdot}ml^{-1}$
Glucose	5	10	200	400	125	22.5 $mg{\cdot}ml^{-1}$
H_2O		175	4000	6200		
G6PDH			15	30		

G6PDH is ordered as 1.1 mg/ml and diluted 1 part enzyme with 2 parts H_20.

HK is ordered as 2 mg/ml and diluted 1 part enzyme with 1 part H_20.

CPK is ordered as a lyophilised enzyme and 15 mg/ml is dissolved in 0.5% $NaHCO_3$ + 0.05% BSA. Dilute 2 parts enzyme with 1 part H_2O.

MUSCLE CREATINE

Reagents	Conc. mmol/l	Volume / 300 µl	x20 (µl)	x40 (µl)	Stock mmol/l	Mass / Volume
Glycine	100	100	2000	4000	320	2.4 g· 100 ml^{-1}
$Mg(Ac)_2 \cdot 4\ H_20$ pH 9.0-9.1	5				16	0.4 g· 100 ml^{-1}
KCl	30	5	100	200	2000	15.0 g· 100 ml^{-1}
$ATP \cdot Na_2 \cdot 3.5\ H_20$	1.5	20	400	800	25	15.4 $mg{\cdot}ml^{-1}$
PEP – tricyclohexyl – ammonium salt	1.0	15	300	600	25	11.6 $mg{\cdot}ml^{-1}$
$NADH \cdot Na_2 \cdot 2\ H_20$	0.15	5	100	200	12	9.0 $mg{\cdot}ml^{-1}$
LDH		0.5	10	20		5.0 $mg{\cdot}ml^{-1}$
PK		0.5	10	20		10.0 $mg{\cdot}ml^{-1}$
H_2O		155	3100	6200		

CPK is ordered as a lyophilised enzyme and 15 mg/ml is dissolved in 0.5% $NaHCO_3$ + 0.05% BSA.

MUSCLE GLYCOGEN

Reagents	**Conc. mmol/l**	**Volume / 250 µl**	**x20 (µl)**	**x40 (µl)**	**Stock mmol/l**	**Mass / Volume**
Triethanolamine Adjust to pH 8.2	100	80	1600	3200	375	7.0 g· 100 ml^{-1}
KOH	40				150	0.8 g· 100 ml^{-1}
$Mg(Ac)_2 \cdot 4\ H_20$	30				112.5	2.4 g· 100 ml^{-1}
$EDTA \cdot Na_2 \cdot 2\ H_20$	1				3.75	0.14 g· 100 ml^{-1}
DTT	1	5	100	200	60	9.36 $mg{\cdot}ml^{-1}$
$NAD \cdot Na_2 \cdot 4\ H_20$	1	10	200	400	30	19.92 $mg{\cdot}ml^{-1}$
$ATP \cdot Na_2 \cdot 3.5\ H_20$	0.75	5	100	200	45	27.72 $mg{\cdot}ml^{-1}$
H_2O		150	3000	6000		
G6PDH		2	40	80		

G6PDH is ordered as 1.1 mg/ml and diluted 1 part enzyme with 2 parts H_20.

HK is ordered as 2 mg/ml and diluted 1 part enzyme with 1 part H_20.

MUSCLE LACTATE

Reagents	Conc (mmol/l)	Volume (ml)	Mass / volume
2-Amino-2-methyl-1-propanol pH 10.0	1000	10	8.9 g·100 ml^{-1}
Hydrazine	1000	5	
NAD^+	100	0.05	70 $mg \cdot ml^{-1}$

Lactate dehydrogenase (LDH; bovine heart). Dilute to 550 U/ml with 20 mM Tris containing 0.02% albumin.

PDC ASSAY

Extraction buffer

Chemical	**Conc (mM)**	**g·100 ml^{-1}**
Sucrose	200	6.844
KCl	50	0.3732
$MgCl_2 \cdot 6H_2O$	5	0.102
EGTA	5	0.1904
Tris	50	0.606
NaF (dissolve before adding to rest of solution)	50	0.21
DCA	5	0.06445
Triton X-100	0.1%	100 µl
Set to pH 7.8 with 1 M HCl		

Incubation buffer

Chemical	**Conc (mM)**	**g·100 ml^{-1}**
Tris·HCl	100	1.75
Na_2EDTA	0.5	0.0268
$MgCl_2 \cdot 6H_2O$	1.0	0.0293
Set to pH 7.8 with 1 M HCl		

Mix 9 parts incubation buffer with 1 part 1mM NAD^+, 1 part 1 mM TPP, and 1 part 0.5 mM CoASH.