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Development of a liquid chromatography-mass spectrometry method to investigate branched chain amino acid and acylcarnitine metabolism in type 2 diabetes

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Abstract

The global prevalence of obesity and type 2 diabetes (T2D) continues to rise at an alarming rate. Despite the well-established association between obesity and an increased risk of developing T2D, the mechanisms underlying the pathogenesis of T2D with obesity remain unclear. Skeletal muscle is a major site for the disposal of ingested carbohydrate in healthy individuals. It is generally accepted that chronic overnutrition leads to accumulation of fat and fatty acid metabolites within the skeletal muscle which are believed to play a pivotal role in the progression of insulin resistance to carbohydrate metabolism which is one of the pathological hallmarks of T2D. Whilst several metabolites have been implicated, there appears to be no consensus over which metabolite is mediating skeletal muscle insulin resistance.

In recent years, circulating concentrations of acylcarnitines, which are intermediates in glucose, fatty acid and branched chain amino acids (BCAAs) metabolism have been identified as potential novel biomarkers of insulin resistance and T2D. Furthermore, fatty acid derived acylcarnitines have since been shown to impair insulin signalling *in vitro.* Interestingly, BCAAs and their associated short-chain acylcarnitines appear to be more closely associated with insulin resistance than any marker of fatty acid metabolism, giving rise to the hypothesis that BCAAs and their catabolites may also play a causative role in the development of skeletal muscle insulin resistance. However, the simultaneous and quantitative assessment of acylcarnitines, BCAAs and related metabolites in human skeletal muscle is lacking or limited to one

metabolite group. Therefore, the main aim of this this thesis was to develop a quantitative analytical method for the assessment of BCAAs and acylcarnitines in human muscle to extend upon much of published literature which has been limited to investigations in fasting plasma samples and to explore their role in the development of insulin resistance and T2D.

In Chapter 3 a novel liquid chromatography coupled to high resolution mass spectrometry method was developed and optimised to enable the quantitative assessment of a full range of BCAA and fatty acid derived acylcarnitines, BCAAs and related catabolites in both human plasma and muscle samples. The commonly cited challenges of metabolite quantification from biological tissues were systematically addressed using stable isotope internal standards allowing metabolite concentrations to be determined with a high degree of confidence.

In Chapter 4, the method was validated by quantitatively assessing BCAAs and acylcarnitine concentrations in fasting plasma and skeletal muscle samples of patients with T2D and an age matched obese control subjects. The results revealed striking elevations of BCAAs and BCAA derived acylcarnitines in both plasma and muscle of patients with T2D compared to control subjects. Furthermore, these metabolites were significantly correlated with fasting blood glucose. Surprisingly, no significant differences in fatty acid derived acylcarnitines were observed between groups in either plasma or muscle. These data show that plasma profiles may not always reflect muscle profiles as suggested by previous reports.

In Chapter 5, skeletal muscle BCAA and acylcarnitine metabolism was investigated in the fasted and insulin stimulated state. In order to determine if the elevations observed in fasting state in the previous persist in the face of insulin thereby allowing some indication of whether they could be causative of insulin resistance. A group of young and old lean, and old overweight/obese individuals was investigated in a cross sectional design. Ageing was associated with a (30%) decline in muscle BCAA content and decreased insulin sensitivity. And increased adiposity was associated with a (20%) increase in BCAA content. In response to insulin infusion, there was an attenuated decline in muscle BCAA and BCAA catabolite content in the old lean group only. In addition, fatty acid derived acylcarnitines were suppressed in all groups despite differing glucose disposal during insulin infusion. These findings appear to dissociate muscle BCAA content, ageing and insulin resistance and suggest fatty acid derived acylcarnitnes may not be associated with insulin resistance.

In Chapter 6, the potential interactions of BCAA and fatty acid metabolism were explored in a group of middle aged and older aged patients with T2D. Middle aged T2D patients had elevated meal derived fatty acid oxidation and endogenous fatty acid delivery to muscle during an oral glucose tolerance test (OGTT) when compared to age and BMI matched control subjects. This was associated with elevated fasting muscle BCAA content and an attenuated suppression of plasma BCAA and BCAA catabolites during the OGTT. Remarkably, these metabolic perturbations were absent in the older aged T2D patients, despite a similar duration of diabetes and insulinaemic responses to OGTT.

Collectively, the work in this thesis provides quantitative assessment of muscle BCAA catabolism and fatty acid metabolism in humans. T2D and obesity-induced insulin resistance are characterised by elevated BCAAs and BCAA derived acylcarnitines but ageing *per se* exerts the opposite effects. In addition, the results of this thesis suggest that fatty acid derived acylcarnitines may not be associated with muscle insulin resistance *in vivo*. The potential role of BCAA derived acylcarnitines in insulin resistance and their role as biomarkers of progression to T2D requires further investigation.

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Publications

Journal articles

Robert Jones, **Pardeep Pabla**, Joanne Mallinson, Aline Nixon, Tariq Taylor, Andrew Bennett, and Kostas Tsintzas. 2020. “Two Weeks of Early Time-Restricted Feeding (ETRF) Improves Skeletal Muscle Insulin and Anabolic Sensitivity in Healthy Men.” The American Journal of Clinical Nutrition 112 (4): 1015–28.

Kostas Tsintzas, Robert Jones, **Pardeep Pabla**, Joanne Mallinson, David A. Barrett, Dong Hyun Kim, Scott Cooper, et al. 2020. “Effect of Acute and Short-Term Dietary Fat Ingestion on Postprandial Skeletal Muscle Protein Synthesis Rates in Middle-Aged, Overweight, and Obese Men.” American Journal of Physiology - Endocrinology and Metabolism 318 (3): E417–29.

Abstracts

Pardeep Pabla, Dong-Hyun Kim, David Barrett, Francis Stephens, and Kostas Tsintzas. Targeted metabolomics in human skeletal muscle and plasma reveals distinct differences in key biomarkers of Type 2 diabetes. Presented at Physiology 2019. Aberdeen, UK.

Pardeep Pabla, Dong-Hyun Kim, David Barrett, and Kostas Tsintzas. A novel targeted LC-MS/MS method for absolute quantification of acylcarnitines in human skeletal muscle: Implications for insulin resistance. Presented at MetaboMeeting 2018, University of Nottingham, UK.

Declarations

All of the development and validation of the analytical method in chapter 3 was performed by myself. The method was applied to biological samples obtained from various human *in vivo* studies conducted within our research group. All the sample processing, metabolite extraction, metabolite data acquisition and data processing was performed by myself. I assisted with sample collection in all the studies, with the exception of samples used in **chapter 5**. Where others contributed to the data presented in this thesis is declared below.

In **chapter 3**, Dr **Robert Jones** and I recruited participants and conducted all *in vivo* testing, **Aline Nixon** performed cannulations and Drs **Tariq Taylor** and **Natalie Shur** performed skeletal muscle biopsy procedures to obtain samples used for confirmation of method suitability of the analytical method.

In **chapter 4** some of participants in the control group were recruited by Professor **Kostas Tsintzas** and I assisted with sample collection and analysis.

In **chapter 5** all participant recruitment, assessment of body composition, blood and muscle biopsy collection, and the hyperinsulinaemic- euglycaemic clamp was performed by Dr **Carolyn Chee**.

In **chapters 4 and 6**, all patients with Type 2 diabetes were recruited by Dr **Christopher Gaffney** who also performed assessments of body composition, dietary intake. Professor **Francis Stephens** analysed meal derived fatty acid oxidation data.

Analysis of serum insulin in **chapters 4 and 6** and plasma free fatty acids in **chapter 6** was performed by **Sally Cordon**. Skeletal muscle biopsy and

cannulation procedures in those chapters were performed by Drs **Tariq Taylor** and **Ciaran Doherty**

All studies and *in vivo* procedures were performed in the David Greenfield Human Physiology Unit, Queen's Medical Centre, University of Nottingham.

I declare that the thesis is the result of my own work which has been undertaken during my period of registration for this degree at The University of Nottingham.

Signed:

Date:

Acknowledgements

I would like to express my sincerest gratitude to my primary supervisor, Professor Kostas Tsintzas for his continual support, guidance and friendship from the beginning of my MSc and throughout my PhD. Through his wealth of knowledge on all matters scientific (and otherwise, especially BMWs), meticulous nature and attention to detail, he has taught me so many important life lessons. I hope we can continue to work together for many years to come. Secondly, to Dr Dong-Hyun Kim who introduced me to world of metabolomics. His patient supervision and encouragement were vital in helping me find my feet in a new area of science and enabled me to grow as a researcher. Thirdly, I would like to thank Professor David Barrett for his invaluable advice and input even after his retirement. I feel privileged to have profited so generously from his vast analytical experience which gave me the confidence to overcome the many challenges of quantitative metabolomics. Finally, I owe a huge thank you to Professor Francis Stephens who was always there to point me in the right direction. His mentorship and scientific rigour inspire to me to pursue a career in research.

I am indebted to Drs Catharine Ortori and Salah Abdelrazig for introducing me to world of liquid chromatography coupled to mass spectrometry and their technical support throughout my PhD. Cath, for her thorough training and seemingly never-ending analytical knowledge and patience. Salah, for his countless and creative analogies that made some of the most complex analytical aspects understandable to a relative novice like me. I will remember those stressful days (and nights) spent panicking by Q Exactive for a long time.

I am incredibly lucky to have made so many new friends along the way with whom I was able to share the journey with. To everyone in the offices of D83, C15 and the extended members of the CAB and metabolic and molecular physiology research groups over the years, thank you for your help and warm welcome, it made the process so much more enjoyable. In particular, Robbie, Simon and Andy for all the discussions, laughs, lab days and beers. Tariq, thanks for being a big brother and for constantly challenging me but most importantly for always being there, especially when times got really hard.

I also greatly appreciate the assistance of the staff of the David Greenfield Human Physiology Unit for their support in completing the studies that contributed to this thesis. I also acknowledge Dr Scott Cooper for this patience and training in several laboratory techniques.

Finally, a huge heartfelt thank you to my family, for their enduring love, support and sacrifice not just during my studies but always, and to my friends who accept and believe in me.

To those no longer with us, I hope I can make you proud. The work in this thesis is dedicated to you.

Table of Contents

List of Abbreviations

ACC	Acetyl coenzyme A carboxylase
Akt	Protein Kinase B
ANOVA	Analysis of Variance
BCAA	Branched chain amino acid
BCAT	Branched chain amino transferase
BCKA	Branched chain keto acid
BCKDH	Branched chain keto dehydrogenase
BCKDK	Branched chain keto dehydrogenase kinase
BMI	Body mass index
BSA	Bovine serum albumin
CACT	Carnitine acylcarnitine translocase
CD36	Cluster of differentiation 36
CoA	Coenzyme A
CoASH	Free coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CV	coefficient of variance
DAG	Diacylglycerols
DEXA	Dual Energy X-ray Absorptiometry
DM/ dm	Dry mass
DNA	Deoxyribonucleic acid
EI	Electron impact
EMCL	Extramyocellular lipid
ESI	Electrospray ionization
FABPpm	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FDA	Food and Drug Administration
FFA	Free-Fatty Acid
G6P	Glucose 6 phosphate
GC	Gas chromatography
GDR	Glucose disposal rate
GFR	Glomerular filtration rate
GIR	Glucose infusion rate
GLUT4	Glucose Transporter 4
GSK3	Glycogen synthase kinase 3
HFD	High fat diet
HGP	Hepatic glucose production
HILIC	Hydrophilic interaction chromatography
HK	Hexokinase
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance

HPLC	High-performance liquid chromatography
IMCL	Intramyocellular Lipid
IMTG	Intramuscular triglyceride
INSR	Insulin receptor
IRS-1	Insulin receptor substrate
KH_2PO_4	Potassium phosphate
LC	Liquid chromatography
LCFA	Long chain fatty acids
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass/charge ratio
MCD	Malonyl CoA decarboxylase
MCFA	Medium chain fatty acids
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger Ribonucleic Acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide Adenine Dinucleotide (Oxidised)
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NAFLD	Non alcoholic fatty liver disease
NFkB	Nuclear factor kappa β
OGTT	Oral Glucose Tolerance Test
PBS	Phosphate buffered saline
PDC	Pyruvate Dehydrogenase Complex
PDCa	Pyruvate Dehydrogenase Complex (active)
PDK	Pyruvate Dehydrogenase Kinase
PFK	Phosphofructokinase
PFP	Pentafluorophenyl
PI3K	Phosphatidylinositol (PI)-3 kinase
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PP2Cm	Protein Phosphatase 2
QC	Quality control
RP	Reversed phase
RQ	Respiratory Quotient
SD	Standard Deviation
SEM	Standard Error of the Mean
SPE	Solid phase extraction
SSD	System suitability standard
T2D	Type 2 diabetes
TAG	Triacylglycerol/Triglyceride

TCA	Tricarboxylic acid cycle
TST-T	Tris-Buffered Saline with 0.1% Tween 20
UHPLC	Ultra-high-performance liquid chromatography
v:v	Volume:Volume
VLDL	Very low-density lipoprotein
w:v	Weight:Volume
WHO	World Health Organisation

For a full list of abbreviations for all analytes quantified with the method developed in this thesis see **Table 3.2**

1 Chapter 1 General introduction

1.1 Obesity and Type 2 diabetes

1.1.1 Obesity

Obesity can be defined as an abnormal or excessive body fat accumulation in the adipose tissue and other organs, with multiple organ specific pathophysiological consequences (González-Muniesa et al. 2017). Implicit with this definition is the link between body fat and ill health. The World Health Organization (WHO) defines overweight as a body mass index (BMI) calculated as weight (kg) divided by height squared (m^2) equal to or greater than 25 kg/m^2 and obese as a BMI equal to or greater than 30 kg/m^2. Considered by some as a 21st century epidemic, primarily due to lifestyle trends and excessive energy intake, the prevalence of obesity is increasing at an alarming rate worldwide (Di Cesare et al. 2016). The projected global number of people who will be overweight or obese is estimated at 1.4 billion and 570 million, respectively, by 2030 (Kelly et al. 2008).

Although it is generally accepted that increased fat deposition is primarily driven by an imbalance between energy intake and energy expenditure, with some arguing a particular role of physical inactivity i.e., reduced energy expenditure (Prentice and Jebb 1995), it is likely that multiple environmental and genetic factors also contribute to an individual's propensity for weight gain (Ravussin 1995). Indeed, circumstances such as socioeconomic status, environment and personal behaviours, and genotype–phenotype interactions must be considered to understand the development of obesity, as all these factors affect food intake, nutrient turnover, thermogenesis, lipid utilization of fatty acids away from storage and towards oxidation, and differential fat

storage in regional adipose depots versus non-adipose tissues. Regardless of the cause, obesity is associated with increased risk of mortality (Manson et al. 1995) and the development of several cardiometabolic diseases, such as type 2 diabetes mellitus (discussed below), cardiovascular diseases, some types of cancer and other adverse pathological conditions (Williams et al. 2015). Thus, obesity is associated with a significant increase in morbidity and mortality and is a major public health problem.

Another difficulty encountered in studying obesity is the marked heterogeneity of individuals with obesity. Examples of obesity types at both ends of the continuum are subcutaneous obesity, in which excess subcutaneous fat is found around the hip and thigh areas (pear-like body shape or gynoid obesity, which is more common in women), and visceral obesity, in which fat (mainly mesenteric adipose tissue) is mainly concentrated in the abdominal region (apple-like body shape or android obesity). Visceral obesity is more common in men and tends to be more pernicious in health terms, particularly affecting cardiovascular disease risk factors (González-Muniesa et al. 2017). Furthermore, others have suggested that visceral adipose tissue may be an innocent bystander and simply reflects inappropriate fat storage within organs such as liver and muscle, also known as ectopic fat (Frayn et al.1997; Fabbrini et al. 2009). Therefore, both fat distribution as well as overall level of fat accumulation, is key in mediating the adverse health consequences of obesity.

1.1.2 Type 2 diabetes

Type 2 diabetes (T2D) mellitus is a disease characterized by dysregulation of carbohydrate, lipid and protein metabolism, and results from impaired insulin secretion by pancreatic islet β-cells, resistance to the metabolic effects of the

hormone insulin on peripheral tissues (termed insulin resistance) or a combination of both. As a result, a state of chronically elevated blood glucose concentrations (hyperglycaemia) ensues. Although only a subset of obese people develops T2D, obesity is a major risk factor for T2D, and rates of T2D prevalence have paralleled those of obesity (Kahn, Hull, and Utzschneider 2006). Skeletal muscle insulin resistance is considered to be an important initiating or primary defect that is evident year or even decades before β-cell failure and overt hyperglycaemia develops (DeFronzo and Tripathy 2009). However, the importance of muscle insulin resistance in the development of T2D is debated by others. For example in the proposed twin cycle hypothesis of the etiology of T2D (Taylor 2013), pre-existing muscle insulin resistance and raised circulating insulin levels, results in hepatic (liver) and pancreatic fat accumulation. Indeed, elevated circulating insulin leads to hepatic de novo lipogenesis (Schwarz et al. 2003), that is the conversion of non-fat precursors to fatty acids and subsequent accumulation of fat in the liver leading to hepatic insulin resistance, and this may be more important for the progression from insulin resistance to clinically overt T2D (this is discussed further below).

For some obese individuals, the pancreatic islet β-cells increase insulin release sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance. Clinically overt T2D manifests only when β-cells are unable to secrete sufficient amounts of insulin to offset the insulin resistance (Ferrannini and Mari 2014). Interestingly, β-cell dysfunction exists in individuals who are at high risk of developing the disease even when their glucose levels are still normal (Kahn, 2001). Several mechanisms have been speculated to explain β-cell decline, these include

ageing, β-cell exhaustion due to the increased secretory demand arising from insulin resistance, desensitization of the β-cell due to the elevations in glucose, lipid accumulation (also referred to as lipotoxicity) and a reduction in β-cell mass (Kahn, 2001).

1.1.2.1 Reversal of T2D

Although it was previously believed that T2D was inexorably progressive, recent work has clearly demonstrated reversibility of T2D (especially within the first 6 years of diagnosis). This was demonstrated in a study in which eleven patients with T2D with an average BMI ~ 33 kg/m^2 were subject to a very low calorie diet comprising of only 600 Kcal per day, for eight weeks (Lim et al. 2011). Remarkably, fasting plasma glucose, which is determine primarily by liver insulin sensitivity, declined after just one week. This was accompanied by a 30% reduction in liver fat which reached 70% by eight weeks. Furthermore, pancreatic fat was lowered by ~25% and normal insulin secretion was restored. Insulin sensitivity also increased, which was primarily attributed to an increased capability to supress hepatic glucose production and not increased peripheral glucose update (by muscle). These findings substantiated the twin cycle hypothesis suggested by Taylor and colleagues (Taylor 2013) (Figure 1.1) Nevertheless, alterations in muscle insulin sensitivity contribute to the initial development of the pre-existing insulin resistance that exacerbates the accumulation of fat in other organs. Therefore, the understanding of how skeletal muscle insulin resistance occurs is vital (and discussed later).

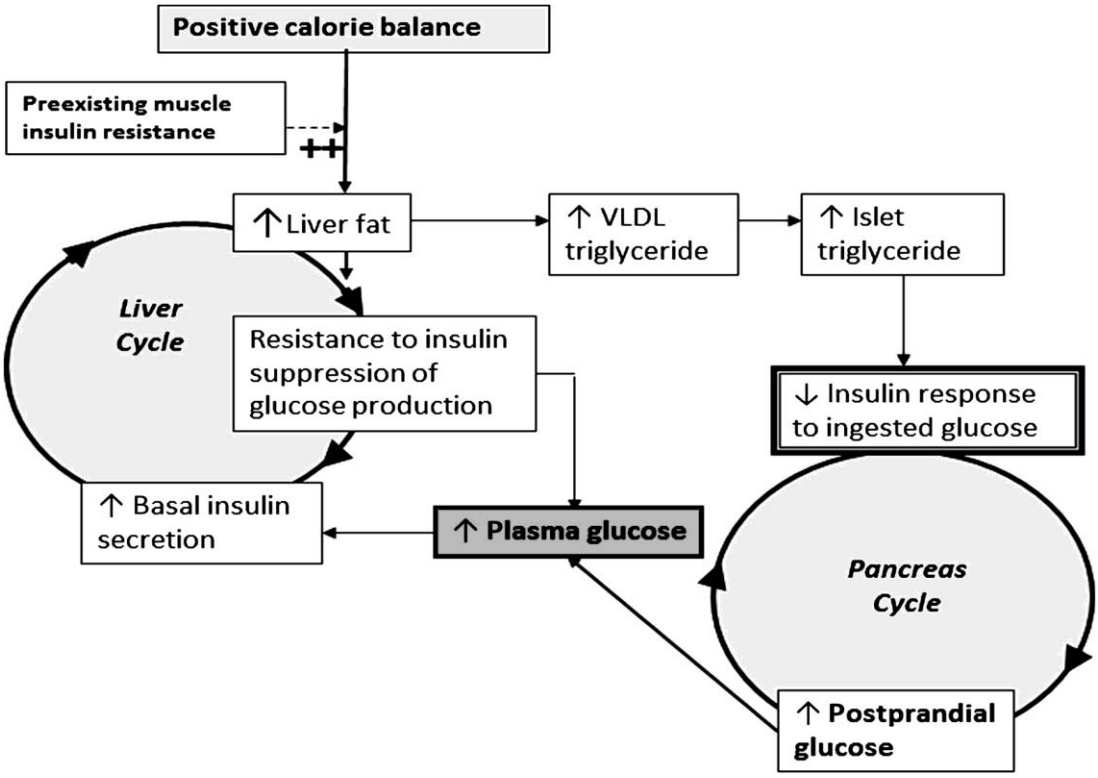


Figure 1.1 The twin cycle hypothesis proposed by R Taylor (2013). During long-term intake of more calories than are expended each day, any excess carbohydrate undergoes de novo lipogenesis, which particularly promotes fat accumulation in the liver. Because insulin stimulates de novo lipogenesis, individuals with a degree of insulin resistance (determined by family or lifestyle factors) will accumulate liver fat more readily than others because of higher plasma insulin levels. In turn, the increased liver fat will cause relative resistance to insulin suppression of hepatic glucose production Over many years, a modest increase in fasting plasma glucose level will stimulate increased basal insulin secretion rates to maintain euglycemia. The consequent hyperinsulinemia will further increase the conversion of excess calories to liver fat. A cycle of hyperinsulinemia and blunted suppression of hepatic glucose production becomes established. Fatty liver leads to increased export of VLDL triacylglycerol which will increase fat delivery to all tissues, including the islets. This process is further stimulated by elevated plasma glucose levels Excess fatty acid availability in the pancreatic islet would be expected to impair the acute insulin secretion in response to ingested food, and at a certain level of fatty acid exposure, postprandial hyperglycemia will supervene. The hyperglycemia will further increase insulin secretion rates, with consequent enhancement of hepatic lipogenesis, spinning the liver cycle faster and driving the pancreas cycle. Eventually, the fatty acid and glucose inhibitory effects on the islets reach a trigger level that leads to a relatively sudden onset of clinical diabetes

1.1.3 Insulin signalling in muscle

Insulin exerts all its known physiological effects by binding to the insulin receptor (INSR) on the plasma membrane of target cells. In muscle, the primary action of insulin is to augment glucose uptake through increased translocation of GLUT4 stored in intracellular vesicles to the plasma membrane (Shepherd and Kahn 1999). INSR is a heterotetrameric receptor tyrosine kinase formed from two extracellular α subunits, which bind insulin and (intracellular) two membrane-spanning β subunits, each of which contains a tyrosine kinase domain. Upon insulin binding at the α site, there is conformational change in the β subunit leading to phosphorylation of three tyrosine molecules on the insulin receptor. Once the insulin receptor has been phosphorylated, insulin receptor substrate (IRS)-1 moves to the cell membrane and becomes phosphorylated. Tyrosine phosphorylation of IRS-1 results in activation of the p85 regulatory subunit of phosphatidylinositol (PI)-3 kinase and activates the p110 catalytic subunit, leading to an increase in phosphatidylinositol-3,4,5 triphosphate. The latter acts as a secondary messenger by travelling along the cell membrane to activate PDK1, which is crucial in the activation of downstream protein kinase B (also called Akt) and phosphorylation of Akt substrate 160 (AS160), which facilitates the translocation of GLUT4 to the sarcolemma and subsequent entry of glucose into the cell. (Figure 1.2). It also activates glycolytic enzymes including hexokinase and phosphorylates glycogen synthase kinase 3 (GSK3) (Cross et al. 1995). The latter action prevents this enzyme from deactivating glycogen synthase and hence promotes increased glycogen synthesis. Akt also plays a

key role in the regulation of cell growth, via its stimulatory effects on the mammalian target of rapamycin (mTOR) pathway (Haar et al. 2007)

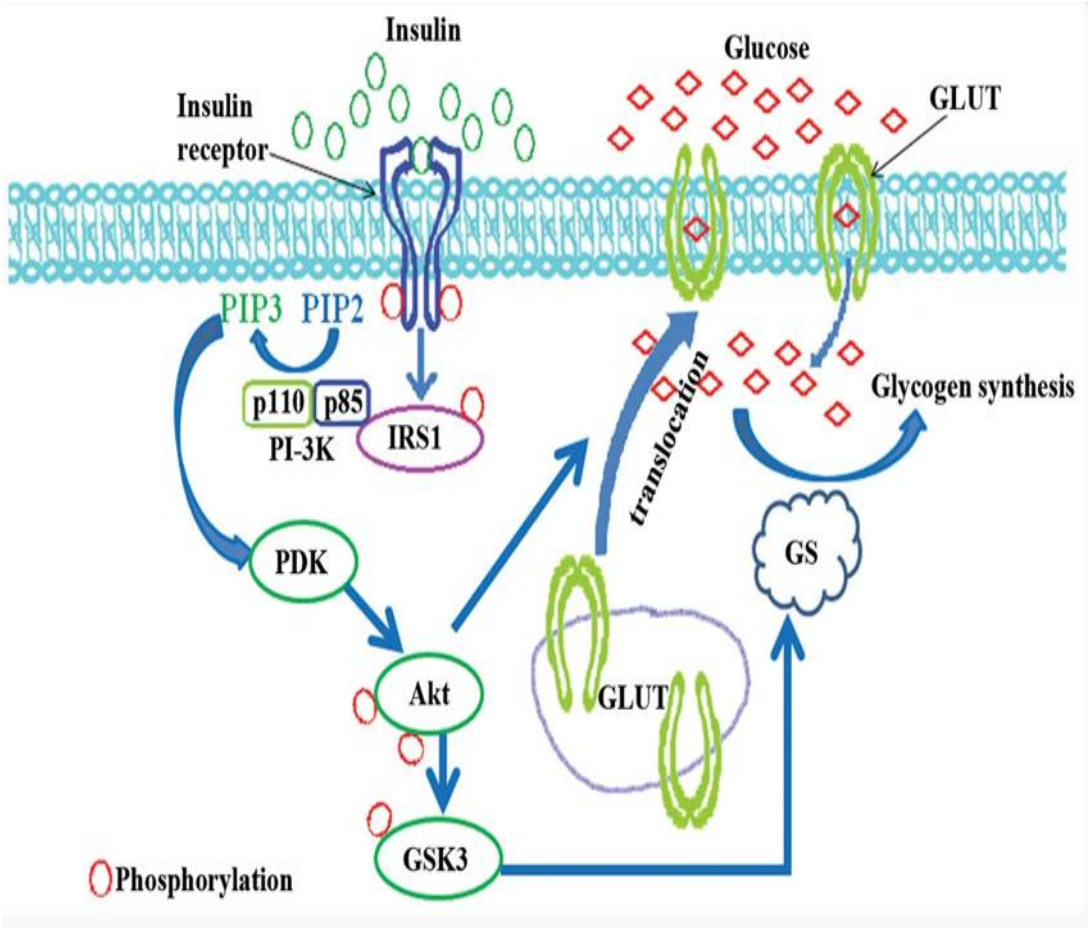


Figure 1.2 The insulin signalling pathway in skeletal muscle and its involvement in the regulation of glucose transport and glycogen synthesis. Taken from Yu and Chai (2015)

1.1.3.1 Contraction mediated glucose uptake in muscle

It is important to note that muscle contraction can also regulate glucose uptake into the muscle cell. The molecular signalling mechanisms that lead to GLUT4 translocation during muscle contraction are not well understood. It is generally believed that contractions stimulate GLUT4 translocation via a molecular

mechanism distinct from that of insulin (Richter and Hargreaves 2013). However, it appears these two pathways at least partially converge in their distal parts, and there are now a number of signalling molecules involved in GLUT4 translocation that are activated both by insulin and muscle contractions. This is supported by the observation that the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin blocks insulin-induced PI3K activation in perfused rat skeletal muscle, also inhibits contraction induced glucose uptake (Wojtaszewski et al. 1996). The various signalling mechanisms by which contraction regulates glucose uptake through GLUT4 are unclear at present but may be related to contract induced calcium release from the sarcoplasmic reticulum, regulation of cytoskeletal components such as the actin cytoskeleton, nitric oxide production, reactive oxygen species and other downstream targets (Richter and Hargreaves 2013). Evidence for the importance of any one component over the other as of yet is unknown. The significance of contraction induced glucose uptake will be important to consider later when physical inactivity induce insulin resistance is discussed. Another insulin independent mechanism that can regulate GLUT4 translocation (and independently of insulin) is through the AMP-activated protein kinase (AMPK). AMPK is an enzyme that is activated in situations where there are changes in the cellular energy status such as muscle contraction and hypoxia (Musi and Goodyear 2003). This pathway is activated by phosphorylation of AMPK by an upstream kinase. AMPK then acts on yet unidentified targets to initiate a singling cascade which results in GLUT4 translocation (Musi and Goodyear 2003).

1.1.4 Basis of ectopic fat accumulation

As stated, earlier distribution of fat in the body appears to be more important than overall fatness in the development of insulin resistance. Increased adiposity/obesity is a major risk factor of T2D. Indeed, 80-90 % of T2D patients are overweight or obese (Smyth and Heron 2006). However, many individuals with high levels of obesity maintain normal glucose tolerance and do not develop insulin resistance and T2D. One study measured 314 individuals and quantified total body, skeletal muscle and liver fat with magnetic spectroscopy and assessed insulin sensitivity with an oral glucose tolerance test. It was demonstrated that despite being matched for BMI and total body fat, the accumulation of fat in the muscle and liver differentiated insulin sensitive and insulin resistant obese individuals. In fact, the obese insulin sensitive group were more insulin sensitive than a group with lower BMI (25-29) and total body fat but more intramuscular fat (Stefan et al. 2009). Another compelling line of support for this comes from a study in which two groups of obese individuals matched for body composition, but different liver fat content (one obese group had < 4 % total fat in the liver and the other obese group had >15 %) were subject to overfeeding until both groups gained ~6 % total body mass. After weight gain, liver (hepatic), muscle and adipose tissue insulin sensitivity were assessed. Although both groups gained equal weight only the obese group with higher liver fat content had worsened insulin sensitivity and this was accompanied by the finding that adipose tissue genes involved in lipogenesis (fat storage) increased in the obese group with the lower, but not higher liver fat content (Fabbrini et al. 2015). The data clearly demonstrate body fat distribution is vital in the pathogenesis of insulin resistance and T2D.

One postulated reason for this could be explained by the adipose tissue expandability capacity (Figure 1.3), in which individuals have set capacity to expand and store excess energy and once the limit has been reached, fat spills over into the circulation and is inappropriately stored ectopically in tissues such as muscle, liver and pancreas (Gray and Vidal-Puig 2007; Brøns and Grunnet 2017a). Indeed, it has been shown in a study that measured adipose tissue blood flow and arterial-venous blood sampling across the tissue together with isotopically labelled fat ingestion, that obese individuals had substantially depressed adipose tissue storage of meal derived fat (McQuaid et al. 2011b). As a result, more of meal derived fat appeared in the very low-density lipoprotein (VLDL) fraction which suggests that the meal derived fat was rerouted to the liver and subsequently to other tissues.

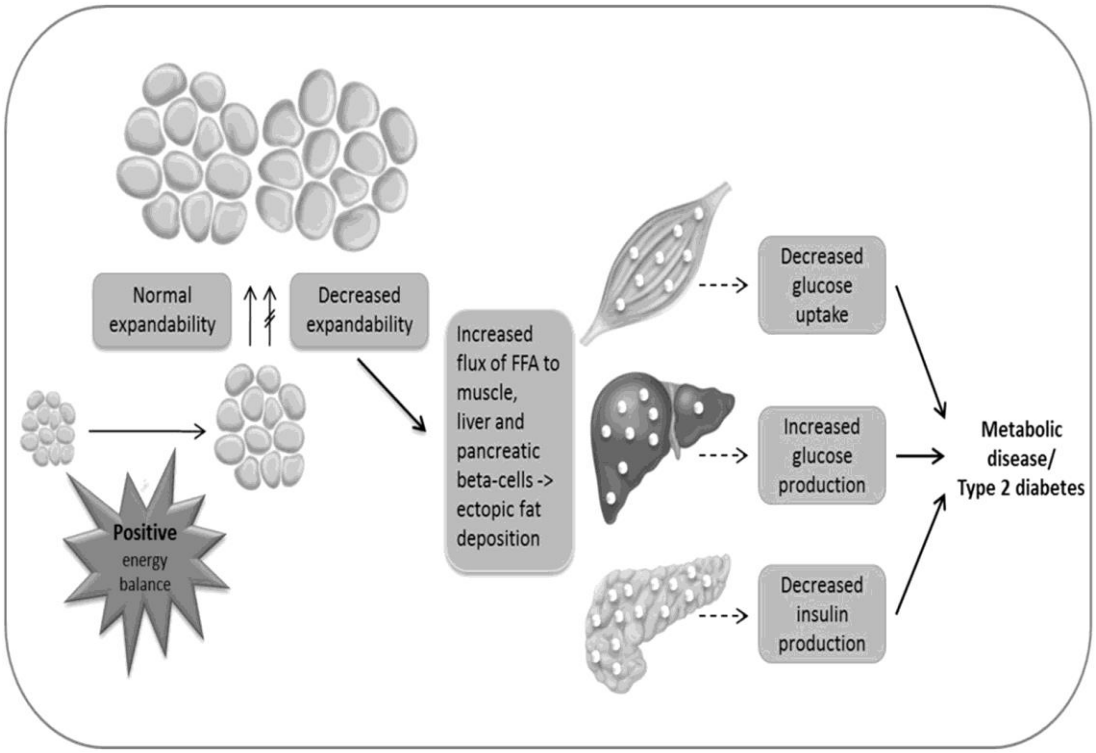


Figure 1.3 The proposed hypothesis for limited adipose tissue expandability. When the body is in a positive energy balance, the adipose tissue will expand to handle the excess energy. If the adipose tissue is not capable of expanding sufficiently, there will be a spillover of (free fatty acids) FFA to non-adipose tissue leading to harmful effects in liver, muscle and pancreas. Figure from Brøns and Grunnet (2017).

1.2 Skeletal muscle metabolism

1.2.1 Skeletal muscle fat metabolism

1.2.1.1 Fatty acid transport across the membrane

The provision of fatty acids to muscle originates from several sources, including chylomicrons, carrying exogenously supplied dietary lipids and VLDLs carrying endogenously supplied lipids from the liver, and as fatty acids bound to albumin directly from the adipose tissue. On the surface of the capillaries, these blood-borne TAGs are hydrolysed by lipoprotein lipase, thereby providing the fatty acids for utilisation by muscle. In contrast to the original belief that fatty acid entry into muscle tissues was determined solely by their delivery to and the diffusion across the plasma membrane of muscle, it has been demonstrated that fatty acid uptake occurs via a highly regulated, protein mediated mechanism involving one or more fatty acid transporters (Holloway et al. 2017). This system is acutely and chronically regulated by physiologic and metabolic stimuli and appears to be dysregulated in insulin-resistant muscle. With selected metabolic stimuli (i.e., muscle contraction and insulin), these fatty acid transporters can be induced to translocate within minutes from its intracellular depot to the plasma membrane, thereby increasing fatty acid influx into muscle cells. Three main types of tissue specific transport/binding proteins have been identified, the plasma membrane fatty acid binding protein ($FABP_{pm}$), cluster of differentiation 36 (CD36) transporting protein which are ubiquitously expressed and fatty acid transport protein (FATP) which has some tissue specificity (Bonen et al. 2007). Evidence has revealed that obesity (Fabbrini et al. 2009) and T2D (Bonen et al. 2004) is associated with increased expression of CD36. In T2D it would appear that

more of muscle CD36 pool may be relocated to the membrane rather than just increased expression (Bonen et al. 2004; C. Aguer et al. 2010).

After fatty acids enter the muscle cell cytosol, they are converted to a fatty acyl-CoA derivative by the action of ATP linked fatty acyl-CoA synthetase in preparation for β-oxidation, the major pathway by which fatty acids are oxidised. Alternatively, they can be stored in the intramuscular triglyceride (TAG) store. To take part in the β-oxidation pathway, cytosolic long-chain acyl-CoA must be transported across the otherwise impermeable inner mitochondrial membrane. Therefore, carrier mediated transport is required in the form of free carnitine.

1.2.1.2 Carnitine

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) is a naturally occurring quaternary amine compound, synthesised from the amino acids lysine and methionine. Being a zwitterionic molecule, carnitine contains a positively charged amine and a negatively charged carboxyl group, as well as a centrally positioned hydroxyl group. The hydroxyl group functions as a binding site for acyl residues and is fundamental to the two primary biochemical roles of carnitine in skeletal muscle which are the translocation of fatty acids across the mitochondrial membrane and the buffering of excess acetyl groups from high glycolytic flux (such as during exercise) (Stephens, Constantin-Teodosiu, and Greenhaff 2007). Around 95% of whole-body carnitine stores are sequestered within skeletal muscle (Brass 1995).

1.2.1.3 Translocation into mitochondria and β-oxidation with carnitine

Once activated to their respective CoA esters via acyl-CoA synthetase, long chain fatty acyl-CoAs must be converted to acylcarnitine esters before they are able to permeate the mitochondrial membranes and access the matrix enzymes of the β-oxidation pathway (Fritz and Yue 1963). Carnitine palmitoyltransferase 1 (CPT1), situated within the outer mitochondrial membrane (Murthy and Pande 1987)), catalyses the reversible esterification of carnitine with long-chain acyl-CoA to form long-chain acylcarnitine. CPT1 is considered to be the rate-limiting enzyme for long-chain fatty acid entry into the mitochondria and oxidation (McGarry and Brown, 1997). Cytosolic acylcarnitine is then transported into the mitochondrial matrix in a simultaneous 1:1 exchange with intramitochondrial free carnitine via the carnitine acylcarnitine translocase (CACT), which is situated within the mitochondrial inner membrane. Some evidence from isolated mitochondria from human skeletal muscle has suggested that the fatty acid translocase FAT/CD36, which is located in the outer mitochondrial membrane, translocates acylcarnitine from CPT1 to CACT (Bezaire et al. 2006). Once inside the mitochondrial matrix, acylcarnitine is transesterified back to free carnitine and long-chain acyl-CoA in a reaction catalysed by carnitine palmitoyltransferase 2 (CPT2), which is situated on the matrix side of the inner mitochondrial membrane (Woeltje et al. 1987)). The intramitochondrial long-chain acyl-CoA is then oxidized and cleaved by the β-oxidation pathway to form the ultimate product, acetyl-CoA, which enters tricarboxylic cycle to be oxidized (Figure 1.4). Evidence from inherited diseases affecting fatty acid oxidation, revealed that the process is bidirectional; acylcarnitines can be

formed in the mitochondrial matrix from acyl-CoA and carnitine and are exported into the cytosol and released into plasma. Almost all acyl-CoA can be found as its respective carnitine ester in the circulation with a pronounced increase in physiological and pathophysiological conditions (Rinaldo, Cowan, and Matern, 2008).

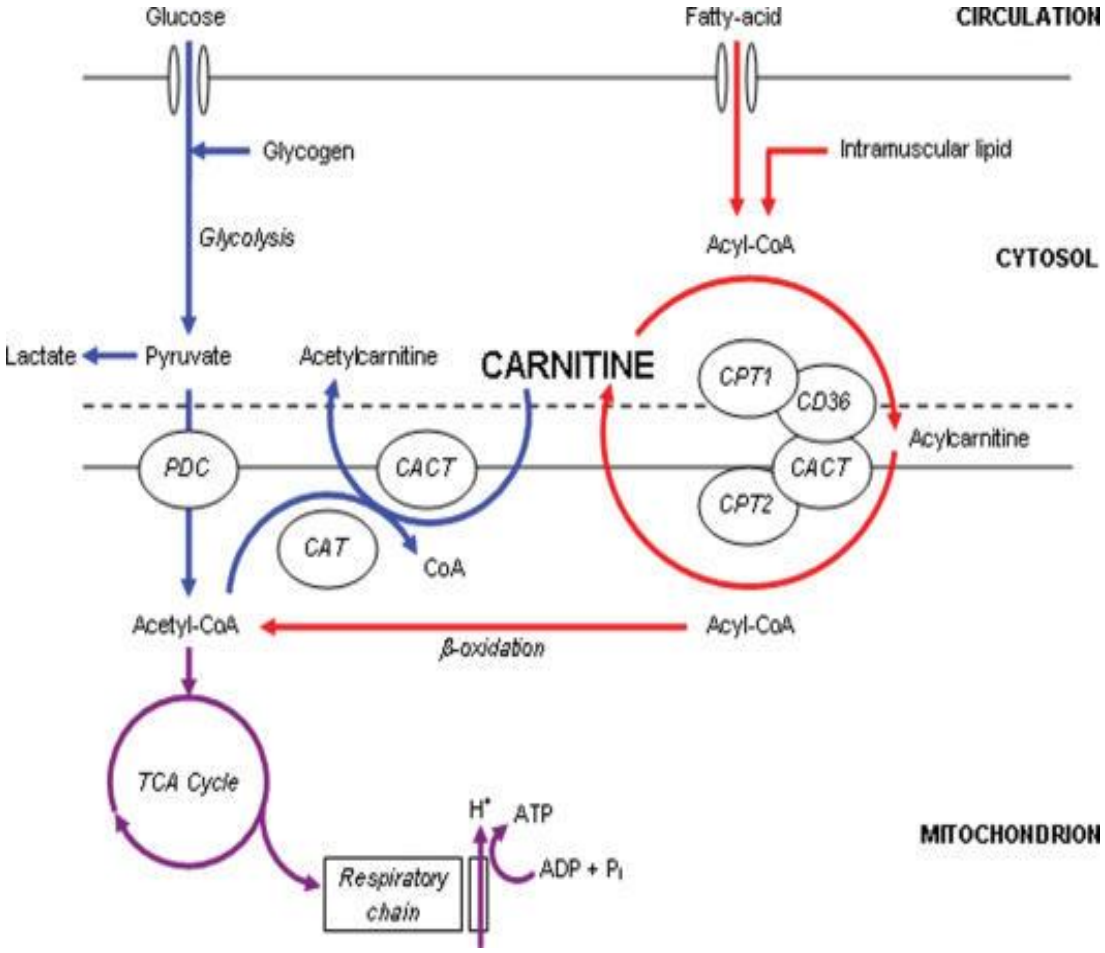


Figure 1.4 An overview of muscle fat (red) and glucose (blue) metabolism in skeletal muscle prior to the entry into the tricarboxylic (TCA) cycle (purple). PDC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; CAT, carnitine acetyltransferase; CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase; CD36, fatty acid translocase. Taken from (Stephens, Constantin-Teodosiu, and Greenhaff 2007)

1.2.2 Skeletal muscle glucose metabolism

As stated earlier, glucose transport into the cell is mediated via the GLUT4 transporter. Upon entry into the cell, glucose is rapidly phosphorylated to form glucose-6-phosphate (G-6-P), a reaction catalysed by hexokinase which is an irrreversible step. Phosphorylation effectively traps glucose in the cell and commits the G-6-P molecule to its intracellular fate of either storage in the form of glycogen or oxidation through glycolysis to form cytosolic pyruvate that can enter the TCA cycle. Under different experimental conditions the fate of G-6-P may change. Under physiological hyperinsulinaemia both oxidation and glycogen storage contribute to glucose disposal, but 'under conditions of sustained hyperinsulinaemia (such as those observed during a hyperinsulaemic clamp) glycogen storage predominates as the site of glucose disposal (Thiebaud et al. 1982).

1.2.2.1 The pyruvate dehydrogenase complex (PDC)

Depending on the redox state of the cell, pyruvate can either be reduced to lactate via the lactate dehydrogenase reaction or converted to acetyl-CoA by the PDC. The PDC is a mitochondrial membrane-bound multi-enzyme complex that catalyses the irreversible decarboxylation of pyruvate to acetyl-CoA and is considered one of the rate-controlling reactions in carbohydrate oxidation. The catalytic activity of the PDC is regulated by covalent transformation between its active and inactive forms (Linn et al., 1969), as well as by end-product inhibition. Transformation of the PDC to its active form (PDCa) is determined by the competing activities of the PDC phosphatase (activator) and kinase (deactivator), whilst *in-vitro* observations suggest end-product inhibition to be principally regulated by the ratios of acetyl-CoA to

CoASH (free co-enzyme A) and NADH to NAD (Pettit et al, 1975). Flux through the PDC reaction ultimately determines the fate of pyruvate and is thus considered a branch-point between mitochondrial and non-mitochondrial substrate utilisation. Therefore, an intracellular accumulation of products that result in the inhibition of PDCa could subsequently create a ‘back-log’ leading to a mismatch between cytosolic reactions and mitochondrial oxidation.

1.2.3 Interaction of fat and glucose in muscle

Some of earliest work investigating the relationship between fat and carbohydrate metabolism was performed by Randle and colleagues when they proposed the glucose-fatty acid cycle (Randle et al. 1963). In isolated heart and skeletal muscle preparations, Randle and colleagues demonstrated that the utilization of one nutrient inhibited the use of the other directly and without hormonal mediation (Figure 1.5). It appears that increased concentrations of intercellular accumulation of products from fatty acid oxidation inhibit key enzymes in the glucose metabolism pathway such phosphofructokinase (PFK), and the PDC, the rate-limiting enzyme in the conversion of pyruvate to acetyl-CoA. The extent of inhibition is graded and increases along the glycolytic pathway, being most severe at the level of PDC and less severe at the level of PFK. This sequence occurs because the initial event, triggered by fatty acid oxidation, is an increase in the mitochondrial ratios of acetyl-CoA/ CoA and NADH/ NAD, both of which inhibit PDC activity. It was proposed that these changes lead to an accumulation of cytosolic citrate, which in turn inhibits PFK, followed by an increase in glucose 6-phosphate, which eventually inhibits hexokinase. Consequently, there would be an accumulation of intracellular glucose and inhibition of its cellular uptake due to impaired

phosphorylation at the hexokinase step. Whilst this mechanism regulates muscle fuel usage actually, whether this mechanism operates in human skeletal muscle chronically (specifically in pathological states) remains uncertain (Samuel, Petersen, and Shulman 2010)

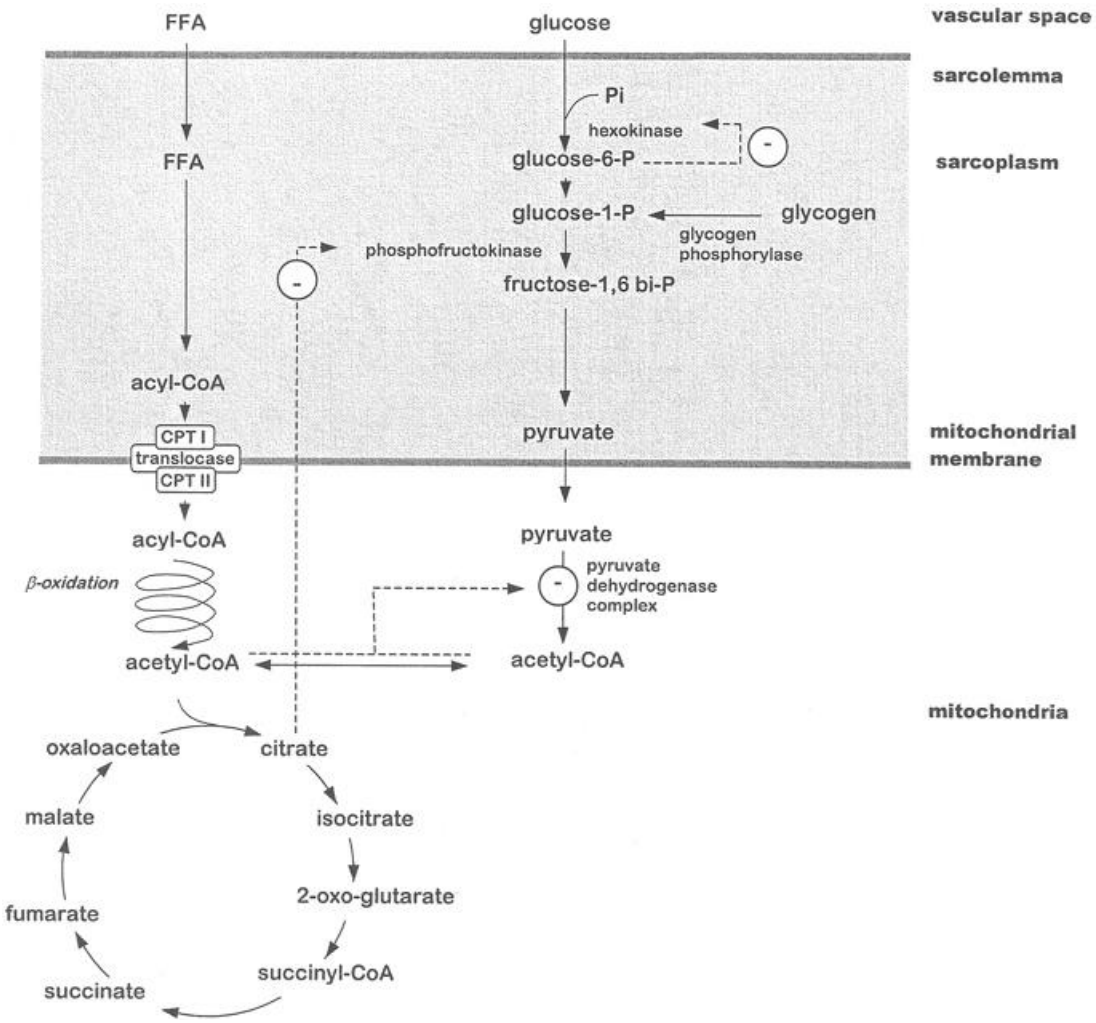


Figure 1.5 The glucose-FA or Randle cycle describes potential mechanisms involved in the interaction between fat and carbohydrate metabolism. In this model, FA availability seems to be the dominant factor. Briefly, increased FA availability would increase acetyl-CoA concentrations; accumulation of acetyl-CoA would result in inhibition of pyruvate dehydrogenase (PDH); accumulation of citrate would inhibit phosphofructokinase (PFK); and accumulation of glucose-6-phosphate (G6P) would reduce hexokinase (HK) activity. Ultimately, this would inhibit carbohydrate metabolism with increasing availability and oxidation of FA. Taken from Jeukendrup (2002)

1.2.3.1 Glucose can also regulate fat oxidation

Since the early work of Randle and colleagues it has now known that high glucose availability can regulate fat oxidation, most likely through the actions of Malonyl coenzyme A (malonyl-CoA) (Mcgarry, Mannaerts, and Foster, 1977). Malonyl-CoA is the product of the acetyl coenzyme A carboxylase (ACC) reaction. ACC is activated by high levels of citrate and high levels of its precursor substrate cytosolic acetyl-CoA. Malonyl-CoA is found in a variety of tissues including heart, liver, adipose, and skeletal muscle (Mcgarry, Mannaerts, and Foster, 1977). in the liver, malonyl-CoA is both an intermediate in the de novo synthesis of fatty acids and an allosteric inhibitor of (CPT 1), the enzyme that regulates the rate at which long-chain fatty acyl (LCFA)-CoAs enter the mitochondria where they are oxidized. However, in tissues such as skeletal and cardiac muscle, in which the de novo synthesis of fatty acids is minimal, the regulation of CPT 1 is its dominant role. Evidence for this mechanism comes from studies which have measured the oxidation of isotopically labelled medium chain (octanoate) fatty acids (MCFA) which enter the mitochondria independent of CPT 1 and LCFAs (oleate) whilst maintaining physiological hyperglycaemia and hyperinsulinemia (Sidossis et al. 1996, 1999; Rasmussen et al. 2002). Through this elegant design, it has been demonstrated that skeletal muscle malonyl-CoA is increased during physiological hyperglycaemia with hyperinsulinemia and that this increase is associated with the inhibition of functional CPT-1 activity and reduced muscle LCFA oxidation but not MCFA oxidation (Rasmussen et al. 2002). Interestingly, muscle uptake of LCFA was unchanged between the fasting and hyperglycaemia with hyperinsulinemia states, suggesting that increase in

malonyl-CoA shunted LCFA away from oxidation and toward storage inside the muscle cell. It has since been demonstrated that skeletal muscle of obese individuals and patients with T2D has elevated malonyl-CoA compared to lean controls (Bandyopadhyay et al. 2006). This was accompanied by reduced palmitate (a LCFA) oxidation and increased lipogenesis in tissue homogenates. However, it is important to note that it would appear that muscle malonyl-CoA concentration may not regulate long-chain fatty acid oxidation during exercise, despite high PDC flux during exercise, and therefore the marked accumulation of acetyl groups (constantin-teodosiu et al. 1991). It is possible that the sensitivity of CPT1 to malonyl-CoA paradoxically decreases in response to exercise (Stephens, Constantin-Teodosiu, and Greenhaff 2007).Thus, under different physiological circumstances and availability, both fatty acids and glucose can regulate the oxidation of the other substrate.

1.2.4 Skeletal muscle protein metabolism

Human skeletal muscle is in a constant state of turnover. This involves the opposing anabolic process of muscle protein synthesis (MPS) and catabolic process of muscle protein breakdown (MPB). The dynamic equilibrium between these ultimately determines net muscle protein balance. Thus, a significant rise in MPS (anabolism) and/or a reduction in MPB (catabolism), such that net protein balance remains positive can result in the accretion of skeletal muscle proteins. Conversely, a negative net protein balance, arising from a reduction in MPS and/or increase in MPB, will result in a loss of skeletal muscle protein.Net protein balance is maintained by ingestion of protein-containing meals which results in systemic hyperaminoacidemia that is stimulatory for the synthesis of new proteins (Breen and Phillips 2011).

1.2.4.1 Insulin and muscle protein metabolism

The main *in vivo* effect of insulin with regards to protein metabolism is the inhibition of protein breakdown and oxidation (Tessari et al. 1986; Abdulla et al. 2016) but there is contention over its ability to stimulate protein synthesis, especially independent of maintained amino acid availability. In a specific experimental conditions , insulin may stimulate muscle protein synthesis when concentrations are raised to supraphysiological concentrations or when insulin is infused locally across a limb where local hypoaminoacidaemia may not occur (Fujita et al. 2006; Trommelen et al. 2015). However, it is unlikely that this experimental condition would occur in vivo. Ageing appears to be associated with a blunted MPB response to insulin (Wilkes et al. 2009) and this response appears to be independent of body of composition.

1.2.4.2 Interactions of lipid and protein metabolism

Work from this group has shown a direct impact of increasing lipid availability and muscle protein metabolism. It has been shown that inducing insulin resistance through lipid infusion during a hyperinsulineamic- euglycaemic clamp resulted in a completely attenuated muscle protein synthetic response to a 21g bolus of amino acids (Stephens et al. 2015). This blunted anabolic response was attributed to reduced activation of the mTOR pathway which is they key pathway involved in the regulation of protein synthesis. The induction of insulin resistance appears to be key in mediating this blunted response as the consumption of an oral lipid emulsion prior to the coingestion of milk protein and dextrose (a more physiological representation of the former report) resulted in an increase amino acid incorporation into skeletal muscle. (Tsintzas et al. 2020). Interestingly, inducing insulin resistance through lipid infusion

during a hyperinsulineamic- euglycaemic clamp has also been shown to be accompanied, by the accumulation of branched chain amino acid derived catabolites in muscle biopsies obtained at the end of the clamp (Stephens et al. 2014). Thus, models of acute lipid induced insulin resistance appears to be associated with both a reduced synthetic response and perturbations in branched chain amino acid metabolism. Indeed, it has been shown that obese middle-aged men have a reduced protein synthetic response under hyperinsulineamic hyperaminoacideamic-euglycemic clamp (representing the post-prandial state) conditions. However this blunting was offset by a decline in MPB, despite lower postprandial leg glucose disposal (Murton et al. 2015). Therefore, obese non -frail middle-aged men do not appear to be an increased risk of muscle mass loss but clearly have a diminished muscle synthetic capacity.

1.3 Lipid induced insulin resistance in muscle

The early work of Randle and colleagues clearly provided the framework of our understanding of the reciprocal relationship between fat and glucose, certainly as basis to acutely regulate substrate usage. However, whether this mechanism is responsible for the impairments in glucose utilisation seen in chronic progressive conditions such as T2D remains uncertain. Despite several decades of work in this specific area the specific mechanism by which lipids or specific lipid species impair glucose uptake is still largely unknown. An overview of the (often conflicting) opinions in this area is provided below.

It has long been demonstrated that elevated circulating fatty acids are present in obesity and T2D and plasma fatty acids are negatively correlated with insulin resistance (Perseghin et al. 1997). However, a stronger predictor of insulin resistance appears to be the amount of triglyceride stored in the muscle (Pan et al. 1997; Perseghin et al. 1999). These findings clearly implicate increased fatty acid availability and storage with the development of insulin resistance. Lipid infusions combined with heparin to activate lipoprotein lipase and raise plasma concentrations of free fatty acids during a euglycaemic-hyperinsulinaemic clamp is an experimental model for studying the direct impact of increased fatty acid availability on muscle metabolism (Boden 1996).

Under these circumstances some studies have shown that the intramuscular concentrations of G-6-P and glucose were lower compared to infusions where fatty acids were absent (Roden et al. 1996; Dresner et al. 1999). Furthermore, in patients with T2D, intramyocellular concentrations of glucose were much lower than healthy controls during a euglycaemic-hyperinsulinaemic clamp

(Cline et al. 1999). These findings were also accompanied by reduced activity of key signalling molecules in the insulin signalling cascade, namely IRS-1–associated PI 3-kinase activity (Dresner et al. 1999). Taken together these findings suggest impairments in insulin mediated glucose transport across the cell are primarily responsible for reduced glucose uptake and are in contrast with the mechanisms described by the original hypothesis put forward in the glucose-fatty acid cycle hypothesis (Randle et al. 1963). A strength of these studies is that they used a non-invasive magnetic resonance imaging method to sequentially measure the accumulation of these metabolites over time. Whilst these findings implicate impairments in insulin mediated glucose transport as the main factor underlying lipid induced insulin resistance, those studies did not measure other elements of the glucose-fatty acid cycle including PDC activity directly. In combination with the findings that obesity and T2D is characterised by reduced oxidative capacity got fatty acid oxidation (Kelley and Simoneau 1994; Petersen et al. 2003) led to the consensus that lipid oversupply (likely mediated by increased delivery from the adipose tissue) is not matched by skeletal muscle oxidative capacity. Thus, lipid is diverted away from oxidation to storage as TAG within muscle.

1.3.1 Accumulation of lipids in muscle

Intramuscular lipid can be found in several distinct metabolic depots within muscle tissue, in particular in the interstitium as extramyocellular lipid (EMCL) or within muscle fibres as intramyocellular lipid (IMCL). EMCL is quantitatively the largest lipid pool within muscle and, together with the intermuscular lipid depot found under the facia between individual muscles, has consistently been correlated with insulin resistance in large cross-sectional studies. The much

smaller IMCL pool is predominantly composed of intramuscular triglyceride (IMTG) droplets and phospholipids, but also includes diacylglycerols (DAG) and ceramides, which are mechanistically linked to insulin resistance since accumulation can interfere with intracellular insulin signalling (Bosma et al. 2012).

It is now well known that IMTG nor IMCLs *per se* do not cause insulin resistance, this is evidenced by the finding that highly trained endurance athletes have similar IMTG content to individuals with T2D yet are very insulin sensitive (Goodpaster et al. 2001). Well trained athletes also exhibit mitochondrial adaptations that increase capacity for fat oxidation and likely have much higher turnover rates, since IMCLs are a key fuel source during light to moderate intensity PA (Schrauwen-Hinderling et al. 2003). There is a need to distinguish between subcellular fractions of lipid, with obese T2D individuals having three times higher subsarcolemmal lipid content but similar levels of intermyofibrillar lipids compared to normoglyceamic obese controls.

Exercise training studies have reported increased IMCL content, including the number of droplets residing close to mitochondria (Tarnopolsky et al. 2007) which likely function as a local energy store. Interestingly, the training-induced improvements of insulin sensitivity in overweight/obese insulin resistant individuals have been associated with increased IMCL content but reduced lipid intermediates (Dubé et al. 2008). When considered together, these findings suggest that the accumulation of IMCL per se does not cause insulin resistance and may simply act as a biomarker of dysfunctional glucose metabolism in individuals with obesity and T2D. Instead, the focus has largely

shifted to the accumulation of lipid intermediates including long-chain fatty acyl-CoAs, acylcarnitines, ceramides and DAGs.

1.3.1.1 DAGs

DAG can be formed from the hydrolysis of TAG or synthesised from glycerol combining with long-chain fatty acyl CoA. Lipid infusion induces a transient increase in muscle DAG content that precedes the activation of protein kinase C (PKC) theta (θ). This was associated with impaired insulin-stimulated activation of IRS-1 and supressed downstream activity of PI3K/Akt (Szendroedi et al. 2014). Accumulation of DAGs and subsequent activation of PKCθ were augmented in individuals with obesity or T2D. This could reflect differential lipid partitioning caused by an increased ratio of TAG hydrolase to DAG hydrolase (Moro et al., 2009) or reduced levels of hormone sensitive lipase (Blaak, 2004). The proposed role of PKCθ is supported in rodent knockout models which are protected from lipid-induced insulin resistance (Kim et al., 2004).

Rodents overexpressing DAG acetyl transferase, which increases the conversion of DAG into TAG, are protected from lipid-induced IR (Liu et al., 2007). A similar overexpression model observed a paradoxical increase in DAG content, accompanied by an improvement in insulin sensitivity (Timmers et al., 2011). This suggests a more complex role for DAGs and the importance of considering the turnover rate (balance between supply and oxidation). This may explain the increased DAG content in highly trained and obese individuals (Amati et al., 2011). Exercise has been shown to increase DAG acetyl transferase (Liu et al., 2007), which suggests the increased DAG content in highly trained individuals may be from increased supply, whereas it may reflect

reduced oxidation of fatty acids in the obese population. This could explain why the DAG composition varies considerably between these groups.

1.3.1.2 Ceramides

Ceramides are sphingolipids that can be derived from palmitate and are strongly associated with insulin resistance. Previous research has shown inverse correlations between ceramide content and insulin sensitivity (Adams et al., 2004; Straczkowski et al., 2004). Elevated levels of ceramides (both saturated and unsaturated) are found in obese, but not well-trained individuals and this is likely due to higher skeletal muscle turnover and therefore less accumulation (Amati et al., 2011). This suggests ceramide synthesis is linked to lipid oversupply, but it has also been shown that inflammatory agonists augment ceramide production (Holland et al., 2011). This is important to consider as obesity and insulin resistance are widely considered as pro-inflammatory states. Although in a group of females with obesity, elevated ceramide levels were only found in those with insulin resistance (Coen et al., 2010), which could suggest a more specific role for ceramides mediating insulin signalling. Interestingly, exercise-induced improvements in insulin sensitivity of obese adults were correlated with the reduction in ceramide but not DAG content (Dubé et al., 2008).

Supplying long-chain saturated fatty acids such as palmitate (C16:0) or stearate (C18:0) increases ceramide synthesis both in vitro (Chavez et al., 2003) and in vivo (Blachnio-Zabielska et al., 2010). Both chemically inhibiting (Holland et al., 2007) and reducing the expression of serine palmitoyl transferase (Watson et al., 2009), a key enzyme in the synthesis of ceramide from palmitate, ameliorates lipid-induced insulin resistance. The relatively low

abundance of saturated fatty acids (SFA) in intralipid infusions may therefore explain why some studies do not see an acute increase in ceramide content despite inducing IR (Itani et al., 2002). Recent findings using genetic ablation to block the synthesis of specific chain lengths of ceramides, implicate C18:0 as most abundant in skeletal muscle and disruptive to glucose homeostasis (Turpin-Nolan et al., 2019). Mechanistically, ceramides are thought to exert their effect by impaired insulin-stimulated activation of Akt through activation of PKC (Fox et al., 2007) and hyperactivation of protein phosphatase 2A (PP2A) (Dobrowsky et al., 1993).

1.3.1.3 PDC and PDKs

Studies from this group and others have shown that the intravenous infusion of lipid emulsion combined with heparin in healthy individuals during euglycaemic hyperinsulinaemia results in impaired insulin-stimulated oxidative and nonoxidative glucose disposal, with both inhibition of the PDC by increased fatty acid β-oxidation and/or lipid intermediate accumulation as a primary event (Tsintzas et al. 2007; Stephens et al. 2014). Lipid infusion during a glucose clamp was accompanied by increased fat oxidation, decreased skeletal muscle PDC activity, accumulation of long chain-Acyl CoAs and acetylcarnitine as well as increased expression of muscle PD kinase (PDK) (which phosphorylates and inactivates PDC and thus inhibits glucose oxidation). The increased muscle acetylcarnitine under these circumstances, is a reflection of excessive Acetyl-CoA production (from increased fatty acid availably and oxidation), which itself is an allosteric inhibitor of PDC (Klyuyeva, Tuganova, and Popov 2008). Furthermore, these conditions are not necessarily accompanied by decreased proximal insulin signalling (Tsintzas

et al. 2007; Høeg et al. 2011; Storgaard et al. 2004). These findings suggest that both impairments in oxidative disposal and insulin mediated glucose transport contribute to lipid induced insulin resistance.

1.3.2 Mitochondrial overload hypothesis

In contrast to the work linking the diversion of fat away from the mitochondrial oxidation and into the accumulation of lipid intermediates that may impair insulin signalling and glucose uptake, other work has suggested the excessive mitochondrial entry of fatty acids as the primary driver of the development of insulin resistance (An et al. 2004; Koves et al. 2008). The mechanistic basis of this work originates from a study which subjected Wistar rats to a high fat diet (HFD) for 12 weeks and observed an accumulation of fatty acids and acylcarnitines in the fed state in both the circulating pool and within skeletal muscle (Koves et al. 2008). Whereas lean control rats showed a reduction in a fatty acid derived acylcarnitines in response to feeding, this response was absent in the HFD group suggesting continual mitochondrial fatty acid entry and an inability to switch substrate usage to carbohydrate in response to feeding. These findings were also observed in diabetic fatty rats and this was accompanied by a reduction in TCA intermediates. These findings suggest that excessive fatty acid availability leads to an increased entry of long chain Acyl-CoAs into the mitochondria and elevated β-oxidation which is not matched by a similar increase in TCA intermediates and TCA flux and thus an accumulation of acylcarnitines in muscle due to ‘incomplete’ fatty acid oxidation (i.e fatty acids are not completely oxidised to CO_2 and accumulate and may be exported into the plasma). Moreover, in a subsequent experiment, knockout mice lacking malonyl CoA decarboxylase (MCD), which degrades

the natural inhibiter (malonyl-CoA) of CPT1 activity and β-oxidation, had markedly lower acylcarnitine levels in muscle and were protected against diet-induced insulin resistance, despite high levels of long chain Acyl-CoA. However, the longer-term consequences of such a genetic manoeuvre were not investigated. The authors proposed that strategies that reduced rather than enhanced muscle fatty acid oxidation be considered a therapeutic treatment of insulin resistance. Although these findings did not implicate acylcarnitines as directly causative of insulin resistance, they show that reduced accumulation of acylcarnitines in the mitochondria can improve insulin sensitivity. This work led to the hypothesis that with obesity, mitochondria become congested by elevated carbon supply and cannot adequately switch their fuel preference in different physiological states (Muoio 2014). A schematic overview of some of the mechanisms pertaining to lipid induced insulin resistance is shown in Figure 1.7

1.3.2.1 Acylcarnitines and insulin resistance

Since the work implicating acylcarnitines in the development of insulin resistance, studies have also observed elevated concentrations of even chain length acylcarnitines in the plasma of obese subjects and T2D patients (Adams et al. 2009; Mihalik et al. 2010). These findings appear to substantiate the notion that obesity and T2D is a state of incomplete fatty acid oxidation. Acylcarnitines have also been shown to cause insulin resistance *in vitro.* Murine C2C12 myotubes were treated for 18 h with C4:0, C14:0, or C16:0 acylcarnitine at 3 different concentrations (5, 10, and 25 μmol/L). Treatment with C4:0 acylcarnitine at all concentrations resulted in a significant decreased Akt (Ser473) phosphorylation in response to insulin. Treatment with 5–10

mmol/L C14:0 or C16:0 acylcarnitines significantly decreased Akt (Ser473) phosphorylation in response to insulin stimulation (Aguer et al. 2015). In addition, C12 and C14 acylcarnitine treatment (20 μmol for 15 hours) of murine monocyte cells has been shown to activate nuclear factor kappa β (NFkB) which is a protein transcription factor that can promote pathways that lead to serine phosphorylation of insulin receptor substrate-1 and a proinflammatory state leading reactive oxygen species production and further promote insulin resistance (Adams et al. 2009).These findings gave rise to yet another lipid intermediate that could cause insulin resistance.

Whilst acylcarnitines per se have been shown to induce insulin resistance in vitro (Aguer et al. 2015), little is known about their intramuscular profiles in human skeletal muscle from diabetic patients. This is important as numerous studies have reported on elevated plasma acylcarnitines in human studies with the assumption that they reflect skeletal muscle metabolism (Mihalik et al. 2010). However, more recent studies measuring transorgan fluxes in pigs (Schooneman et al. 2015) and humans (Xu et al. 2016) have demonstrated that liver is a major contributor to the plasma pool under fasting conditions and inadequately reflect skeletal muscle metabolism (Schooneman et al. 2014). Furthermore, in response to an OGTT the only tissue that appeared to reflect plasma acylcarnitine profiles was the heart (Makrecka-Kuka et al. 2017) providing further evidence that plasma acylcarnitines may not be an appropriate biomarker for skeletal muscle insulin resistance. Little is known about the skeletal muscle acylcarnitine profiles in obese and T2D humans nor if they reflect ‘incomplete or excessive’ fat oxidation.

1.3.2.2 Increasing or decreasing muscle fatty acid oxidation?

It should be noted that the strategy to divert fatty acids away from oxidation and particularly in the muscle may appear an attractive option, but it is important to consider the potential longer-term consequences of such a strategy. Indeed, carnitine depletion with meldonium in rodents increased carbohydrate and reduced fat oxidation at the whole body level (Porter et al. 2017). Conversely, carnitine feeding in humans for 12 weeks led to 20% increase in muscle total carnitine and 6% increase in energy expenditure compared to a carbohydrate consuming control group. Furthermore, the increase in energy expenditure was able to offset an increase in fat mass seen in the carbohydrate control group (Stephens et al. 2013). Therefore, whilst excessive fatty acid flux through β-oxidation can be detrimental to insulin action, whether strategies to reduce fatty acid oxidation in the face of increased fat availability are appropriate is questionable. A recent study investigated the long-term effects of reduced fatty acid oxidation in mice by using the drug etomoxir, an inhibitor of CPT-1 (Lundsgaard et al. 2019). Similar to previous findings, acute increases in glucose oxidation and peripheral glucose disposal were observed. However, circulating fatty acid and lipid accumulation in the liver and heart increased within hours, and after several days the mice developed hepatic steatosis and glucose intolerance and exhibited lower whole body insulin sensitivity (Lundsgaard et al. 2019). These findings demonstrate inhibiting fatty acid oxidation by diverting fatty acids away from CPT-1 may not be a viable long-term strategy to treat insulin resistance, especially under conditions of elevated or even maintained dietary fat availability.

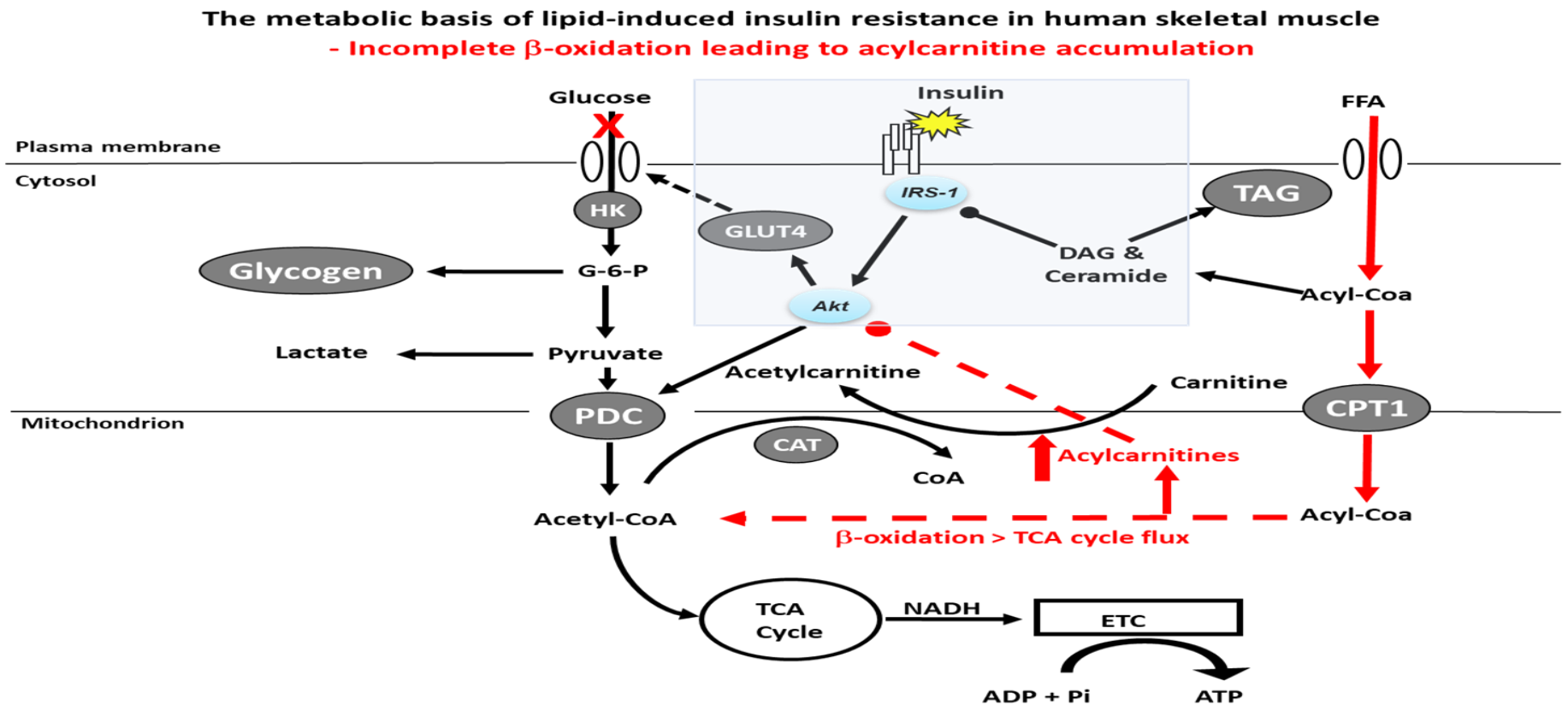


Figure 1.6 An overview of potential pathways in muscle by which perturbed lipid metabolism leads to insulin resistance**,** see text above for details.

1.3.3 Metabolic inflexibility of insulin resistant muscle

Work by Kelley and colleagues, characterised muscle fuel metabolism in healthy, obese and insulin resistant individuals. One of the key characteristics of skeletal muscle is its ability to switch fuel utilisation in response to the availability of different substrates. One measure of muscle fuel usage is respiratory quotient (RQ) which is a measure of carbon dioxide production as a ratio to oxygen consumption. At a RQ of 1 glucose is the sole fuel (6 carbons from glucose oxidised to 6 molecules of oxygen) and an RQ of 0.7 is marker of fat oxidation. As RQ increased from 0.7 to 1, the contribution of glucose to the fuel mixture is increasing. Measuring RQ in arterial and venous blood across a limb, permits the calculation of muscle specific fuel usage, assuming the contribution of adipose tissue, skin and bone do not contribute significantly. Lean, healthy subjects shifted from a low RQ (0.83) in the fasted state to a high RQ (0.99) during a hyperinsulinaemic-euglycaemic clamp demonstrating a clear shift from fat to carbohydrate use with increased glucose availability. In contrast, in obese subjects, despite rates of fatty acid uptake that were equivalent to those of lean subjects, fasting rates of fatty acid oxidation by leg tissues were significantly lower. Fasting RQ was 0.9 (suggesting higher glucose use) and in response to insulin did not change and therefore there was an impaired ability to switch to glucose usage (Kelley et al. 1999; Kelley and Mandarino 1990). These observations led the authors to ask the question whether impairments in muscle mitochondria oxidative capacity could precede insulin resistance and possibly inherent impairments of mitochondria predispose individuals to accumulate lipid and lipid species in muscle which then cause insulin resistance. Indeed, reduced mitochondrial enzyme content

was observed in cross sectional comparisons between lean and insulin resistant individuals (Colberg et al. 1995; Kelley et al. 1999). It should be noted that later investigations have shown that metabolic inflexibility with regards to mitochondria does not explain the difference in fuel use and that the impairments lay at the level of glucose transport into the cell. When adjusting the difference in RQ between healthy lean and T2D groups for glucose disposal rate, the differences in RQ between groups were abolished (i.e. the reason RQ does not change in response to insulin is because T2D are insulin resistant and do not transport the glucose into the cell for subsequent oxidation) (Galgani et al. 2008). This suggests that the metabolic inflexibility to glucose observed in T2D cannot not be solely explained by a primary impairment in glucose oxidation but may also be the consequence of impaired glucose transport.

1.3.4 Mitochondrial dysfunction

Inherent defects in mitochondrial activity and function could in part explain why some individuals develop insulin resistance. However, when directly measuring mitochondrial respiration in muscle samples, the lower oxygen flux capacity in T2D patients is normalised to that of age-matched non-diabetic controls, when expressed relative to mitochondrial deoxyribonucleic acid (DNA) levels (Boushel et al. 2007). Thus, whilst it appears that mitochondrial content is lower, perhaps due to increased physical inactivity/sedentary lifestyle, muscle mitochondrial function at rest remains largely uncompromised. Additionally, direct assessment of hepatic mitochondrial oxidation by magnetic resonance spectroscopy revealed no difference in mitochondrial flux between healthy BMI matched control subjects and non-

alcohol fatty liver disease (NAFLD) patients (Petersen et al. 2016), a disease characterised by excessive hepatic fat accumulation. These data suggest that tissue accumulation of lipids and lipid related species is most cases is a result of an imbalance between supply and oxidative capacity of tissues, the latter is most likely a reflection of physical activity levels. Work from this group has shown that old inactive obese subjects with low aerobic capacity had an equivalent rate of fatty acid disappearance from plasma into muscle but a reduced oxidation of plasma derived fatty acids and increased accumulation of IMCL compared to age-matched lean subjects following a single bout of exercise at 50 % of VO_2 max (Chee et al. 2016). These findings clearly show the implications of a reduced oxidative capacity relative to fatty acid supply, which subsequently causes an accumulation of IMCL.

1.3.5 Inactivity induced insulin resistance

It should be noted that are physiological states in which the induction of muscle insulin resistance can be induced without parallel increases in IMCL. During periods of physical inactivity it would appear that muscle insulin resistance can precede accumulation of IMCL and the lack of muscle contraction explains the reduction in glucose uptake (Dirks, Wall, and Stephens 2020). For example, one week of bed rest can reduce whole body glucose disposal by 30-40 % (Stuart et al. 1988; Dirks et al. 2016). Importantly, this was assessed by hyperinsulinaemic–euglycaemic conditions, which considered the gold standard assessment of muscle insulin sensitivity (DeFronzo, Tobin, and Andres 1979). Remarkably, reduction in glucose tolerance can be observed within only a few days of the induction of physical inactivity (Dirks et al. 2018) The rapid development of insulin resistance suggests that the removal of

muscle contraction *per se* disuse-induced insulin resistance, likely due to a lack of stimulus for translocation of the facilitative glucose transporter, GLUT4, to the plasma membrane via various signalling pathways, which is the most adjacent physiological mechanism preceding muscle glucose uptake (as stated in Section 1.1.3.1). Indeed, the aforementioned reports displayed reductions in gene expression of GLUT4 following bed rest (Dirks et al. 2018). Moreover, in this experimental model the reduction in insulin sensitivity was not accompanied by an accumulation in IMTG content nor lipid intermediate species such as ceramides (Dirks et al. 2016). However a trend for increased DAG has been observed following a week of bed rest induced inactivity (Dirks et al. 2018). Taken together, these data suggest that IMCL accumulation follows (rather than precedes) the development of insulin resistance, perhaps suggesting that insulin resistance may be a cause, not a consequence, of IMCL accumulation. Collectively these findings give rise to question as to whether IMCL are causative of insulin resistance or are in innocent bystander of reduced muscle energy expenditure with physical inactivity.

1.3.6 Summary

To summarise, there are clearly several (and sometimes conflicting) opinions on specific mechanisms underpinning the pathophysiology of lipid induced insulin resistance. This could in part be explained by the range of mechanistic models used to investigate this area and a lack of control of confounding factors such as physical activity. Given that models of acute but extreme physical inactivity such as bedrest or limb immobilisation clearly show the induction of insulin resistance may even precede lipid accumulation in the muscle (Dirks, Wall, and Stephens 2020), further questions which factor is

primarily responsible for the reduced glucose uptake. Furthermore, although several studies implicate the tissue accumulation of lipid and lipid species with aberrant insulin action, there appears to be no consensus on which lipid specie(s) constitute the primary cause of lipid induced insulin resistance. Moreover, previous aspects not previously investigated such as sub cellular localisation of lipid may also be a key factor (Kahn et al. 2021). One way to reconcile all these contrasting views would be to measure each aspect (physical activity and the subcellular accumulation of each lipid intermediated implicated in the development of insulin resistance) using time course studies. Of course, availability of methods is likely to preclude this type of analysis.

1.4 Branched chain amino acids

1.4.1 Overview and synthesis

The branched-chain amino acids (BCAAs), valine, leucine, and isoleucine are essential amino acids that cannot be synthesised in the human body and therefore must be obtained from the diet. BCAAs are synthesized in bacteria, plants, and fungi. The synthesis of valine and isoleucine is carried out by the same enzymes, and leucine is created from α-ketoisovalerate, a transamination precursor of valine (Figure 1.7a). The carbons in valine (and leucine) are derived from the readily available and abundant pyruvate, but isoleucine carbons are derived from the relatively rare threonine. Despite the absence of synthesis in animals, BCAAs are abundant in the human body, constituting approximately 35 % of all essential amino acids in most mammals and greater than 30 % of muscle proteins (Harper, Miller, and Block 1984). The functional R groups of all three BCAAs are branched (hence their name), small, and hydrophobic, rendering them critical components of most proteins (Chou and Fasman 1973; Dill 1990).

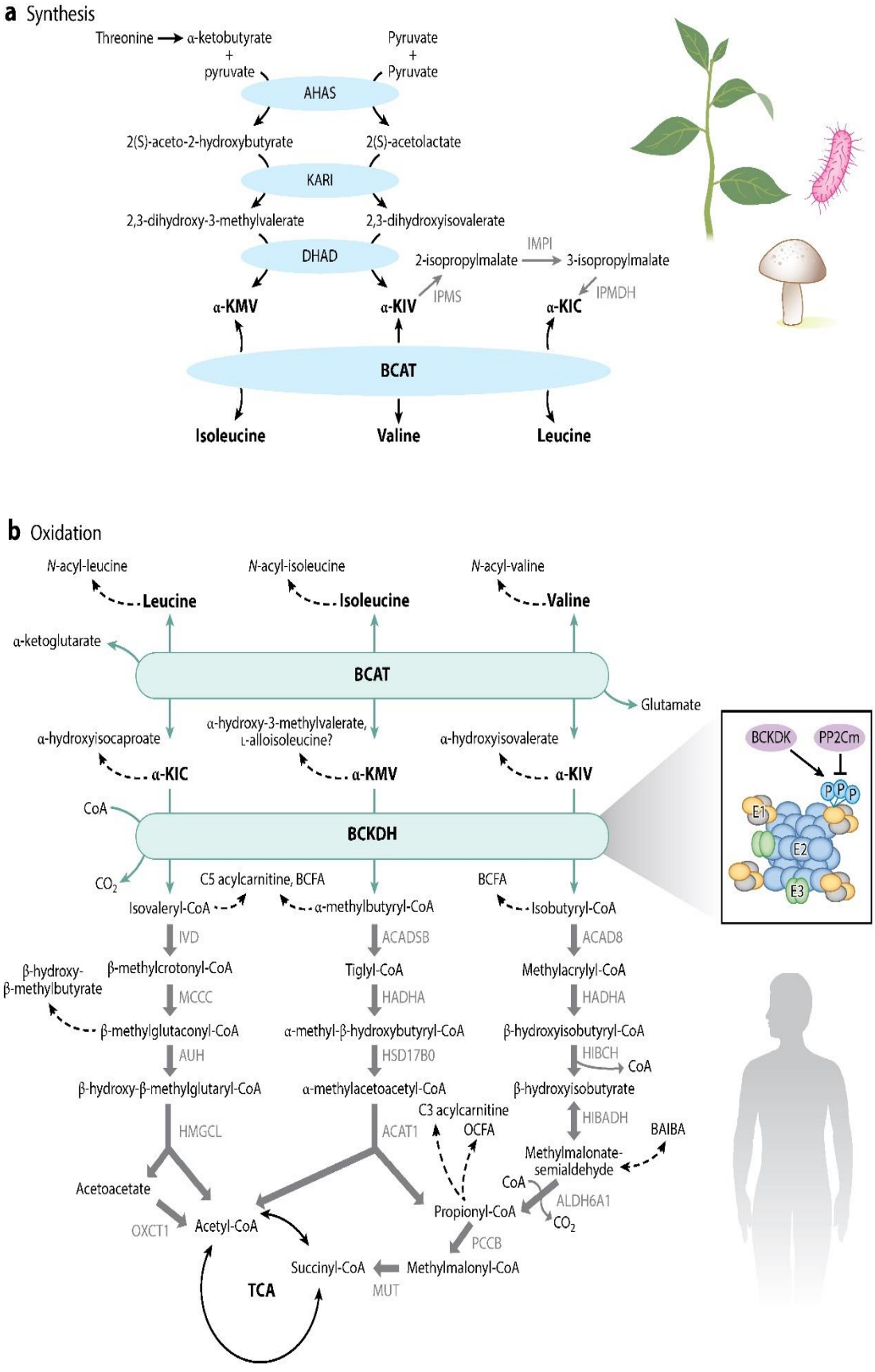

a Synthesis
Threonine
α-ketobutyrate
+
pyruvate
Pyruvate
+
Pyruvate
AHAS
2(S)-aceto-2-hydroxybutyrate
2(S)-acetolactate
KARI
2,3-dihydroxy-3-methylvalerate
2,3-dihydroxyisovalerate
DHAD
2-isopropylmalate
IMPI
3-isopropylmalate
IPMS
IPMDH
α-KMV
α-KIV
α-KIC
BCAT
Isoleucine
Valine
Leucine
b Oxidation
N-acyl-leucine
N-acyl-isoleucine
N-acyl-valine
Leucine
Isoleucine
Valine
α-ketoglutarate
BCAT
Glutamate
α-hydroxyisocaproate
α-hydroxy-3-methylvalerate,
L-alloisoleucine?
α-hydroxyisovalerate
α-KIC
α-KMV
α-KIV
CoA
BCKDH
CO2
C5 acylcarnitine, BCFA
BCFA
Isovaleryl-CoA
α-methylbutyryl-CoA
Isobutyryl-CoA
IVD
ACADSB
ACAD8
β-hydroxy-
β-methylbutyrate
β-methylcrotonyl-CoA
Tiglyl-CoA
Methylacrylyl-CoA
MCCC
HADHA
HADHA
β-methylglutaconyl-CoA
α-methyl-β-hydroxybutyryl-CoA
β-hydroxyisobutyryl-CoA
AUH
HSD17B0
HIBCH
CoA
β-hydroxy-β-methylglutaryl-CoA
α-methylacetoacetyl-CoA
β-hydroxyisobutyrate
C3 acylcarnitine
OCFA
HIBADH
BAIBA
HMGCL
ACAT1
Methylmalonate-
semialdehyde
CoA
Acetoacetate
ALDH6A1
CO2
OXCT1
Acetyl-CoA
Propionyl-CoA
PCCB
Succinyl-CoA
Methylmalonyl-CoA
TCA
MUT
BCKDK
PP2Cm
P
E1
E2
E3

Figure 1.7 BCAA synthesis and catabolism. Synthesis (a) occurs in plants, bacteria, and fungi. Oxidation (b) occurs in plants, bacteria, fungi, and animals. All three BCAAs share the BCAT and BCKDH steps, after which catabolism of each BCAA is unique. The BCKDH complex is composed of a core of 24 E2 subunits, which are docked by E1 heterotetamers and E3 dimers. BCKDK inhibits E1 via phosphorylation, which is reversed by PP2Cm. Abbreviations: ACAD8, acyl-CoA dehydrogenase family member 8; ACADSB, short/branched chain acyl-CoA dehydrogenase; ACAT1, acetyl-CoA acetyltransferase 1; AHAS, acetohydroxyacid synthase; α-KIC, α-ketoisocaproic acid; α-KIV, α-ketoisovaleric acid; α-KMV, α-ketomethylvaleric acid; ALDH6A1, aldehyde dehydrogenase 6 family member A1; AUH, AU RNA-binding protein/enoyl-coenzyme A hydratase; BAIBA, beta-amino-isobutyric acid; BCAA, branched chain amino acid; BCAT, branched chain amino transferase; BCFA, branched chain fatty acid; BCKDH, branched chain amino acid dehydrogenase; BCKDK, BCKDH kinase; CoA, coenzyme A; DHAD, dihydroxyacid dehydratase; HADHA, hydroxyacyl-CoA dehydrogenase subunit alpha; HIBADH, 3-hydroxyisobutyrate dehydrogenase; HIBCH, 3-hydroxyisobutyryl-CoA hydrolase; HMGCL, 3-hydroxymethyl-3-methylglutaryl-CoA lyase; HSD17B0, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; IPMDH, isopropylmalate dehydrogenase; IPMI, isopropylmalate isomerase; IPMS, isopropylmalate synthase; IVD, isovaleryl-CoA dehydrogenase; MCCC, methylcrotonoyl-CoA carboxylase; MUT, methylmalonyl-CoA mutase; OCFA, odd-chain fatty acid; OXCT1, 3-oxoacid CoA transferase; P, phosphorylation; PCCB, propionyl-CoA carboxylase subunit beta. Taken from Neinast et al. (2018)

1.4.2 BCAA catabolism

The clinical importance of BCAA catabolism is demonstrated in patients with in-born errors of metabolism such as maple syrup urine disease, a rare autosomal recessive disorder caused by mutations in the branched chain α-ketoacid dehydrogenase (BCKDH) enzyme complex. The body is not able to catabolise BCAAs leading to supraphysiological levels of BCAAs and BCKAs in the blood. As a result, these individuals experience hypotonia and ketoacidosis as well as serious neurological issues such as developmental delay, hallucinations, seizures, and coma (Siddik and Shin 2019).

1.4.2.1 Transamination

The catabolic pathways of the BCAA have several features in common. All three BCAAs are initially transaminated by branched chain amino transferase (BCAT) to form branched chain ketoacids (BCKAs). The products of this reaction are α-ketoisocaproate (KIC), α-keto-b-methylvalerate (KMV), and α-ketoisovalerate (KIV) and are formed from leucine, isoleucine and valine transamination, respectively. In this readily reversible reaction, α-ketoglutarate (αKG) acts as the nitrogen acceptor to yield glutamate (Ichihara 1975) (Figure 1.8). Glutamate then acts as an amino group source to form alanine from pyruvate or as a substrate for ammonia detoxification to glutamine. Glutamine, alanine, and a significant portion of the BCKA can be released from muscles to the blood.

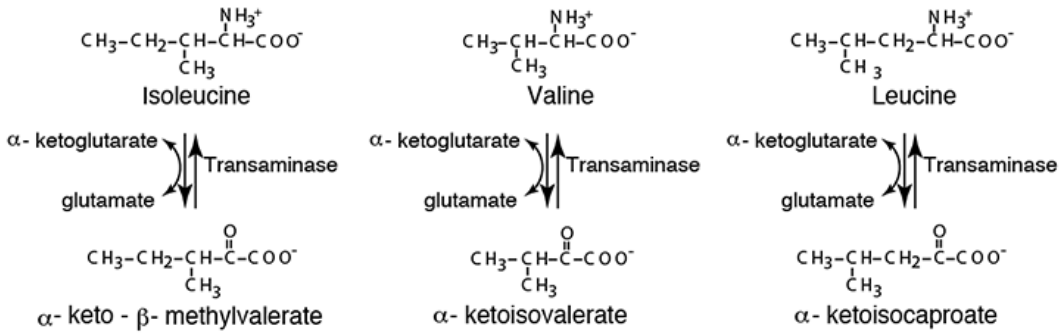


Figure 1.8 The reversible transamination reaction catalysed by branched chain amino transferases (BCATs).

1.4.2.2 Branched chain α-ketoacid dehydrogenase complex

The next steps of BCAA catabolism involve the irreversible oxidation of BCKAs and is catalysed by the branched chain α-ketoacid dehydrogenase (BCKDH) complex. This enzyme is located on the inner surface of the inner membrane of the mitochondria and shares many attributes with the pyruvate dehydrogenase (PDH) complex. Like the PDH complex, BCKDH catalyses an oxidative decarboxylation, releasing CO2 and NADH and covalently adding a coenzyme A (CoA) group to the oxidized BCKA product (Johnson and Connelly 1972). This step commits the BCAA carbon skeleton to the degradative pathway (Figure 1.8b).

The BCKDH complex consists of 3 catalytic components: a heterotetrameric BCKA decarboxylase (E1), a homo-24-meric dihydrolipoyltransacylase (E2), and homodimeric dihydrolipoamide dehydrogenase (E3). BCKDH activity is also regulated by post-translational covalent modification involving phosphorylation and dephosphorylation of its E1-α subunit. The inhibitory regulation of the BCKDH complex occurs via the inactivating phosphorylation

at Ser293 on the E1-α subunit by the BCKDH kinase (BCKDK), resulting in decreased BCKDH complex activity. The complementary activating dephosphorylation is carried out by protein phosphatase, PP2Cm (also known as PPM1K) (Harris et al. 2005). BCKDK is allosterically suppressed by BCKAs (its greatest for (α-KIC) thus allowing elevations in BCKAs to promote their own oxidation (Randle 1983). Efficient product inhibition of BCKDH also occurs by NADH and acyl-CoAs.

Oxidation of branched-chain amino acids in muscle serves two functions. The first is the generation of ATP and therefore can be used for energy provision, and the second is the synthesis of glutamine, which effluxes from the muscle. Work investigating the fate of BCAA derived carbon within muscle using radioactive isotopes revealed that only a small proportion (5%) ultimately enters the TCA cycle for oxidation and the majority are converted to glutamate (Goldberg 1978; Wagenmakers, Salden, and Veerkamp 1985).

After BCKDH decarboxylation, subsequent catabolism of BCAAs resembles fatty acid oxidation, and indeed, these two processes share a number of enzymatic subunits. Each set of reactions is mostly unique to each BCAA, and all occur inside the mitochondrial matrix. Ultimately, BCAA carbons are either lost as CO_2 or enter the tricarboxylic acid (TCA) cycle. Specifically, valine (5C) loses 2 carbons to CO_2 and contributes 3 carbons to the TCA as succinyl-CoA; leucine (6C) loses 1 carbon to CO_2 and contributes 5 carbons to the TCA in acetyl-CoAs; and isoleucine (6C) loses 1 carbon to CO_2 and contributes 5 carbons to the TCA as acetyl-CoA and succinyl-CoA. Short chain acylcarnitine can be formed in the catabolism of BCAAs, in the same manor described for fatty acids. Chain lengths of 3,4 and 5 carbons (some steps are shown in figure

1.7b but for a detailed schematic see (**Figure 4.4**). Valine is considered glucogenic (i.e., succinyl-CoA is anaplerotic), whereas leucine is considered ketogenic, and isoleucine is both keto and glucogenic. As of yet, little is known about the relative activities and kinetics of enzymes involved in the catabolic pathways for BCAAs in human tissues. This is important as, short chain acyl CoA products and associated short chain acylcarnitines could theoretically increase with both increased tissue flux through BCKDH or reduced enzyme activity of catabolic enzymes or both and no studies have addressed this directly to date nor quantitatively assessed BCAA and catabolites in muscle to give an indication of flux.

1.4.2.3 Distribution of BCAA catabolism

One unique aspect of BCAAs is that several organs contribute to their catabolism and there appears to be significant interorgan exchange. Consequently, assessment of whole body BCAA catabolism, especially in humans has been extremely challenging. Furthermore, the characterisation of activities of the BCAT and BCKDH enzymes has largely been performed in ex vivo preparations (Harper, Miller, and Block 1984; Sitryawan et al. 1998b). what has been established is that BCAT and the BCKDH complex are broadly expressed in human tissues, indicating wide tissue ability for BCAAs transamination and oxidation.

BCAT activity in human tissues is highest for kidney, followed by brain, stomach, and heart. A lesser and similar grade of activity is present in liver, small intestine, and colon while the lowest activity is showed by muscle and adipose tissue. The highest enzymatic activity of the human BCKDH complex has been found in kidney, followed by liver, brain, and heart. Muscle, stomach,

and colon had similar and lesser activity of the enzyme. Adipose tissue and small intestine had the lowest BCKD complex activity. The highest concentration of human BCKDH kinase (responsible for the inactivation of the complex) has been found in skeletal muscle while kidney possesses the lowest activity state (Sitryawan et al. 1998) When taking into account the organ weight as a proportion of body weight, the skeletal muscle has the highest quantitative capacity for both BCAAs transamination and oxidation. However, the BCKD kinase concentration is highest in muscle tissue, denoting the capacity of skeletal muscle to restrain the oxidation of BCKAs as well (Sitryawan et al. 1998; Brosnan and Brosnan, 2006). These results are only suggestive because other factors such as rates of blood flow, and transport into cells, are also important to consider. The muscle capacity for transamination appears to be far greater than oxidation. Assessment of leucine kinetics in humans has shown that the rate of transamination is much greater than KIC oxidation (Matthews et al. 1981), fitting with the model of high BCAT activity but highly constrained BCKDH activity in human muscle. However, the situation is reversed in the liver (at least in rodents) and the general consensus is that muscle transaminates BCAAs and releases significant quantities of BCKAs which are taken up by the liver. There is generally a lack of data on the fate of BCAAs in humans most likely due to the challenges of obtaining tissue samples and cannulation across the liver.

In perhaps in the most comprehensive integrative study to date, which investigated the whole body fate of BCAAs in rodents using a stable isotopes and tissue level flux analysis in rodents has shown that indeed muscle, liver and adipose tissue house the largest capacity to oxidise BCAAs but also that

tissue oxidative flux correlates very poorly with phosphorylation status of the BCKDH (Neinast et al. 2018).

1.4.3 BCAAs Obesity and Insulin resistance

Elevated BCAAs were first associated with obesity almost 50 years ago when Felig and colleagues made the observation that obesity was associated with elevated circulating BCAAs and infusion of insulin did not fully suppress their efflux from the forearm in obese individuals (Felig, Marliss, and Cahill 1969; Felig 1975). Despite these early observation, little regard was paid to perturbed BCAA metabolism and its potential contribution to insulin resistance.

Interest in BCAA being implicated in the progression of insulin resistance was renewed in the last decade by a study that performed comprehensive metabolic profiling of plasma from lean and obese subjects (Newgard, An, Bain, Muehlbauer, Stevens, Lien, Haqq, Shah, Arlotto, Slentz, Rochon, et al. 2009). An unbiased metabolomic and principal component analysis revealed the three BCAAs, the aromatic acids phenylalanine and tyrosine and C_3 and C_5 BCAA-derived acylcarnitine species were the components that most strongly dissociated between lean and obese individuals. Furthermore, evaluation of the association between this BCAA-related metabolite component and HOMA-IR revealed a significant linear relationship ($r = 0.58$, $p<0.0001$), even after adjusting for obese vs. lean status using a partial Spearman correlation coefficient ($r=0.33$, $p<0.0001$). This independent relationship persisted even after adjustment for age, race, and gender. Surprisingly, even though elevations in free fatty acids were also observed in obese plasma, these were not identified as a component that differentiated obese from lean subjects. Furthermore, although adjustments were made for

age, race, and gender, the obese subjects were also less physical active (as assessed using the international physical activity questionnaire) and an adjustment for physical activity was not considered. Thus, it is unknown to what extent physical inactivity could be accountable for the associations seen. The association between BCAA and insulin resistance was strengthened when it was later demonstrated that plasma baseline BCAA concentrations could predict the onset of T2D many years before clinical diagnosis in a longitudinal study (Wang et al. 2011). These findings validated BCAAs as new biomarkers for T2D development and interest in BCAA as a causative rather simply a reflective component of T2D was renewed. Moreover, Mendelian genetics studies revealed that polymorphisms near the PPM2 gene (encoding for the BCKDH phosphatase) that affect BCAA levels also increase the risk of insulin resistance (Lotta et al. 2016) However, these studies still provided no mechanistic link between BCAAs, obesity and insulin resistance.

1.4.4 Evidence from animal studies

Work in animals has focused primarily on the manipulation of dietary BCAA intake. Rodents fed a high fat diet supplemented with BCAAs had an overaccumulation of even chained acylcarnitines species (arising from fatty acid oxidation) as well as specific accumulation of C_3 and C_5 acylcarnitine species (arising from BCAA catabolism) in skeletal muscle. In contrast, BCAA supplementation of a standard diet caused no accumulation of acylcarnitine species and the animals did not become insulin resistant (Newgard et al. 2009). The authors suggested that against a background of a high fat diet, excess BCAAs may compete with fatty acid for oxidation and saturate the capacity for mitochondrial fuel oxidation in muscle leading to incomplete fatty

acid oxidation and development of insulin resistance, which corroborates with previous findings in humans from the same group (Koves et al. 2008) This suggestion is difficult to reconcile with the data outlined above suggesting only a small fraction of BCAA carbon enters the TCA cycle (Goldberg 1978; Wagenmakers, Salden, and Veerkamp 1985). Whilst these studies did not demonstrate a causal role of amino acids and their catabolites in inducing skeletal muscle insulin resistance, their results did suggest for the first time that aberrant BCAA metabolism may either contribute to or result from the development of insulin resistance.

1.4.4.1 Impaired BCAA catabolism in animal models of obesity

One consistent finding from rodent models of obesity is that, through a yet unidentified mechanism, obesity results in down regulated transcription of BCAA catabolic genes in adipose tissue and the liver (She et al. 2007; Zhou et al. 2019) as well as increased phosphorylation of the BCKDH enzyme in these tissues (She et al. 2007; Lackey et al. 2013) and this was associated with elevated plasma BCAA concentrations (She, Horn, et al. 2007). Indeed, the modulation of adipose tissue through adipose tissue transplantation of healthy adipose tissue into mice lacking the enzyme BCAT, reduced circulating BCAA concentrations (Herman et al. 2010). However, when assessing BCAA flux across the subcutaneous adipose tissue in lean, obese insulin sensitive and obese insulin resistance humans, there were no differences in uptake or release (Lackey et al. 2013). These findings questioned the significance of adipose tissue to human BCAA metabolism. The metabolic consequences of reduced BCAA catabolism in those tissues seems unclear but some have suggested that it results in shift in BCAA

catabolism to the muscle where they compete with fatty acids for oxidation (White et al. 2016).

More recently another study has demonstrated a relationship between skeletal muscle BCAA and fat oxidation. Using a transcriptomics approach, it was shown that skeletal muscle of insulin resistant and T2D individuals had lower mRNA expression (activity was not measured) of BCAA catabolic enzymes (mut and ALDH6A1, see figure 1.7b) (Lerin et al. 2016). Interestingly high fat feeding of rodents for 4 months or rendering mice inactive through denervation also resulted in similar reductions in BCAA catabolic gene expression (Lerin et al. 2016). Untargeted metabolomics analysis revealed reduced muscle BCKA content despite similar BCAA levels in insulin resistant individuals compared to insulin sensitive controls. This could in part be explained by reduced levels of the nitrogen accepter (αKG) possibly reflecting reduced transamination. However, there was also increased relative expression of C_5 acylcarnitine which would suggest increased catabolic flux. Given that these data were relative expression of metabolites with no quantification it is difficult to assess what scenario this represents (i.e increased or decreased catabolic flux). Heterogenous gene deletion of the enzyme mutase (mut), which converts methylmalonyl-CoA to succinyl-CoA (that can be incorporated into the TCA cycle or enter complex II of the electron transport chain), in mice resulted in weight gain and impaired glucose tolerance. The authors attributed this effect to reduced fatty acid oxidation, which was based on increased relative expression of acylcarnitines in muscle and elevated TAG content in muscle. Thus, reduced BCAA flux resulted in ‘incomplete’ fatty acid oxidation, resulting in the accumulation of lipid in muscle and probably caused insulin

resistance. There are several limitations to this work, firstly the heterogenous knock out model could have had several offsite effects on whole body metabolism and no quantitative assessment of fatty acid oxidation was made. Other reports have also shown a reduced mRNA expression of BCAA catabolic enzymes in muscle of patients with T2D together with increased plasma BCAA and BCKA concentrations (Hernández-Alvarez et al. 2017). These findings were reproduced in a mouse model of T2D (using the db/db mouse) suggesting that T2D is state of reduced muscle BCAA catabolism and oxidation although no assessment of BCAA complete oxidation were made. However it has subsequently been shown with the use of stable isotope tracers and direct measures of tissue oxidative flux that the db/db mouse does in fact have increased muscle BCAA oxidation (Neinast et al. 2018). Furthermore, this same report has shown that post translation modifications (i.e the phosphorylation status) of the BCKDH enzyme, considered the rate limiting step in BCAA oxidation, does not correlate with tissue oxidation. These findings question the extent at which mRNA expression reflects tissue oxidation given that post translation modification does not appear to. These are serious limitations of the work outlined in the former reports that have not been considered.

Collectively, the data from animal and untargeted metabolomics, and assumptions from mRNA expression data from human works appears to suggest that obesity and insulin resistance are a state of impaired BCAA catabolism. Thus, it has been suggested that strategies to increase BCAA catabolism are warranted to as a treatment for obesity (Zhou et al. 2019). Furthermore, animal studies have shown that both excessive and impaired

BCAA oxidation in muscle can lead to reduced fatty acid oxidation. The differences appear to be a result of different analytical methods used for assessment and static measures with untargeted analysis (and hence no information on actual concentration) and gene expression data. Furthermore, these studies have failed to address the question, if BCAA catabolism is impaired in muscle then what is the reason for the increased BCAA catabolites in muscle of insulin resistant individuals. Therefore, a more likely explanation for those findings is that there may be a mismatch within the mitochondria between catabolism and complete oxidation which may be in a feedback mechanism be inhibiting further flux in the BCAA catabolism pathway. To address this, quantitative assessments of this pathway to give an indication of concentrations are required.

1.4.5 Evidence from human studies

Direct evidence from human studies appears to contradict the animal work. Reports that have investigated human BCAA metabolism appear to confirm the earliest observations that pre-existing insulin resistance leads to perturbed BCAA metabolism (Piro et al. 2020; Tan et al. 2020). In fact, since the earlier associative reports linking BCAA to the development of T2D (Wang et al. 2011), more recent mendelian genome wide association reports have demonstrated that higher BCAA levels do not have a causal effect on insulin resistance, rather insulin resistance likely drives higher fasting BCAAs (Wang et al. 2017; Mahendran et al. 2017).

Targeted profiling of visceral adipose tissue in humans has shown a down regulation of the three BCAA levels in the visceral adipose tissue of patients with the metabolic syndrome and otherwise healthy obese subjects compared

to healthy lean controls together with an up regulation of their metabolic derivatives, the short chain acyl-carnitines (C_3-C_5) (Piro et al. 2020). In addition, elevated levels of kynurenine, a catabolic product of tryptophan was also seen to be elevated, adding further support for increased not impaired catabolism in human adipose tissue. These findings appear to be in contradiction with previous studies where a lower expression of BCAA catabolic enzymes has been observed (She, Horn, et al. 2007).

Another recent study investigated whole body leucine flux, using stable isotope tracers (U-$^{13}C_6$) leucine and concentrations of circulating BCAAs, BCKAs and short chain acylcarnitines in morbidly obese humans before and after gastric bypass surgery and directly tested the hypothesis from animal work that BCAA catabolism is impaired with obesity and insulin resistance (Tan et al. 2020). In contrast to animal reports, an elevation in whole body leucine flux (a measure of leucine transamination to KIC), leucine oxidation (a measure of $^{13}CO_2$ production) and non-oxidative disposal of leucine (a measure of protein synthesis) were all elevated in insulin resistance morbidly obese subjects. Furthermore, these observations were also accompanied by increased plasma BCAA, BCKA and short chain acylcarnitines, demonstrating that elevations of the metabolites can reflect accelerated BCAA catabolism and not just impaired BCAA catabolism as previous studies which have only measured relative expression of enzymes and non-quantitative concentrations of BCAA related metabolites have suggested (Lerin et al. 2016; Zhou et al. 2019). Following gastric bypass surgery and an increase in insulin sensitivity, the elevations in leucine flux and plasma BCAA profiles all significantly

reduced providing further evidence that impaired BCAA metabolism is a consequence of the insulin resistant state.

Another question that remains to be addressed is what are the consequences of elevated short chain acylcarnitines in humans? Although a number of studies have assumed that skeletal muscle insulin resistance may be a consequence of reduced BCAA catabolism in other tissues, which effectively burdens skeletal muscle with excess BCAAs, there are very few studies that have investigated human skeletal muscle BCAA metabolism in this context. One study from this group did show that in response to lipid infusion during a hyperinsulinaemic clamp, short chain BCAA derived acylcarnitines (C_3-C_5) were elevated in muscle when compared to saline control (Stephens et al. 2014). These acylcarnitine species were negatively correlated with glucose disposal and muscle glycogen accumulation during the clamp. Given that high levels of insulin (during the clamp) are expected to supress protein catabolism (Tessari et al. 1986), these findings provided a novel scenario in which lipid induced insulin resistance (through high lipid oxidation under clamp conditions) may directly impact upon BCAA catabolism, increasing oxidation which could exacerbate insulin resistance. In support of this, it has been shown the valine catabolite 3-HIB is elevated in muscle of patients with T2D (Jang et al. 2016). Furthermore, in cultured myotubes it was shown that this metabolite can leave the muscle cell and drive endothelial transport of fatty acids into the muscle in a paracrine fashion (Jang et al. 2016). Therefore one proposed model from these studies is that lipid overspill into muscle resulting in insulin resistance, increases BCAA catabolism (Stephens et al. 2014). Indeed postprandial lipid supply to muscle is increased in insulin resistant individuals

(van Hees et al. 2011). In turn, excessive BCAA catabolism in muscle may in turn lead to further influx of fatty acid into the muscle (Jang et al. 2016), potentially leading to a vicious cycle whereby insulin resistance promotes BCAA catabolism in muscle which in turn worsens insulin resistance. However, mechanistic data to support this concept is lacking and there has been a lack of experimental evidence on alterations in muscle *per se* as primary cite of perturbed BCAA metabolism.

The lack of quantitative assessment of BCAAs, related catabolites and fatty acid oxidation in human muscle and plasma has precluded the assessment of how these pathways are interacting. Furthermore, there appears to be direct contradictions between rodent and human work. Given that the more robust integrative physiology reports suggest that insulin resistance drives excessive BCAA catabolism. This is an important area of research that has been under investigated thus far. One way to address the issues outline above is to develop targeted metabolomic methods to assess tissue concentrations of metabolites. The premise of metabolomics is discussed in the next section below. Moreover, much of the work in this area has been limited to the fasting state, if BCAAs or any related catabolite was truly causative of insulin resistance and not simply just a biomarker then the well documented elevations in these metabolites should persist in the insulin stimulated state as opposed to being suppressed. However, how the muscle profiles of these metabolites change in the face of insulin (across varying degrees of insulin sensitivity) has not been assessed thus far.

1.5 Metabolomics

1.5.1 Metabolomics and the metabolome

Metabolomics, which is the profiling of metabolites in biofluids, cells and tissues, is routinely applied as a tool for biomarker discovery (Nicholson, Lindon, and Holmes 1999). Perhaps more importantly, the inherent sensitivity of metabolomics enables subtle alterations in biological pathways to be detected to provide insight into the mechanisms that underlie various physiological conditions and aberrant processes, including diseases. Metabolites can be defined as small molecular weight chemicals involved in metabolism (such as glucose and ethanol). Metabolites are the substrates and products of metabolism that drive essential cellular functions, such as energy production and storage, signal transduction and apoptosis. Collectively, the total number of metabolites in a sample is referred to as the metabolome.

1.5.2 Use of metabolomics for chronic diseases.

In the case of many chronic metabolic diseases, the condition may well be present or in progression for years before they become clinically apparent. For example, by the time relative insulin deficiency manifests as hyperglycaemia and a diagnosis of type 2 diabetes is made, considerable pancreatic beta cell insufficiency has already occurred (Tabák et al. 2009). Whilst clinical and laboratory-based predictors such as glucose tolerance tests may aid in identifying disease risk, they must often be performed in close proximity to the onset of overt disease and may provide little insight regarding the underlying mechanisms. For diseases such as chronic kidney disease (CKD), which is defined as the presence of kidney damage for at least 3 months, independent

of the underlying chronic renal disease, current diagnostic methods have numerous limitations (Hocher and Adamski 2017). In CKD current diagnostic methods such as urinary albumin excretion and/or estimated glomerular filtration rate (GFR) are also used to determine the clinical stage of CKD and inform decisions on subsequent treatment. However, estimates of GFR that are based on creatinine are influenced by biological confounders such as age, sex and in particular muscle mass. As creatinine is produced by muscle metabolism, changes in muscle mass can result in false estimates of GFR (Ferguson et al. 2015).

The identification of novel biomarkers may negate the requirement for invasive procedures such as tissue biopsy, which may have many associated complications. Furthermore, metabolites may have unanticipated roles as regulatory signals with hormone-like functions or effectors of the disease process itself. Therefore, metabolite concentrations could presage the onset of overt disease and therefore aid in the identification of at-risk individuals by adding information over standard clinical markers, which in the case of some diseases may not be reliable. Once biomarkers have been identified, the next steps involve validation of the biomarkers using targeted approaches to provide absolute quantification of metabolites in samples. Metabolomics analysis works very well with many types of sample of human or animal origin, including body fluids (urine, serum, plasma, dried blood spots, saliva, cerebrospinal, lung lavage or seminal fluid), tissue (including biopsy samples), stool, cell culture or exhaled breath.

1.5.3 Qualitative and Quantitative metabolomics

The two orthogonal approaches used in metabolomics are targeted and non-targeted metabolomics.

Untargeted metabolomics

Untargeted metabolomic methods are global in scope and have the aim to simultaneously measure as many metabolites as possible from biological samples without bias. Untargeted analyses are most effective when implemented in a high-resolution mass spectrometer, to facilitate structural characterization of the metabolites (i.e. which metabolite family they belong to and any biochemical isomers) Its unbiased nature allows it to examine the relationship between interconnected metabolites from multiple pathways.

Targeted metabolomics

Targeted metabolomic analyses measure the concentrations of a predefined set of metabolites. A standard curve for a concentration range of the metabolite of interest is prepared, so that accurate quantification can be gained. This type of analysis can be used to obtain exact concentrations of metabolites identified by untargeted metabolomics, providing analytical validation (Roberts et al. 2012).

1.5.4 Overview of metabolomics work flow

The main methodologies that are used for untargeted and targeted metabolite recovery and identification are mass spectrometry-based metabolomics. Mass spectrometry (MS) can be defined as an analytical method by which ionised molecules are detected according to their mass-to-charge (m/z). MS can be combined with a chromatography method, as thousands of ions can be

present in metabolomic experiments. Chromatographic separation before entering the mass spectrometer minimizes signal suppression and allows for greater sensitivity.

1.5.4.1 Sample extraction

The first step in any metabolomics involves metabolite extraction from the sample. In general, metabolites of interest are extracted by liquid extraction with one solvent, aqueous or organic, or with a combination of solvents (Lees, and Sloane 1953) Sample preparation and sample introduction methods for the analysis of biological samples can include, but are not limited to, direct injection, liquid-liquid extraction (LLE), solid-phase extraction (SPE), supercritical fluid extraction, accelerated solvent extraction, microwave-assisted extraction, protein precipitation, and membrane methods, such as dialysis or ultracentrifugation. Each method has its own relative merits and is appropriate for certain metabolites (Dettmer, Aronov, and Hammock 2007)

1.5.4.2 Sample injection & chromatography

As stated above, MS systems are often coupled with a chromatography approach to enable the separation of metabolites in the sample matrix. Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase (which can be either gas or liquid). The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system,

while others pass rapidly into mobile phase, and leave the system faster (Coskun 2016)

The main chromatography approaches utilise either liquid chromatography (LC) or gas chromatography (GC). Liquid chromatography followed by MS (LC/MS) enables the detection of the most metabolites and has therefore been the technique of choice for global metabolite profiling work. Analysis of a wide range of metabolites, ranging from high to low molecular weight, and from hydrophilic to hydrophobic, can be performed by selecting the appropriate column and mobile phases (Putri et al. 2013)

During chromatography, aliquots of separated metabolites are applied to ionization chambers; only ionized metabolites will be analysed. The MS provides information on mass/charge ratio (m/z), which is used to calculate the mass of individual metabolites. Reversed phase chromatography is a standard tool for the separation of medium polar and nonpolar analytes, whereas hydrophilic interaction chromatography (HILIC) is used for the analysis of highly polar compounds.

1.5.4.3 Analyte ionisation

After chromatographic separation, analytes are ionised before MS analysis but the efficacy of this process differs between metabolites and some might not be suitable for analysis in the mass spectrometer (Baldwin 2005) The most often used ionization methods in the field of metabolomics are electrospray ionization (ESI) and electron impact (EI) ionization. ESI is the favourite ionization technique for HPLC-MS for multiple reasons. It adequately ionizes molecules in the liquid phase and can universally be used for small molecules

(<1,000 amu) as well as for large molecules such as peptides and proteins. Moreover, ESI is a soft ionization technique, so it does not induce a significant fragmentation of the molecular ions. A drawback in using ESI is that its ionization efficiency is negatively affected by the presence of salts, so the chromatography methods are limited to the use of only volatile buffers such as ammonium acetate or ammonium formate. In addition, ion suppression can occur when co-eluting metabolites compete for a limited number of molecular ions with low electron or when proton affinity metabolites are obscured, or not detected at all. EI is the ionization method of choice for GC-MS based analysis. EI is a hard ionization method as it causes fragmentation of metabolites and it enables detection with minimal matrix effects due to co-eluting metabolites (Nagana Gowda and Djukovic 2014). In order to obtain a broad coverage of the metabolome, ionization must be performed in positive and negative mode. Depending on the mass analyser used, detection in positive and negative mode can be performed simultaneously in a single run (Idborg-Björkman et al. 2003) thus, reducing the time needed for analysis and eliminating possible variability due to injection errors.

1.5.4.4 Mass spectrometry

Metabolite detection with high resolution and sensitivity is generally desired. However, achieving both goals in a single MS detection mode is challenging because as a general rule higher sensitivity leads to lower resolution and vice versa. There are various options including single (MS) or tandem (MS/MS) mass analyzers to choose from, each of which has different sensitivity and resolution performance. The single-configuration mass analyzers include the quadrupole (Q), linear ion trap (LIT), quadrupole ion trap (QIT), time of flight

(TOF), Fourier transform ion cyclotron resonance (FTICR), and Orbitrap. Quadrupole and ion trap analyzers offer high sensitivity, but limited resolution whereas TOF, FTICR, and Orbitrap offer high mass resolution. Mass analyzers arranged in a tandem configuration include triple-quadrupole ion trap (QTrap), triple quadrupole (TQ), quadrupole-TOF (Q-TOF), and linear-quadrupole ion trap-Orbitrap (LTQ-Orbitrap). Because of their high sensitivity and selectivity, TQ and QTrap analyzers are the most common MS spectrometers hyphenated to LC and employed in targeted metabolic studies, while Q-TOF, LTQ-Orbitrap, and FTICR analyzers are more suitable for global profiling and metabolite identification (including isotopomer analysis) due to their higher mass-resolving power. An overview of the metabolomics workflow with and an example of triple quadrupole MS is given in Figure 1.9.

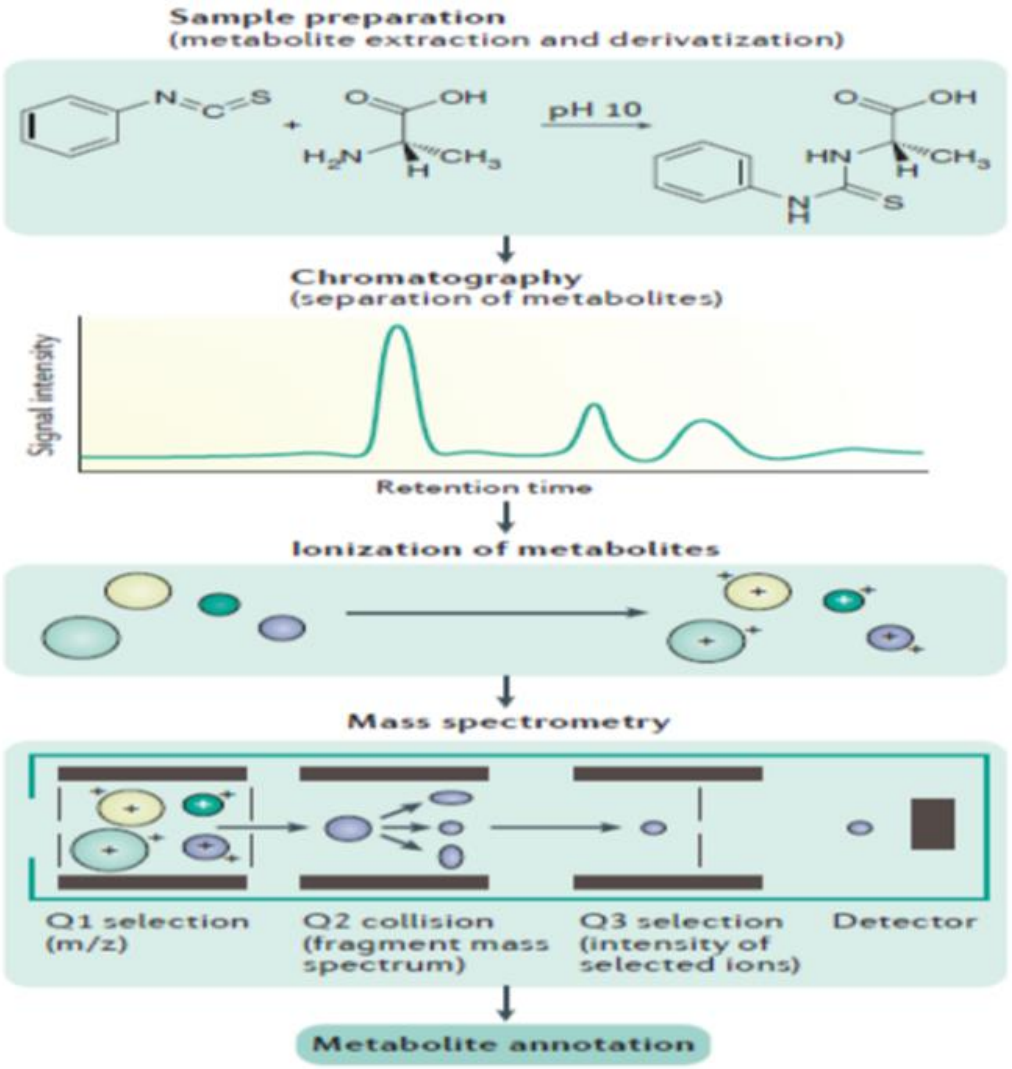


Figure 1.9 Metabolites have to be extracted from the sample (for example using organic solvents) to facilitate further analytics. Some metabolites such as amino acids require derivatization steps to increase the mobility of their ions before MS. To increase resolution and minimize matrix effects, metabolites are separated using high performance liquid or gas chromatography. The individual peaks observed during chromatography may contain several metabolites. During chromatography, aliquots of separated metabolites are applied to ionization chambers; only ionized metabolites will be analysed later in the process. The MS provides information on mass/charge ratio (m/z), which is used to calculate the mass of individual metabolites. The mass spectrometer can be constructed in various ways; the schematic depicts a tandem mass spectrometer run in targeted mode. All parts of the mass spectrometer operate under a vacuum (depicted by the green line). Ionized molecules are pre-selected by m/z ratio in the first quadrupole (Q1), which consists of four metal rods (black bars, for clarity only two rods are shown) arranged in parallel around one axis. In the second quadrupole (Q2) molecules released sequentially from Q1 are fragmented by collision with neutral gas. Each molecule fragments into a characteristic pattern, which can be used to unequivocally identify the molecule. To quantify the molecule of interest its fragment is selected in quadrupole 3 (Q3) and quantified in a detector. This procedure is repeated for every molecule of interest. Metabolite annotation is based on molecular mass, validation by fragmentation spectra and retention time during chromatography. Figure taken from (Hocher and Adamski 2017).

1.5.5 Challenges associated with metabolite quantification in biological samples

A commonly cited challenge of quantifying tissue metabolites is the lack of an appropriate 'blank' which can mirror the complexity of the matrix. This is important as the potential of 'matrix effects' to enhance or suppress analyte signal can lead to inaccurate quantification. Two analytical approaches are generally used to produce standard curves for metabolite quantification, the method of standard addition or using a metabolite free representative proxy (or surrogate) matrix in the traditional standard addition approach, a known quantity of analyte is added directly to aliquots of the matrix of interest and the signal response to increasing amounts of analyte is measured. This approach is important as coeluting compounds in the biological matrix, which may not be present in the proxy matrix, may be a source of signal suppression or enhancement and thus may lead to erroneous calculations of analyte concentrations in biological samples (Andersen 2017). Whilst it is important to determine these matrix effects, the use of the method of standard addition approach has certain limitations and may be problematic for calibration purposes. For example, the BCAAs have endogenous concentrations in the µM range and free carnitine has an endogenous intramuscular concentration in the mmol/kg (dry mass). Therefore, the calibration standards at the lowest points on the calibration curve may not result in detectable responses above the high endogenous baseline such as when spiking nM calibrants upon a high µM background. In addition, the higher points on the calibration curve may lead to detector saturation and therefore the calibration is no longer in the linear range. These disadvantages can be overcome by using a proxy matrix

and isotopically labelled standards which a structurally the same as the analytes of interest (Stokvis, Rosing, and Beijnen 2005). Aqueous solutions of bovine serum albumin (BSA) have previously been used as substitutes for biological matrices such as plasma (Minkler et al. 2015) and liver (Xu et al. 2020). Access to tissues of interest, lack of internal standards and analytical instruments is likely to be the reason there is dearth in quantitative assessment of metabolites relating to BCAA catabolism in human muscle.

1.6 Aims and objectives

The first aim of this thesis was to develop an LC-MS/MS method for the quantitative assessment of acylcarnitines, BCAAs and related metabolites in human skeletal muscle and plasma samples. The commonly cited challenges with targeted metabolomics in biological samples were addressed using both the method of standard addition within the biological matrix and a substitute matrix using isotopically labelled internal standards.

The second aim of this thesis was to provide quantitative assessments of muscle and plasma BCAA and acylcarnitine concentrations in patients with T2D. Simultaneously profiling muscle and plasma enabled the determination of the extent plasma profiles are reflective of tissue profiles

The third aim of this thesis was to extend upon much of the current data which is limited to the fasted state by determining how the fasting muscle concentration of acylcarnitines, BCAAs and related metabolites change with age and insulin resistance in response to insulin infusion.

To date, no studies have quantitively assessed BCAA catabolites together with markers of fatty acid oxidation in both plasma and muscle of obese subjects and patients with T2D. In chapter 6, the potential interactions of fatty acid and BCAA metabolism were explored with the use of an isotopically labelled fatty acid drink and the application of the analytical method. The impact of age with T2D was also investigated.

2 Chapter 2 General methods

2.1 Human volunteers

2.1.1 Ethical approval

All studies and sample collection protocols were approved by the University of Nottingham Faculty of Medicine & Health Sciences Research Ethics and the NHS Research Ethics Committees and met the regulations outlined in the Declaration of Helsinki. All studies were performed at the David Greenfield Human Physiology Unit, University of Nottingham.

In Chapter 3, muscle and plasma samples used for method development and assessment of method suitability in biological samples were obtained from a study assessing the effects of time restricted feeding in young healthy males (Ethics approval Ref. Number. 19–1705). The study was also registered at clinicaltrials.gov as NCT03969745.

In Chapter 4, samples used were from two separate studies, the first investigating the acute effect of feeding on muscle protein synthesis (Ethics approval Ref. Number 14/EM/0136) and the second was a clinical trial involving patients with Type 2 diabetes (Ethics Ref. No 14/EM/0136 and ClinicalTrials.gov Identifier: NCT02197299). Diabetics patients recruited as a part of the same clinical trial study were also studied in Chapter 6.

In Chapter 5, samples were from a larger study investigating the skeletal muscle lipid metabolism and insulin sensitivity in young and elderly subject (Ethics approval Ref. Number E13102011BMS).

2.1.2 General screening for healthy volunteers

Volunteers were medically screened and completed a health questionnaire, blood screen of full blood count, urea and electrolytes, liver function tests coagulation, lipid profile, thyroid function test and electrocardiograph (ECG). Subjects were excluded if they had a history of diabetes, cardiovascular, metabolic or respiratory disease. Those who did not meet inclusion criteria were excluded from further participation.

2.1.3 Patient recruitment

To achieve the recruitment targets 25 GP surgeries in Nottingham City, Nottinghamshire County, and Derbyshire Count were contacted, who provided a patient list. Subjects were contacted (by mail) if they were male patients aged 18-65 years old, with type 2 diabetes that were not taking insulin or sulfonylureas. Those who met the initial criteria were invited for a general health screening as described above and excluded based on the following exclusion criteria.

- Malignancy (excluding localised basal and squamous cell skin cancer)
- Metabolic diseases (stable treated hypothyroidism allowed)
- Active cardiovascular disease (current angina, myocardial infarct, or coronary artery surgery/angioplasty within 12 months)
- Primary muscle disorders
- Cerebrovascular disease
- Neurological disease e.g. epilepsy, Parkinsons disease
- Active respiratory disease
- Active gastrointestinal or liver disease
- Renal impairment (eGFR <60 ml/min)
- Clotting dysfunction
- Anti-diabetes medication other than metformin
- Other medications that may affect glucose tolerance (e.g. corticosteroids), muscle metabolism, or safety (e.g. anticoagulants)
- Any other conditions in addition to the above that the investigators consider may affect study measurements or safety

- Abnormalities on screening blood tests that in the view of the investigators are clinically significant

All participants provided written informed consent. The Informed Consent Form was signed and dated by the participant before they entered the trial.

2.2 Assessment of insulin sensitivity and body composition

2.2.1 Hyperinsulineamic- euglycaemic clamp

The hyperinsulineamic-euglycaemic glucose clamp technique, originally developed by DeFronzo and colleagues (R. A. DeFronzo, Tobin, and Andres 1979b), is widely accepted as the gold standard method of directly determining metabolic insulin sensitivity *in vivo*, particularly when the assessment of insulin sensitivity *per se* is of primary interest and feasibility is not an issue. After an overnight fast, insulin is infused intravenously at a constant rate (dose per body surface area per minute). As a result of the constant infusion, a new steady-state insulin level that is above the fasting level (hyperinsulinaemia) is achieved. Consequently, glucose disposal in insulin sensitive tissues such as skeletal muscle and adipose tissue is increased, whereas hepatic glucose production (HGP) is suppressed. A bedside glucose analyser is used to frequently monitor blood glucose levels at 5 min intervals while 20% glucose is given intravenously at a variable rate to “clamp” blood glucose concentrations in the normal range (euglycaemia). This concentration is a predetermined level of glycaemia within normal range of glycaemia; a fasting level of 4.5mmol/L was chosen for chapter 5. Because 80–90% of the infused glucose is taken up by skeletal muscle under conditions of euglycaemic

hyperinsulinaemia, insulin sensitivity measured with the insulin clamp technique primarily reflects skeletal muscle (Eleuterio Ferrannini et al. 1988). After ~2 hours of constant insulin infusion, steady-state conditions can typically be achieved for plasma insulin, blood glucose, and the glucose infusion rate (GIR). Assuming that the hyperinsulinaemic state is sufficient to completely suppress HGP, and since there is no net change in blood glucose concentrations under steady-state clamp conditions, the GIR must be equal to the glucose disposal rate (M). Thus, whole body glucose disposal at a given level of hyperinsulinaemia can be determined directly. M is typically normalized to body weight or fat-free mass to generate an estimate of insulin sensitivity. The validity of insulin sensitivity utilising the glucose clamp technique depends on steady state conditions being achieved. Therefore, the values for glucose disposal rates were taken during a period of at least 30 minutes to more than 1-hour post insulin start during which the coefficient of variation for blood glucose, plasma insulin and GIR is < 5%. The contribution of HGP can also be directly assessed (allowing appropriate adjustments to M) with the use of labelled glucose tracers.

There are some limitations of the hyperinsulineamic-euglycaemic clamp technique that should be considered. The main limitations of this approach are that it is time consuming, labour intensive, expensive and requires an experienced operator to manage the technical difficulties. Thus, for epidemiological studies, large clinical investigations, or routine clinical applications the glucose clamp is not appropriate. In addition, if measuring insulin sensitivity/resistance is not a primary study outcome, then the cost/benefit ratio for the clamp may not be favourable. Another limitation is that

the clamp utilizes steady-state insulin levels that may be supraphysiological. This results in a reversal of the normal portal to peripheral insulin gradient. Thus, the glucose clamp may not accurately reflect insulin action and glucose kinetics sunder physiological conditions that a dynamic test such as an oral meal or oral glucose load may determine (Muniyappa et al. 2008)

2.2.2 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) used in chapter 6 is a simple test widely used in clinical practice to diagnose glucose intolerance and T2D. In the overnight fasted state, a standard oral glucose load (75g) is consumed and blood sampling for blood glucose and insulin are measure at (15-30 min) intervals for 120 mins (Man et al. 2005). The OGTT reflects the efficiency of the body to dispose of glucose after an oral glucose load or meal. During an OGTT, significant (~30–40%) amounts of glucose are taken up by the splanchnic bed (liver), and HGP is less completely suppressed than during the hyperinsulinaemic- euglycaaemic clamp technique. As a result, the plasma glucose concentration during OGTT is affected by both hepatic and peripheral (primarily muscle) insulin resistance. Although the OGTT reflects glucose tolerance rather than insulin resistance *per se*, it has the advantage of being representative of the normal physiological response to a glucose load.

2.2.3 Homeostasis model assessment of insulin resistance

The homeostasis model assessment of insulin resistance (HOMA-IR) is a simplistic model of the interaction between glucose and insulin dynamics which is underpinned by the assumption that an increase in fasting blood

glucose will result in a corresponding increase in insulin (Muniyappa et al. 2008). The HOMA-IR is calculated as follows:

$$HOMA - IR = \frac{I_0 \times G_0}{22.5}$$

Where I_0 is equal to fasting insulin concentration (μU/ml), G_0 is equal to fasting glucose (mmoll/L). The denominator of 22.5 is a normalising factor, the product of normal fasting plasma insulin of 5 μU/ml and normal fasting plasma glucose of 4.5 mmol/L typical of a “normal” healthy individua = 22.5. Therefore, for an individual with “normal” insulin sensitivity, HOMA-IR = 1.

2.2.4 Dual Energy X-ray Absorptiometry

In Chapters 5 and 6, whole-body and regional body composition was assessed by Dual Energy X-ray Absorptiometry (DEXA) scans (Lunar Prodigy DEXA; GE Medical Systems, Bedford, UK)**.** All participants filled out a questionnaire prior to each scan to assess suitability and wore light clothing free from any metallic items. Scans were undertaken with the participant motionless in a supine position with arms placed by their side and hands pronated. All regional analysis was performed using specialised software (enCORE; GE Medical Systems, Chicago, IL, USA). The scanner passes two low-dose X-ray beams through the body (40 and 70 keV). Due to the different elemental composition of fat, muscle tissue and bones, the X-rays pass through regions of the body at different rates. Regional determination of fat, lean mass and bone mineral density can then be calculated by linear decomposition of the measured X-ray beam attenuation (Pietrobelli et al. 1996). DEXA offers an accurate and convenient, non-invasive estimation of body composition. Limitations stem from the assumptions that there is a constant fractional composition of lean

tissue (water, protein and mineral content), which can vary with hydration states. To limit the influence of nutrition and hydration status, all scans were undertaken in the morning at approximately 08:00 after an overnight fast and voiding of the bladder.

2.3 Blood sampling and analysis

2.3.1 Blood collection and arterialisation

In all Chapters, volunteers were cannulated to enable regular arterialised-venous blood sampling. A sterile 12G cannula was placed retrograde into a dorsal vein on the back of the hand. Lidocaine (1%) was injected (approximately 1 ml) subcutaneously prior to cannula insertion to minimise discomfort. The cannula was kept patent by a slow running 0.9% saline infusion. To arterialise the blood, this hand was then placed in an air-warming unit set to heat the hand to 55°C and kept there for the remainder of the visit. This technique has minimal effects on core temperature and blood flow distribution to the contralateral forearm (Gallen and Macdonald 1990).

Due to the inherent risks associated with direct cannulation of an artery, the heated hand technique was used. As the skin temperature of the hand is warmed to above 37℃ there is an increased blood flow from vasodilation of the forearm muscle and opening of the arteriovenous anastomoses. Capillary beds open to increase blood flow to the skin and disperse heat, the heat-induced vasodilatation reduces blood transit time and results in arterialised-venous blood that is similar in concentrations of metabolites to arterial blood. (McGuire et al. 1976)

Arterial metabolite concentrations do not differ substantially across the body (i.e., in different arteries) whereas venous samples can differ depending on the sampling site (i.e the organ that drains into that venous compartment).

2.3.2 Blood glucose concentration

Immediately after collection, whole blood glucose concentrations were determined using the glucose oxidase method (YSI 2300 STAT PLUS Glucose and Lactate Analyser, Yellow Springs Instruments, Ohio, USA). Glucose oxidase catalyses the conversion of glucose to hydrogen peroxide (H_2O_2), which is subsequently oxidised and releases electrons. When the reaction is in dynamic equilibrium, the electron flow is linearly proportional to H_2O_2 concentration, which is determined by the initial blood glucose concentration. The YSI analyser was calibrated on the morning of each study visit using an external glucose standard of 10 mM.

2.3.3 Isolation of plasma

Blood was dispensed into a sodium heparin coated vacutainer containing egtazic acid (EGTA)-glutathione (7.5 $\mu l \cdot ml^{-1}$ of whole blood). Vacutainers were gently mixed prior to being spun at 4400 g for 10 min at 4℃ in a centrifuge. Plasma from the sodium heparin tube was transferred to tubes containing tetrahydralipostatin to inhibit lipolytic activity and stored at – 80^0C until analysis.

2.3.4 Plasma free fatty acids

In Chapter 6, plasma free fatty acids (FFA) were quantified using an enzymatic colorimetric assay (NEFA-HR (2), Wako, Osaka, JP) on a benchtop clinical chemistry analyser (ABX Pentra 400, Horiba Ltd., Kyoto, JP). In brief, FFA

present in the samples was converted to acyl-CoA which was subsequently oxidised to form hydrogen peroxide. The latter undergoes an oxidation condensation reaction catalysed by peroxidase to form a blue-purple pigment, which was quantified for absorbance at 546/660 nm. An internal standard of oleic acid was used to obtain a calibration curve, from which FFA concentrations could be derived from.

2.3.5 Isolation of serum

Blood was dispensed into a vacutainer containing clotting activation factors and left to clot at room temperature for ≥ 20 min. Vacutainers were spun at 4400 g for 10 min at 4℃, before the supernatant was transferred into an Eppendorf tube and stored at – 80^0C until analysis.

2.3.6 Serum insulin

In chapters 4 and 6, serum insulin concentrations were determined using a solid phase ^{125}I radioimmunoassay kit (Human Insulin Assay HI-14K, Merck Millipore, Burlington, MI, USA). This assay utilises ^{125}I-labelled human insulin, diluted with human insulin antiserum, to determine insulin concentrations using the double antibody technique. The unlabelled (endogenous) insulin in the sample competes with the ^{125}I-labelled insulin antigen for the limited and fixed number of binding sites on the antibody lining the test tubes. After 20 to 24 h, the test tubes were decanted, blotted, and left inverted for 30 min to dry, prior to measuring gamma radiation in a scintillation counter (COBRA II auto gamma, Canberra Packard, Melbourne, AU). Serum insulin values were determined using a standard curve of known insulin concentrations (0 to 200 $\mu U \cdot ml^{-1}$) normalised to the blank. All participants samples were measured on

the same assay and two quality control samples were included in each assay, to ensure they fit within the concentration range provided by the supplier.

2.4 Muscle analysis

2.4.1 Muscle biopsy procedure

In Chapters 3, 4 ,5 and 6, skeletal muscle samples were obtained from the vastus lateralis in the fasted state using the Bergstrom needle biopsy technique (Bergström., 1975) and immediately frozen in liquid nitrogen-cooled 2-methylbutane and subsequently stored in liquid nitrogen until analysis. In chapter 5 a vastus lateralis biopsy was also obtained immediately post a 3-hour hyperinsulineamic-euglycaemic clamp and in chapter 6, following a 2-hour OGTT. Initially, the area was cleaned with an antiseptic iodine solution prior to administering local anaesthetic (2% lidocaine) to the skin and surrounding fascia. After a short time, a small incision (<1 cm) was made with a sterile scalpel prior to insertion of a five-millimetre Bergström needle (Bignell Surgical Instruments Ltd, Sussex, UK); suction assistance was used to improve sample yield.

2.4.2 Muscle protein expression

In Chapters 5 and 6 western blotting was used to quantify target protein expression. Western blotting, also known as immunoblotting, is a well-established and routinely used analytical technique used for the detection of specific proteins and phosphorylation status of proteins in a sample of tissue extract. The principles of Western blotting relay on forming a protein to antibody complex through specific binding of a primary antibody to a membrane-bound target protein separated by its molecular weight. Detection

of the primary antibody is generally achieved through a primary antibody specific secondary antibody.

2.4.3 Protein extraction

For samples in Chapter 6, one portion of frozen wet muscle (~30 mg) was extracted in HEPES buffer (50 mM HEPES, 10% glycerol, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 150 mM NaCl, 1% Triton x-100, pH 7.5). Sodium orthovanadate and sodium fluoride were added as phosphatase inhibitors and a protease inhibitor cocktail (Cat# P8340, Sigma-Aldrich, St. Louis, MO, USA) was added on the day. Each sample was homogenised on ice for 30 sec and left on ice for 20 min prior to centrifugation at 12,000 x g for 15 min at 4℃. The supernatant was then transferred to a fresh tube, prior to quantifying total protein concentrations with the Pierce bicinchoninic acid assay

In Chapter 5 proteins were extracted using the Trizol RNA extraction method. As a result, messenger RNA (mRNA) expression and protein content could be determined from the same part of each tissue sample. The mRNA data was not analysed for any results presented in this thesis.

One 20 mg portion of wet muscle was transferred from liquid nitrogen into 800 µl of TRI reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing 200 µg of glycogen, to extract ribonucleic acid (RNA). Samples were then homogenised for approximately 30 sec using a polytron. The homogenate was incubated at room temperature for a minimum of 5 min, prior to the addition of 160 µl chloroform:isoamyl alcohol (49:1). Samples were mixed by repeated inversions for 30 sec before centrifugation at 12,000 x g for 15 min at 4℃. Subsequently, 400 µl of the aqueous phase was transferred to an Eppendorf

containing an equal volume of ice-cold isopropanol. These samples were incubated at −20℃ overnight. The mixture was then spun at 12,000 x g 4℃ for 15 mins and resulting pellet washed in 75% ethanol. This was spun at 10,000 x g for 10 mins, prior to removing the supernatant and drying the pellet using a water aspirator. Proteins were precipitated from the phenol-ethanol supernatant by adding 1.2 ml isopropanol and incubating for 10 min. Samples were then spun at 12,000 x g at 4℃ for 10 min to pellet the proteins, before washing 3 times with 0.3 M guanidine HCl in 95% ethanol. A final wash of 100% ethanol was performed, prior to spinning samples at 7500 x g for 5 min. The supernatant was discarded and pellet air dried, before resuspending in 400 µl of 8 M urea (deionised with amberlite ion exchange resin), 50 mM Tris and 4% SDS buffer. To aid with resuspension, samples were heated to 35℃ and pipetted up and down. Next, samples were sonicated on ice for 3 x 10 sec at 20 kHz, before a final spin at 10,000 x g for 10 min to isolate soluble proteins. The supernatant was transferred to a fresh tube prior to quantifying total protein concentrations with the Pierce bicinchoninic acid assay.

2.4.4 Protein quantification

The Pierce bicinchoninic acid assay (Thermo Fisher Scientific) was used to quantify total protein concentrations against a range (0 to 2000 $\mu g \cdot ml^{-1}$) of albumin concentrations. Typically, samples were diluted 1 in 10 with water before loading and the standards were diluted in the same buffer used for the respective extraction. All samples were measured in duplicate and the plate was incubated for 30 min at 37℃ before quantifying absorbance on a spectrophotometer (Spectra Max 190, Molecular Devices) at 562 nm. From this a standard curve was derived and the diluted concentrations of the

samples were calculated. To ensure equal protein content, all samples were diluted to a uniform concentration with the appropriate extraction buffer and then mixed with 4 x SDS loading buffer (containing bromophenol blue). This mixture was heated for 5 min at 95℃ to fully denature proteins, before being left to cool at room temperature. Dilution and denaturing of proteins were performed on the day of the analysis.

2.4.5 Electrophoresis/SDS-PAGE and Western blotting

Gels were hand cast on the day at either a fixed % of acrylamide or a gradient (5 to 20%) depending on the molecular weight(s) of the proteins being probed for. A standard 4% stacking buffer was used, and plates were inserted into an electrophoresis cell (Atto, Tokyo, JP) filled with 1x Tris-Glycine-SDS running buffer. Ten µl of protein ladder (Precision Plus Protein, Bio-Rad, Hercules, CA, USA) was loaded on each gel. The gels were run at a constant amplitude of either 40 (one gel) or 80 (two gels) mA for approximately 2 h to separate proteins via electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride membrane in an electrophoresis transfer cell (Bio-Rad, Hercules, CA, USA) filled with Tris-Glycine-Methanol transfer buffer. Transfer of proteins was completed using an overnight transfer (≥ 16h) at a constant amplitude of 40 mA.

Adequate transfer of proteins was visually inspected using a ponceau-S stain, this was extensively washed out in distilled water in tris-buffered saline containing 0.1% (w/v) tween 20 (TBS-T). Membranes were then blocked, typically in 5% (w/v) skimmed milk power or bovine serum albumin (BSA) diluted in TBS-T for 1 h at room temperature. After blocking, membranes were

washed 3 x 10 min in TBS-T, before incubation with the appropriate primary antibody on a rocking table overnight at 4℃

The next day, membranes were again washed 3 x 10 min in TBS-T prior to incubation with an anti-host (of animal primary antibody was raised in) horseradish peroxidase-linked secondary antibody (Dako, Glostrup, DK) for 1 h at room temperature. A final wash step was performed prior to the addition of enhanced chemiluminescence (Amersham ECL Prime, GE Healthcare Life Sciences, Buckinghamshire, UK) solution for 5 min. Protein expression was then visualised in a dark room using X-ray film (Hyperfilm, Amersham Biosciences, Buckinghamshire, UK) for a variety of different exposure times. Films were then digitally scanned and intensity of band(s) quantified using 2D densitometry software (AIDA Image Analyser). Proteins of interest were normalised against an endogenous loading control of α-actin expression. The exact dilutions of primary and secondary antibodies and blocking solutions is provided in the appropriate method(s) section in the experimental chapters (5 and 6).

2.5 BCAAs, BCKAs, free carnitine and acylcarnitines in muscle and plasma

Muscle and plasma samples were collected and stored as described above. All details including sample preparation, metabolite extraction and quantification using liquid chromatography coupled to high resolution mass spectrometry are described in full in Chapter 3 below.

3 Chapter 3 Development and validation of a liquid chromatography coupled to high resolution mass spectrometry method for quantification of acylcarnitines, branched chain amino and keto acids in human skeletal muscle and plasma.

3.1 Introduction

3.1.1 Overview of acylcarnitine metabolism

Acylcarnitines are intermediary metabolites formed during fatty acid, carbohydrate and amino acid metabolism from the esterification of free carnitine to acyl-CoA species. Acylcarnitines species can vary in their chain length according to the different acyl groups attached, often characterised by short medium and long chain acylcarnitines.

Long chain acylcarnitines are formed via the action of carnitine palmitoyltransferase 1 (CPT1) which facilitates the translocation of cytosolic long-chain fatty acyl-CoA groups into the mitochondrial matrix for β-oxidation (Fritz and Yue, 1963). Once inside the mitochondrial matrix, acylcarnitines are transesterified back to free carnitine and long-chain acyl-CoA in a reaction catalysed by carnitine palmitoyltransferase 2 (CPT2). The intramitochondrial long chain acyl CoA can then undergo β-oxidation; in a series of enzymatic steps, the long chain acyl-CoA is cleaved by 2 carbons to ultimately form the 2-carbon compound Acetyl- CoA, which can enter the tricarboxylic acid (TCA) cycle for complete oxidation. The corresponding medium and short chain acylcarnitine esters can be formed during each step in the β-oxidation pathway.

Acetylcarnitine, the shortest acylcarnitine species (formed directly from Acetyl-CoA) can be formed from both carbohydrate and fatty acid metabolism. Under conditions of high glycolytic flux (such as high intensity exercise) Acetyl-CoA formation from pyruvate oxidation, catalysed by the pyruvate dehydrogenase complex (PDC), is in excess of its utilization by the TCA cycle (i.e., its rate of

condensation with oxaloacetate is less than its rate of of formation) leading to its subsequent accumulation. Free carnitine thereby fulfils a key role as an Acetyl group buffer to maintain a viable free muscle CoA pool.(Stephens, Constantin-Teodosiu, and Greenhaff 2007)

It is know well known that acylcarnitines are exported from the mitochondria into the cytosol (Lysiak-Szydlowska et al. 1986) and ultimately into the plasma. The efflux of acylcarnitine may be important to prevent the accumulation of toxic CoA products within the mitochondria in states of elevated or impaired fatty acid oxidation. Indeed, the profiling of plasma acylcarnitines has been routinely performed for the screening and diagnosis of genetic disorders of fatty acid oxidation, organic acid related diseases and renal failure (Rinaldo, Cowan and Matern, 2008).

Acylcarnitines have also been identified as potential biomarkers in a number of chronic metabolic diseases involving derangement in energy metabolism, including type 2 diabetes (T2D) (Sun et al. 2016). It has been suggested that elevations in plasma acylcarnitines arise due to a mismatch between lipid supply and oxidative capacity in skeletal muscle resulting in their export and accumulation in plasma (Mihalik et al. 2010; Adams et al. 2009). It is thought that this oversupply of lipid leads to mitochondrial overload and stress which in turn leads to skeletal muscle insulin resistance, one of the pathological hallmarks of type 2 diabetes (Koves et al. 2008). Whilst elevated plasma acylcarnitines have been observed in obese and T2D subjects, plasma acylcarnitine profiles may not accurately reflect skeletal muscle metabolism (Schooneman et al. 2014). Direct and concurrent comparisons of muscle tissue and plasma in human subjects are therefore warranted.

3.1.2 Branched chain amino acids and acylcarnitines

Several lines of emerging evidence have demonstrated a robust association between circulating branched chain amino acids (BCAAs) and the development of insulin resistance (Newgard 2012). Furthermore, plasma BCAA concentrations are a strong predictor of the future development of T2D (Wang et al. 2011). Interestingly, BCAAs and related metabolites of BCAA catabolism, short, odd chain acylcarnitines (C_3 and C_5 moieties) were shown to be more closely related to insulin resistance than any lipid related analyte as assessed by principal component analysis of over 100 metabolites in plasma of obese insulin resistant individuals compared to lean controls (Newgard et al,2009) The fact that products of BCAA catabolism as well as BCAAs *per se* have been associated with insulin resistance suggests alterations in BCAA catabolic flux is likely to be implicated in the development of the condition. Despite these strong associations, the origin of these elevated metabolites in plasma is unknown. Recent evidence has demonstrated that BCAA catabolism is impaired in skeletal muscle of insulin resistant individuals (Lerin et al. 2016) and that skeletal muscle may contribute to the circulating BCAA pool following the induction of insulin resistance in rats (David et al. 2019). These data point to an important and under investigated role of skeletal muscle BCAA metabolism and the need for further investigation in humans using quantitative assessments of metabolites involved in BCAA catabolism.

3.1.3 Previous methods for the quantitative analysis of acylcarnitines, BCAAs and BCKAs

The detection and quantification of acylcarnitines is a useful tool to investigate alterations in energy metabolism in several diseases. Accordingly, a broad spectrum of methods for the analysis of carnitine and its acyl derivatives have been used, including radioenzymatic methods, spectrophotometry and high-performance liquid chromatography (HPLC) linked to ultraviolet (UV) detectors. However, these methods have a number of draw backs including laborious and time consuming sample preparation as well as a lack of specificity and sensitivity to allow the coverage of a range of acylcarnitines (Möder, Kießling, and Löster 2005).

There are several challenges encountered in the detection of acylcarnitines and BCAAs in biological samples, particularly if these are to be quantified simultaneously with a single extraction protocol. These include, complexity of the composition of different biological matrices resulting in matrix interference of the metabolites to be quantified, a range of polarities from the polar short chains to low polar long chains species, the presence of a number of isomers of metabolites found in biological samples and limited number of commercially available standards for accurate and confident determination of identities (S. Li, Gao, and Jiang 2019; Mansour, Wei, and Danielson 2013).

The most common currently used method to overcome the challenges outlined above is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Both reversed phase (RP) and hydrophilic interaction liquid chromatography (HILIC) methods have been developed (Minkler et al. 2008;

Peng et al. 2013). These methods allow for time dependant retention and separation of a range of metabolites, (including structural isomers of certain species) coupled to the highly selective and sensitive triple quadrupole mass spectrometer for detection (Giesbertz et al. 2015; Minkler et al. 2015) More recent work has also utilised high resolution mass spectrometry to quantify a range of acylcarnitines enabling accurate quantification without the need of any complex sample preparation nor the synthesis of authentic standards (Xiang et al. 2017). In addition, some very recent work has demonstrated that BCAAs and the products of the first step of their catabolism, branched chain keto acids (BCKAs) can also be quantified using reversed phase LC-MS with some methods enabling simultaneous quantification of both BCAA and BCKA in plasma (Zhang et al. 2018.; Li et al. 2016; Sargsyan and Trchounian 2020).

A list of recent publications utilising these methodologies is shown in Table 3.1.

Table 3.1 Recent studies with validated LC-MS methods for the detection of acylcarnitines and/or BCAAs and BCKAs

Reference	**Matrix**	**Analytes measured**	**Sample preparation**	**Detection Method**
(Minkler et al. 2008)	Various biological tissues	Acylcarnitines	SPE and derivatisation	RPLC – MS/MS
(Miller et al. 2012)	Dried blood spots	Amino acids and acylcarnitines		HILIC-MS/MS
(Peng et al. 2013)	Human plasma	Acylcarnitines	Liquid extraction	HILIC-MS/MS
(Kivilompolo et al. 2013)	Human serum and rat tissue	Acylcarnitines	Liquid extraction	HILIC-MS/MS
(Minkler et al. 2015)	Rat plasma, urine and	Butyrobetaine, and Acylcarnitines	SPE and derivatisation	RPLC – MS/MS

	skeletal muscle			
(Giesbertz et al. 2015)	Rat plasma and liver	Acylcarnitines	Derivatisation to butyl esters	RPLC – MS/MS
(Xiang et al. 2017)	Rat plasma and urine	Acylcarnitines	Liquid extraction	RPLC and HILIC – MS/MS
(Zhang et al. 2018.)	Pig serum and muscle	BCKA	Liquid extraction	RPLC – MS/MS
(Li et al. 2016)	Human plasma	BCAA and BCKA	Liquid extraction	RPLC – MS/MS
(Sargsyan and Trchounian 2020)	Human plasma	BCAA and BCKA	Liquid extraction	RPLC – MS/MS

Branched chain amino acids (BCAA), branched chain keto acids (BCKA), solid phase extraction (SPE), reversed phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC).

The studies in Table 3.1 demonstrate that acylcarnitines, BCAA and BCKA can be successfully extracted and quantified in several biological matrices. Acylcarnitines have been successfully derivatised to butyl esters in previous work, but this process may result in partial hydrolysis of acylcarnitines leading to inaccurate quantification (D. W. Johnson 1999). Although these limitations have been largely overcome by the use of alternative derivatisation agents (Minkler et al. 2015) , any sample extraction procedure that involves complex protocols or steps to increase sample enrichment of chemically similar analytes (such as SPE) may result in the loss of other chemically dissimilar analytes because of its specificity. Therefore, the applicability of specific extraction methods is often limited to one type of metabolite (e.g. acylcarnitines) not allowing for simultaneous determination of upstream metabolites such as BCAAs and BCKAs. It is likely that with the correct combination of extraction solvents and LC-MS conditions that all metabolites of interest could be simultaneously quantified. The impact of matrix effects on analyte quantification can be accounted for with use of isotopically labelled internal standards with full method validation.

3.1.4 Justification of method development

Despite the comprehensive metabolite profiling and coverage with previously established methods (Giesbertz et al. 2015; Minkler et al. 2015; Xiang et al. 2017), there currently is no available method for the simultaneous quantification of a range of carnitine and its acylated species, BCAAs and BCKAs with a single extraction method in multiple human tissues from the same donors. Furthermore, despite their increasing association with insulin resistance and type 2 diabetes (Koves et al. 2008; Céline Aguer et al. 2015a),

quantitative analysis of these metabolites in skeletal muscle samples of type 2 diabetic patients have not been performed. Studies that have attempted to simultaneously investigate these metabolites have been limited to either untargeted metabolomic studies or have only been able to measure the relative concentration of these metabolites (Zhou et al. 2019).

3.1.5 Aims

Therefore, the aim of this chapter was to

- To develop and optimise a liquid chromatography coupled to high resolution mass spectrometry method using a single extraction procedure to enable the simultaneous targeted quantification of a range of acylcarnitines, BCAAs and their BCKAs in both human skeletal muscle and plasma.

3.2 Methods

3.2.1 Materials and chemicals

A range of acylcarnitine standards (C_2 - C_{16}) were purchased from Tocris (Bristol, UK).C_4 and C_5 acylcarnitine, and all isotopically labelled (deuterated d_9-C_0, d_3- C_2, d_3-C_3, d_3-C_4, d_9-C_5, d_3-C_8, d_9-C_{14} and d_3-C_{16}.) acylcarnitines, (uniformly labelled carbon -13, U- ^{13}C) BCAA and BCKA internal standards were purchased from CK isotopes, (Newtown Unthank, UK.) Unlabelled free carnitine, BCAAs and their respective BCKA standards, bovine serum albumin (BSA), potassium phosphate (KH_2PO_4), LC-MS grade formic acid and ammonium carbonate were purchased from Sigma-Aldrich (Gillingham, U.K.).LC-MS grade methanol, acetonitrile (VWR, Leicestershire, UK) and isopropanol (Fisher Scientific Ltd, Loughborough, UK), and were used throughout. All full list of standard names and details including retentions times, accurate masses and allocated analyte internal standards are given in Table 3.2.

3.2.2 Sample preparation and metabolite extraction

One portion of frozen muscle (~30mg) was freeze-dried, separated free of visible blood, fat and connective tissue. Wet muscle portions were placed into liquid nitrogen chilled Eppendorf tubes with pierced caps and placed upright in a carboard box containing liquid nitrogen. The box was then placed into the freeze dryer pre-set at -56^{o} C and sealed under vacuum at low pressure for at least 24 hours. This enables the water in the biological sample to transition from the solid phase directly to the vapour phase without passing through the liquid phase. This allows the effect of any changes in water content on analyte

concentration and sample stability to be eliminated compared to wet tissue. Furthermore, connective tissue can account for up to 40-50 percent of the sample dry weight therefore it is necessary to freeze-dry the sample before powdering in order to remove the connective tissue and accurately collect muscle tissue (Harris, Hultman, and Nordesjö 1974). Indeed, the requirement of a fine powdered tissue sample for improved acylcarnitine extraction from muscle has been reported on previously (Sun et al. 2006). The dry sample was then powdered at room temperature using a pestle and mortar. All powdered samples were weighed out on a five decimal place balance.

Analytes were extracted using modified versions of the methods described by Sun et al (Sun et al. 2006) and Stephens et al (Stephens et al. 2014). Powdered muscle samples were vigorously vortexed for 5 min following the addition of 500 µL of isopropanol/ 1 M KH_2PO_4 buffer (1:1 v: v). 50 µL of internal standard mixture (containing deuterated acylcarnitines and U- ^{13}C BCAA and BCKA) was spiked into the extraction solvent prior to the addition to muscle powder. Then, samples were vigorously vortexed for a further 5 min following the addition of 500 µL of acetonitrile and centrifuged for 20 min at 14,000 *g* at 4° C. The supernatant was removed and evaporated to dryness (~ 4 hours for tissue extractions and ~ 7 hours for standards) under vacuum centrifuge at room temperature. Dried samples were resuspended in 100 µL of methanol: water (1:1 v: v) and gently mixed for 5 min at 4° C. After centrifugation at 14,000 *g*, the supernatant was removed and stored at -80° C until analysis. Plasma samples were thawed on ice and a 100 µL aliquot was prepared and processed as described above for skeletal muscle samples.

3.2.3 Liquid chromatography coupled to high resolution mass spectrometry conditions

The analysis was performed using an Dionex UHPLC system (FisherScientific,Waltham, MA) coupled to a high-resolution orbital-trap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Waltham, MA). Two separate injections of the same sample were used for reversed phase C18 and HILIC analysis. The injection volume was 5 µL, and samples were maintained at 4 °C during the analysis.

Acylcarnitines and BCKAs were separated using an ACE PFP - C18 column (100 x 2.1 mm, 2 µm pore size, Avantor, Theale, Reading, UK) maintained at 40°C with a flow rate of 300 µL/min. The mobile phases were A: water with 0.1 % formic acid and B: methanol with 0.1% formic acid. The gradient started at 0.5% B and increased to 90% B over 13 min and followed by equilibration to give a total run time of 17 min.

A ZIC-pHILIC column (4.6 × 150 mm, 5 µm particle size, Merck Sequant, Watford, U.K.), maintained at 45 °C with a flow rate of 300 µL/min, was used for the separation of BCAAs and free carnitine. Mobile phase A was composed of 20 mM ammonium carbonate in water (pH 9.1), and mobile phase B was composed of acetonitrile. The gradient started with 20% A and increased to 95% A over 16 min. This was followed by equilibration to give a 24 min run time.

MS was performed in simultaneous ESI+ and ESI− full-scan modes with spray voltages of 3.5 (ESI+) and 2.5 kV (ESI−) and capillary voltages of 40 (ESI+) and −30 V (ESI−). In both modes, the sheath-, auxiliary-, and sweep-gas flow

rates were 40,11,2 arbitrary units, respectively, and the capillary and heater temperatures were 300 and 400 °C, respectively. Automated gain control (AGC) was targeted at 1×10^4. The isolation width of the precursor ion was set at 0.7 *(m/z*). Mass resolution was set at 70,000 from *m/z* 100 to 600.

3.2.4 Preparation of standards

Free carnitine and acylcarnitine standards were prepared at concentrations of 2 mmol/L in 100% methanol. BCAAs and BCKAs were prepared at 4 and 2 mmol/L, respectively, in methanol/water 1:1 (v: v). The stock solutions were combined and diluted to create calibration standards across an appropriate concentration range for each analyte (see results sections for ranges) using methanol/water 1:1 (v: v). Stocks and calibration standards were stored at -80^o C until use. 1 mg of each BCAA and BCKA internal standard was weighed out at on 5 decimal place balance and diluted in 1 mL methanol/water 1:1 (v: v). The standards were combined and diluted to give a final concentration in each sample of ~ 1 µmol/L. The acylcarnitine internal standards (NSK-B, CK isotopes, Newtown Unthank, UK) were dissolved in 1 mL of LC-MS grade methanol and stored in accordance with the manufacturer's guidance.

3.2.5 Method validation in the biological matrix

3.2.5.1 Use of isotopically labelled acylcarnitine standards for method validation

To account for the issues involved with quantifying analytes in tissue samples (outlined in chapter 1, section 1.5.5), the method of addition approach was used in the current work. The isotopically labelled standards (deuterated and U- ^{13}C) were used as a substitute for the authentic unlabelled standard and

spiked into equal amounts of powdered skeletal muscle to construct 10 point calibrations curves, with a triplicate at each point (as opposed to using internal standards). Several skeletal muscle biopsy samples from healthy control subjects were freeze dried and powdered (as described in section 3.2.2). Powdered muscle from each sample was subsequently mixed into one homogeneous pool using a pestle and mortar, the pool was collected, vigorously vortexed and decanted, and this process was repeated upon the addition of each powdered sample. Aliquots of this pool were used throughout the validation process. This approach was repeated for all analytes at the same concentrations in a proxy matrix (7.5 % BSA in PBS) to determine whether the assay could extract analytes equally from the two different biological matrices.

3.2.5.2 Use of unlabelled standards and isotopically labelled internal standards for matrix comparison

The use of the method of addition is essential to determine the effects of the matrix on sample processing factors such as analyte recovery, precision, accuracy and ion suppression. However, the routine use of this approach is not practical due to the large amount of muscle powder required each time for calibration and the inherent limitations described previously. Therefore, in a separate experiment both skeletal muscle and 7.5% BSA in PBS were spiked with each unlabelled standard expressed as a ratio a specific isotopically labelled standard to determine if their respective response was different between the two matrices and whether the more convenient proxy matrix of BSA was a valid substitution of human skeletal muscle as well as plasma for which it has been validated by others previously (Minkler et al. 2008, 2015).

This would determine if the internal standards could account for any ion suppression in skeletal muscle. In all cases calibration standards were analysed using the same procedure as was described for samples and the same calibrant range was spiked into each matrix. For example, a 100 µL calibrant at 1 µmol/L was spiked into BSA and extracted, dried and reconstituted into a final volume of 100 µL, representing a calibration concentration of 1 µmol/L. The equivalent calibrant spiked into ~ 1mg of muscle tissue and reconstituted in a final volume of 100 µL was then corrected by applying a dilution factor (to convert µmol/ 100 µL to µmol/L) and then divided by tissue mass in Kg to calculate the final concentration value expressed as 100 µmol/ kg dry mass (dm).

3.2.6 Validation of the proxy matrix as a substitute for human plasma

The anticipated biological range of acylcarnitines was initially established from previous publications investigating changes in human plasma concentrations in response to insulin infusion and weight loss (Mihalik et al. 2010; Schooneman et al. 2016). Based on this range of acylcarnitine concentrations, ten-point calibration standards (n=10) were analysed and compared between human control plasma and the BSA proxy matrix. Independent plasma samples from six separate healthy subjects were then analysed and the calculated concentrations from both approaches were compared. Free carnitine, BCAAs and BCKAs have a high endogenous plasma concentration in the µmol range and therefore these analytes were validated in the BSA solution using unlabelled and isotopically labelled standard peak area ratios.

3.2.7 Method validation experiments and parameters

In both of the biological matrices studied, accuracy, precision and recovery were determined at three (low, middle and high) levels with six replicates at each point. The analyte concentration at each level was based on typical concentrations reported in previously published studies demonstrating that acylcarnitines may change between two and five fold following insulin infusion (Stephens et al. 2014; Mihalik et al. 2010). Accuracy and precision criteria were satisfied where the coefficient of variance (CV) of the 6 replicates were within 15 %. Recovery was assessed by comparing the analyte response between a sample spiked pre-extraction with the response of the analyte spiked post extraction at the sample reconstitution step.

Stability of the samples during the analysis was determined by a repeat injection of a pooled QC sample stored in the autosampler at 4^{o} C over a long acquisition time (24-60 hours) and measuring the CV of internal standard ratios of the injections (within run stability). Stability of the analytes was also determined by subjecting QC samples to a single freeze-thaw cycle following freezer storage at -80^{0} C. Repeatability of the extraction assay was determined by extracting separate aliquots of a pooled powdered skeletal muscle and human plasma QC and measuring the CV of the quantified sample concentrations after correction for tissue volume. Matrix effects were determined by comparing the response of the analyte added at the post extraction point with the response of a standard directly injected without any sample extraction. A value greater than 100 % indicates that the analyte response is being enhanced by the matrix and conversely, a value below 100 % indicates ion suppression in the matrix.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by stable isotope dilution of the internal standards and extraction from the matrix. LOD and LOQ were measured at a signal to noise ratio of 3 and 10, respectively. Where possible, the Food and Drug Administration (FDA) analytical procedures and methods validation for drugs and biologics guidance for Industry was followed (FDA and CDER, 2018).

3.2.8 Extraction efficiency experiment

When determining tissue concentration, correcting for tissue mass can be a source of error. Due to the varying methods and tissue yield of human muscle biopsy techniques together with the requirement of large amounts of wet tissue for assays routinely performed in this laboratory it is not always possible to obtain equal amounts of dry powder for analysis from biopsy samples. Dried samples for metabolite quantification are advantageous as any changes in muscle water content and their effects on analyte concentrations are eliminated. In the current work, 1 fixed volume of extraction solvent was used for all samples. In the biological application (subsequent) chapter, the average skeletal muscle tissue mass was ~ 5 mg dry weight, which has been shown to be optimal previously (Stephens et al. 2014). To determine if lower tissue weights result in an overestimation of concentration or if doubling tissue weights saturates the extraction solvent, one biopsy was powdered and aliquoted at 3 different weights (1,5 and 9 mg) in triplicate. The calculated dry muscle concentration was compared to determine if the assay procedure was appropriate for a range of different tissue weights without the need to correct the extraction volume.

3.2.9 Application of method to human samples for confirmation of method suitability

The method was applied to seven fasting skeletal muscle samples and six fasting plasma samples from healthy young male subjects [age, 23 ± 1 y, BMI (in kg· m^2), 24.0 ± 0.6; mean ± SEM] that were recruited as a part of another study (University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee, Ref. No. 19–1705). Samples were obtained prior to any intervention that would impact upon fasting skeletal muscle metabolism, full details of that study are available (Jones et al. 2020). The aim of this was to determine if the method resulted in analyte concentrations consistent with previously reported literature, either using mass spectrometry or other established methods for a particular analyte. Data are report as mean ± SEM

3.2.10 Peak extraction, analyte confirmation and quantification

Raw data collected from LC-MS were processed on Thermo Xcalibur Processing Quan Browser (version 4.1) software. The ratio of high-resolution LC–MS peak areas of the analyte/internal standard were calculated and were used to construct calibration curves of peak area ratio against analyte concentration. For acylcarnitines the identity of each species reported in the biological application was confirmed by screening for the accurate mass parent ion and the product ion common to all acylcarnitines (*m/z* 85).

In the current approach tandem mass spectrometry (MS/MS) for the acquisition of product ion spectra was used for analyte confirmation. MS/MS was performed in data dependant mode in which a pre-selected inclusion list

of ions which were fragmented if a threshold frequency was exceeded. During MS acquisition, the top 5 eluting ions in terms of spectral peak intensity were selected for fragmentation and product ion analysis (MS/MS). A possible limitation of this approach could be low abundance, but relevant analytes may not be selected for MS/MS and consequently not identified and quantified. However, the analytes of interest in the current work have a relatively high endogenous abundance and the pathways they relate to are constantly in flux and the use of a targeted inclusion list largely overcome this limitation (See Appendix A)

For species without a representative standard, analyte concentrations were determined using the closest structurally related and/or nearest eluting analyte standard curve. For skeletal muscle quantification a correction factor of tissue mass was applied which was then expressed as a concentration of µmol per kg dry weight.

Table 3.2 List of detected analyte names, accurate mass and retention times in the current method. The abbreviation STD refers to that analyte having an authentic unlabelled standard used for calibration

Analyte	**Abbreviation**	**Accurate mass**	**internal standard**	**Retention time (min)**
Free carnitine STD	C0	162.1124	d9- C0	9.66
Acylcarnitines				
Acetylcarnitine STD	C2	204.123	d3- C2	2.76
Propionoylcarnitine STD	C3	218.1387	d3-C3	3.72
Malonylcarnitine	C3-DC	248.1125	d3- C2	9.06
Isobutyrylcarnitine	ISO C4	232.1543	d3-C4	4.55
Butryrylcarnitine STD	C4	232.1543	d3-C4	4.64
Hydroxybutyryl/ hydroxyisobutyryl carnitine	C4-OH	248.1488	d3- C2	3.18/3.32
Succinyl/Methylmalonyl carnitine	C4-DC	262.1281	d3- C2	8.90
Isovalerylcarnitine STD	C5	246.17	d9-C5	5.52
2-methylbutyroylcarnitine	Iso C5	246.17	d9-C5	5.36
2-Methylcrotonoyl carnitine	C5:1	244.1538	d9-C5	5.21
3-Hydroxyisovaleryl carnitine	C5-OH	262.1649	d9-C5	3.85
Glutarylcarnitine	C5-DC	276.1438	d9-C5	3.67
Hexanoylcarnitine STD	C6	260.1856	d9-C5	6.66
Adipoyl/3-Methylglutaryl carnitine	C6-DC		d9-C5	4.28/4.48
3-Hydroxyhexanoylcarnitine	C6-OH	276.1806	d9-C5	5.07
Octanoyl-carnitine STD	C8	288.2169	d3-C8	8.31
3-Hydroxyoctanoyl carnitine	C8-OH	304.211	d3-C8	6.90
Octenoylcarnitine	C8:1	286.2014	d3-C8	7.49
Decanoylcarnitine STD	C10	316.2482	d3-C8	9.46
Decenoylcarnitine	C10:1	314.2370	d3-C8	8.99
Decadienoylcarnitine	C10:2	312.2162	d3-C8	8.49
Decatrienoylcarnitine	C10:3	310.2011	d3-C8	8.42

3-hydroxydecanoylcarnitine	C10-OH	332.2431	d3-C8	8.49
Lauroylcarnitine STD	C12	344.2795	d9-C14	10.27
Dodecenoylcarnitine	C12:1	342.2630	d9-C14	9.88
Myristoylcarnitine STD	C14	372.3108	d9-C14	10.87
Tetradecenoylcarnitine	C14:1	370.2952	d9-C14	10.53
Tetradecandienoylcarnitine	C14:2	368.2795	d9-C14	10.21
3-hydroxytetradecenoyl-carnitine	C14-OH	388.3057	d9-C14	10.40
Palmitoylcarnitine STD	C16	400.3421	d3-C16	11.49
Hexadecenoylcarnitine	C16:1	398.3262	d3-C16	11.02
Hexadecadienylcarnitine	C16:2	396.3108	d3-C16	10.72
3-hyrdoxyhexadecanoyl-carnitine	C16-OH	416.3370	d3-C16	10.93
Octadecanoylcarnnitine	C18	428.3734	d3-C16	12.27
Oleylcarnitine	C18:1	426.3578	d3-C16	11.62
Linoleoylcarnitine	C18:2	424.3421	d3-C16	11.25
3-hyrdoxystearoylcarnitine	C18-OH	444.3677	d3-C16	11.55
Arachidoylcarntine	C20	456.4047	d3-C16	13.27
BCAAs				
Leucine STD		132.1019	U- ^{13}C Leucine	8.53
Isoleucine STD		132.1019	U- ^{13}C Isoleucine	8.81
Valine STD		118.0860	U- ^{13}C Valine	9.47
BCKAs				
α-ketoisocaproic acid STD	KIC	129.0557	U- ^{13}C KIC	5.04
α-keto-β-methylvaleric acid STD	KMV	129.0557	U- ^{13}C KMV	4.61
α-ketoisovaleric acid STD	KIV	115.040	U- ^{13}C KIV	2.84

3.3 Results and discussion

3.3.1 Optimisation of analyte separation and mass spectrometry detection

3.3.1.1 Reversed phase C18 chromatography.

Chromatographic separation of acylcarnitines and BCKAs was achieved using a (PFP) C18 column. This resulted in good separation over a 17 min run time and good peak shape of short, medium, and long chain acylcarnitines including isomers of species related specifically to BCAA metabolism (Figure 3.1 A). Analyte retention time increased with increasing carbon number. A coelution of analyte and IS was achieved, which is important to compensate for matrix effects and varying ionisation efficiencies during gradient elution (Figure 3.1 B).

In the current method baseline separation of C_4 and iso C_4 (butryryl and isobutryryl- carnitine) was achieved in plasma samples allowing the distinction of the two isomers. However, within the skeletal muscle sample matrix the two isomers could not be distinguished. The reason for this lack of separation may, in part, be related to the much greater concentration of intramuscular butryryl-carnitine relative to isobutryryl-carnitine, leading to the smaller peak being masked by the larger one. Therefore, a separate re-analysis of skeletal muscle samples would be required to confidently separate these two C_4 carnitine isomers. As a result, all intramuscular C_4 concentrations are presented as a total of this species whereas in plasma the two species are presented individually.

Similar to previous work also utilising a C18 column (Zhang et al. 2018) , the current method was able to separate all 3 BCKAs with excellent baseline separation for the isomers, KIC and KMV (Figure 3.3). This confirms the ability of the method to accurately determine the independent catabolic fate of leucine and isoleucine. The three BCKAs all eluted within 6 mins of the total 17 min run time and if the aim of future work is focused solely on BCKA quantification this method could be optimised to enable a rapid, high throughput analysis approach. Recent publications have been able to successfully and simultaneously quantify the BCAAs and BCKAs in human plasma using ultra high performance liquid chromatography with reversed phased columns with run times < 10 mins (Li et al. 2016; Sargsyan and Trchounian 2020). However, the current method extends on the previous work by allowing the simultaneous quantification of BCKAs and acylcarnitines arising from further BCAA catabolism as well as acylcarnitines species arising from fatty acid and glucose metabolism.

3.3.1.2 HILIC Chromatography

BCAAs were separated using a HILIC column employing a mobile phase composition and gradient commonly used in our lab for the identification of a range of polar metabolites (Schatschneider et al. 2018)Whilst performance was excellent for valine, baseline separation was not achieved for leucine and isoleucine. However, their respective U- ^{13}C resolved in an identical manner (Figure 3.4). Therefore, the midpoint of the peaks was selected as the cut-off point for manual peak integration and this criterion was applied to all samples across the validation procedure and for quantification of sample concentrations. Using this criterion satisfied all parameters of method

validation (see next sections for validation data). Furthermore, the reported sample concentrations are consistent with the established literature for quantification of plasma BCAAs using methods have derivatised amino acids to improve separation (Fuchs et al. 2019)

In the current work free carnitine and short chain acylcarnitines were also well retained and separated with the HILIC column and mobile phase combination. In particular, the species C_2, C_3, C_3-DC, C_4-OH and C_4-DC were retained and detected with HILIC method. Carnitine and acylcarnitines vary in their polarity with short chains being considerably more polar than the long chain species. HILIC columns permit excellent retention of small polar molecules and have been used with success by others for the retention of free and short chains (Xiang et al. 2017) and a full range of acylcarnitines (Peng et al. 2013).

Free carnitine and the shortest acylcarnitine, C_2, were both retained on the C18 column. However free carnitine eluted within 1 minute of the total run time and C_2 peak showed peak fronting with an almost split peak (Figure 3.1) likely to due to the high concentration of this analyte relative to the other acylcarnitines. However, both free carnitine and C_2 were retained with better, sharper peak shapes on the HILIC column despite their mmol concentration (Figure 3.4 B). The calibration curve gradients of C_2 were identical between C18 and HILIC columns and peak area ratios from biological samples were also identical. This suggests that despite the poorer peak shape of C_2 on the C18 column, retention of the analyte was not compromised. In accordance with this, C_2 satisfied all analytical validation parameters with the C18 column.

3.3.1.3 MS/MS fragmentation

Fragmentation of acylcarnitines yields a common fragment of *m/z* 85.0283 which is considered the most abundant and diagnostic product ion (Chace et al. 1997; Cansu et al. 2011)Other common fragments include *m/z* 60.081 and 144.101. Others fragments specific to certain species, as described by recent work that utilised parallel reaction monitoring (PRM) (Xiang et al. 2017) were also monitored and used to confirm the identity of different acylcarnitines. (Figure 3.2).

3.3.1.4 Potential strategies to improve analyte detection

A possible methodological approach to improve confidence in isomeric species quantification is to utilise the PRM capabilities of the Orbital trap mass spectrometer. Monitoring of all product ions produced by fragmenting an authentic standard of those analytes at different collision energies to determine if, for example, leucine or isoleucine produces a unique fragment which can in turn be used to quantify. Indeed, this approach was investigated but with no success using the current analytical conditions and was not investigated further (data not shown). A recent publication utilised a similar approach using a triple quadrupole instrument and monitoring separation mass transitions for leucine and isoleucine allowing detection and quantification without the need for chromatographic separation (Sargsyan and Trchounian 2020). This strategy could also be used for identification of the isomeric C_4 acylcarnitines within skeletal muscle in the previous section. However, this approach would require the availability of an authentic standard for the species which was not available during the current work.

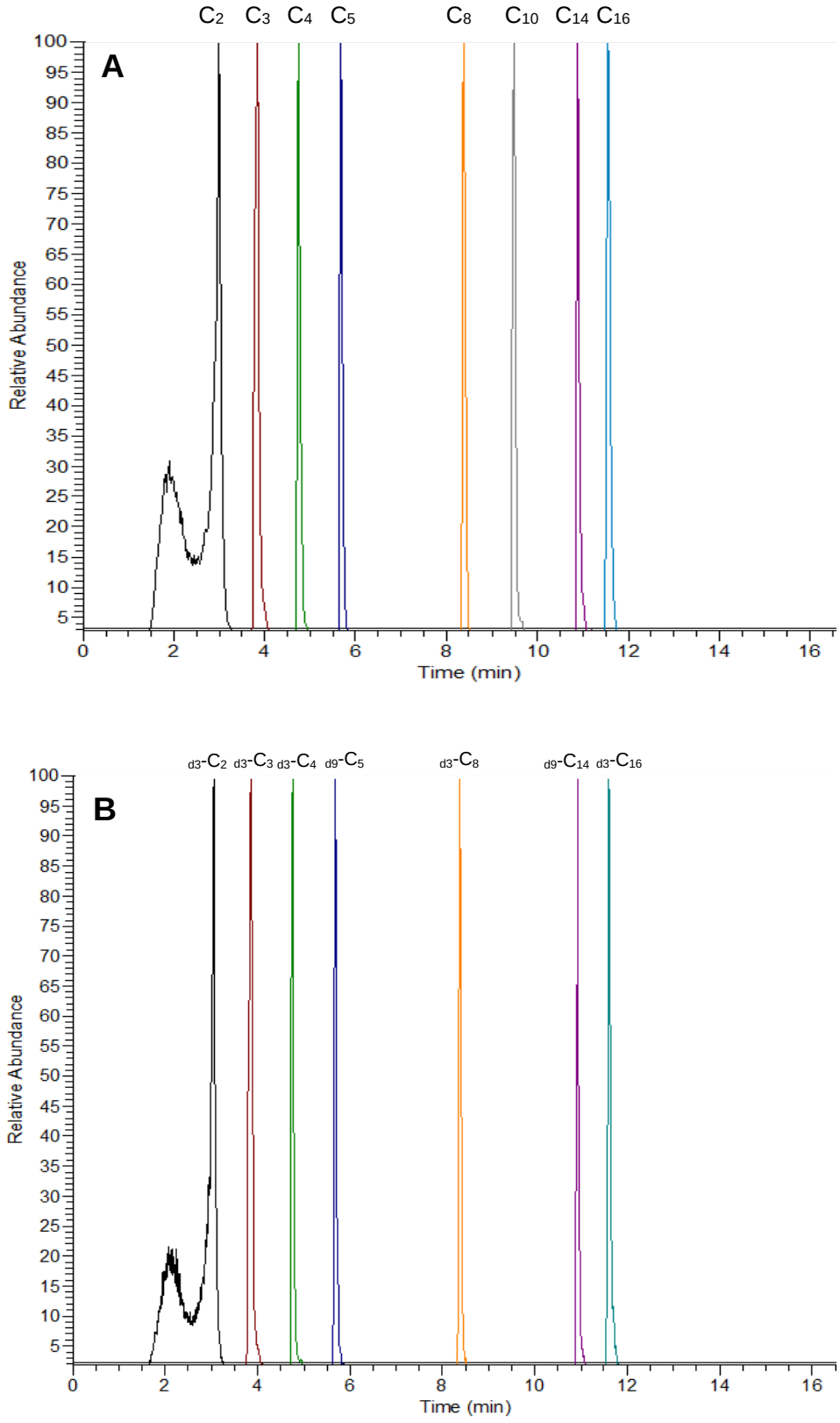


Figure 3.1 Extracted total ion chromatograms for eight of the ten acylcarnitine standards at a concentration of 250 nmol (A) and deuterated acylcarnitine standards used for method validation and as internal standards in biological samples for quantification at a concentration of 80 nmol (B). Chromatograms acquired a using revered phased C18 column coupled to high resolution mass spectrometry.

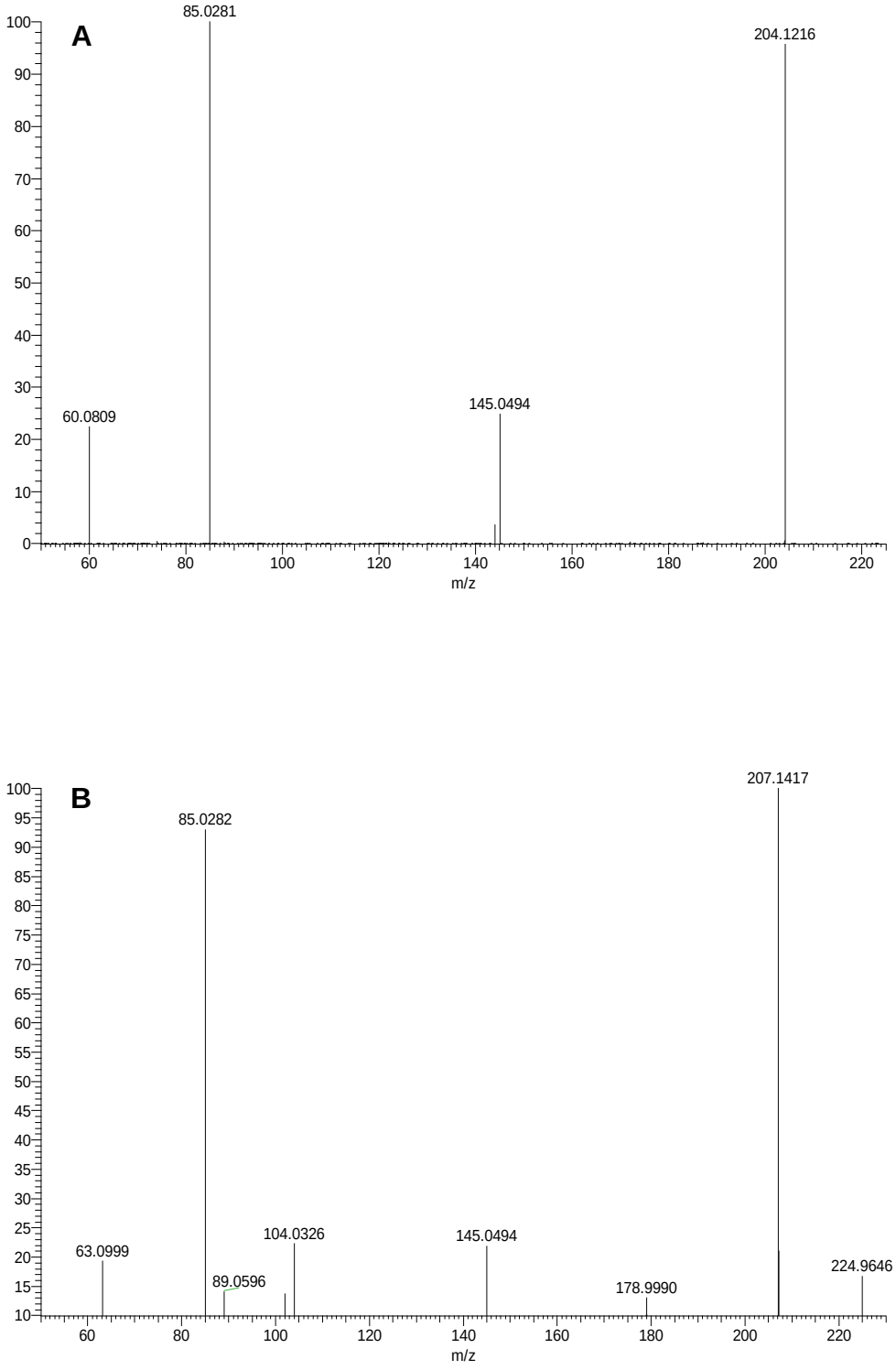


Figure 3.2 Product ion spectra of C_2, acetylcarnitine with a precursor ion of *m/z* 204.123 (A) and its respective internal standard d_3-C_2, acetylcarnitine with a precursor ion of *m/z* 207.1417 (B). Spectra acquired using data dependant mass spectrometry with product ion of *m/z* 85.028 used for acylcarnitine species confirmation.

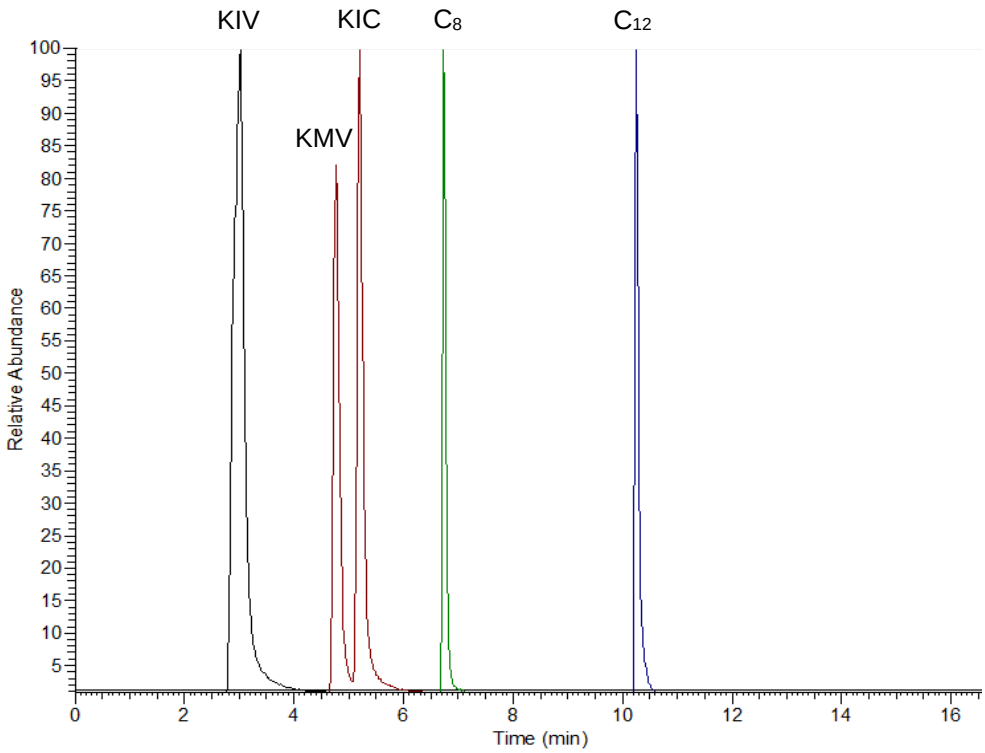


Figure 3.3 Extracted total ion chromatograms of the three branched chain keto acids (BCKAs) at a concentration of 1 µmol and the two remaining unlabelled acylcarnitine standards at a concentration of 250 nmol (not displayed in **Figure 2.1**) from a calibration standard spiked into 7.5% BSA in PBS. Analytes were separated using a reversed phase C18 column and detected in negative mode for BCKAs and in positive mode for acylcarnitines.

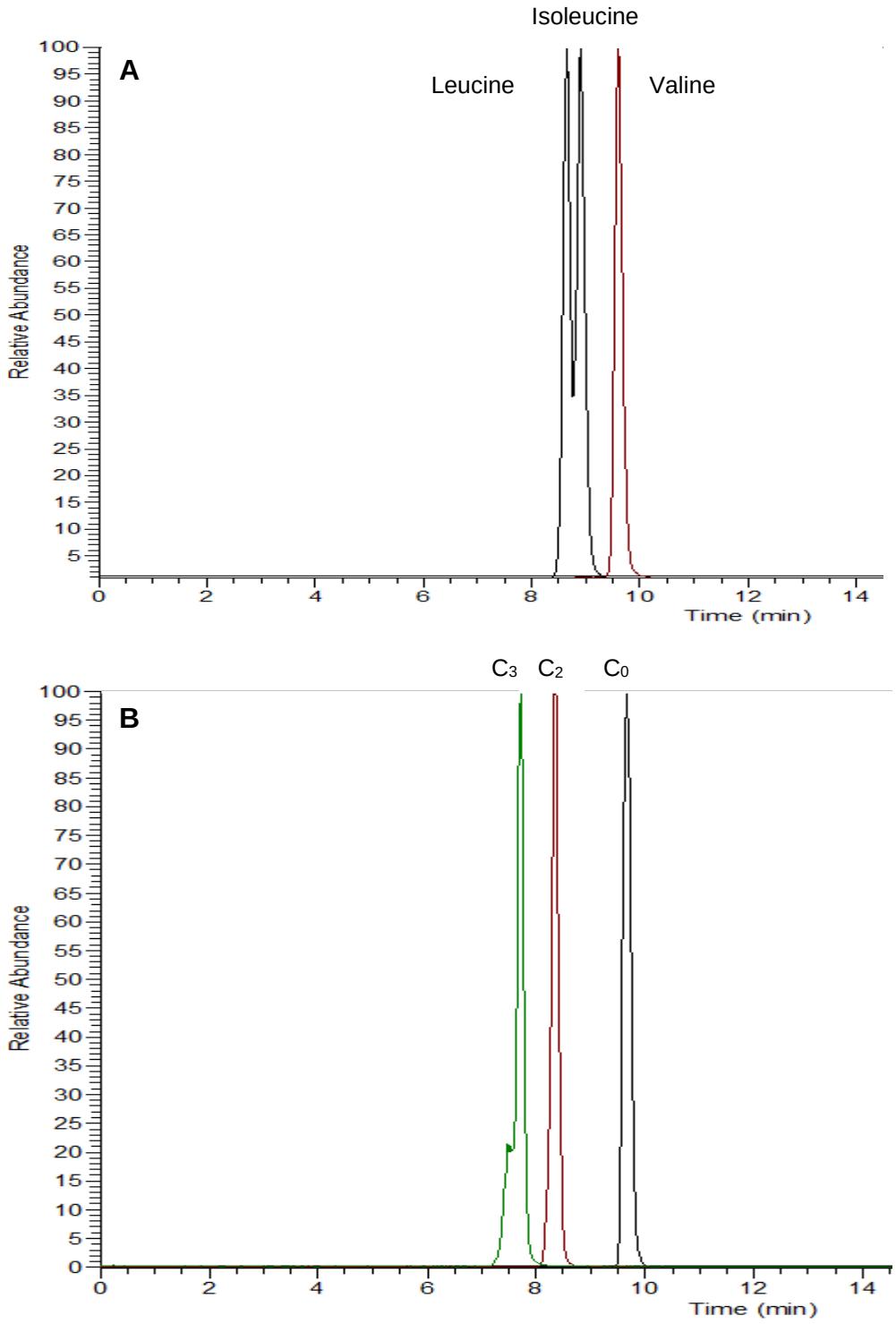


Figure 3.4 Extracted total ion chromatograms (TICs) of the three branched chain amino acids (BCAAs) separated and quantified using hydrophilic interaction liquid chromatography (HILIC) coupled to high resolution mass spectrometry from a calibration standard spiked into 7.5 % BSA in PBS at 100 µM and detected in positive mode (A). Free (C_0), acetyl (C_2) and propionoyl (C_3) carntine TICs which were also detected using the HILIC method, extracted from a pooled skeletal muscle QC sample. 14 mins of the total 24 min run time shown.

3.3.2 Biological matrix validation with isotopically labelled standards

3.3.2.1 Validation using skeletal muscle with the standard addition method

Validation data for this approach in skeletal muscle is shown in Table 3.3. All analytes showed excellent linearity (R^2 > 0.99) across their respective calibration ranges. Precision ranged from 1.4 to 14.1 % for analytes and all were within the 15% FDA criteria for method validation. With the exception of C_{14} and C_{16} acylcarnitine at their lowest calibration level, all analytes satisfied the criteria for method accuracy and were within 15 % of the true calibration value. C_{14} acylcarnitine and the two BCKAs KMV and KIC were within 20 % of the true value at their lowest calibration point which is considered acceptable when analyte concentration is close to or at the LOQ.

Long chain acylcarnitine recovery was lower than the shorter chains species. Therefore, a correction factor was applied to accuracy to account for the differing recovery levels for analytes. Only the highest concentration levels for long chain acylcarnitines were impacted by the lower recovery but these concentrations are higher than typical concentrations found in human muscle samples. It was noted that C_{14} and C_{16} analyte standards did not completely dissolve in methanol: water (1:1) above the nmol range and this is an important consideration when preparing the high concentration stock and calibration solutions as well as in the choice of reconstitution solvents for acylcarnitine analysis owing to the range of polarity of these analytes.

The BCKAs also had lower recovery at the low and mid-level but this recovery did not compromise the accuracy or precision of the measurements. Two 5-point (rather than one 10 point) calibrations curves were constructed for these analytes to cover the low-mid range and the mid-high range to minimise the effects on quantification. The low recoveries observed for the BCKAs could be due an incompatibility of the organic solvents used for extraction (acetonitrile and isopropanol) and the highly polar BCKAs but this was not investigated further. However, the use of solvents in the current method satisfies all the parameters of validation across the biological range of the analytes.

Table 3.3 Method validation data from the standard addition method using labelled isotopes as a substitute for the authentic unlabelled standards spiked into powdered human skeletal muscle. Calibration curves were prepared in triplicate and precision, accuracy and recovery were assessed with 6 replicates at each level. A matrix effect less than 100 % indicates ion suppression and greater than 100 % indicating ion enhancement. Calibration range for BCAAs and BCKAs are µmol/mg.

Analyte (Isotope)	Slope (R^2)	Calibration range (µmol/mg)	LOD (nmol/mg)	LOQ (nmol/mg)	Concentration (nmol/mg)	Precision (CV) n = 6	Accuracy (%) n = 6	Recovery (%) n= 6	Matrix effect (%)
(d_9) - C_0	3 x 10^7 (0.999)	0.1-21.7	50	100	100	8.8	93.9	88.0	15.3
					5.5 x 10^3	8.3	99.1	87.1	23.1
					21.7 x 10^3	4.6	99.7	86.1	31.0
(d3) - C_2	6 x 10^7 (0.994)	0.01-5.5	5	20	23.8	11.6	97.8	85.2	81.1
					1350	1.9	102	95.8	64.3
					5500	2.9	100	97.6	81.0
(d3) - C_3	3 x 10^8 (0.999)	0.005-5	1	5	5	3.5	99.3	96.7	88.8
					250	14.1	104	97.3	109
					1000	1.4	96.6	92.8	96.9
(d3) - C_4	5 x 10^8 (0.998)	0.005-5	1	5	5	6.3	92.0	95.5	86.9
					250	3.9	109	101	121
					1000	2.8	95.6	93.7	95.1
(d9) - C_5	6 x 10^8 (0.997)	0.005-5	1	5	5	5.1	91.4	97.1	83.8
					250	3.0	104	100	120
					1000	2.6	91.4	92.1	91.0
(d3) - C_8	7 x 10^8 (0.999)	0.005-5	0.5	1	5	3.5	95.2	102	62.0
					250	4.0	97.1	102	69.8
					1000	2.4	86.3	92.9	64.1
(d9) - C_{14}	5 x 10^8 (0.994)	0.005-5	0.5	1	5	4.7	83.8	91.2	37.3
					250	13.8	102	83.5	38.0
					1000	7.2	104	70.0	38.9

(d3) - C_{16}	3 x 10^8 (0.993)	0.01-5	0.5	5	10	8.8	231	96.1	27.6
					500	10.1	109	63.1	28.1
					2000	12.6	119	48.6	24.9
BCAA		**(µmol/mg)**	**(µmol/mg)**	**(µmol/mg)**	**(µmol/mg)**				
(U-^{13}C) Leucine	4 x 10^7 (0.998)	1-350	0.3	0.6	0.75	6.5	94.3	94.3	80.1
					3.5	7.6	87.0	87.0	85.3
					300	9.5	97.8	97.8	86.7
(U-^{13}C) Isoleucine	5 x 10^7 (0.996)	1-350	0.3	0.6	0.75	5.8	96.0	96.0	84.1
					3.5	7.9	88.3	88.3	83.8
					300	8.0	99.3	99.3	91.3
(U-^{13}C) Valine	2 x 10^7 (0.99)	1-400	0.4	0.6	1	5.8	97.2	97.2	78.5
					4.5	3.7	85.2	85.2	81.5
					400	10.3	99.8	99.8	85.3
BCKA									
(U-^{13}C) KIC	6 x 10^7 (0.997)	0.2-100	0.1	0.2	0.25	13.2	103	43.5	116
					1	13.5	86.0	31.6	97.1
					100	2.3	99.0	87.2	96.6
(U-^{13}C) KMV	5 x 10^7 (0.995)	0.2-100	0.1	0.2	0.25	12.0	120	55.2	120
					1	10.5	93.0	42.2	100
					100	2.6	99.0	88.0	96.7
(U-^{13}C) KIV	1 x 10^7 (0.99)	0.4-200	0.1	0.2	0.25	10.2	118	46.2	100
					1	13.6	60.0	35.5	120
					100	1.8	101	84.3	90.2

Limit of detection (LOD), limit of quantification (LOQ), branched chain amino acids (BCAA), branched chain keto acids (BCKA). α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV).

3.3.2.2 Validation using proxy matrix method (7.5 % BSA in PBS)

The isotope addition of carnitine and acylcarnitines was also performed in a proxy matrix (BSA) with the identical calibrants to assess the suitability of this matrix as a substitute for skeletal muscle. Data for this approach is shown in Table 3.4. The slopes of curves were similar between skeletal muscle and BSA with very similar linearity ($R^2 > 0.99$), precision (2 – 9.3 %) and accuracy (72.5 – 136 %). Analyte recovery was between 80.2 and 114 % for all acylcarnitines.

There was a large range in the matrix effect on acylcarnitines in both matrices, with ion suppression increasing with longer chain lengths. These data highlight the importance of internal standards to account for these matrix effects to enable absolute quantification in biological samples

.

Table 3.4 Method validation data using isotopically labelled acylcarnitine standards spiked into the proxy matrix of 7.5 % BSA in PBS. Calibration curves were prepared in triplicate and precision, accuracy and recovery were assessed with 6 replicates at each level. A matrix effect less than 100 % indicates ion suppression and greater than 100 % indicating ion enhancement.

Analyte (Isotope)	Slope (R^2)	Calibration range (µmol/L)	LOD (nmol/L)	LOQ (nmol/L)	Concentration (nmol/L)	Precision (CV) n = 6	Accuracy (%) n = 6	Recovery (%) n = 6	Matrix effect (%)
(d_9) - C_0	8 x 10^7 (0.991)	0.1-21.7	10	20	100	4.7	105	89.3	42.5
					5.5 x 10^3	1.1	112	91.7	80.0
					21.7 x 10^3	4.1	108	91.1	83.0
(d_3) - C_2	8 x 10^7 (0.995)	0.01-5.5	1	20	23.8	2.9	142	94.4	77.5
					1350	2.2	114	93.3	89.6
					5500	3.9	89.2	95.3	89.3
(d_3) - C_3	3 x 10^8 (0.998)	0.005-5	0.5	1	5	3.3	102	96.5	67.1
					250	8.5	108	91.3	67.3
					1000	2.8	95.8	94.8	74.2
(d_3) - C_4	4 x 10^8 (0.997)	0.005-5	0.5	1	5	4.6	87.0	91.2	70.5
					250	2.2	111	96.0	72.1
					1000	2.8	96.4	93.1	71.9
(d_9) - C_5	5 x 10^8 (0.996)	0.005-5	0.5	1	5	4.6	95.6	96.4	67.7
					250	3.6	111	94.9	87.9
					1000	4.5	101	96.6	76.6
(d_3) - C_8	4 x 10^8 (0.997)	0.005-5	1	5	5	5.3	96.7	97.6	66.7
					250	3.6	102	96.2	77.7
					1000	5.0	99.1	96.8	81.2
(d_9) - C_{14}	3 x 10^8 (0.994)	0.005-5	0.5	1	5	2.0	72.5	99.4	48.9
					250	3.2	91.2	93.0	57.4
					1000	5.1	91.6	103	56.0
(d_3) - C_{16}	2 x 10^8 (0.997)	0.01-5	0.5	5	10	5.4	136	114	41.3
					500	6.8	94.5	85.0	27.9
					2000	9.3	95.6	80.2	38.2

Limit of detection (LOD), limit of quantification (LOQ),

3.3.3 Use of the proxy BSA in PBS matrix as a valid substitute for analyte quantification in powdered skeletal muscle and plasma

3.3.3.1 Skeletal muscle

The acylcarnitine peak area ratio response was almost identical between skeletal muscle and BSA with all analytes demonstrating excellent linearity (Table 3.5). These results indicate that the use of isotopically labelled internal standards is sufficient to account for the effect of the matrix on analyte detection and quantification. A representation of the likeness of the two matrices' calibration is shown in Figure 3.5. Taken together with the method validation data from the isotope addition method outlined above, this method can extract acylcarnitines equally efficaciously from both skeletal muscle and BSA, meeting the validation parameters outlined in section 3.2.7.

3.3.3.2 Plasma

This approach was also carried out across the biological range in human plasma to compare the analyte response to the proxy matrix. Similar to skeletal muscle, the analyte response was very similar between human plasma and BSA (Figure 3.6). Six independent plasma samples were also analysed, and the concentration of each analyte was calculated and compared between the two matrices. Corrections for endogenous analyte concentrations in human plasma were made by subtracting the endogenous concentration from each calibration standard. With the exception of C_{16} acylcarnitine (8.6%), the CV of calculated concentrations between the two matrices was within 5% for all acylcarnitines. These data confirm that BSA is an appropriate substitute for

human plasma and can be used for calibration purposes. The data for these experiments are also displayed in Table 3.5.

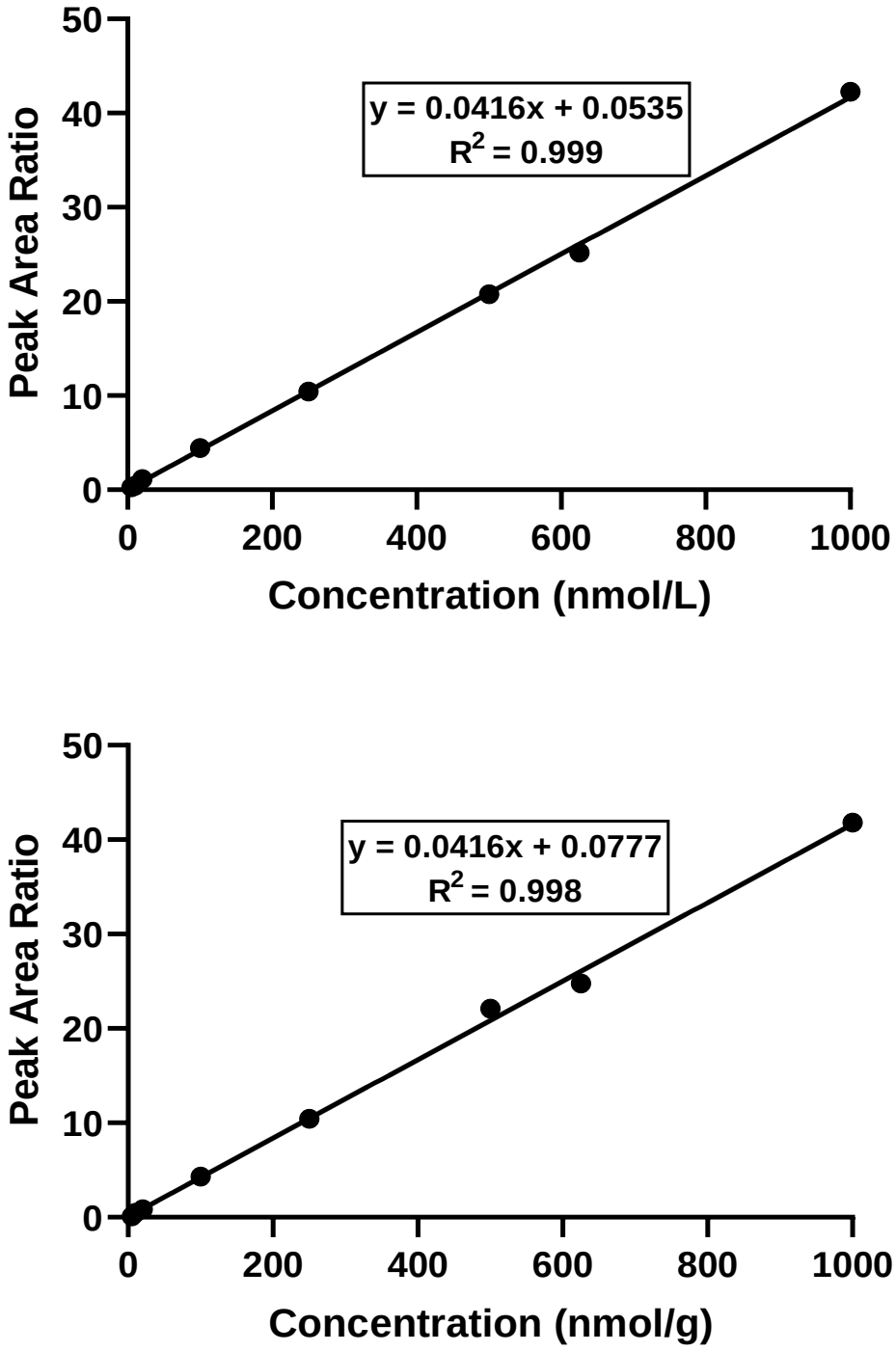


Figure 3.5 Calibration curves for C_4 butyrylcarnitine from a range of 1 nmol/ L to 1 µmol/L spiked into the proxy matrix of 7.5 % BSA in PBS (A) and same calibrants spiked into powdered human skeletal muscle (B) expressed as nmol/g

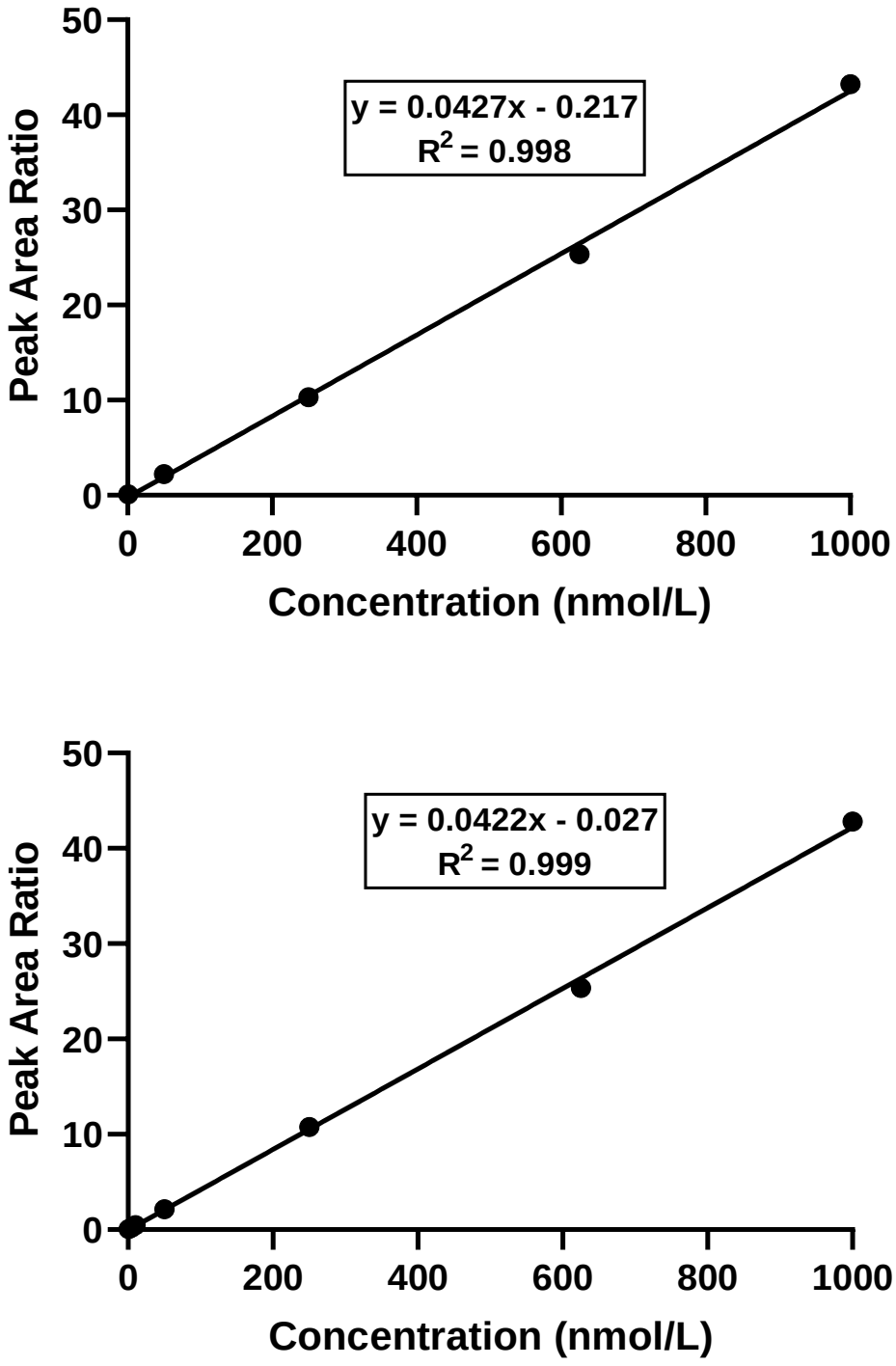


Figure 3.6 Calibration curves for C_8 octanoylcarnitine from a range of 1 nmol/L to 1 µmol/L spiked into the proxy matrix of 7.5 % BSA in PBS (A) and same calibrants spiked into 100 µL aliquots of human plasma (B)

Table 3.5 Comparisons of skeletal muscle and plasma to proxy matrix (7.5 % BSA in PBS) spiked with unlabelled standards. Responses are expressed as peak area ratios to isotopically labelled internal standards with calibration ranges specific to each matrix. CV of plasma samples relate to variation of 6 sample concentrations calculated with calibration in plasma and calibration in BSA.

		Skeletal muscle			Plasma			
Analyte	**Internal standard**	**Calibration range µmol/ mg(L)**	**Slope Muscle (R^2)**	**Slope BSA (R^2)**	**Calibration range µmol/L**	**Slope Plasma (R^2)**	**Slope BSA (R^2)**	**Sample CV % N = 6**
C_2	d_3-C_2	10 - 250	14.3 (0.981)	13.6 (0.999)	0.01 - 50	8.4 (0.996)	8.6 (0.999)	3.0
C_3	d_3-C_3	0.005 - 12	37.9 (0.997)	37.8 (0.996)	0.001-1	42.7 (0.999)	40.8 (0.999)	4.5
C_4	d_3-C_4	0.005 – 25	40.8 (0.999)	39.4 (0.999)	0.001-1	40.1 (0.999)	39.8 (0.999)	1.7
C_5	d_9-C_5	0.005 – 25	36.9 (0.999)	36.5 (0.999)	0.001-1	33.3 (0.999)	33.0 (0.999)	0.8
C_6	d_9-C_5	0.005 – 25	34.9 (0.999)	31.8 (0.998)	0.001-1	40.9 (0.999)	41.9 (0.999)	2.5
C_8	d_3-C_8	0.005 - 12	35.4 (0.998)	35.4 (0.998)	0.001-1	42.5 (0.999)	42.2 (0.999)	0.5
C_{10}	d_3-C_8	0.005 - 5	32.7 (0.997)	32.4 (0.999)	0.001-1	55.1 (0.999)	54.6 (0.999)	0.6
C_{12}	d_3-C_8	0.005 - 5	18.5 (0.997)	22.6 (0.999)	0.001-1	61.8 (0.998)	63.3 (0.999)	4.2
C_{14}	d_9-C_{14}	0.005 - 5	37.2 (0.999)	37.5 (0.999)	0.001-1	42.4 (0.998)	42.2 (0.999)	0.4
C_{16}	d_9-C_{14}	0.01 - 5	19.9 (0.998)	21.1 (0.999)	0.001-1	13.5 (0.997)	15.8 (0.999)	8.6

3.3.4 Method validation for analytes with high endogenous concentrations

The spiking of authentic unlabelled free carnitine (C_0), BCAA and BCKA standards into skeletal muscle and/or plasma was not appropriate for calibration due to relatively high endogenous concentrations of these metabolites. However, the data from sections 3.3.2 – 3.3.4 demonstrated that the method can extract analytes from two matrices with equally efficiency and that BSA in PBS is an appropriate substitution of both skeletal muscle and plasma. The use of labelled internal standards can correct for any matrix effects that may impact upon method validation and calculation of analyte concentrations in biological samples. Therefore, a full validation of these analytes using unlabelled standard/ internal standard peak area ratios was performed in BSA in accordance with same criteria outlined in section 3.2.7 and across the same calibration range outlined in Table 3.3. Data from these experiments is shown in Table 3.6. Free carnitine (C_0), BCAAs and BCKAs showed excellent linearity, precision, accuracy. As seen with the isotopes, recovery of BCKA particularly at the low and mid-point was 50 % or less but this did not affect precision or accuracy. Previous work has shown high recoveries of BCKA albeit at higher concentration and using a more polar extraction solvent mixture of methanol/ water (Zhang *et al.*, 2018).

It should be noted that the current method covers a range of analytes with varying degrees of polarity using a single extraction procedure which is likely to have an impact on some validation parameters such as recovery and accuracy, particularly at extreme ends of polarity. These data highlight the importance of internal standards to correct for such challenges. One strength

of the current method is the number of analyte specific internal standards used allowing for greater confidence in each analyte's quantification. Of note, despite lower recoveries of some analytes, quantitative performance of the method was not impacted over the vast range of concentrations measured. This is of particular significance as if/when this method is applied to other tissues (e.g. adipose tissue) with varying levels of endogenous abundance, the method remains valid over the biological range of that tissue.

Table 3.6 Method validation data for free carnitine, branched chain amino and keto acids spiked into 7.5 % BSA in PBS using peak area ratio of unlabelled standards and isotopically internal standards. Calibration ranges were appropriate for both skeletal muscle and plasma analyte concentrations.

Analyte	Internal standard	Calibration range (µmol/L)	Slope	R^2	Concentration (µmol/L)	Precision (%) n = 6	Accuracy (%) N = 6	Recovery (%) n = 6	Matrix effect (%)
C_0	d_9-C_0	1-1000	1.38	0.998	2.5	1.4	110	94.9	88.1
					50	0.8	112	113	74.3
					500	2.5	112	120	74.6
Leucine	U-^{13}C Leucine	0.3-300	0.619	0.999	0.75	2.3	86.9	90.3	148
					3.5	1.0	106	95.6	128
					300	2.7	102	83.2	88.5
Isoleucine	U-^{13}C Isoleucine	0.3-300	0.644	0.999	0.75	2.6	97.4	94.8	132
					3.5	1.6	107	101	112
					300	1.3	101	80.5	86.1
Valine	U-^{13}C Valine	0.8-400	0.407	0.998	1	5.2	103	84.7	86.7
					4.5	1.6	109	93.6	73.3
					400	2.2	103	85.2	81.9
KIC	U-^{13}C KIC	0.1-100	0.719	0.999	0.25	3.1	85.0	49.0	147
					1	1.2	111	46.9	129
					100	1.7	100	74.1	120
KMV	U-^{13}C KMV	0.1-100	0.816	0.999	0.25	3.4	96.1	53.3	118
					1	1.2	114	52.3	113
					100	0.8	102	75.1	109
KIV	U-^{13}C KIV	0.2-300	0.629	0.999	0.4	2.7	113	27.0	128
					3	1.4	114	28.4	118
					300	1.4	100	70.4	114

α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV)

3.3.5 Sample stability and extraction repeatability

With exception of C_{12} acylcarnitine, all analytes had an intra-day stability between 90 and 110% (referenced to time 0) For acylcarnitines and BCKAs this stability relates to an acquisition time of 48 hours and ~ 56 hours for BCAA and the samples was kept in the autosampler at 4^{o} C throughout. Skeletal muscle QCs following a freeze thaw cycle in which the QCs were stored at -80^{o} C between analysis did not appear to have any effect on stability (free-thaw stability ranged between 95 and 106 %). This is an important observation as the workflow of the current method requires two separate injections of the sample to cover all the analytes to be quantified (i.e. one injection for C18 and another for HILIC column). Sample stability and repeatability data are displayed in Table 3.7.

Each analyte repeatability was below 12 % apart from KIV in skeletal muscle which had a CV of 17 %. C12 acylcarnitine had an RSD > 10 % in both plasma and muscle. This may be of importance if multiple batches of samples are to be extracted over a period before analysis. To reduce any impact of sample degradation over the acquisition a standard curve was run at the beginning and just prior to the conclusion of each quantitative analytical run.

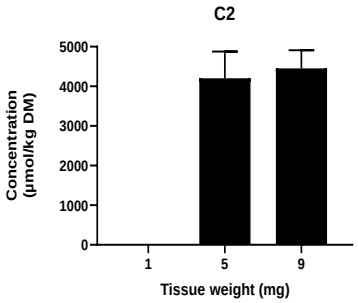
Table 3.7 Method stability data from intra-day and following a freeze-thaw cycle in skeletal muscle presented as percentage with CV of the replicates. Freeze-thaw stability data not available for plasma. Analyte repeatability refers to the variation in calculated analyte concentration from 9 replicates of the same human skeletal muscle or plasma sample extracted on the same day.

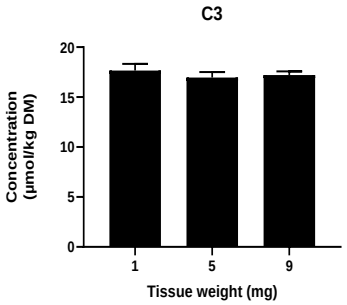
	Skeletal muscle					Plasma		
Analyte	**Intra-day Stability (%)**	**CV (%) n = 7**	**Freeze-thaw stability (%)**	**CV (%) n = 6**	**Repeatability CV (%) n = 9**	**Intra-day stability %**	**CV (%) n = 11**	**Repeatability CV (%) n = 9**
C_0	101.5	4.7	100.8	2.0	5.6	98	1.1	1.5
Acylcarnitines								
C_2	99.3	1.4	99.7	1.7	5.8	103.4	1.8	0.7
C_3	102.5	1.4	99.2	2.1	4.7	99.2	2.5	2.9
C_4	104.0	2.5	101.6	1.0	5.5	104.9	5.1	4.0
C_5	93.9	4.4	97.6	4.8	6.2	95.1	3.2	4.9
C_6	90.7	5.6	106.2	6.3	6.5	91.0	6.2	4.2
C_8	100.9	1.4	99.8	0.6	4.0	98.3	1.3	2.3
C_{10}	93	6.0	103.2	2.2	5.3	91.2	6.1	11.1
C_{12}	89.2	7.3	100.2	2.6	11.4	85.6	11.6	10.0
C_{14}	98	1.5	97.3	1.9	6.1	91.1	3.2	5.3
C_{16}	100.2	0.5	98.0	2.4	5.5	94.2	3.2	3.5
BCAA								
Leucine	94	2.7	100.7	1.8	3.5	97.5	2.1	2.9
Isoleucine	109.1	5.7	101.5	6.6	5.7	95.4	4.8	3.3
Valine	100.5	1.8	99.5	0.9	4.3	97.0	1.7	3.5
BCKA								
KIC	100.9	1.7	99.6	1.1	7.7	98.0	1.7	2.6
KMV	99.1	1.1	99	1.1	6.0	94.2	3.3	3.7
KIV	109.6	6.3	95.9	2.9	17.7	98.5	1.6	2.3

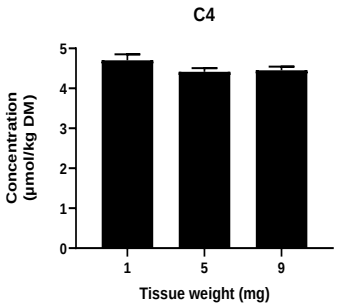
α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV).

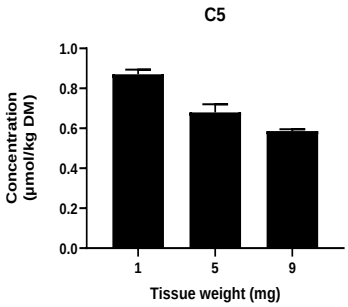
3.3.6 Effect of sample weight on extraction efficiency

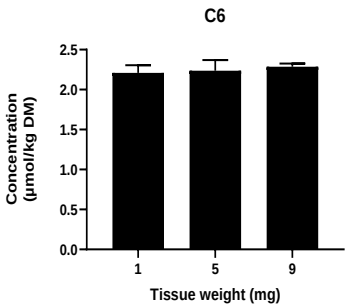
C_2 acylcarnitine and KIV were not detected in1 mg of tissue. KIC, KMV, C_{12} and C_5 had a CV of greater than 20 % with higher error in all cases attributable to a higher concentration being calculated at 1 mg vs 5 and 9 mg. Doubling the tissue volume had little effect on any metabolite with almost identical concentrations at 5 and 9 mg. Based on this data, it is recommended that low tissue volumes are avoided to minimise any errors in calculations. Alternatively, adjustments in extraction or reconstitution volumes could be made where appropriate but this was not investigated in the current work. Data for the extraction efficiency experiments is show in Figure 3.7.

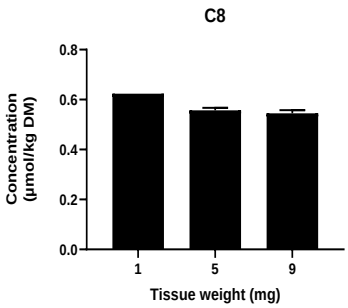

C2
Concentration (µmol/kg DM)
5000
4000
3000
2000
1000
0
1
5
9
Tissue weight (mg)

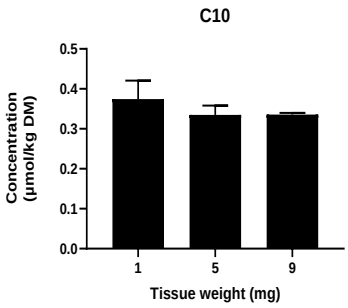

C3
Concentration (µmol/kg DM)
20
15
10
5
0
1
5
9
Tissue weight (mg)

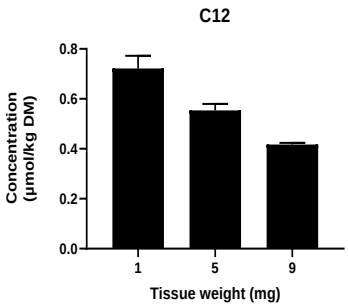

C4
Concentration (µmol/kg DM)
5
4
3
2
1
0
1
5
9
Tissue weight (mg)

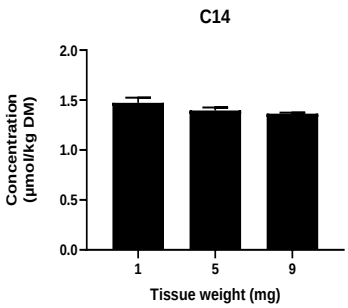

C5
Concentration (µmol/kg DM)
1.0
0.8
0.6
0.4
0.2
0.0
1
5
9
Tissue weight (mg)

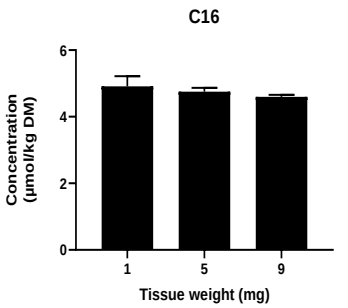

C6
Concentration (µmol/kg DM)
2.5
2.0
1.5
1.0
0.5
0.0
1
5
9
Tissue weight (mg)


C8
Concentration (µmol/kg DM)
0.8
0.6
0.4
0.2
0.0
1
5
9
Tissue weight (mg)


C10
Concentration (µmol/kg DM)
0.5
0.4
0.3
0.2
0.1
0.0
1
5
9
Tissue weight (mg)


C12
Concentration (µmol/kg DM)
0.8
0.6
0.4
0.2
0.0
1
5
9
Tissue weight (mg)


C14
Concentration (µmol/kg DM)
2.0
1.5
1.0
0.5
0.0
1
5
9
Tissue weight (mg)


C16
Concentration (µmol/kg DM)
6
4
2
0
1
5
9
Tissue weight (mg)

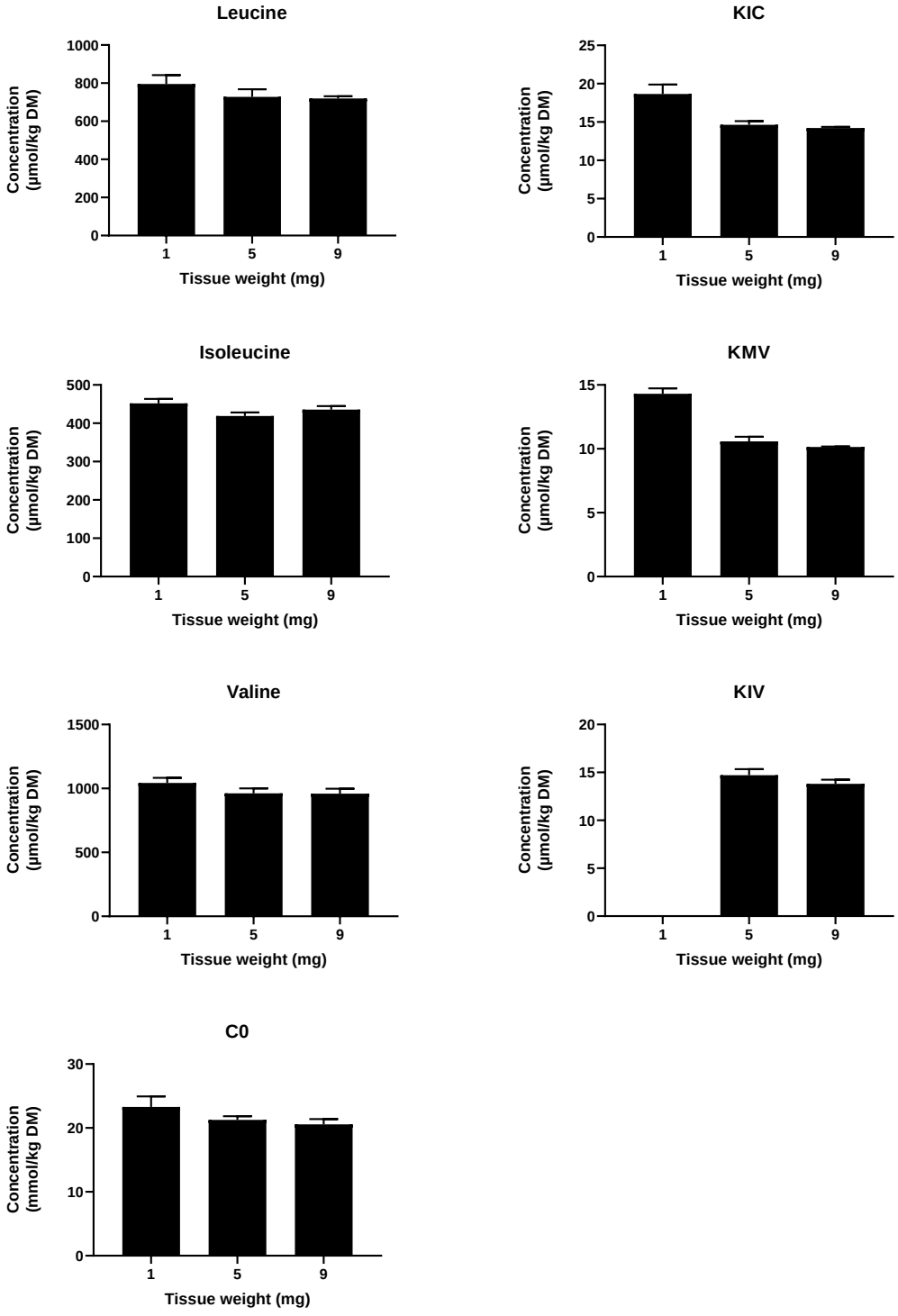


Figure 3.7 Comparison of calculated concentrations of each analyte extracted in triplicate from tissue weights of 1, 5 and 9 mg. Missing bars indicate that the analytes was undetected at a tissue weight of 1 mg. Data are mean ± SD. α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV).

3.3.7 Application of method to human samples for confirmation of method suitability

3.3.7.1 Muscle metabolites

Muscle free carnitine content has been extensively investigated, particularly in young male healthy as it pertains to its key role in the regulation of substrate metabolism during muscular contraction (Stephens, Constantin-Teodosiu, and Greenhaff 2007). The most common and established method used to measure muscle free carnitine is the radioisotopic method which can measure both free and acylated carnitine (Cederblad et al. 1990) but cannot discriminate acyl chain lengths. The reported muscle carnitine content across multiple previously published literature with a subject cohort that was very similar to these samples (i.e. young healthy males) is ~15-18 mmol/Kg (dry mass) with the sum of muscle free carnitine and the highest abundant acylated carnitine species, acetylcarnitine being ~20 mmol/Kg (dry mass) (Harris, Foster, and Hultman 1987; Sahlin 1990; Constantin-Teodosiu, Cederblad, and Hultman 1992). The average reported muscle free carnitine content in this study was 16.5 ± 1.7 mmol/kg which is consistent with the aforementioned studies using the established radioisotopic assay and a recent study that used LC-MS to determine intramuscular carnitine (Xu et al. 2016). In addition, muscle acetylcarnitine concentration was 2.1 ± 0.7 mmol/kg which is also consistent with concentrations determined by the radioisoptoic assay and LC-MS. Furthermore, the ratio of acetyl to free (0.13) carnitine was also in agreement with that reported previously (Constantin-Teodosiu, Cederblad, and Hultman 1992). The calculated concentration of muscle free carnitine from the current

method also strongly correlated with concentration calculated using the radioisotopic assay (see Appendix B).

Intramuscular BCAA concentrations have been reported on previously. Of the three BCAA, valine is of the highest abundance followed by leucine with isoleucine being of the lowest abundance (Bergström et al. 1974; Bergström, Fürst, and Hultman 1985). Indeed, the concentrations of BCAAs in the current showed the same pattern with values of 1133 ± 67, 591 ± 40 and 337 ± 27 µmol/Kg for valine, leucine and isoleucine, respectively. Previous reports have expressed intramuscular BCAA concentrations in terms of wet muscle as opposed to dry mass (as in the current work). Due to the relatively large amount of water in skeletal muscle, wet muscle analyte concentrations are ~ four times lower than when expressed in dry tissue. In accordance, the BCAA concentrations measured are 3-5 times lower than the wet weight concentrations reported by others (Glover et al. 2008) but consistent with those measured in dry tissue by HPLC (Wilkinson et al. 2006).

3.3.7.2 Plasma metabolites

As with skeletal muscle, multiple reports have demonstrated that plasma BCAA concentrations follow the same pattern as skeletal muscle, with valine being the most abundant and isoleucine the lowest (Bergström, Fürst, and Hultman 1985; Fuchs et al. 2019). In healthy young males, BCAA plasma concentrations are in the µmol range. The average calculated concentrations in the six plasma samples were 253 ± 15. 124 ± 5 and 79 ± 4 valine, leucine and isoleucine, respectively. These concentrations are in agreement with previously published work which can has measure plasma BCAA concentration using amino acid chromatography (Bergström et al. 1974),

HPLC (Sahlin, Katz, and Broberg 1990) and LC-MS/MS (Schmidt et al. 2016; Li et al. 2016) which have all reported very similar concentrations in subjects of similar age and body weight.

Plasma free and acetylcarnitine have been reported to be in the µmol range as determined radioistopically (Stephens et al. 2006), direct infusion MS/MS (Schooneman et al. 2016) or LC-MS (G. Xu et al. 2016). Additionally, acylcarnitines from species lengths of three carbons and above have been reported to be in the nmol range. Concentrations in this study were 51.4 ± 3.3 and 6.6 ± 1.4 µmol/L for free and acetyl carnitine, respectively. Acylcarnitine concentrations from species length 3 to 16 carbons ranged from 10 – 500 nmol/L with C_3 as the most abundant plasma acylcarnitine

3.3.8 General discussion

The aim of this chapter to develop and analytically validate a LC-MS method that could enable the simultaneous quantification of (acyl)carnitines, BCAA and BCKAs. These analytes have been individually and collectively associated with several chronic metabolic diseases (Newgard 2012; Gaggini et al. 2018). The retention of a range of acylcarnitine species lengths is important to make inferences about the metabolism of carbohydrate lipid and amino acids. An increasing area of active research in human physiology is the interaction of lipid and amino metabolism in skeletal muscle and the potential implications in the development of insulin resistance (Stephens et al. 2014, 2015) Whilst methods currently exist to quantify these analytes in range of matrices a validated method to quantify them from a single extraction procedure has not been developed previously.

A commonly cited challenge of quantifying tissue metabolites is the lack of an appropriate ‘blank’ which can mirror the complexity of the matrix. This is important as the potential of ‘matrix effects’ to enhance or suppress analyte signal can lead to inaccurate quantification. In addition, the method of standard addition approach within the biological matrix can also provide challenges to calibration when the endogenous concentration of an analyte is high (as is the case with the analytes of interest in this study) and thus spiking relatively small amount of the analyte does not result in a detector response above the baseline. These issues were addressed systematically in this chapter. First, isotopically labelled standards were used as substitutes of each analyte and spiked into powdered skeletal muscle and BSA and this approach satisfied the necessary validation parameters (Tables 3.4 and 3.5) However, this did reveal low recoveries of BCKAs and long chain acylcarnitines. These low recoveries did not impact on the analytical validation parameters and were easily accounted for by either adjusting calibration ranges and accuracy for analyte recovery. These results demonstrate that the single extraction procedure used in the current work is sufficient to extract a range of analytes with varying degrees of polarity and satisfy all the validation parameters required for confident quantification in biological samples. Following method validation within the biological matrix, isotopically labelled standards were used as internal standards to correct for differing matrix effects on analytes between skeletal muscle and BSA. For calibration and analyte quantification, increasing amounts of unlabelled standard expressed as a ratio to a fixed amount internal standard was spiked into skeletal muscle, plasma and BSA. This approach demonstrated an almost identical response within the between the matrices

(illustrated in Figures 3.4 and 3.5 and outlined in Table 3.5) confirming that the use of internal standards was appropriate to correct for matrix effects and result in accurate metabolite quantification. In support of this, when this method was applied to some biological samples (section 3.2.7), calculated concentrations of analytes such intramuscular free carnitine and plasma BCAAs were consistent with previous published studies which have used methodologies that were established and validated almost half a century ago (Bergström et al. 1974; Cederblad et al. 1990).

The current work is a different approach to much of the previous work which has monitored pre-selected parent/product ion transitions using triple quadrupole instruments. This method is regarded as the gold standard for analyte quantification and has formed the basis for much of work in the area of acylcarnitine quantification (Minkler et al. 2008; Giesbertz et al. 2015). However, these methods also require authentic standards for metabolite identification and retention time alignment. The use of high-resolution MS allows the determination of analytes based on accurate mass of compounds, together with MS/MS fragmentation for confirmation. Indeed, a high-resolution approach has been used to successfully develop and validate a method for acylcarnitine quantification in animal plasma and urine (Xiang et al. 2017). The work in this chapter adds further support for the use of high-resolution mass spectrometry for quantitative metabolomics.

3.3.9 Limitations

Some limitations of the current work include the inability to separate certain isomers. For example, baseline separation was not achieved for leucine and isoleucine. However, the use of a consistent peak integration cut off point

resulted in satisfaction of all validation parameters with excellent repeatability and reproducibility of these analytes. In skeletal muscle, the separation of isomers of C_4 acylcarnitnes was not possible with this method on either column and may require further optimisation of LC conditions or even longer C18 columns. In addition, although laborious sample processing steps such as sample derivatisation and SPE were avoided with the current method, these steps may aid the separation and identification of structural isomeric/isobaric species (Giesbertz et al. 2015). The coverage of acylcarnitines in the current method is not as expansive as others. However, the detection of novel acylcarnitine species was not the aim of this work. The relatively smaller number of species were quantified with high confidence due to the large number of authentic and isotopically labelled internal standards used in the current work.

3.4 Conclusions

In conclusion the work in this chapter describes a fully validated method for the simultaneous quantification of acylcarnitines, BCAAs and BCKAs, key metabolites with established associations with obesity, insulin resistance and type 2 diabetes. Using the method of standard addition with isotopically labelled standards it was possible to address the matrix effect of biological samples to allow accurate and confident quantification of these key metabolites in both muscle and plasma. This method avoids laborious and tedious sample preparation and owing to the high sensitivity of this method, concentrations in samples are well above LOD and LOQ. The method was then applied to human samples and showed concentrations consistent with previous studies.

4 Chapter 4 Quantitative metabolomics in human skeletal muscle and plasma reveals distinct differences in key biomarkers of Type 2 diabetes

4.1 Introduction

Elevated plasma acylcarnitines (Mihalik et al. 2010), branched chain amino acid (BCAAs) (Newgard et al. 2009) and branched chain keto acids (BCKAs) (Menni et al. 2013) have all been associated with insulin resistance and the development of Type 2 diabetes. Furthermore, their use as possible early biomarkers for the development of type 2 diabetes has been validated by population based studies that show these metabolites successfully predict future disease (Wang et al. 2011; Sun et al. 2016). Despite these robust relationships between plasma profiles and disease progression, little is known about the origin of the metabolites in plasma nor which tissue's metabolism they reflect. Furthermore, it has not been established if they can be causative of insulin resistance *in vivo* or whether simply reflect an insulin resistant state.

In contrast to short-chain acylcarnitines that can be produced from glucose, amino acids, and fatty acid degradation, medium- and long-chain acylcarnitines are derived exclusively from fatty acid metabolism. Long-chain species are mainly synthesized and metabolized in mitochondria; therefore, concentrations of long-chain acylcarnitines are used as markers of mitochondrial fatty acid oxidation. Indeed, the presence of acylcarnitines in plasma is routinely used a diagnostic measure of inborn errors of fatty acid metabolism (Rinaldo, Cowan, and Matern, 2009).

It has been proposed that the plasma concentrations of acylcarnitines can predict the intracellular energy metabolism pattern and can be used as a marker of metabolic dysfunction in obesity and type 2 diabetes (Adams et al. 2009; Mihalik et al. 2010) For example, elevated even number chain lengths

(C_2-C_{20}) of acylcarnitines have been proposed to reflect mitochondrial overload when lipid availability is in excess of the capacity of the mitochondria to utilise fatty acids, leading to 'incomplete' fatty acid oxidation and accumulation of acylcarnitines (due to reduced oxidation) in muscle and subsequently into the circulating pool in plasma (Koves et al. 2008). Whether or not acylcarnitines simply reflect the lipotoxcity associated with obesity and lipid accumulation in muscle or play some causative role in human remains unknown at present (Schooneman et al. 2013). Moreover, the majority of acylcarnitine profiling thus far in human studies has been limited to plasma concentrations and not tissue content (Adams et al. 2009; Mihalik et al. 2010).

Despite the established association of intracellular lipid accumulation and intermediates with insulin resistance of glucose metabolism (Samuel, Petersen, and Shulman 2010), there are several lines of emerging evidence that plasma BCAAs and products of their catabolism such as BCKAs (produced from BCAA transamination) and odd number carbon chain lengths (C_3-C_5) acylcarnitines (products of oxidative metabolism of BCAAs) may be more closely associated with insulin resistance than lipid markers (Newgard et al. 2009; Menni et al. 2013). These findings have given rise to the hypothesis that BCAAs and associated metabolites may cause insulin resistance in skeletal muscle (White et al. 2016). Alternatively, it has since been suggested that altered skeletal muscle metabolism may contribute to those species in the circulation (Lerin et al. 2016; David et al. 2019).

Whilst a number of studies have measured circulating concentrations of acylcarnitines, BCAAs and related metabolites (Mihalik et al. 2010; Newgard 2012) and attempted to make inferences on skeletal muscle metabolism, it has

been demonstrated that plasma metabolite profiles do not accurately muscle tissue profiles (Schooneman et al. 2014). Therefore, studies investigating both tissue and plasma profiles are essential to draw conclusions from plasma profiles. This is vital given that more recent data has shown that acylcarnitines (Céline Aguer et al. 2015a), BCAAs and BCKAs (Zhou et al. 2019) can all impair insulin signalling *in vitro*. Therefore, quantitative assessments of these metabolites in human tissue are important to determine if (or at all) acylcarnitines, BCAA and BCKAs can contribute to insulin resistance in human skeletal muscle.

In recent years, much attention has been paid to the development of LC-MS/MS based methods for identification and quantification of acylcarnitines, BCAAs and BCKAs in biological tissues. Whilst these methods provide large coverage of acylcarnitines, they often require complicated and laborious steps of sample preparation which may limit them to one metabolite type. Although in recent years, more rapid and simpler methods have allowed for the simultaneous quantification of BCAAs and BCKAs in plasma, a method to quantify all three groups of these important and related metabolites (ie acylcarnitines, BCAAs and BCKAs) was lacking. In the previous chapter a quantitative method with a single and simple extraction protocol was developed and optimised to simultaneously measure all three metabolite types in both plasma and skeletal muscle tissue.

4.1.1 Aims

- To apply and validate the optimised method from the previous chapter, using plasma and skeletal muscle samples from both Type 2 diabetic patients and control subjects for absolute quantification of BCAAs, BCKAs and short-, medium- and long-chain acylcarnitines.
- To compare the signature of all three types of metabolites between plasma and muscle and examine to what extent plasma concentrations reflect skeletal muscle tissue metabolism.

4.2 Methods

4.2.1 Participant characteristics

The samples used in this chapter were obtained from two separate studies involving overweight/obese subjects and patients with type 2 diabetes. Samples from a total of ten overweight/obese patients with type 2 diabetes (T2D) and fourteen middle-aged normoglycemic controls (CON) were used in this study. Sample collection was approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (E15012015) and the NHS Research Ethics Committee (East Midlands REC reference No 14/EM/0136). Subject characteristics with details on sample amounts are shown in Table 4.1

Table 4.1 Subject characteristics of CON and T2D participants and number of samples used for the analysis of each sample type.

	CON (n=14)	**T2D (n=10)**
Age (years)	45.1 ± 1.9	47.7 ± 1.5
BMI (Kg/m^2)	31.9 ± 1.3	30.1 ± 1.3
Fasting glucose (mmol/L)	4.6 ± 0.1	6.2 ± 0.5 ****
Fasting serum insulin (mU/L)	19.6 ± 4.1	17.9 ± 2.3
HOMA-IR	4.1 ± 0.9	5.0 ± 0.9
Skeletal muscle biopsy (n)	14	10
Plasma (n)	7	10

Body mass index (BMI). **p < 0.01 Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

4.2.2 Sample collection

In all cases subjects arrived at the laboratory at 08:00 after an overnight fast (10–12 h), having abstained from heavy exercise and alcohol for the previous 48 h. Diabetic patients were instructed to not take any of their diabetic medication on the day of the experimental visit. Subjects then rested in bed in a semi-supine position, and an intravenous cannula was inserted retrograde into the superficial hand vein of one arm to obtain an arterialized venous blood sampling. Arterialization of the venous sample was achieved by heating the hand to 50-55^{o} C using hot-air warming unit (Gallen and Macdonald 1990).

An arterialised fasting state blood sample was obtained, and blood glucose concentration was determined immediately as described in chapter 2 section 2.3.1. The blood sample was then dispensed into appropriate vacutainers for the isolation of plasma (chapter 2, section 2.3.3) and serum (chapter 2, section 2.3.5) for the determination of plasma acylcarnitine, BCAAs and BCKAs and serum insulin (chapter 2, section 2.3.6). Skeletal muscle samples were obtained from the vastus lateralis in the fasted state using the Bergstrom needle biopsy technique (Bergström 1975) and immediately frozen in liquid nitrogen-cooled 2-methylbutane and subsequently stored in liquid nitrogen until analysis.

4.2.3 Preparation of samples, calibrants and QCs for LC-MS analysis

One portion of frozen skeletal muscle was freeze-dried and powdered, and a 100 µL aliquot of plasma was processed and prepared for extraction as described in Chapter 3, section 3.2.2. Calibrants were prepared from stock

solutions (described in chapter 3 section 3.2.4) and diluted to produce 10-point standard curves to cover appropriate biological ranges for skeletal muscle and plasma. Calibrants were then spiked into 7.5 % BSA in PBS and processed in an identical manner to biological samples.

Biological samples were extracted in batches with no more than 30 samples per batch. Within each batch at least 2 repeatability QC samples were also extracted in an identical manner to the biological samples. Repeatability QC samples were aliquots from a single homogeneous QC reference sample (either muscle or plasma) used to determine any variability in the extraction procedure between batches. For all the work presented in this thesis, samples within any analytical run were all extracted in the same day. All samples and calibrants were reconstituted in 100 µL of methanol:water (1:1, v:v), at this step, a 10 µL aliquot of each sample was used to create a pooled QC sample of all biological samples to be quantified. This pooled QC was injected repeatedly to account for any variation in retention times or MS performance over the duration of the analytical run.

4.2.4 LC-MS setup and analysis

Analytes were separated and analysed using both RP and HILIC chromatography linked to high resolution MS as described in chapter 3, section 3.2.3. For LC-MS analysis, the column was equilibrated with the mobile phase and a system suitability standard (SSD), comprising of an unextracted calibrant was injected for confirmation of chromatographic separation, MS detection and MS/MS fragmentation. Then, an appropriate number of solvent blanks (methanol:water 1:1) were injected to ensure no carry over from the SSD. The ten-point calibration curve was then injected followed by solvent

blanks to account for any carry over from the highest calibrant point. Prior to any biological sample injection, the column was conditioned with at least 5 injections of the pooled QC and finally the first of the repeatability QCs. Biological samples were injected in a randomised order and interspersed with a pooled QC and the subsequent repeatability QC every ten samples. Following the injections of all biological samples, the calibration curve was injected again at the of the analytical run. All samples, QCs and calibrants were stored at -80^{o} C between analysis. Samples were analysed on the C18 column first and then on the HILIC column.

To confirm that the method was suitable to quantify each set of biological samples with confidence, analytical acceptance criteria were applied to the calibrants and QC samples. The CV of both the pooled QC and calculated concentrations from the repeatability QC was assessed with at least 6 replicates in every analytical run and variability within 15% for each analyte was deemed acceptable. The concentration of each biological sample was calculated from both calibration curves (ran at the beginning and end of each acquisition).

4.2.5 Peak extraction and statistical analysis

Raw data collected from LC-MS was processed on Thermo Xcalibur Processing Quan Browser (version 4.1) software for analyte quantification as described in chapter 3, section 3.2.10. Differences in muscle and plasma metabolite concentrations between CON and T2D were assessed using a Student's unpaired *t* test. Due to sample availability, only 7 of the 14 subjects in CON had a corresponding plasma value and therefore correlations between muscle and plasma were only performed in these 7 subjects. Pearson's

correlation coefficients were used to analyse associations between muscle and plasma analyte concentrations in only those subjects (from both groups) with both a muscle and corresponding fasting plasma sample (n = 17). Correlation analysis was also performed on data pooled from both groups to investigate associations between fasting blood glucose and plasma (n =14), and muscle analyte concentrations (n= 21). Biological data are mean ± SEM unless stated otherwise. Statistical significance was accepted at $p < 0.05$. When referring to chain lengths AC were grouped as short (C_3-C_5), medium (C_6-C_{10}) and long-chain (C_{12}-C_{20}). Summed BCAA refers to the summation of leucine, isoleucine and valine concentrations. Summed BCKA refers to the summation of α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV) concentrations.

4.3 Results

4.3.1 Assessment of method performance and QCs

Within plasma, variation in the repeatability QC and the pooled QC (a measure of variation of the system) were within 10% for every analyte. For muscle analysis, all analytes repeatability QC and the pooled QC were less than 11%, except for C_{10} which had a repeatability QC variation of 16.5%. In general, variation in both types of QCs was greater in muscle than plasma, though it should be noted that muscle is a far more heterogenous in composition than plasma. The percentage difference between the two slopes was less than 8% for muscle analysis. For all standard curves regardless of matrix, the R^2 was above 0.99. Taken together, the results from the QC analysis demonstrate the analytical method met all QC criteria to quantify all analytes across multiple batches accurately. Details of all analytical performance parameters is given in Table 4.2.

Table 4.2. Assessment of the analytical run for the quantification of analytes in biological samples. Calibration curves were analysed at the beginning and end of each analytical run. Repeatability QCs are aliquots of one the same sample and pooled QCs are repeat injections of the same sample over the analysis time. Data for the second calibration curves for BCAA in muscle were not obtained due to technical difficulties with the LC-MS system.

	Skeletal muscle					Plasma				
Analyte	**Slope Pre**	**Slope Post**	**Slope difference (%)**	**Repeatability QC (%) N = 6**	**Pooled QC (%) N = 7**	**Slope Pre**	**Slope Post**	**Slope difference (%)**	**Repeatability QC (%) N = 8**	**Pooled QC (%) N = 14**
C_2	6.01	6.01	0.8	8.5	3.4	8.8	8.6	2.5	0.7	1.8
C_3	30.1	31.4	4.2	10.7	1.9	36.3	37.4	3.1	2.9	2.5
C_4	33.4	34.2	2.1	9.8	4.7	32.9	30.8	6.4	4	5.1
C_5	24.6	24.0	2.4	4.2	5.1	25.0	24.5	2.1	4.9	3.2
C_6	31.1	30.78	1.3	10.6	5.4	34.3	34.9	1.9	4.2	6.2
C_8	35.1	33.5	4.8	10.7	3.1	39.1	38.0	2.6	2.3	1.3
C_{10}	38.4	37.6	2.1	16.5	5.8	55.6	52.6	5.5	8.3	6.1
C_{12}	41.1	42.8	2.5	10.7	10.9	73.4	58.1	20.9	5.5	9.1
C_{14}	31.9	32.9	3.3	7.4	2.3	40.1	37.6	6.3	5.3	3.2
C_{16}	15.6	15.1	3.4	6.5	3.5	15.7	15.1	3.5	3.5	3.2
KIC	0.75	0.80	7.3	6.6	5.1	0.86	0.82	5.5	2.6	1.7
KMV	0.91	0.9	1.5	8.2	2.6	0.88	0.80	9.7	3.7	3.3
KIV	0.73	0.73	0.7	7.3	2.7	0.73	0.69	6.2	2.3	1.6
Leucine	-	-	-	8.3	2.4	0.80	0.80	0.1	2.9	2.1
Isoleucine	-	-	-	3.4	2.2	0.80	0.80	1.4	3.3	4.8
Valine	-	-	-	5	4.6	0.51	0.49	2.3	3.5	1.7

4.3.2 Plasma metabolites

4.3.2.1 Plasma BCAAs and BCKAs

Plasma leucine (16%) and valine (15%) concentrations were higher in T2D vs CON, p = 0.04 and 0.03, respectively. There was also a trend for isoleucine concentration (p = 0.10) to be (13.6 %) higher in T2D vs CON. Plasma BCKAs were similar between T2D and CON. Numerical elevations in KIC and KIV approached statistical significance p = 0.09 and 0.13, respectively. The sum of the three BCKAs was also numerically but not significantly higher in T2D than CON (p = 0.13). Plasma BCAA and BCKA data are shown in Figure 4.1.

4.3.2.2 Plasma carnitine and acylcarnitine profile

There were no differences in plasma free carnitine or acetylcarnitine (C_2) (Table 4.3). The sum of short-chain (but not medium- or long-chain) acylcarnitines were higher in T2D vs CON (p = 0.02) and this was driven by significant elevations in C_3 (p = 0.01), iso C4 (p =0.04), C_4 (p <0.001) and C_5:1 (p = 0.03) species. C_4-DC, C_5-OH and iso C_5 tended to be higher in T2D (p = 0.06, 0.08 and 0.10, respectively). There were no differences in any other plasma medium- or long-chain length acylcarnitines between the two groups (Figure 4.2).

4.3.3 Muscle metabolites

4.3.3.1 Muscle BCAAs and BCKAs

Muscle leucine (27%), isoleucine (21) and valine (26%) concentrations were higher in T2D than CON ($p = 0.01$, 0.04 and 0.01, respectively). Muscle BCKAs were not different between the two groups but there was a trend for lower levels of KIC in T2D than CON ($p = 0.1$). Muscle BCAA and BCKA data are shown in Figure 4.1.

4.3.3.2 Muscle free carnitine and acylcarnitines

Muscle free carnitine and acetylcarnitine concentrations were not different between groups. The sum of short-chain (but not medium- or long-chain) acylcarnitines were higher in T2D than CON ($p = 0.02$). This elevation was driven by increased concentrations in C_3-DC ($p = 0.02$), C_4-OH ($p = 0.03$), C_4-DC and C_5:1 ($p=0.01$ for both) species. There were no differences between groups in the concentrations of medium- or long-chain species (Figure 4.2).

An illustration of muscle BCAA catabolic flux is shown in Figure 4.3 and a diagram of the biochemical pathway of each BCAA's catabolism indicating where each acylcarnitine species can be formed is shown in Figure 4.4.

Table 4.3 Free carnitine, acetylcarnitine and sum of short-, medium- and long-chain acylcarnitine concentrations in human plasma and muscle samples from obese individuals with type 2 diabetes (T2D) and without T2D (CON)

	CON	**T2D**
Plasma	n=7	n =10
Free carnitine (µmol/L)	41.0 ± 3.9	43.0 ± 1.9
Acetylcarnitine (C2) (µmol/L)	8.1 ± 0.5	8.5 ± 1.2
Short-chain (C3-C5) (µmol/L)	0.7 ± 0.1	1.0 ± 0.1*****
Medium-chain (C6-C10) (µmol/L)	0.5 ± 0.1	0.5 ± 0.1
Long-chain (C12-C18) (µmol/L)	0.6 ± 0.02	0.5 ± 0.1
Muscle	n=14	n=10
Free carnitine (mmol/kg dry mass)	11.8 ± 0.6	13.3 ± 1.4
Acetylcarnitine (C2) (mmol/kg dry mass)	1.5 ± 0.3	1.9 ± 0.3
Short-chain (C3-C5) (µmol/kg dry mass)	40.9 ± 4.0	53.6 ± 4.2*****
Medium-chain (C6-C10) (µmol/kg dry mass)	10.2 ± 1.3	12.9 ± 3.8
Long-chain (C12-C18) (µmol/kg dry mass)	54.3 ± 13.2	59.2 ± 19.9

Normoglycemic controls, CON. Type 2 diabetic patients, T2D. * $p < 0.05$

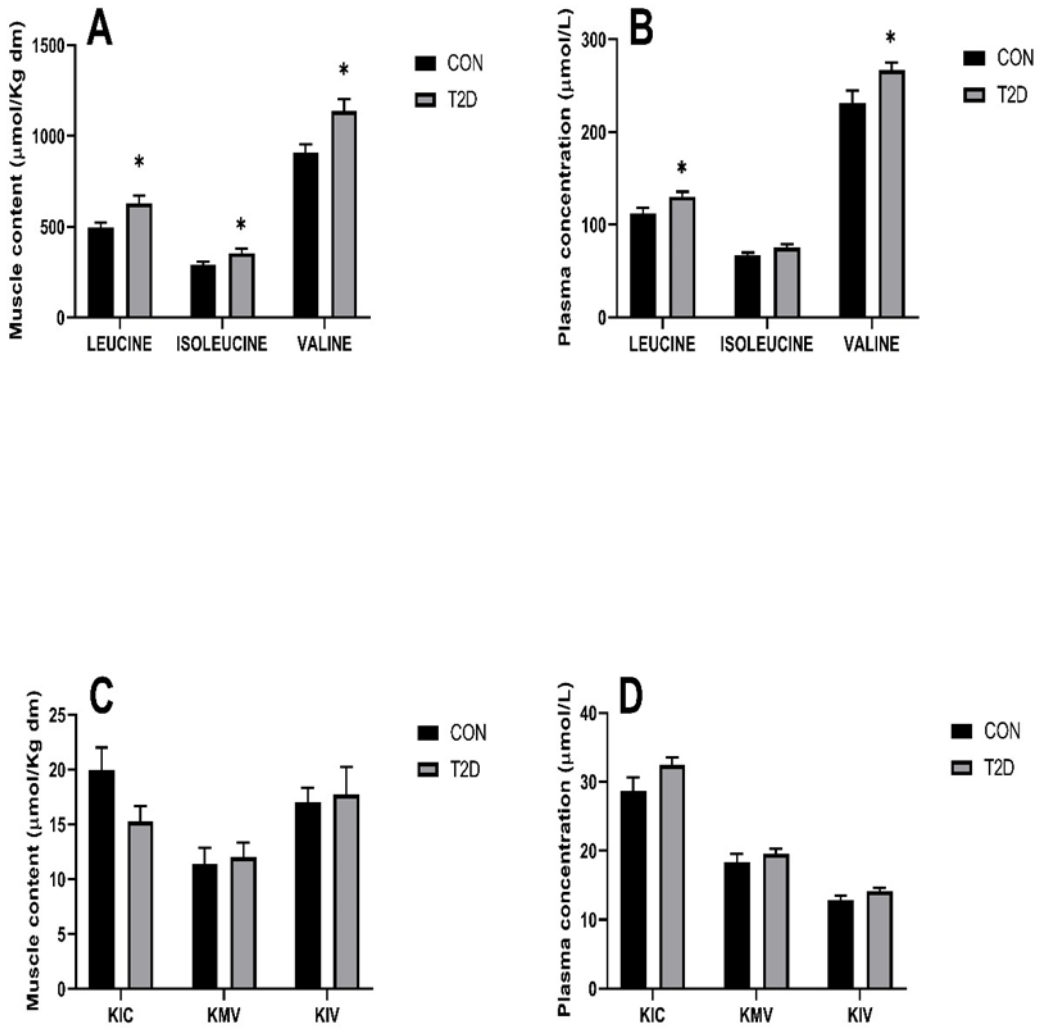


Figure 4.1 Muscle (A) and plasma (B) branched chain amino acid (BCAA) concentrations. Muscle (C) and plasma (D) branched chain keto acid (BCKA) concentrations. α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV). CON, control. T2D, type 2 diabetic patients. Data are mean ± SEM. * $p < 0.05$ from CON. For muscle n = 14 and n = 10 for CON and T2D, respectively. For plasma n = 7 and n=10 for CON and T2D, respectively.

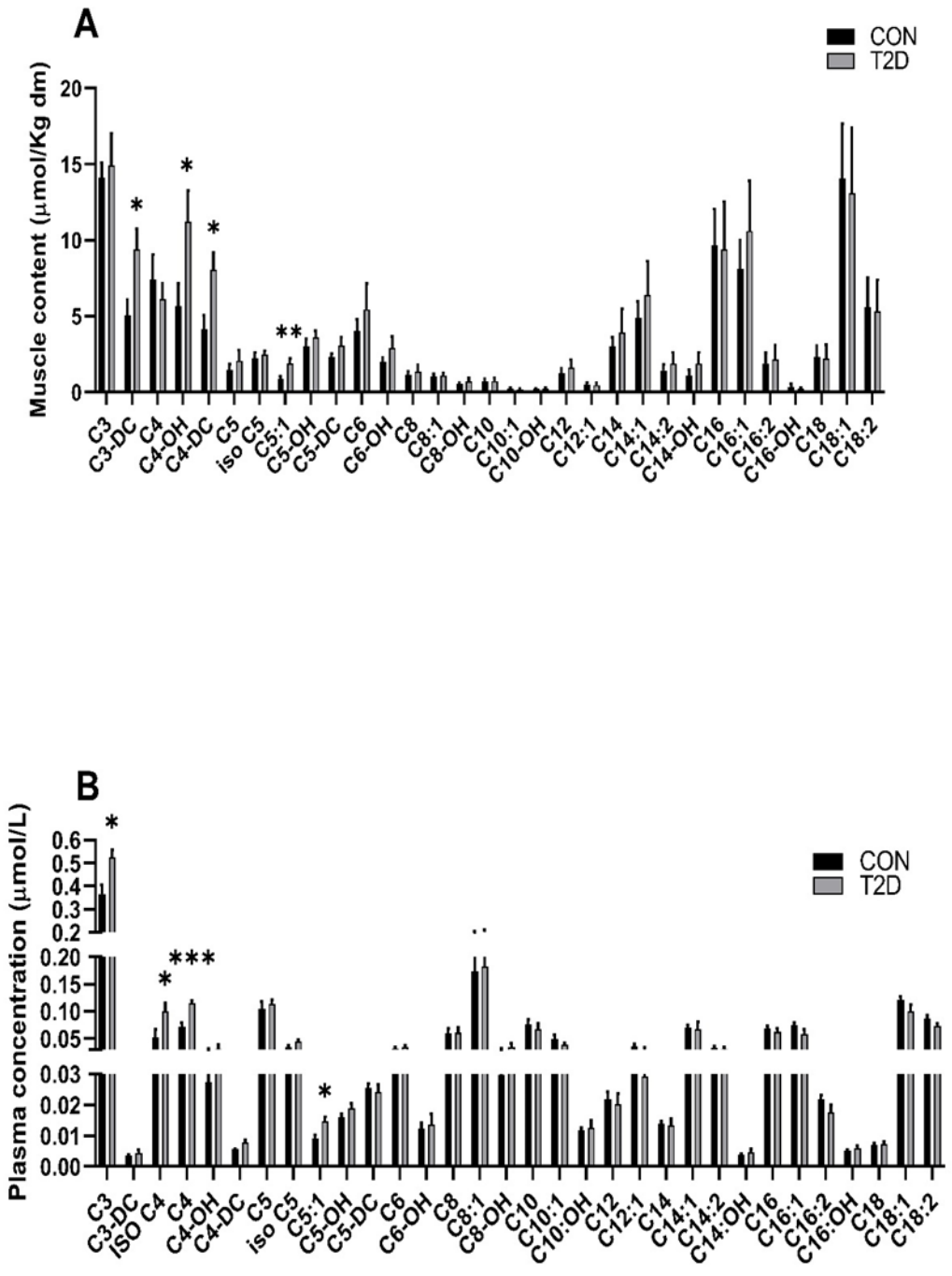


Figure 4.2 Acylcarnitine profile in muscle (A) and plasma (B). CON, control. T2D, type 2 diabetic patients. Data are mean ± SEM. * $p < 0.05$, *** $p < 0.001$ from CON compared by t test. For muscle n = 14 and n = 10 for CON and T2D, respectively. For plasma n = 7 and 10 for CON and T2D, respectively.

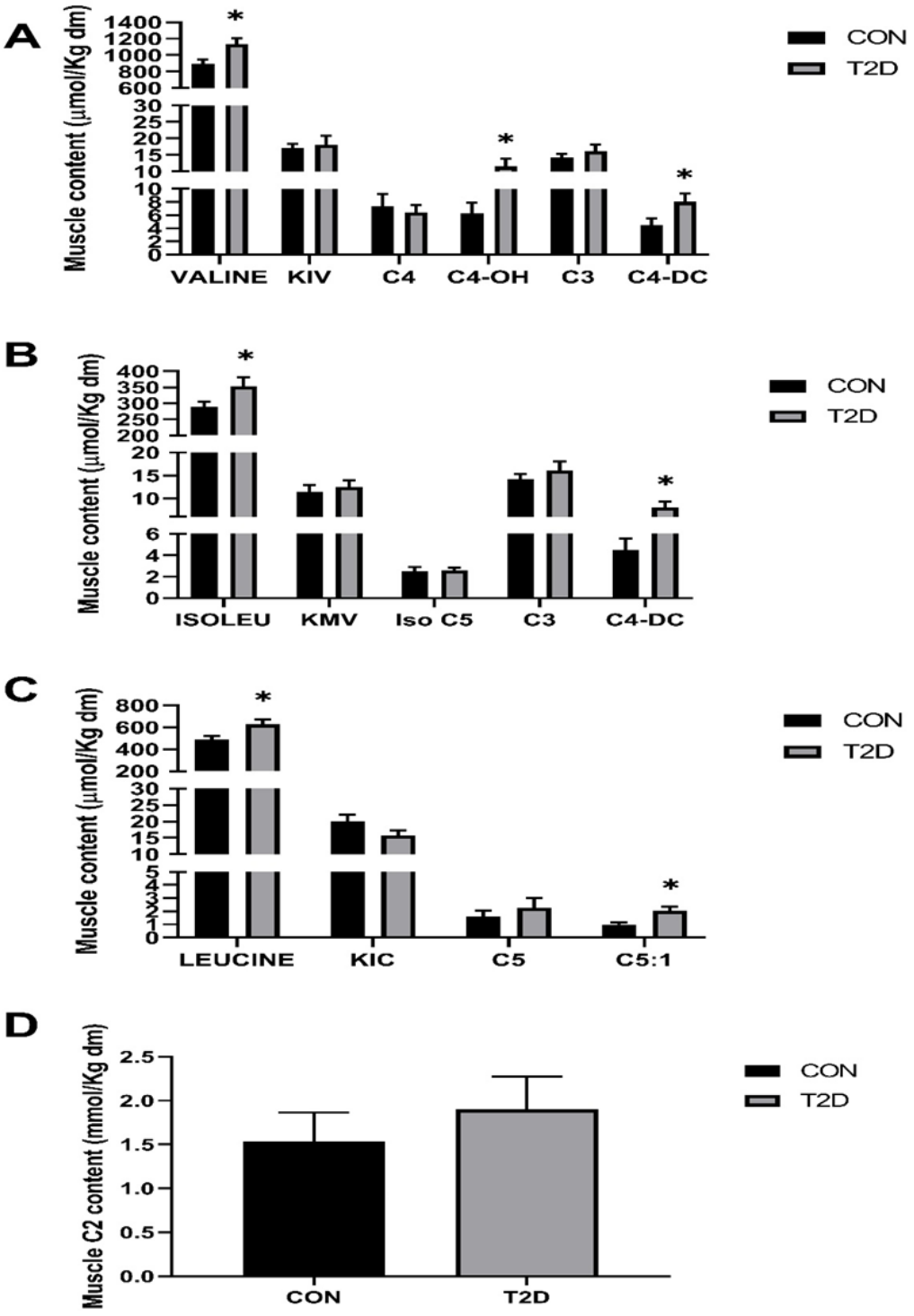


Figure 4.3 Skeletal muscle quantitative concentrations of valine (A), isoleucine (B) and leucine (C) and subsequent metabolites formed during their respective catabolism to branched chain keto acids and metabolite specific acylcarnitine species. Muscle C2, acetylcarnitine, the final product of both leucine and isoleucine catabolism in muscle (D). α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV). CON, control. T2D, type 2 diabetic patients. Data are mean ± SEM, n = 14 and n = 10 for CON and T2D, respectively. * $P < 0.05$ CON vs T2D. A schematic of BCAA catabolism showing where each acylcarnitine species is formed is shown below (Figure 4.4).

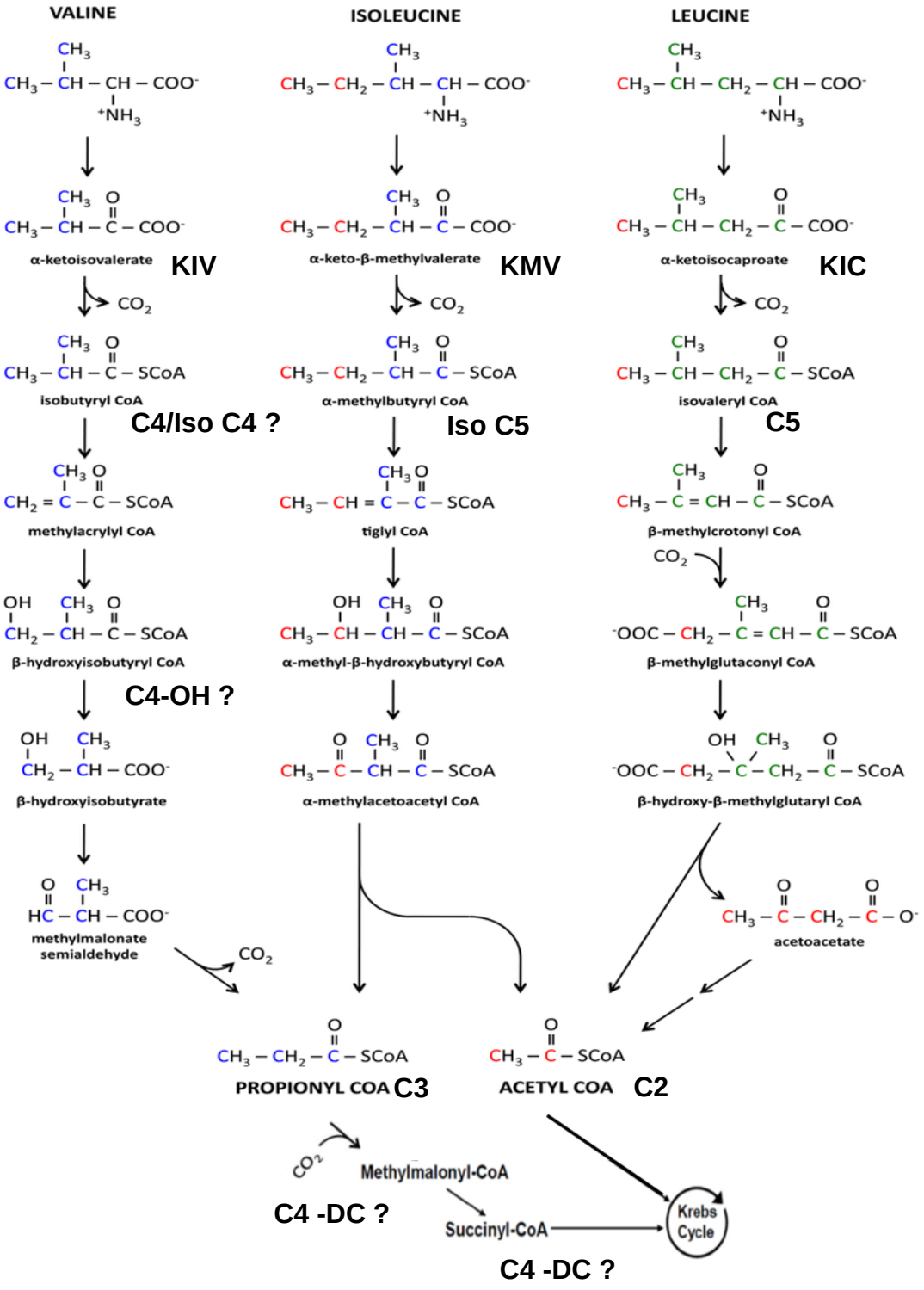


Figure 4.4 Schematic diagram of each branched chain amino acid's specific catabolic pathway. An abbreviation of quantified acylcarnitine species with the current analytical method is shown in bold next to the corresponding CoA ester from which it is formed. A question mark next to C4-OH and C4-DC denotes that these species can also be formed from other CoA esters from other metabolic pathways so may represent multiple species.

4.3.4 Correlations between muscle, plasma and fasting blood glucose

All 3 BCAAs and C_5:1 acylcarnitine were moderate-strongly correlated between muscle and plasma when data from both groups were pooled (leucine, r =0.58; p = 0.01; isoleucine, r = 0.72; p = 0.001; valine, r = 0.68; p = 0.003; C_5:1, r = 0.82; p < 0.001).

Plasma BCAAs and C_5:1, C_3 and C_5-OH were positively correlated with fasting blood glucose (Table 4.4), whereas Iso C_4 showed a weaker correlation, r = 0.5 (p = 0.07). Muscle C_5:1 also correlated with fasting blood glucose (Table 4.3), whereas the sum of intramuscular BCAA showed a weaker association r = 0.4 (p = 0.10) with fasting blood glucose.

There were no correlations between any metabolite in either muscle or plasma and fasting serum insulin or HOMA-IR.

Table 4.4 Correlations between analytes and fasting blood glucose concentration on data pooled from both groups (n = 14 for plasma and n = 21 for muscle).

Analyte	**r**	**p**
Plasma leucine	0.71	0.005
Plasma isoleucine	0.75	0.002
Plasma Valine	0.79	0.0007
Plasma KIC	0.7	0.005
Plasma KMC	0.69	0.006
Plasma KIV	0.72	0.003
Plasma C5:1	0.54	0.04
Plasma C3	0.72	0.004
Plasma C5-0H	0.54	0.04
Muscle C5:1	0.45	0.04

α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV).

4.4 Discussion

The aim of this chapter was to apply and validate the analytical method that was developed in the previous chapter for the simultaneous quantitative and targeted profiling of acylcarnitines, BCAAs and BCKAs in both plasma and skeletal muscle. The results of this study reveal elevated plasma and muscle BCAA and short chain acylcarnitine in patients with T2D when compared with their obese non-diabetic counterparts. However, there were no differences in plasma and muscle BCKAs or medium- and long-chain acylcarnitines between groups.

On average, the plasma BCAA concentrations were ~15 % higher in patients with T2D compared with normoglycaemic control subjects that were matched for age and BMI. These elevations are in line with several reports linking increased circulating BCAAs with insulin resistance across a range of population cohorts (Newgard et al. 2009; Fiehn et al. 2010; Alfaqih et al. 2018; Chen et al. 2016). The fact that plasma BCAA levels can be used to predict the future development of Type 2 diabetes (Wang et al. 2011) highlights the importance of accurate and validated measurements of these metabolites in at risk populations. In line with this, each plasma BCAA was strongly correlated with fasting blood glucose (an index of fasting insulin resistance) across all participants from both groups ($r > 0.7$ for each BCAA, Table 4.3). These findings extend upon much of the previous untargeted metabolomic approaches to provide quantitative assessment of changes in those metabolites. If quantitative methods could be applied to determine plasma BCAAs concentrations in subjects progressing from impaired fasting glucose to clinically diagnosed T2D then this could lead to the potential for BCAAs to

be used as diagnostic biomarkers in disease progression. However, large scale studies would be needed to validate this.

Plasma levels of BCKAs (the reversible products of BCAA transamination) were not significantly different between T2D and CON but there was a trend for elevated KIC and KIV (arising from leucine and valine catabolism, respectively). BCKAs have also been associated with T2D and impaired fasting glucose (Menni et al. 2013). Indeed, despite the lack of significant elevations in the present study, each BCKA was positively correlated with fasting blood glucose. BCAA and BCKAs have both been independently associated with the development of insulin resistance and T2D suggesting that perturbations in BCAA catabolism are likely key sites of aberrant metabolism in insulin resistance states. In support of this statement elevated plasma short-chain acylcarnitines were also observed in T2D in the current study. These species are produced when BCKAs are catabolised irreversibly and committed to their oxidative fate. Plasma C_3, iso C_4 and C_5:1 (all BCAA derived) were all significantly elevated in T2D, which is consistent with previously published studies on obese (Newgard et al. 2009) and diabetic patients (Mihalik et al. 2010; Bene et al. 2012) that demonstrated elevated C_3 and C_5 species in plasma, providing further evidence for increased BCAA catabolism in obese humans. Furthermore, in the present study as with BCAA plasma C_3, $C_{5:1}$ and $C_{5\text{-}OH}$ also showed positive correlations with fasting blood glucose. Collectively, the findings from this study provide further compelling evidence of the association of a BCAA-related plasma signature and insulin resistance in T2D.

The method used in the current study was also able profile a full range (short, medium, and long) of acylcarnitines. In contrast to previous studies (Adams et al. 2009; Mihalik et al. 2010), elevations of medium and long chain acylcarnitines (derived exclusively from fatty acid metabolism) in plasma were not observed in T2D patients. One possible explanation for these discrepancies could be related to the fact that the control group in this group was matched to the T2D group for age and obesity. It is possible that if a lean age-matched control group had also been included, then a further differentiation in the profile of other medium-or long-chain acylcarnitines may have surfaced. Indeed, the aforementioned study investigated a lean, obese and an obese with T2D group (Mihalik et al. 2010). In that study both the obese and T2D groups exhibited elevated plasma long-chain acylcarnitines compared to lean age matched control with no difference between the obese and obese T2D subjects. The accumulation of even chain acylcarnitines with obesity and T2D is thought to represent incomplete β-oxidation of fatty acid intermediates resulting in the formation of corresponding acylcarnitines and subsequent export into the plasma (Koves et al. 2008). Given that the control group in this study was also obese, the results in the current study most likely reflect the effect of insulin resistance *per se* rather than increased adiposity. It is important to note that not all studies profiling circulating fasting acylcarnitines have found differences between control and T2D subjects. On the contrary, reduced medium and long chain acylcarnitines have also been observed in T2D patients compared to healthy lean controls (Bene et al. 2012). This could suggest reduced rather than incomplete fatty acid oxidation in T2D. Clearly there are discrepancies in plasma acylcarnitines profiles across

different cohorts. This is important to consider as numerous studies have reported on elevated plasma acylcarnitines in human studies with the assumption that they reflect skeletal muscle metabolism (Mihalik et al. 2010). However, more recent studies measuring transorgan fluxes in pigs (Schooneman et al. 2015) and humans (G. Xu et al. 2016) have demonstrated that liver is the major contributor to the plasma pool under fasting conditions and plasma inadequately reflect skeletal muscle metabolism (Schooneman et al. 2014). Therefore, the inconsistent findings when measuring fasting circulating acylcarnitine profiles could be reconciled by accounting for liver metabolism. Interestingly, in the flux studies it was shown that liver and the gut can also produce C_3 and C_5 acylcarnitines and is releasing them into the blood during fasting states, suggesting liver may be the key site of origin of the elevated plasma acylcarnitines observed in the current study.

The origin of elevated plasma BCAAs and associated short-chain acylcarnitines has been the subject of much research over the past decade. Although elevated circulating BCAA in obese subjects was observed many decades ago (Felig, Marliss, and Cahill 1969) it is only more recently that these metabolites have been implicated in the progression of insulin resistance (Newgard et al. 2009) Data from animal studies has led to the hypothesis that obesity leads to reduced adipose tissue and liver BCAA catabolism and spill over from the adipose tissue into the circulation (She, Van Horn, et al. 2007) Consequently, skeletal muscle may take up and catabolises this excess supply of BCAA and these BCAAs may compete with fatty acid oxidation within the mitochondria, the latter point being supported by the findings of elevated BCAA derived acylcarnitines in skeletal muscle of rodents fed a high fat diet,

supplemented with BCAAs (Newgard 2012). However, direct evidence for this in humans is lacking. One of the novel elements of the developed analytical method in the current work is that it enabled the quantitative assessment of BCAA catabolic products from the level of the BCAA to short chain acylcarnitines formed during catabolism (Figure 4.3). BCAA concentrations were several fold higher than their catabolic products, indeed valine was an order of magnitude higher than its short chain acylcarnitine products. Furthermore, leucine and isoleucine can both form Acetyl-CoA before entering the TCA/krebs cycle for complete oxidation. However, products of leucine and isoleucine catabolism were in the μmol/kg dry mass range where as acetylcarnitine which is formed form Acetyl-CoA was in the mmol/kg dry mass range (an order of magnitude higher). These quantitative assessments of BCAA catabolites suggest only a minor contribution of BCAAs to energy provision, especially during the fasted state in humans. This is important to consider as Acetyl-CoA is the end product of β- oxidation of fatty acids and is likely the main source of Acetyl-CoA and acetylcarnitine, not BCAAs. In support of this, it has been demonstrated using stable isotope tracers in rodents that BCAAs only contribute ~ 5 % of total carbons to the TCA cycle and fatty acids are the major contributor of carbons to the TCA cycle in muscle (Neinast et al. 2020). Taken together, these findings argue against the suggested hypothesis that excessive BCAA catabolism in muscle and compete with fatty acid oxidation in muscle.

Recent evidence has demonstrated that BCAA catabolism may be impaired in skeletal muscle of insulin resistant individuals (Lerin et al. 2016) and that skeletal muscle may directly contribute to the circulating BCAA pool following

the induction of insulin resistance in rats (David et al. 2019). However, data from our group has demonstrated that muscle BCAA derived short-chain acylcarnitines were more closely related with muscle insulin resistance and accumulated in excess (suggesting that catabolism is increased not impaired) during an euglycaemic-hyperinsulineamic clamp with concurrent lipid infusion, an experimental model of lipid-induced insulin resistance (Stephens et al. 2014). Collectively, the human studies in the literature implicate skeletal muscle as an important site of origin of the elevated levels of circulating BCAA often observed in insulin resistant subjects and warrants further investigation.

In agreement with the altered plasma profile of BCAA observed in patients with T2D in the present study, their intramuscular BCAA content was also ~25% higher when compared with CON. This was not accompanied by changes in their respective muscle BCKA content. However, there was trend for reduced muscle KIC in T2D. This could imply an impairment in muscle BCAA catabolism, leading to accumulation of BCAAs and possible export into the plasma and thus muscle may be a contributing pool of elevated BCAAs seen in plasma. In support of this notion, a strong correlation between plasma and skeletal muscle BCAA concentrations. However, to confirm to what extent muscle, or indeed any other tissue including adipose, contributes to the plasma pool of BCAA, studies of their arterio-venous balance across multiple organs are required.

There were also significant elevations of some short-chain acylcarnitines (including C_3-DC, C_4-OH, C_4-DC and C_5:1) were also observed in skeletal muscle from individuals withT2D. $C_{5:1}$ is formed from leucine metabolism, whereas C_4-DC can be formed from both valine and isoleucine catabolism and

could possibly represent an inability to utilise succinate. It should also be noted that C_4-OH (hydroxybutyryl/hydroxyisobutyryl) can be derived from either ketone (hydroxybutyryl) or valine catabolism (hydroxyisobutyryl). The current analytical method did not allow for sufficient discrimination between the two isomers. Given that in the current study other species (C_4-DC) relating to valine catabolism were also elevated, a possible contribution of valine catabolism to the production of C_4-OH species without chromatographic separation of the isomers cannot be ruled out. Interestingly, elevated hydroxyisobutyrate (which is formed during valine catabolism in the step after hydroxyisobutyryl-CoA and the production of C_4-OH, see **Figure 4.4)** has been observed in diabetic muscle supporting the notion that BCAA catabolism may be elevated in diabetic skeletal muscle (Jang et al. 2016). Furthermore, hydroxyisobutyrate may also leave the muscle cell and drive vascular fatty acid transport into the muscle cell and promote lipid accumulation and subsequently insulin resistance. Taken together, these data suggest a degree of excess BCAA catabolism as a result of obesity related insulin resistance. The fact that intramuscular BCAA content remained elevated despite concomitant increases in their catabolic products could suggest that the accumulation of BCAA may be in excess of their catabolism and possibly through a feedback mechanism may be limiting further catabolism. From the available human data, it could be speculated that insulin resistance leading to excessive proteolysis and BCAA catabolism in muscle leading to the accumulation of short chain acylcarnitines in muscle, given that they likely contribute only minor amounts to muscle energy provision, overtime they may spill over into the circulation, in support of this muscle and plasma C_5:1 had

the strongest of all correlations measured in this study and both muscle and plasma $C_{5:1}$ correlated with fasting blood glucose. Despite studies measuring flux of acylcarnitines in pigs (Schooneman et al. 2015) and humans (G. Xu et al. 2016) have shown liver is the main contributor to fasting acylcarnitine profiles and muscle may be taking up these species, it should be noted that no studies have assessed how BCAA related metabolites or indeed medium and short chain acylcarnitines fluxes are altered in insulin resistant states in humans. Future studies should focus of measuring metabolite flux as well as metabolite concentrations.

The origin of the increased intramuscular BCAAs remains uncertain. Insulin resistance may accelerate muscle protein breakdown and hence contribute to the free amino acid pool of BCAA. Indeed, post absorptive skeletal muscle breakdown rates are greater in patients with T2D but this was matched by increased synthesis (Bell et al. 2006). Therefore, the elevated muscle BCAA measured from a single biopsy giving a ‘snapshot’ of dynamic processes in could reflect greater muscle turnover in T2D. Free intramuscular amino acids were elevated following limb immobilisation (Glover et al. 2008) and it is well established that even short periods of muscle disuse can lead to the rapid development of muscle insulin resistance (Dirks et al. 2019). In addition, recent work has demonstrated leucine release from muscle following 1 week of muscle disuse (Wall et al. 2020). Given that physical inactivity is a major underlying factor in the development of insulin resistance and perturbed lipid metabolism (Chee et al. 2016), it is conceivable that alterations in muscle protein metabolism may contribute to the elevated plasma BCAAs seen in obese and insulin resistance individuals. Further investigations into the activity

of intracellular enzymes are required to elucidate the possible mechanisms. Recent work in animals has demonstrated that the activity of the reversible enzymatic reaction catalysed by the branched chain amino transferase (BCAT), which converts BCAA to their respective BCKA, is reduced with insulin resistance (David et al. 2019), which also increased phosphorylation (and hence inactivation) of the irreversible enzymatic reaction catalysed by the branched chain keto dehydrogenase complex (BCKDH), which converts BCKA into intermediates and ultimately the formation of short-chain acylcarnitines. How the state and expression of this enzyme complex differs in human skeletal muscle between insulin sensitive and insulin resistant individuals and in response to insulin stimulation remains to be determined.

A limitation of this work and others that have characterised fasting concentrations of metabolites, is that it is difficult to assess if they simply reflect an insulin resistant state or were causative of insulin resistance in some way. In this cross-sectional analysis, it is only possible to conclude that T2D is associated with the elevated levels of metabolites observed in plasma and muscle. Whether these metabolites are causative of insulin resistance can only be established by measuring tissue concentrations of such metabolites in the presence of the insulin stimulated state. Currently, no study has characterised how theses metabolites change from the fasted to the insulin stimulated state in human skeletal muscle.

4.5 Conclusions

In conclusion the results of this work demonstrate significant perturbations in BCAA metabolism in patients with T2D which is likely to be associated with

insulin resistance *per se* rather than adiposity. These data are quantitative assessments of metabolites in both muscle and plasma and extend upon previous untargeted and/or semi quantitative published literature by suggesting that BCAAs and products of their catabolism are reflective of each other in plasma and muscle. This warrants further investigation into the role BCAAs may be playing in perturbed metabolism and the development of insulin resistance and T2D and their use as circulating biomarkers of the underlying processes.

5 Chapter 5 The effect of insulin on skeletal muscle branched chain amino acid and acylcarnitine profiles. A comparison of age and obesity.

5.1 Introduction

Elevations in plasma branched chain amino acids (BCAAs) in obesity and type 2 diabetes (T2D) are well established in numerous experimental models (Siddik and Shin 2019). Despite this, the underlying mechanism(s), nor the organs(s) responsible for their elevated levels in plasma is currently unknown. Recent reports in humans and rodents have demonstrated that obesity and insulin resistance may directly perturb skeletal muscle BCAA metabolism (Lerin et al. 2016; David et al. 2019) and possibly even independent of changes in adipose and liver BCAA catabolic capacity (David et al. 2019). Thus, alterations in skeletal muscle BCAA metabolism, independent of concurrent aberrations in other tissues, may contribute to the elevated circulating BCAA observed in obese and insulin resistance states. In support of this notion, in the previous chapter (chapter 4) elevated plasma and muscle BCAAs were observed in patients with (T2D). Therefore, skeletal muscle may be major contributor to elevated BCAA concentrations seen in insulin resistant individuals.

In contrast to the lipotoxicity model which suggests that accumulation of intermediates such diacylglycerides and ceramides arising from an imbalance between lipid delivery and reduced oxidation in muscle may directly impair insulin signalling (Samuel, Petersen, and Shulman 2010), other reports have suggested that excessive mitochondrial entry of lipids leading to the accumulation of acylcarnitines may be causative of insulin resistance (Koves et al. 2008). The majority of the support for the latter hypothesis has come from either rodent or *in vitro* work (Koves et al. 2008; Aguer et al. 2015) or from observations of elevated plasma acylcarnitine concentrations in humans

(Mihalik et al. 2010; Adams et al. 2009). Recent work from this group has shown that lipid infusion during a hyperinsulinaemic euglycaemic clamp, short chain acylcarnitines arising from BCAA catabolism were more closely associated with lipid induced insulin resistance than lipid derived medium or long chain acylcarnitines (Stephens et al. 2014). Based on these findings, it is possible that in pathophysiological states where muscle lipid content is elevated i.e obesity then BCAA catabolism may be increased in conditions where it would normally be supressed, such as during insulin infusion (Stephens et al. 2014).

Ageing per se is also associated with insulin resistance and reduced glucose tolerance (Luzi, Castellino, and DeFronzo 1996). Insulin also exerts an inhibitory effect on protein breakdown and elderly individuals have been shown to have a blunted inhibition of muscle proteolysis in response to insulin (Wilkes et al. 2009). It is well established that ageing is also associated with a blunted anabolic response to amino acids (Cuthbertson et al. 2005; Wall et al. 2015). Thus, with ageing *per se* there could be an increase in free amino acids from muscle breakdown and inability to incorporate readily available amino acids into muscle protein. Whether these amino acids contribute to excessive catabolism in muscle and how, if at all, their availability is associated with muscle insulin resistance is unknown. In chapter 3, a quantitative method to measure BCAA, BCKA and a range of acylcarnitines was developed. This method combined with measurements of BCKDH protein expression would allow the determination of BCAA catabolic flux and how factors such as age and increased adiposity may impact BCAA metabolism in skeletal muscle.

Despite the emerging lines of evidence implicating skeletal muscle as a major contributor to the plasma metabolite signature observed in insulin resistant individuals (i.e., elevated BCAA and a range of acylcarnitines), quantitative assessment skeletal muscle content of all these metabolites in obese, insulin resistant humans has not performed from a single analysis. Furthermore, how these metabolite concentrations change in the insulin stimulated state has not been investigated previously. Therefore, to extend beyond much of the published reports that have measured plasma profiles and made inferences about skeletal muscle (Mihalik et al. 2010) or the reports that have been limited to a single static measure in the fasted state and subsequently hypothesised that BCAA and acylcarnitines are causative of insulin resistance, measurement of these metabolites in the face of insulin are required.

5.1.1 Aims

The aim of this chapter was to investigate the effect of age and adiposity with a specific focus on intramuscular BCAA, BCKA and acylcarnitine concentrations and their respective responses to insulin to determine if any differences may corroborate with reports of perturbation in their metabolism seen in obese and insulin resistant states.

A second aim was to provide further validation of the analytical method developed in chapter 3 to demonstrate its ability to detect expected physiological changes in response to insulin stimulation

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5.2 Methods

5.2.1 Participant characteristics

The samples used in the current study were part of a larger study and some of the data for the YL group has been published previously (Chee et al. 2016). The aim of that study was to assess the effect of ageing and adiposity on skeletal muscle lipid metabolism and insulin sensitivity as opposed to changes in BCAA metabolism.

Samples from seven young lean (YL) and nine old lean (OL) and nine old obese (OO) healthy, recreationally active male volunteers were used in this cross-sectional study, which was approved by the University of Nottingham's medical school ethics committee in accordance with the Declaration of Helsinki (medical ethics approval number E13102011BMS). All participants underwent medical screening, blood testing and gave written informed consent to participate in the study and they were made aware that they were free to withdraw at any point. Participants were excluded if they smoked, had diabetes or other metabolic disorders, cardiovascular disease, blood disorders or abnormal blood tests. Participant characteristics are presented in Table 5.1.

Table 5.1 Participant characteristics of young lean (YL), old lean (OL) and old obese (OO) male volunteers

	YL	**OL**	**OO**
n	7	9	9
Age (years)	21.5 ± 1.0	70.7 ± 0.5 ***	68.8 ± 0.6 $^{\#}$
Body mass (kg)	76.5 ± 4.5	70.7 ± 3.0	86.9 ± 2.2 $^{\#\#\#}$
BMI (kg/m^2)	23.2 ± 1.0	23.4 ± 2.1	29.1 ± 2.1 $^{\#\#\#}$
Lean body mass (kg)	59.3 ± 3.8	51.6 ± 1.4*	55.3 ± 2.0
Leg lean mass (kg)	21.8 ± 1.7	17.2 ± 0.8*	19 ± 0.7
Fat mass (kg)	12.0 ± 1.8	14.5 ± 2.0	26.2 ± 1.1 $^{\#\#\#}$
FMI (kg/m^2)	3.7 ± 0.6	4.8 ± 0.6	8.8 ± 0.5 $^{\#\#\#}$
Leg fat mass (kg)	5.4 ± 0.9	4.9 ± 0.5	7.4 ± 0.6 $^{\#\#}$
Trunk fat mass (kg)	5.6 ± 0.8	8.0 ± 1.4	16.6 ± 0.6 $^{\#\#\#}$
Fasting blood glucose (mmol/L)	4.6 ± 0.1	4.6 ± 0.1	5.1 ± 0.2
GDR (µmol/kg lean body mass/min)	59.8 ± 3.6	43.6 ± 2.1**	27.7 ± 2.5 $^{\#\#\#}$

GDR = Glucose Disposal Rate during the euglycaemic-hyperinsulinaemic clamp. BMI= Body mass index. FMI = fat mass index. Data are mean ± SEM * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, OL compared to YL. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$, OO vs OL.

5.2.2 Experimental protocol

Participants arrived at the laboratory at 0800 after an overnight fast. A DEXA scan (Lunar Prodigy,GE Healthcare, US) was performed to assess body composition (as described in chapter 2, section 2.2.4). They then rested semi supine on a bed while cannulae were inserted retrograde into a superficial vein on the back of the hand for arterialised blood sampling and forearm veins for insulin and glucose infusions. A 3-hour hyperinsulinaemic euglycaemic clamp at 60 mU m^{-2} min^{-1} was carried out to assess insulin sensitivity (protocol described in detail in chapter 2 section 2.2.1).

5.2.3 Sample collection

Skeletal muscle samples were obtained from the vastus lateralis using the Bergstrom needle biopsy technique (Bergström 1975) before and immediately after each clamp and immediately frozen in liquid nitrogen-cooled 2-methylbutane and subsequently stored in liquid nitrogen until analysis. Unfortunately, blood samples were not available for the determination of plasma metabolites and therefore analysis was carried out in muscle only.

5.2.4 BCAA, BCKA and acylcarnitine quantification

BCAAs, BCKAs, free carnitine and range of acylcarnitines were extracted from freeze dried muscle tissue samples as described in chapter 3. Samples, calibrants and QCs were processed and analysed by LC-MS/MS with the same analytical parameters as described in chapter 4. Reproducibility of analytical run was assessed by 7 repeat injections of the same pooled QC same and extraction repeatability was assessed by 9 separate injections of 9 aliquots of the same muscle biopsy sample. Raw data collected from LC-

MS/MS analysis was processed on Thermo Xcalibur Processing Quan Browser (version 4.1) software for analyte quantification as described in chapter 3.

5.2.5 Western blotting

Muscle protein was extracted from one wet portion of frozen skeletal muscle using the mRNA extraction described in Chapter 2 (section 2.4.3) Following protein quantification using the Pierce bicinchoninic acid (PCA) assay, 30 µg of protein was loaded onto fixed 10% acrylamide gel and separated using electrophoresis. Proteins were transferred overnight to a polyvinylidene difluoride membrane. Protein expression was assessed using rabbit polyclonal antibodies for total BCKDH (E1α polypeptide) (Cat. No. # ab138460) and phospho S^{293} BCKDH (Cat. No. # ab200577), purchased from Abcam (Abcam, Cambridge, UK). Membranes were incubated overnight at 4^{o}C with primary antibodies diluted 1:2,500 in 5% BSA/TBS-T (w/v). Specific signal was detected with a horseradish peroxidase-conjugated secondary antibody (1:5,000) swine anti-rabbit horse- radish peroxidase diluted in 5% BSA/TBS-T. Following addition of ECL Prime, protein expression was then visualised in a dark room using X-ray film and normalized to α-actin (1:5,000, Cat. No. #A2066; Sigma, Dorset, UK).

5.2.6 Statistical analysis

The aim of this chapter was to independently compare the effect of age and obesity *per se* on muscle BCAA and acylcarnitine metabolism. Therefore, differences in group participant characterises were compared with separate students unpaired t test (YL vs OL and OL vs OO). The within and between

group differences in the fasted state and in response to the insulin clamp were determined using two separate, two-way mixed ANOVAs (group x pre/post insulin). This design does not permit the direct comparison of YL with the OO group. When a significant main effect was observed, a Sidak post hoc test was performed to identify individual differences. Statistical significance was set at $p < 0.05$, and all values are presented as means ± SEM.

When referring to chain lengths, AC were grouped as short (C_3-C_5), medium (C_6-C_{10}) and long-chain (C_{12}-C_{20}). Summed BCAA refers to the summation of leucine, isoleucine and valine concentrations. Summed BCKA refers to the summation of α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV) concentrations.

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5.3 Results

5.3.1 Participants

YL and OL were matched for BMI and FMI but OL had lower total and leg lean mass and a lower glucose disposal rate during the 3-hour clamp ($p<0.05$, see Table 5.1 for level of significance).

OO had higher BMI, FMI, total body, leg, trunk and total fat mass compared to OL. Glucose disposal during the 3-hour clamp was also markedly reduced in OO compared to OL ($p<0.05$, see Table 5.1 for levels of significance).

5.3.2 Analytical method parameters

For all analyte standard curves, the R^2 was above 0.99. The CV of the pooled QC ranged from 0.5 – 7.3 % for all metabolites (n = 7). The CV of the extraction repeatability QC was below 12 % for all analytes except for KIV which was 17.7 % (n = 9). The analytical method met all the quality control criteria required for accurate quantification of metabolites.

5.3.3 Effect of age and insulin (YL vs OL groups)

BCAA and BCKAs

ANOVA revealed significant ageing ($p = 0.04$), insulin ($p < 0.001$) and interaction ($p = 0.05$) effects on muscle total BCCA content. Fasting muscle total BCAA content was 35% lower in OL vs YL ($p = 0.005$) and insulin resulted in a decrease in total BCAA content by 54 % in YL and 43 % in OL ($p < 0.001$) (Figure 5.1D). Similarly, there were significant ageing ($p = 0.02$), insulin ($p < 0.001$) and their interaction ($p = 0.04$) effects on muscle leucine. Muscle leucine content (Figure 5.1A) followed a similar pattern and was 37 % lower in

OL vs YL in the fasted state ($p = 0.003$) and was reduced 65 and 58 % following insulin in YL and OL, respectively ($p < 0.001$). Fasting muscle isoleucine content was 36% lower in OL vs YL (group effect $p = 0.04$) and declined (72%) in both groups following insulin infusion (insulin effect $p < 0.001$) (Figure 5.1B). There was a main effect of age ($p = 0.04$) on muscle valine, which was 33 % lower in OL vs YL in fasted state ($p = 0.008$) there was also a main effect of insulin ($p < 0.001$), however post hoc analysis revealed that the decline in valine content was only present in YL ($p < 0.001$). Total valine reduced by 42 % in YL but only 25 % in OL (interaction effect $p = 0.06$) (Figure 5.1C).There were no differences between YL and OL in the fasting state muscle content of any of the three BCKAs or their sum (Figure 5.1E-H). Insulin infusion resulted in the reduction in the content of KIC and KMV in YL only (insulin effect for both metabolites $p = 0.03$) but there was no effect of insulin on KIV content in either group.

Acylcarnitine content

There were no significant differences between groups for any single BCAA derived acylcarnitines species. However, ANOVA revealed a main effect of interaction ($p = 0.01$) and insulin ($p = 0.002$) on the sum of short chain acylcarnitines derived from BCAA metabolism was higher in the fasted state in YL vs OL ($p = 0.01$) and insulin suppressed their abundance in YL ($p = 0.001$) only (Figure 5.2A) There were no significant differences in fasting medium chain acylcarnitines (Figure 5.2B) and insulin reduced their concentration in both groups (insulin effect $p < 0.001$). There was trend for fasting long chain acylcarnitines (interaction effect $p = 0.08$) to be higher in YL compared to OL (Figure 5.2C), and insulin suppressed long chain acylcarnitine

content in YL only (insulin effect p = 0.002). There a main effect of age on muscle acetylcarnitine (age effect p = 0.01) which was higher in YL than OL in the fasted state (p = 0.03) and was reduced following insulin stimulation in the YL group only (insulin effect p = 0.003). (Figure 5.3A). A full profile of muscle acylcarnitine concentrations in the fasted and insulin stimulated state can be seen in Appendix C

5.3.4 Effect of increased adiposity (OL vs OO groups)

BCAAs and BCKAs

There was a main of adiposity (p = 0.02) and insulin (p < 0.001) on muscle isoleucine content. Fasting muscle isoleucine content (Figure 5.1B) was 17 % higher in OO vs OL (p = 0.02) and declined (72 %) in both groups (p < 0.001). ANOVA revealed a strong tend for a main effect of adiposity and an adiposity and insulin interaction on muscle leucine (p = 0.06 for both). Fasting muscle leucine content was 19 % higher in OO vs OL (Figure 5.1A) and declined 57 and 66 % in OL and OO, respectively with insulin infusion (p < 0.01). There was a strong trend for an adiposity and insulin interaction effect on muscle valine. Fasting valine was also 19% higher in OO vs OL and declined 45 % in OO (p < 0.001) but only 24 % in OL. (Figure 5.1C). Fasting muscle total BCAA content was 19% higher in OO vs OL (interaction effect p= 0.06) and declined 56 and 43 % in OO and OL, respectively (p<0.001). There were no differences between OL and OO in the fasting muscle content of any of the three BCKAs or their sum (Figure 5.1E-H). Insulin reduced the content of KIC and KMV in OO (P = 0.01) but not in OL and there was a significant interaction effect for KIV (p = 0.03) which revealed a significant reduction in OO following insulin (p =0.04).

Acylcarnitine content

ANOVA revealed a significant interaction effect of adiposity and insulin on total short chain acylcarnitines derived from BCAA metabolism (p = 0.01). Fasting muscle content was higher in OO than OL (p = 0.006; Figure 5.2A) and insulin resulted in their suppression in OO only (p = 0.002). Specifically, muscle succinyl/methylmanoylcarnitine (C_4-DC) (Figure 5.3B) was also elevated in the fasted state in OO when compared to OL (p = 0.004) and was suppressed in OO only with insulin infusion (p= 0.003). The numerical elevations in total medium and long chain acylcarnitines (Figure 5.2B-C) in OO when compared to OL did not reach statistical significance (group effect p = 0.05 and p = 0.09, respectively). Insulin infusion suppressed both medium and long chain acylcarnitines in OO (insulin effect p = 0.003 and p = 0.03, respectively). Muscle acetylcarnitine was not different between the two groups in the fasted state and insulin suppressed its abundance in OO (p = 0.001).

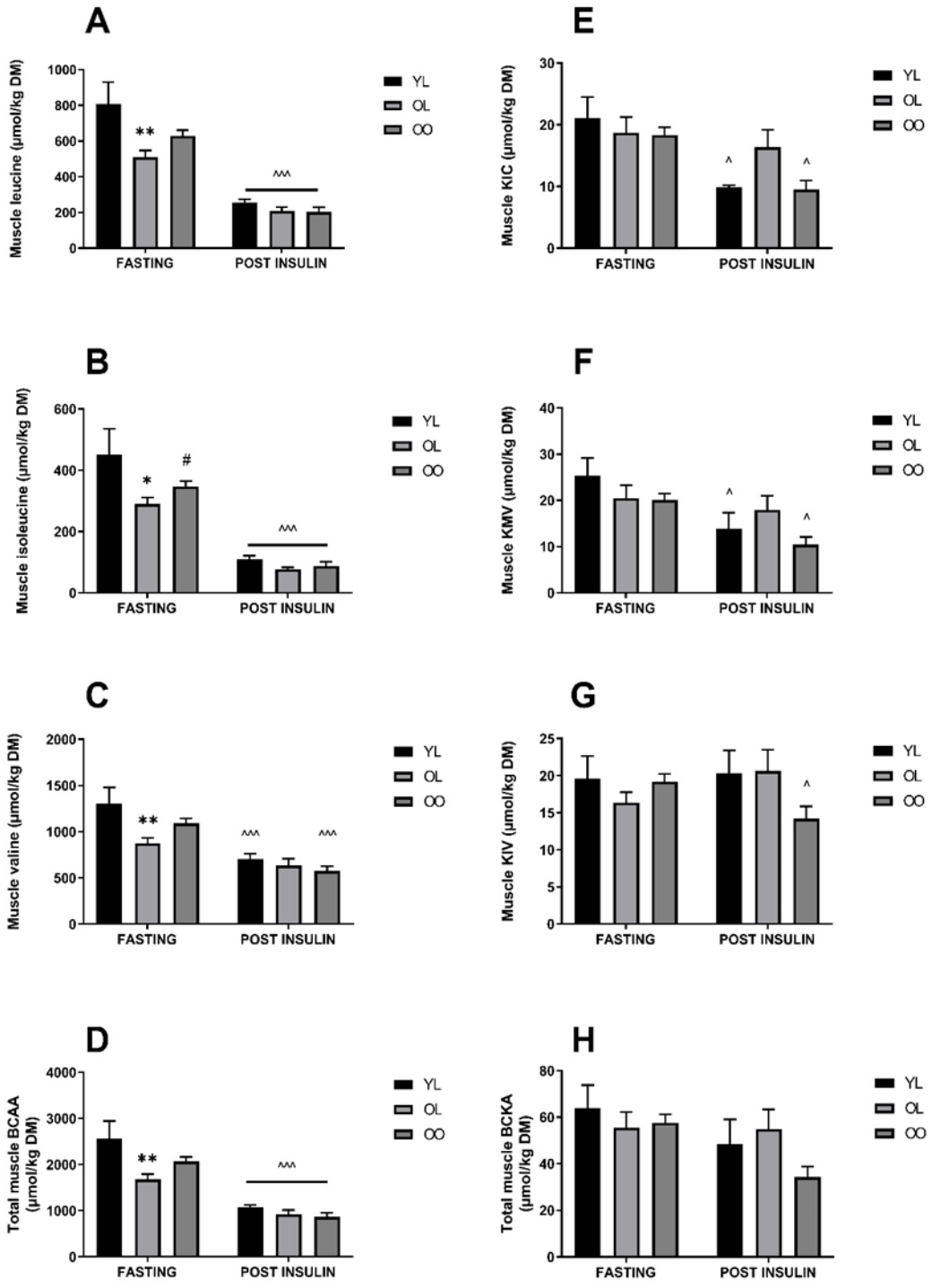

Figure 5.1 5.2 Muscle leucine (A), isoleucine (B) valine (C) and total branched chain amino acid (BCAA) content (D) in the fasted state and post insulin infusion. Muscle KIC (E), KMV (F), KIV (G) and total branched chain keto acid (BCKA) content (H) in the fasted state and post insulin infusion. * $p < 0.05$, OL significantly different from YL. # $p < 0.05$ OO significantly different from OL; ^ $P < 0.05$, ^^ $p < 0.01$ and ^^^ $P < 0.001$ significantly different from the corresponding fasting value. Significance values on figures are of post hoc analysis of significant main effects of age, adiposity, insulin and interactions (see text). Data are mean ± SEM; n = 7 YL, n = 9 OL and n = 9 OO.

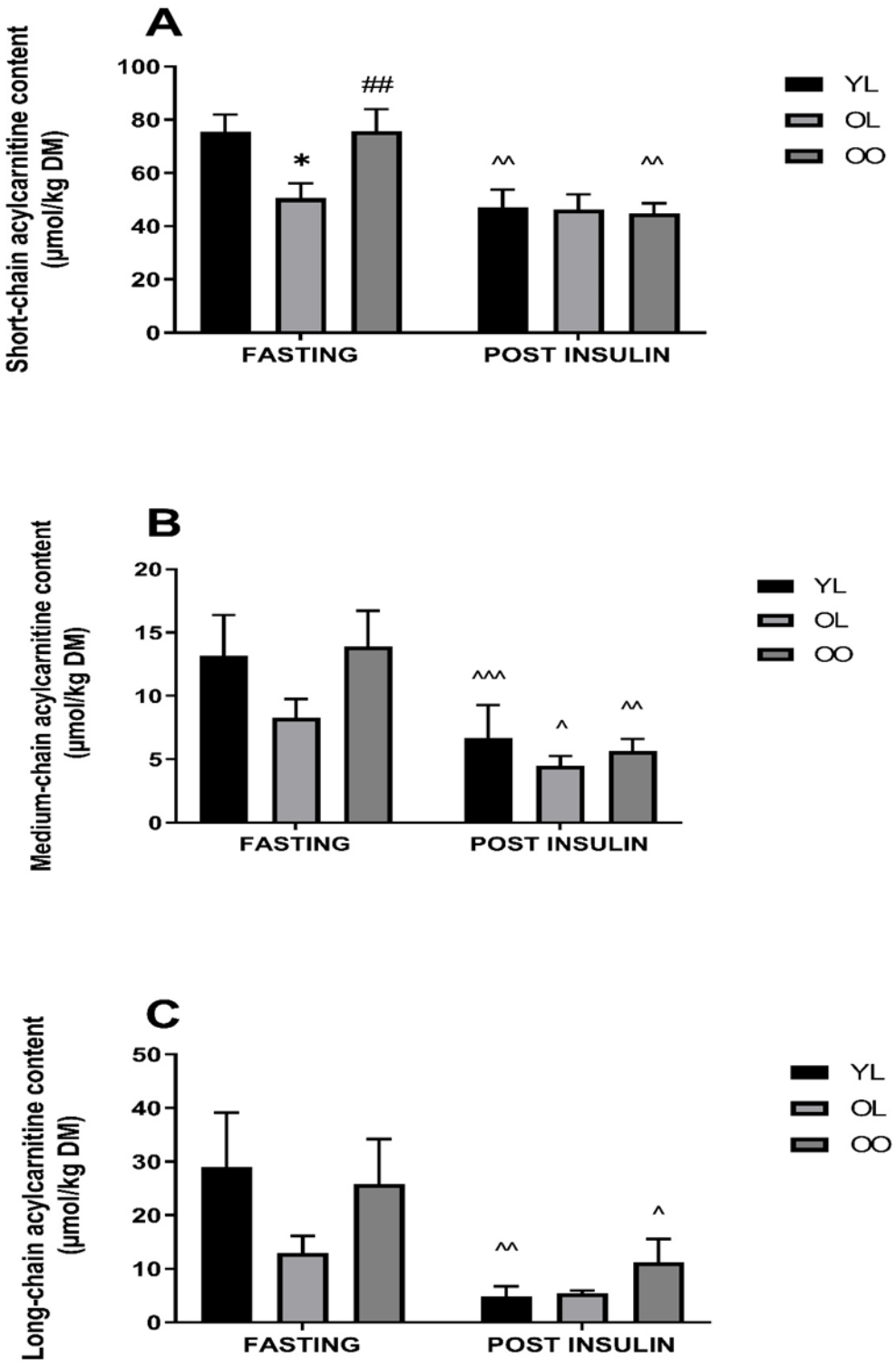

Figure 5.2 Muscle total short (C_3-C_5) chain (A), medium (C_6-C_{10}) chain (B) and long (C_{12}-C_{20}) chain (C) acylcarnitine content in YL, OL and OO subjects in the fasted state and post insulin infusion. * $p < 0.05$ OL different from YL. ## OO different from OL. ^ $P < 0.05$, ^^ $p < 0.01$ and ^^^ $p < 0.001$ significantly different from the corresponding fasting value. Significance values on figures are of post hoc analysis of significant main effects of age, adiposity, insulin and interactions (see text). Data are mean ± SEM; n = 7 YL, n = 9 OL and n = 9 OO.

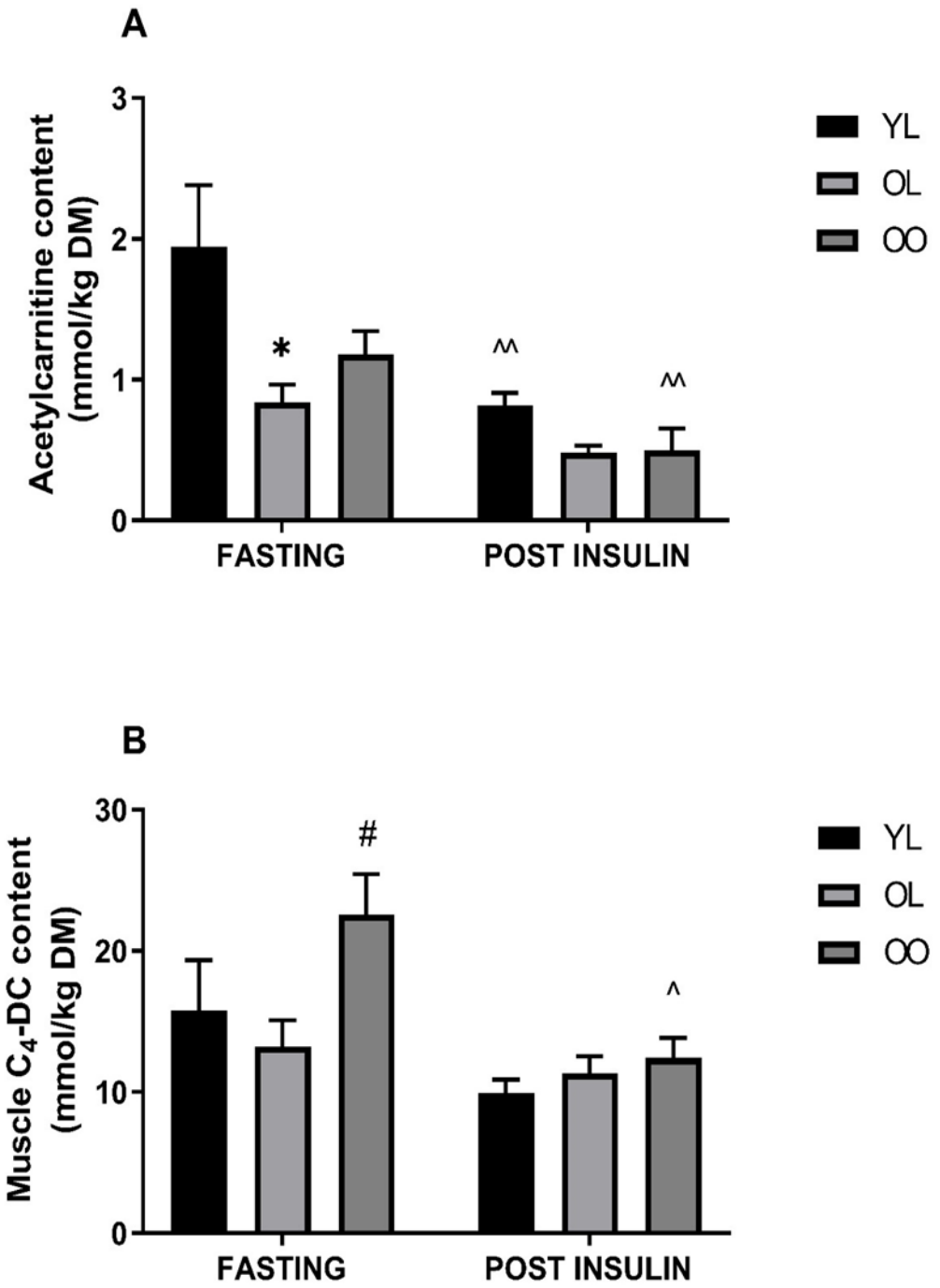

Figure 5.3 Muscle acetylcarnitine (A) and the total of both succinyl and methylmanoylcarnitine, C_4-DC (B) in YL, OL and OO in the fasted state and post insulin infusion. * $p < 0.05$, OL significantly different from YL. # $p < 0.05$ OO significantly different from OL ^ $P < 0.05$, and ^^ $p < 0.01$ significantly different from the corresponding fasting value. Significance values on figures are of post hoc analysis of significant main effects of age, adiposity, insulin and interactions (see text). Data are mean ± SEM; n = 7 YL, n = 9 OL and n = 9 OO.

5.3.5 Muscle BCKDH phosphorylation status

There were no differences between YL and OL or OL and OO in the phosphorylation of the E1a component of the BCKDH complex in the fasted state (Figure 5.4). Furthermore, there was no effect of the insulin clamp on the degree of phosphorylation in any of the groups.

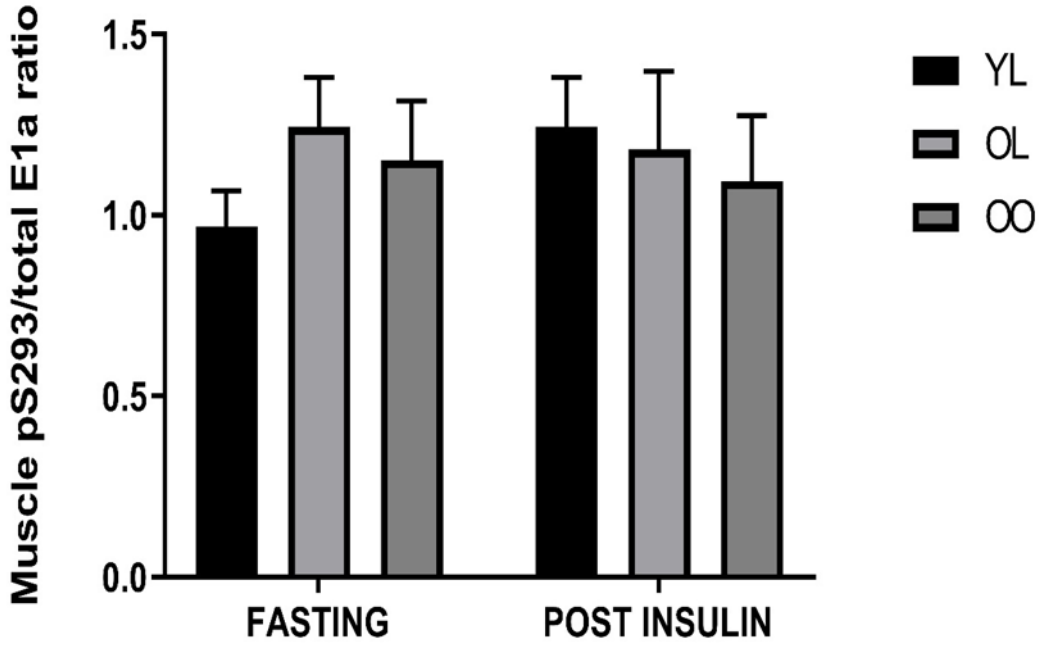

Figure 5.4.Muscle pS293 phosphorylated E1α component of the branched chain keto acid dehydrogenase complex in the fasted state and post insulin infusion. Data are mean ± SEM; n = 5 YL, n = 6 OL and n = 8.

5.4 Discussion

The main findings from this chapter were that ageing is associated with a reduction in fasting intramuscular BCAA content which was observed when comparing young and old lean (albeit slightly more insulin resistant) subjects. Interestingly, the old lean group appeared to exhibit an attenuated suppression of BCAA catabolism in the insulin stimulated state. Moreover, when the old lean group was compared to an age matched overweight and more insulin resistant group, fasting BCAA content was elevated in skeletal muscle of the latter group. Thus, insulin resistance alone cannot explain the decrease in content observed between the old lean group. However, in the acutely insulin stimulated state the overweight group suppressed BCAA, BCKA and short chain acylcarnitines (markers of BCAA catabolism) to a greater extent than the old lean group. These results appear to dissociate age and adiposity-induced insulin resistance from perturbed/excessive muscle BCAA catabolism.

The total muscle BCAA content was approximately 30% lower in older lean subjects compared to their younger BMI matched counterparts. Although total and leg lean mass was also lower in the former group, the BCAA content was expressed per kg of muscle tissue. Therefore, the differences observed are likely independent of differences of body composition and reflective of some level of age-related alterations in muscle protein metabolism that may cause a reduction in the muscle free amino acid pool. Assuming that were no differences in muscle uptake and release of BCAA in the fasted state and given that short chain acylcarnitines (markers of BCAA oxidation) were also significantly lower in the old lean group, the differences could be explained by altered post-absorptive muscle protein turnover rates. Post absorptive leg

protein breakdown rates do not appear to be different between young and older subjects (Wilkes et al. 2009). However, a cross-sectional, retrospective study comparing *in vivo* post-absorptive muscle protein synthesis (MPS) rates determined with stable isotope methodologies between 34 healthy young (22 ± 1 y) and 72 older (75 ± 1 y) men (the largest comparison to date) has shown numerically (but not statistically) higher values in the elderly in fasting muscle protein synthesis rates at a more advanced age (Wall et al. 2015). This may in part, explain the reduced fasting muscle BCAA content in the older subjects compared to their younger counterparts observed in the current study. It has not/cannot be determined to what extent muscle free BCAA content relates to muscle protein synthesis or breakdown and inferences about dynamic processes from static measures should be made with caution. However, work from this group using the same analytical method has demonstrated that increased incorporation of dietary protein into skeletal muscle measured using a stable isotope tracer approach was accompanied by lower muscle BCAA content (possibly due to BCAAs being driven towards protein synthesis) (Tsintzas et al. 2020). This suggests an inverse relationship between muscle free BCAA content and protein synthesis in the postprandial state, although the nature of this relationship in the post-absorptive state is not currently known.

With insulin infusion, in the absence of any concurrent amino acid infusion to maintain euaminoacidaemia (as is the case in the current study), there is a resultant hypoaminocidaemia due to an insulin mediated reduction in proteolysis as well as an increased amino acid cellular uptake (Castellino et al. 1987). Consequently, the lower availability of amino acids in both the

circulation leads to a decline in muscle protein synthesis due to a reduction in substrate availability. However, because the insulin-induced decline in protein degradation exceeds the decrease in protein synthesis, a net protein anabolic effect ensues. In the current study, where no exogenous amino acids were administered, it is likely that the reduction in muscle BCAA content in YL can be attributed mainly to an insulin-stimulated reduction in muscle proteolysis. In the YL group BCKAs (a measure of BCAA transamination) and short chain acylcarnitines (a measure of their oxidation) were all suppressed during the insulin clamp. Indeed, previous reports demonstrating reduced leucine turnover, oxidation and endogenous appearance in the circulation resulting in a less negative net whole body (Castellino et al. 1987; Luzi, Castellino, and DeFronzo 1996) and leg (Wilkes et al. 2009) leucine balance in healthy young individuals during an insulin infusion.

In response to insulin infusion in OL however, BCAA content was decreased in both groups to a similar concentration as YL but the magnitude of the decline was lower in older than younger subjects (evidence by the significant interactions effects). Of the three BCAAs, valine did not (significantly) decrease in OL and the reduction of the BCKAs KIC and KMV were also not observed in OL when compared to YL. This may suggest that valine metabolism should be considered separately and studies using phenylalanine and leucine as an index of total muscle amino acid metabolism may not accurately reflect valine metabolism. In addition, short chain acylcarnitines, specific products of BCAA oxidation did not decrease following insulin infusion in OL suggesting some level of ongoing BCAA oxidation in OL in the insulin-stimulated state. The lack of plasma samples and arteriovenous balance

measurements in this analysis precluded the ability to assess the net efflux of BCKAs or indeed BCAAs into the circulation. Nevertheless, these results highlight a differential fate of intramuscular BCAAs with age in response to insulin. These findings are in agreement with a previous study that demonstrated that older individuals matched for body composition and fasting glucose and insulin concentrations still exhibited an attenuated inhibition of proteolysis in response to insulin (Wilkes et al. 2009). Thus, ageing *per se* appears to be characterised by both lower muscle protein accretion in the postprandial state (Cuthbertson et al. 2005; Wall et al. 2015) and impaired ability to supress proteolysis in the presence of insulin. These findings may in part explain the reduction in lean body and leg mass in the OL group. Whilst the reduced GDR rate during the 3 hour glucose clamp in OL vs YL confirms the previously reported age associated decline in whole body glucose disposal compared to young individuals (Defronzo 1979; Boirie et al. 2001). It should be noted that age *per se* does not necessarily cause insulin resistance (Chee et al. 2016). Much of the 'age' related reduction in muscle metabolic quality, that is, fatty acid oxidation and glucose disposal may be a result of inactivity *per se* (Chee et al. 2016). Unfortunately, data regarding habitual physical activity was not available for the subjects used in the current study. However, future work should aim to replicate the findings of the current study in physical activity matched cohorts.

The finding that adiposity and increased insulin resistance also increased fasting intramuscular BCAA accumulation and some of their catabolites (short chain acylcarnitines) in older individuals when compared to age matched lean subjects could be a result of several factors. Given that the accumulation was

evident in the fasted state suggests that these elevations are likely to be a consequence of factors other than simply differences in dietary protein and energy intake. Previous studies have reported on whole body and skeletal muscle protein turnover in obese subjects. Lower muscle postabsorptive protein synthetic rates have been observed by some (Guillet et al. 2009) but not others (Murton et al. 2015; Beals et al. 2016) when comparing age matched lean and obese men. However, the same reports have all shown a reduced synthetic response to dietary amino acids. Therefore, the elevated BCAA muscle content could represent a chronic, lower postabsorptive incorporation of amino acids into muscle protein (and potential increases in protein breakdown) in the background of worsened insulin resistance resulting in a greater amount of intramuscular free amino acid content.

Interestingly, muscle C_4-DC acylcarnitine was elevated in the OO group when compared to OL. With the current analytical method used, this species represents the sum of both methylmanoylcarnitine and succinylcarnitine. The conversion of methylmanoyl-CoA to succinyl-CoA via the action of methylmanoyl-CoA mutase (mut) is the route by which isoleucine and valine catabolic products enter the TCA cycle or complex 2 of the electron transport chain. A recent report found reduced mRNA levels of mut and other BCAA catabolic enzymes in skeletal muscle of overweight insulin resistant individuals with T2D along with lower relative (not quantitative) expression of BCKAs and some TCA intermediates and accumulation of medium chain acylcarnitines (Lerin et al. 2016). This led to the hypothesis that reduced BCAA catabolic flux with insulin resistance resulted in reduced anaplerosis of TCA intermediates from BCAAs and consequential accumulation of medium and long chain

acylcarnitines, as a result of the inability to completely oxidise fatty acids, which may in turn impair insulin signalling (Lerin et al. 2016). In addition, denervation of rat hindlimb (a rodent model of inactivity) also resulted in reduced mRNA expression of muscle BCAA catabolic enzymes. Furthermore, rodents with a heterogenous mut knockout had increased body weight following 20 weeks of high fat and developed insulin resistance. However, there several limitations to consider with these findings. Firstly, the data was based on untargeted metabolomics with no quantitative assessment of metabolites and mRNA data does not always reflect actual protein content. Secondly, the knockout model resulted in a whole effect and was not specific to muscle. Given that quantitative flux data in rodents (Neinast et al. 2020) and humans (Tan et al. 2020) has shown that obesity and insulin resistance is a state of accelerated BCAA catabolism challenges the aforementioned study. In the previous chapter, this species was also elevated in muscle of obese T2D patients (along with other BCAA derived acylcarnitines, assessed quantitatively) and was also shown to be elevated in the plasma of obese diabetic subjects (Mihalik et al. 2010). It is more likely that there is a degree of increased BCAA catabolism with insulin resistance and adiposity.

It should be noted that succinyl-CoA itself is a TCA intermediate and previous reports have shown a reduced oxidative capacity in muscle of obese individuals and T2D patients (Colberg et al. 1995). The enzyme activity of succinate dehydrogenase was also significantly reduced in all muscle fibre types of those populations compared to lean controls. Therefore, the accumulation of succinylcarnitine could be reflective of reduced oxidative capacity of muscle in OO.

It could be speculated that with both obesity and insulin resistance where both reduced postprandial incorporation of meal derived amino acids into muscle protein (Beals et al. 2016) may lead to the accumulation of intramuscular BCAAs and related metabolites. This effect may be further compounded by reduced physical activity (Glover et al. 2008; Wall et al. 2020). Consequently, BCAA and related metabolites may spill over from muscle into the circulation. If obesity induced insulin resistance progresses to clinically overt T2D this may further exacerbate BCAA metabolism/catabolism through increased proteolysis. Given that both YL and OO had elevated short chain acylcarnitines compared to OL but only OO showed an accumulation of C_4-DC, a distal step in BCAA catabolism, supports the notion that obesity and insulin resistance could be associated with a degree of mismatch between BCAA catabolism and utilisation within the TCA cycle. The current work is the first study to quantitively assess products of BCAA catabolism in human muscle in overweight/obese individuals. The application of the current analytical method in combination with stable isotope tracers to quantitatively determine fluxes through the BCAA and fatty acid metabolism are required to determine the impact the two substrates may have on each other.

In response to insulin infusion, muscle BCAA content was reduced in both OL and OO. Furthermore, there was also a reduction in some BCKAs (notably KIC and KMV) and BCAA derived short chain acylcarnitines in (displaying the same responses to insulin as the YL, despite lower insulin-stimulated glucose disposal) following insulin infusion in OO suggesting a decrease in catabolic flux in line with the decline in BCAA content. The fact that insulin failed to suppress those BCKAs and short chain acylcarnitines in the OL group could

suggest that the OO group was paradoxically more sensitive to the insulin-induced suppression of muscle proteolysis and BCAA oxidative flux. A recent report investigating specifically leg muscle amino acid metabolism demonstrated that leg protein breakdown was not different between obese and lean older men in the postabsorptive state. There was however a blunted synthetic response to insulin and amino acids but importantly a concurrent inhibition of leg protein breakdown resulted in an equal net protein balance when compared to lean controls (Murton et al. 2015). This is in contrast to previous reports showing that in obese individuals there was an impairment in the ability of insulin to supress whole body proteolysis as evidenced by a lower BCAA net balance compared to lean controls (Luzi, Castellino, and DeFronzo 1996; Chevalier et al. 2005; Guillet et al. 2009). The lack of muscle specific data in obese individuals make the findings of this study difficult to determine why the OO reduced markers of catabolism in response to insulin yet an aged matched lean group did not.

Upon insulin infusion medium and long-chain acylcarnitines were suppressed in OO group to similar values of the OL group despite the markedly reduced whole body glucose disposal in the former group. A study from this group demonstrated that during lipid infusion in combination with a hyperinsulinaemic euglycaemic clamp, the accompanying insulin resistance of glucose uptake was not associated with an accumulation of muscle acylcarnitines (Stephens et al. 2014). Rather, insulin resistance was accompanied by increased acetyl-CoA, the final product of β – oxidation and an allosteric inhibitor of the PDC. These findings provided evidence that in humans, excessive and not incomplete fatty acid oxidation causes insulin

resistance. It should be noted that evidence suggesting acylcarnitines directly impair insulin signalling stems from studies using cell culture models (Koves et al. 2008; Adams et al. 2009; Aguer et al. 2015). Though the potential role of acylcarnitines *per se* limiting glucose disposal in humans cannot be ruled out completely, it is likely that a number of factors (including accumulation of DAGs and ceramides) may contribute to insulin resistance seen with excessive fatty acid availability. The finding that some but not all ceramide and DAG species were elevated in the OO group (Chee et al. 2016) supports the latter notion.

Previous studies from this group have shown that short chain acylcarnitines arising from excessive BCAA catabolism were closely associated with the reduced glucose disposal seen with lipid infusion during a euglycaemic hyperinsulinaemic clamp (Stephens et al. 2014). The results from this chapter do not show excessive BCAA catabolism under hyperinsulinaemic conditions despite a lower glucose disposal in OO. Therefore, it could be suggested that without a concomitant elevation in fatty acid availability an aggravating impact on muscle BCAA catabolism may not occur. Fatty acids have been shown to increase the activity of the BCKDH complex in rodents (Paul and Adibi 1992) and therefore the sustained presence of fatty acids (achieved with lipid infusion) may be required to increase BCAA catabolism. In the current study no change in the phosphorylation status of muscle BCDKH was observed before or after insulin infusion. Given that the OO had markedly lower GDR compared to OL, its is likely that there was some degree of elevated lipid availability during the clamp but without an assessment of the amount of fat oxidised it is uncertain as what level of increased fatty acid oxidation is required to increase BCAA catabolism. Of note, in a recent study in rodents

that utilised a stable isotope approach to measure BCAA metabolism in a range of organs, it was shown that mice fed a high fat diet did not elevate BCAA oxidation in muscle, but hyperphagic diabetic (db/db) mice did (Neinast et al. 2018). This may suggest that the severity of obesity and insulin resistance (such as in diagnosed T2D) may be a determining factor in the extent of the excess BCAA catabolism in muscle.

5.5 Conclusion

In conclusion age and obesity appear to have differential effects on skeletal muscle BCAA accumulation and metabolism in humans. The independent impact of age may be greater than the effect of obesity *per se* and future studies involving cohorts with a wide age range should account for this. The fact that muscle medium and long chain acylcarnitines were not associated with reduced glucose disposal rate (an index of insulin resistance) seen with increased overweight/obesity provide further evidence that those species may not be involved in the development of muscle insulin resistance. Further studies matching middle aged and older aged subjects for lean body mass are required to determine if the observed differences in muscle BCAA content persist independent of body composition. Studies investigating muscle BCAA metabolism in combination with fatty acid tracers are required to understand how one substrate may be impacted the other.

6 Chapter 6. The effect of Type 2 diabetes and age on whole body fat oxidation and BCAA metabolism.

6.1 Introduction

Perturbations in BCAA metabolism have long been associated with obesity and insulin resistance (Felig, Marliss, and Cahill 1969). Interest in the interaction between BCAA and insulin resistance has been renewed in recent years based on the findings that a BCAA related signature was more closely associated with insulin resistance than lipid markers (Newgard 2012). Furthermore, baseline circulating BCAA concentrations were able to predict the future onset of type 2 diabetes (T2D) many years before disease progression (Wang et al. 2011). Moreover, Mendelian randomization analysis suggested a causal link between genetic variants associated to impaired BCAA catabolism and higher risk of T2D (Lotta et al. 2016). Therefore, there is growing evidence that high levels of BCAAs and related intermediate metabolites are not only T2D biomarkers but may also contribute to the pathogenesis of insulin resistance and T2D. Despite these associations, the mechanisms by which altered BCAA metabolism may precede and contribute to the development of skeletal muscle insulin resistance are unknown.

Animal work suggests that obesity is associated with reduced BCAA catabolism in adipose tissue and liver (She et al. 2007; Herman et al. 2010; Zhou et al. 2019). Consequently, this may reroute systemic BCAAs to skeletal muscle where excessive BCAA oxidation may compete with fatty acids oxidation in the mitochondria (Newgard 2012; White et al. 2016). However, there is no evidence of reduced BCAA catabolism in human adipose tissue (Lackey et al. 2013b; Piro et al. 2020). Furthermore, the contribution of adipose tissue to BCAA catabolism in humans is likely to be small and the contribution

of BCAAs to mitochondrial oxidation is quantitatively minor in skeletal muscle (Wagenmakers, Salden, and Veerkamp 1985; Neinast et al. 2020).

Previous work from this group has demonstrated that under lipid infusion in combination with hyperinsulinaemic-euglycaemic clamp conditions, BCAA-derived short chain acylcarnitines were positively associated with insulin resistance to tissue glucose disposal (Stephens et al. 2014). More recently, it has been shown that 3-hydroxyisobutyrate (3-HIB), a catabolite of valine, is elevated in muscle of rodent and patients with T2D and this catabolite can leave the muscle cell and increase trans-endothelial flux of fatty acids into muscle in a paracrine manor (Jang et al. 2016). In chapter 4, fasting levels of short chain acylcarnitines from BCAA catabolism were increased in both muscle and plasma of patients with T2D. Therefore, it could be hypothesised that increased muscle BCAA catabolism, secondary to lipid-induced insulin resistance could lead to accumulation of BCAA catabolites in muscle which may in turn exacerbate the pre-existing insulin resistance. This may provide the mechanistic link between the associations of a BCAA related signature with obesity and insulin resistance previously observed. Indeed, more reports have demonstrated that higher BCAA levels do not have a casual effect on insulin resistance, rather insulin resistance drives higher fasting BCAAs (Wang et al. 2017; Mahendran et al. 2017) Alternatively, it has also been suggested that reduced BCAA catabolism in muscle may reduce muscle fatty acid oxidation through reduced anaplerosis of TCA intermediates (Lerin et al. 2016). These findings were based largely on mRNA expression data and untargeted metabolite profiling.

In chapter 5, it was demonstrated that ageing and adiposity-induced insulin resistance exert differential effects on fasting muscle BCAA content, and the ability of insulin to suppress BCAA catabolism. These findings suggest that against the background of ageing, BCAA content may not always reflect insulin resistance. To date, no studies have quantitatively assessed markers of fatty acid and BCAA metabolism simultaneously in humans and how their relationship may be perturbed in T2D.

6.1.1 Aims

- To assess the effect of a chronic state of glucose dysregulation and insulin resistance on whole body meal derived fat oxidation in middle-aged patients with T2D and age- and adiposity-matched controls using an isotopically labelled fatty acid drink.
- To investigate how ageing can modify this effect in a separate group of older patients with T2D with similar disease duration.
- To determine how any differences in fatty acid handling may be related to perturbed skeletal muscle metabolism in both the fasting state and in response to endogenous insulin responses during an oral glucose tolerance test (OGTT).

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6.2 Methods

6.2.1 Participants

Seven middle-aged (MA-T2D) and 7 older-aged patients (OA-T2D) with T2D, matched for disease duration and metformin use, and 7 middle-aged normoglycemic controls (MA-CON) were recruited and volunteered to participate in this cross-sectional study that was part of bigger study investigating the effect of carnitine feeding on muscle metabolism (see chapter 2 for details on patient recruitment). The T2D selected for this study were also matched for body composition to allow the comparison of age per se between the two T2D groups and diabetes per se between the two middle aged groups.The study was approved by the NHS Research Ethics Committee East Midlands, Nottingham and met the regulations outlined in the Declaration of Helsinki. Participant characteristics are shown Table 6.1.

Table 6.1 Participant characteristics of middle aged controls (MA-CON), middle aged Type 2 Diabetic patients (MA-T2D) and older aged Type 2 Diabetic patients (OA-T2D).

	MA-CON	**MA-T2D**	**OA-T2D**
n	7	7	7
Age (years)	44.4 ± 3.5	45.4 ± 1.3	63.4 ± 0.6 ###
Body mass (kg)	98.7 ± 2.9	95.7 ± 3.9	90.8 ± 5.2
BMI (kg/m^2)	31.2 ± 1.5	30.5 ± 1.3	29.6 ± 1.3
Fat free mass (kg)	64 ± 2.2	62.1 ± 1.3	58.7 ± 2.4
Arm lean mass (kg)	7.8 ± 3.7	7.6 ± 5.4	6.6 ± 3.6
Leg lean mass (kg)	20 ± 1.3	19.8 ± 1.1	18.3 ± 1.0
Fat mass (kg)	33.3 ± 2.4	33.8 ± 4.0	30.2 ± 2.9
Trunk fat mass (kg)	20.1 ± 2.0	20.3 ± 2.4	19.0 ± 1.8
Arm fat mass (kg)	2.9 ± 0.3	3.2 ± 0.5	2.9 ± 0.2
Leg fat mass (kg)	9.4 ± 0.3	9.4 ± 1.2	8.0 ± 1.0
Body fat percentage (%)	34.1 ± 2.0	34.7 ± 2.9	34.0 ± 1.3
Fasting blood glucose (mmol/L)	4.4 ± 0.2	6.8 ± 0.7 **	5.8 ± 0.3
Fasting serum insulin (mU/L)	14.0 ± 2.2	18.2 ± 2.6	19.3 ± 3.6
Years since diagnosis		3.7 ± 0.9	5.6 ± 1.7
Metformin treatment (mg/d)		786 ± 241	1000 ± 327

Data are mean ± SEM ** $p < 0.01$, MA-T2D compared to MA-CON. ### $p < 0.001$, OA-T2D vs MA-T2D.

6.2.2 Experimental protocol

This study consisted of a 3-day metabolic monitoring period to assess body composition and determine free-living dietary fat oxidation followed by a laboratory visit day in which subjects underwent a 2-hour OGTT. Blood samples were obtained before, during and after the OGTT, whereas muscle samples were obtained before and after the OGTT.

6.2.2.1 Body composition and free-living measurements

On the first day of the metabolic monitoring period the participant's body composition was assessed by DEXA (chapter 2, section 2.2.4). Throughout the metabolic monitoring period, subjects recorded all food intake in a food diary. Physical activity levels (step counts) were also assessed for all 3 days of the metabolic monitoring period using pedometers (Actiheart, CamNtech inc., Cambridge, UK). On the day before the laboratory visit (day 3 of the metabolic monitoring period), subjects attended the laboratory in a fasted state between 8.00am and 9.00am, having refrained from all exercise including cycling commutes for 24 h before the visit. Subjects voided their bladder into an experimental container and then were weighed and asked to rest on a semi-reclined position. Following this, 15 mg/kg [$^{2}H_{31}$] palmitate and 0.2g/kg ^{18}O-water stable-isotope tracers were suspended in a nutritionally balanced meal replacement drink (330 kcal: 44g carbohydrate, 11g fat, and 14g protein). The [$^{2}H_{31}$] palmitate was added to the meal replacement drink first, which was heated to 50° C to ensure the palmitate dissolved, after which the ^{18}O-water was added. Subjects were allowed 10 minutes to consume the drink; all subjects consumed the drink in this time and in full. Upon completion, a timer was started to accurately determine 10 h after the drink was ingested. Subjects

were then provided with a container to collect all urine for the next 10 h upon leaving the lab. The container was kept cool with ice-packs (~4°C) until delivered to the lab on the following morning (laboratory visit).

The ingestion of [$^{2}H_{31}$]palmitate was to determine free-living dietary fat oxidation in humans. This method has several advantages over the traditional ^{13}C method, including that tracer is not lost through the TCA cycle and that the stable-isotope tracer can be used in free-living conditions. Following oral ingestion, ^{2}H labelled fatty acids such as [$^{2}H_{31}$]-palmitate are metabolised to acetyl CoA releasing NADH. The ^{2}H is liberated as water ($^{2}H_{2}O$) when this NADH is oxidised during oxidative phosphorylation in the mitochondria, and the remaining ^{2}H label is released as $^{2}H_{2}O$ during the oxidation of acetyl CoA in the TCA cycle. The $^{2}H_{2}O$ from both pathways mixes with body water, which is then excreted as urine, where it can be measured either laser-based spectroscopy (Votruba, Zeddun, and Schoeller 2001; A et al. 2004).

6.2.2.2 OGTT

Participants arrived at the laboratory at 08:00 after an overnight fast (10–12 h) having consumed a standardised meal the night before. In order to control for the effects of dietary factors and exercise on whole body and muscle metabolism during the 3-day metabolic monitoring period, the volunteers were provided with prescribed meals (55% of energy from carbohydrate, 30% fat, 15% protein) based on estimated daily energy requirements and asked to refrain from strenuous exercise. The meals were based on habitual foods recorded in the diet diaries to help ensure compliance.Diabetic patients were

instructed to not take any of their diabetic medication on the day of the experiment visit. Subjects then rested in bed in a semi-supine position, and an intravenous cannula was inserted retrograde into the superficial hand vein of one arm for arterialized venous blood sampling. Arterialization of the venous sample was achieved by heating the hand to 50-55^{o}C using hot-air warming unit (Gallen and Macdonald 1990). Indirect calorimetry was performed prior to, and during the final 20 minutes of the OGTT (Cosmed Quark RMR, Rome, IT). Subjects then underwent a standard 2h OGTT, consuming 75g dextrose dissolved in 300ml water

An overview of the metabolic monitoring period and the OGTT is shown schematically in Figure 6.1

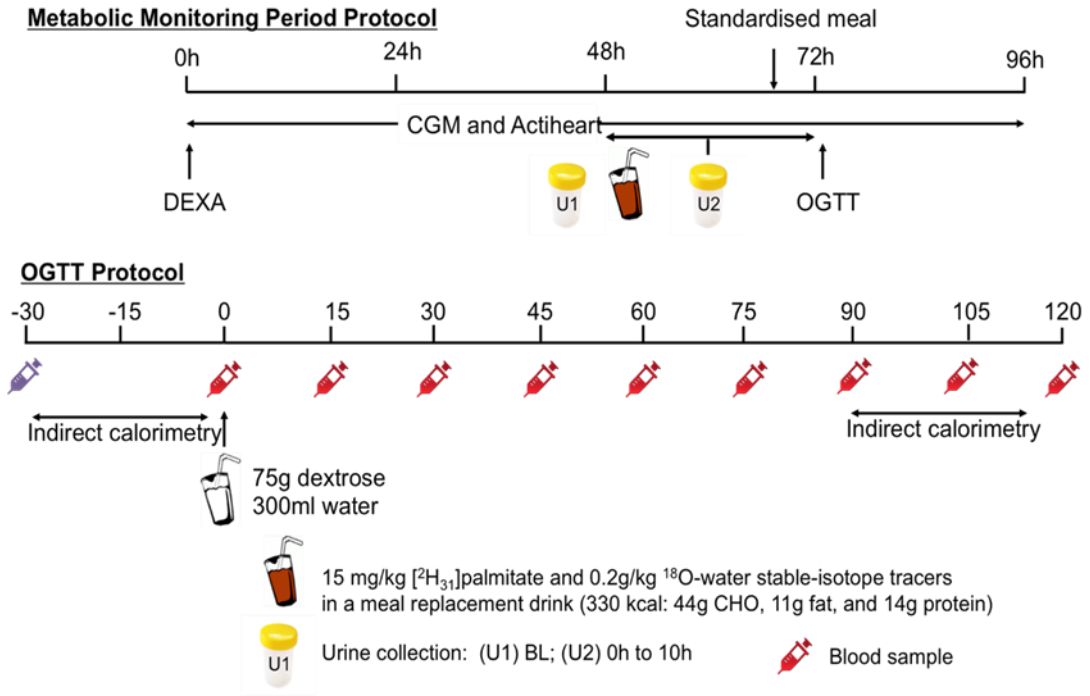

Figure 6.1 Schematic overview of the 3-day metabolic monitoring period (top timeline) and the oral glucose tolerance test (OGTT) during the laboratory visit (bottom timeline). CGM refers to continuous glucose monitoring of free-living

glycaemic profile, but this data was not analysed as a part of the current chapter. Dual-Energy X-Ray Absorptiometry (DEXA).

6.2.3 Sample collection and analysis

Prior to the measurement of enrichment, all urine samples were made acellular. Samples were thawed on ice, transferred to 1.5ml centrifuge tubes, and were then centrifuged at 21,130 RCF for 30 minutes at 4°C (Eppendorf 5424R, Hamburg, Germany). The supernatant was removed and transferred to a glass auto-sampler tube by passing through a 0.25 µm filter. Samples were then kept at 4°C throughout analysis. Urine samples were analysed for $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios by infrared spectroscopy (Elemtex Ltd, UK) to calculate dietary fat oxidation and total body water, respectively (Votruba et al. *Int J Obes* 2001;**25**:1240-4).

Arterialized venous blood samples were collected at every 15 minutes throughout the OGTT and analysed for glucose, insulin and FFA (as described in Chapter 2, section 2.3). Skeletal muscle samples were obtained from the vastus lateralis in the fasted state and immediately following the 2 h OGTT using the Bergstrom needle biopsy technique (Bergström 1975) and immediately frozen in liquid nitrogen-cooled 2-methylbutane and subsequently stored in liquid nitrogen until analysis. Muscle and plasma samples at time 0 and 120 mins were analysed for BCAA, BCKA, free carnitine, acylcarnitine content (as described in chapter 3, section 3.2.2) and muscle protein expression of total BCKDH E1α and phospho S^{293} BCKDH as described in chapter 5 (section 5.3.4).

6.2.4 Statistical analysis

The aim of this chapter was to independently compare the effect of T2D and age per se on fat and BCAA metabolism. Therefore, differences in group participant characterises were compared with separate students paired t test (MA-CON vs MA-T2D and MA-T2D vs OA-T2D). The within and between group differences in the fasted state and in response to OGTT were determined using two separate, two way ANOVAs (2 factors of group (T2D and age) and OGTT) This design does not permit the comparison of MA-CON with the OA-T2D group. When a significant main effect was observed, a Sidak post hoc test was performed to identify individual differences. Statistical significance was set at $P < 0.05$, and all values are presented as means ± SEM. When referring to chain lengths AC were grouped as short (C_3-C_5), medium (C_6- C_{10}) and long-chain (C_{12}-C_{20}). Summed BCAA refers to the summation of leucine, isoleucine and valine concentrations. Summed BCKA refers to the summation of α-ketoisocaproic (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV) concentrations.

6.3 Results

6.3.1 Participant characteristics and free-living measurements

Subjects were well matched for body composition and there were no differences between total and regional lean or fat mass between groups as assessed by DEXA. MA-T2D and OA-T2D were well matched for years since diagnosis and metformin usage. The average daily energy intake in the 3 days prior to the OGTT test day was not different between any groups. Although, MA-CON had a numerically higher total energy intake this was not different to MA-T2D or OA-T2D. The macronutrient composition (expressed as percentage of total energy intake) and physical activity level was not different between groups (Table 6.2).

Table 6.2 Average energy intake and percentage contribution of each macronutrient for the 3 days prior to OGTT

	MA-CON	**MA-T2D**	**OA-T2D**
Average energy intake (Kcal)	2722 ± 338	2077 ± 280	2071 ± 169
Carbohydrate (%)	43 ± 1	43 ± 3	38 ± 2
Fat (%)	37 ± 2	37 ± 2	38 ± 3
Protein (%)	18 ± 2	21 ± 4	16 ± 2
Actiheart activity counts	8.1 ± 2.5	11.1 ± 4.2	9.1 ± 2.9

Data are mean ± SEM. n = 7. Actiheart activity counts are a daily average of a 3-day period.

6.3.2 Dietary fat oxidation

MA-T2D had a higher (10 hour) dietary-derived fat oxidation compared to MA-CON ($p = 0.03$). There was trend ($p = 0.08$) for meal derived fat oxidation to be lower in OA-T2D when compared to MA-T2D. Differences in meal derived fatty acid oxidation are shown in Figure 6.2.

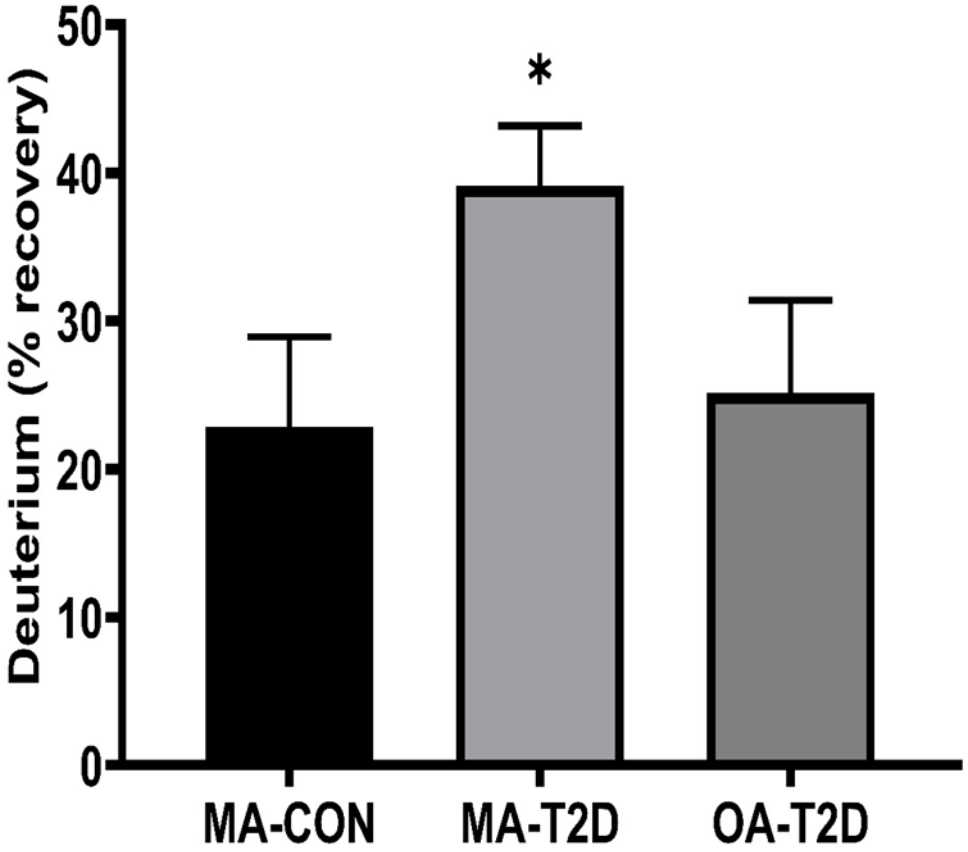

Figure 6.2 10-hour meal derived fat oxidation from a meal replacement drink as assessed by % deuterium recovery from urine samples * $p < 0.05$ MA-T2D compared to MA-CON. Data are mean ± SEM. n = 6.

6.3.3 Response to OGTT

6.3.3.1 Glucose

MA-CON vs MA T2D

Fasting glucose was higher in MA-T2D vs MA-CON (Table 6.1) and increased in response to OGTT in both groups but was higher in at each time point from 30 mins, reaching a peak at 75 minutes at concentrations of 9.3 and 14.5 mmol/L in MA-CON and MA-T2D, respectively (Figure 6.3A). Whereas plasma glucose declined in MA-CON, it remained significantly elevated for the remainder of the OGTT in MA-T2D (interaction effect $p < 0.001$). At the end of the OGTT, plasma glucose decreased to 7.0 and 12.6 mmol/L in MA-CON and MA-T2D, respectively. Accordingly, the total iAUC for glucose was significantly greater in MA-T2D compared to MA- CON over the 120-minute period ($p = 0.004$) (Figure 6.4 A).

MA-T2D vs OA- T2D

There was no difference in fasting blood glucose concentrations between MA-T2D and OA-T2D or at any time point during the OGTT (Figure 6.3A). A peak glucose concentration of 12.9 mmol/L was reached at 90 mins and did not decline thereafter such that glucose concentration was 11.4 mmol/L at the end of the OGTT in OA-T2D. There was no difference in iAUC of glucose during OGTT between the 2 groups (Figure 6.4 A).

6.3.3.2 Serum Insulin

MA-CON vs MA T2D

There was no significant difference in fasting insulin concentrations between the two groups. There was a sharp rise in insulin concentration in MA-CON

and as with glucose, concentrations peaked at 75 minutes at a concentration of 136.2 mU/L (Figure 6.3B). In contrast, the insulin response was blunted in MA-T2D which did not reach statistical significance (main effect of diabetes p = 0.08). Insulin concentrations reached peak values of 66.7 mU/L at 90 mins (Figure 6.3B). Post hoc analysis revealed that insulin concentrations were not significantly different to the fasting value at any point during the OGTT in MA-T2D (interaction effect p = 0.06). However, the iAUC for insulin (Figure 6.4 B) was significantly lower in MA-T2D when compared to MA-CON during OGTT (p = 0.03).

MA-T2D vs OA-T2D

There were no differences in fasting insulin concentrations and no difference in the insulin response to OGTT between MA-T2D and OA-T2D (Figure 6.2B). Serum insulin levels reached a peak concentration of 78.7 mU/L at 105 minutes in OA-T2D but levels were no different to MA-T2D at any time point. Accordingly, there were no differences in iAUC for insulin between the two groups (Figure 6.4 B).

6.3.3.3 Plasma FFA

MA-CON vs MA T2D

There were no differences in fasting plasma FFA concentrations between groups (Figure 6.3C). OGTT resulted in a suppression of FFAs such that final concentrations were 10-fold lower in MA-CON but only 4-fold lower after OGTT in MA-T2D (interaction effect p = 0.01) indicating attenuated suppression of FFA in MA-T2D compared to MA-CON.

MA-T2D vs OA-T2D

Fasting FFA concentrations did not differ between the two groups and despite lower numerical values throughout the OGTT there was no difference in the FFA response to OGTT between the two groups (Figure 6.3C).

The glucose, insulin and FFA response to OGTT are shown in Figure 6.3

6.3.3.4 Energy expenditure

There were no differences in fasting or OGTT energy expenditure between any of the groups. There was significant effect of OGTT in all groups on fat oxidation which declined during OGTT in all groups. The indirect calorimetry data are shown in Table 6.3.

Table 6.3 Indirect calorimetry data for the three groups during the fasted state and final 20 minutes of OGTT. Data are mean ± SEM. n = 5 for MA-CON and n = 7 for MA- and OA- T2D.

	MA -CON		**MA -T2D**		**0A-T2D**	
	Fasting	OGTT	Fasting	OGTT	Fasting	OGTT
Energy expenditure (kj/kg lbm/min)	103 ± 7	106 ± 7	104 ± 2	105 ± 3	95 ± 5	101 ± 5
Fat oxidation (kj/kg lbm/min)	91 ± 26	59 ± 14	84 ± 13	58 ± 7	83 ± 13	46 ± 12
Fat % of EE	83 ± 21	55 ± 13	80 ± 23	54 ± 6	84 ± 10	44 ± 10

Data are mean ± SEM. n = 5 for MA-CON and n = 7 for MA- and OA-T2D; lbm=lean body mass.

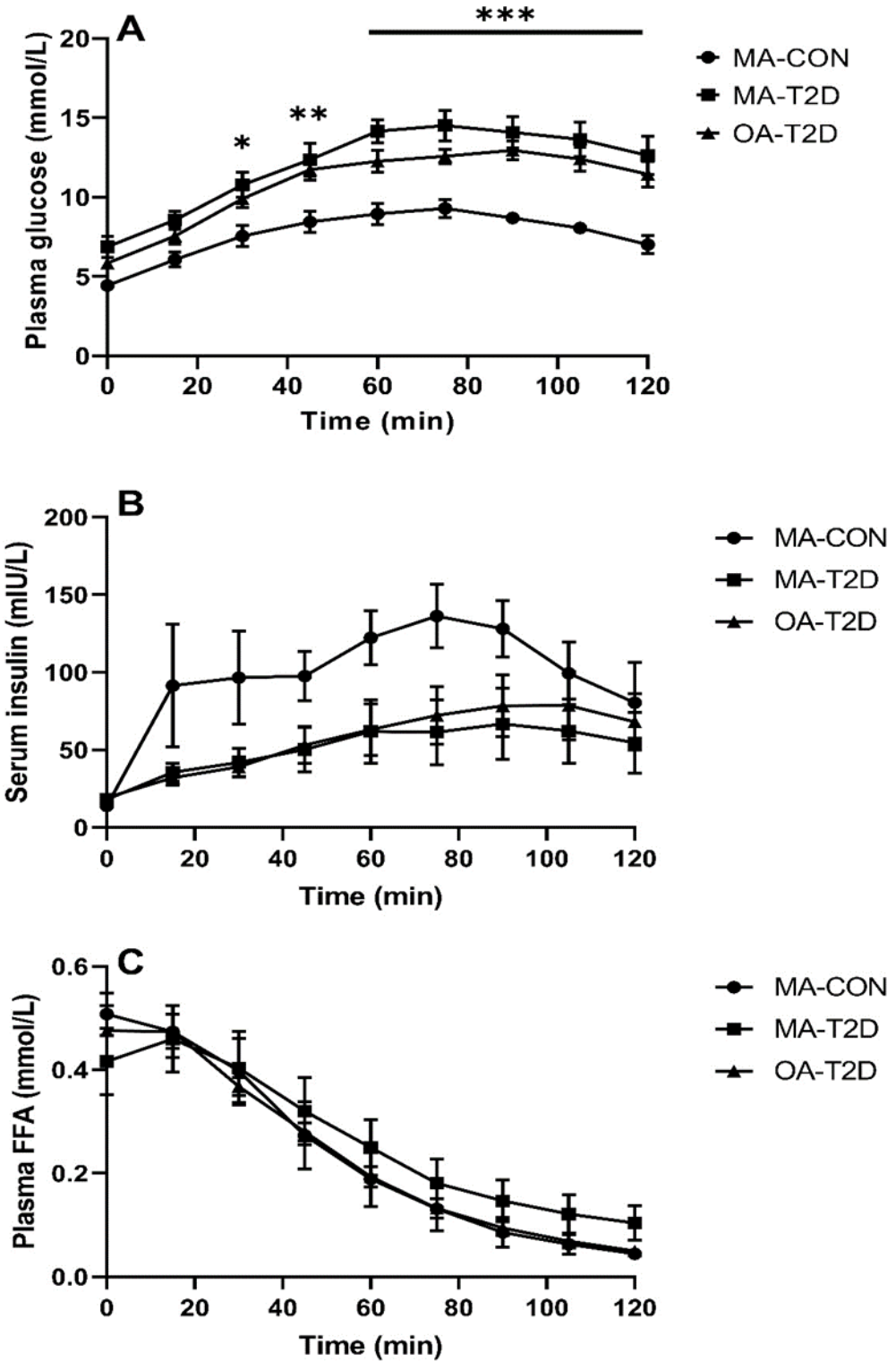

Figure 6.3 Plasma glucose (A), serum insulin (B) and plasma free fatty acid (C) concentrations during an oral glucose tolerance test. * $p < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ MA-T2D different from MA-CON There was also a main effect of interaction ($p = 0.01$) for plasma FFA between MA-CON and MA-T2D. Data are mean ± SEM. n = 7.

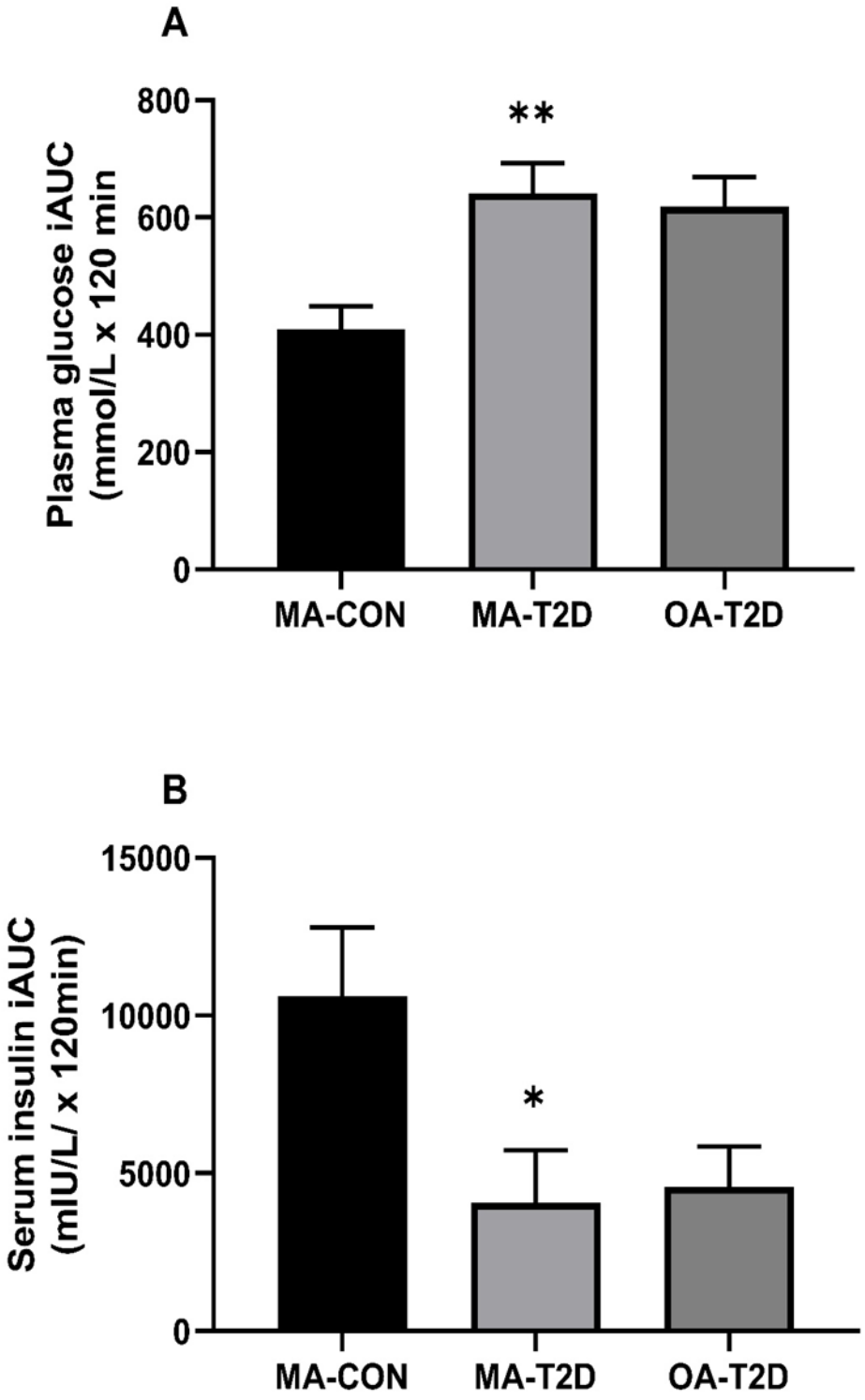

Figure 6.4 Incremental area under the curve (iAUC) for plasma glucose (A) and serum (B) responses over the 120-minute oral glucose tolerance. Data are mean ± SEM n =7. * $p < 0.05$ and ** $P < 0.01$ MA-T2D different from MA-CON.

6.3.4 Muscle metabolites

6.3.4.1 BCAA and BCKA

MA-CON vs MA-T2D

ANOVA revealed a main effect of T2D ($p = 0.003$) and OGTT ($p < 0.001$) on total muscle BCAAs. Fasting total BCAA was 49% higher in MA-T2D compared to MA-CON ($p = 0.008$) and declined 32 % ($p = 0.04$) and 45 % ($p < 0.001$) with OGTT in MA-CON and MA-T2D, respectively (Figure 6.5 H). There was a main effect of T2D and OGTT ($p <0.001$ for both) and strong trend for their interaction ($p = 0.06$) for both muscle leucine and isoleucine. Fasting muscle leucine was 55% higher in MA-T2D vs MA-CON ($p < 0.001$) and declined 43 % ($p = 0.002$) and 51% ($p < 0.001$) with OGTT in MA-CON and MA-T2D, respectively (Figure 6.5 A). Fasting muscle isoleucine was 47% higher in MA-T2D vs MA-CON ($p = 0.006$) and declined 43 % ($p < 0.001$) and 54% ($p < 0.001$) with OGTT in MA-CON and MA-T2D, respectively (Figure 6.5 B). There was a main of T2D ($p = 0.009$) and OGTT ($p = 0.002$) on muscle valine. Fasting concentrations were 46 % higher in MA-T2D ($p = 0.003$) and declined numerically but not significantly 23 % and 38% % ($p = 0.005$) with OGTT in MA-CON and MA-T2D, respectively (Figure 6.5 C).

There were no differences in the fasting intramuscular concentrations of any of the three BCKAs (Figure 6.5E-H). There was a main effect of OGTT on muscle KIC and post hoc analysis revealed a trend for a decline in concentration in MA-CON ($p = 0.07$). Muscle KMV was reduced following OGTT in MA-T2D but not in MA-CON (interaction effect $p=0.02$).

MA-T2D vs OA-T2D

There was strong trend for fasting muscle leucine to be (20%) lower and its suppression during OGTT to be lower in OA-T2D compared to MA-T2D (interaction effect $p = 0.06$) (Figure 6.5A). Fasting concentrations declined 51 % ($p < 0.001$) and 34% ($p = 0.08$) in MA-T2D and OA-T2D, respectively. There was significant interaction effect for muscle isoleucine ($p = 0.04$) driven by (20%) lower fasting isoleucine ($p = 0.05$) and attenuated suppression during OGTT in OA-T2D (38, $p = 0.001$) %vs MA-T2D (54%, $p <0.001$) (Figure 6.5B). There were no differences in fasting muscle valine or total BCAA content between groups (despite an 18% numerical increase for both) and OGTT reduced their content ($p = 0.004$ and $p = 0.04$ for MA-T2D and OA-T2D, respectively) (Figure 6.5C-D). There were no differences between groups in any of the BCKAs in the fasted state and there was no effect of OGTT in either group (Figure 6.5 E-H).

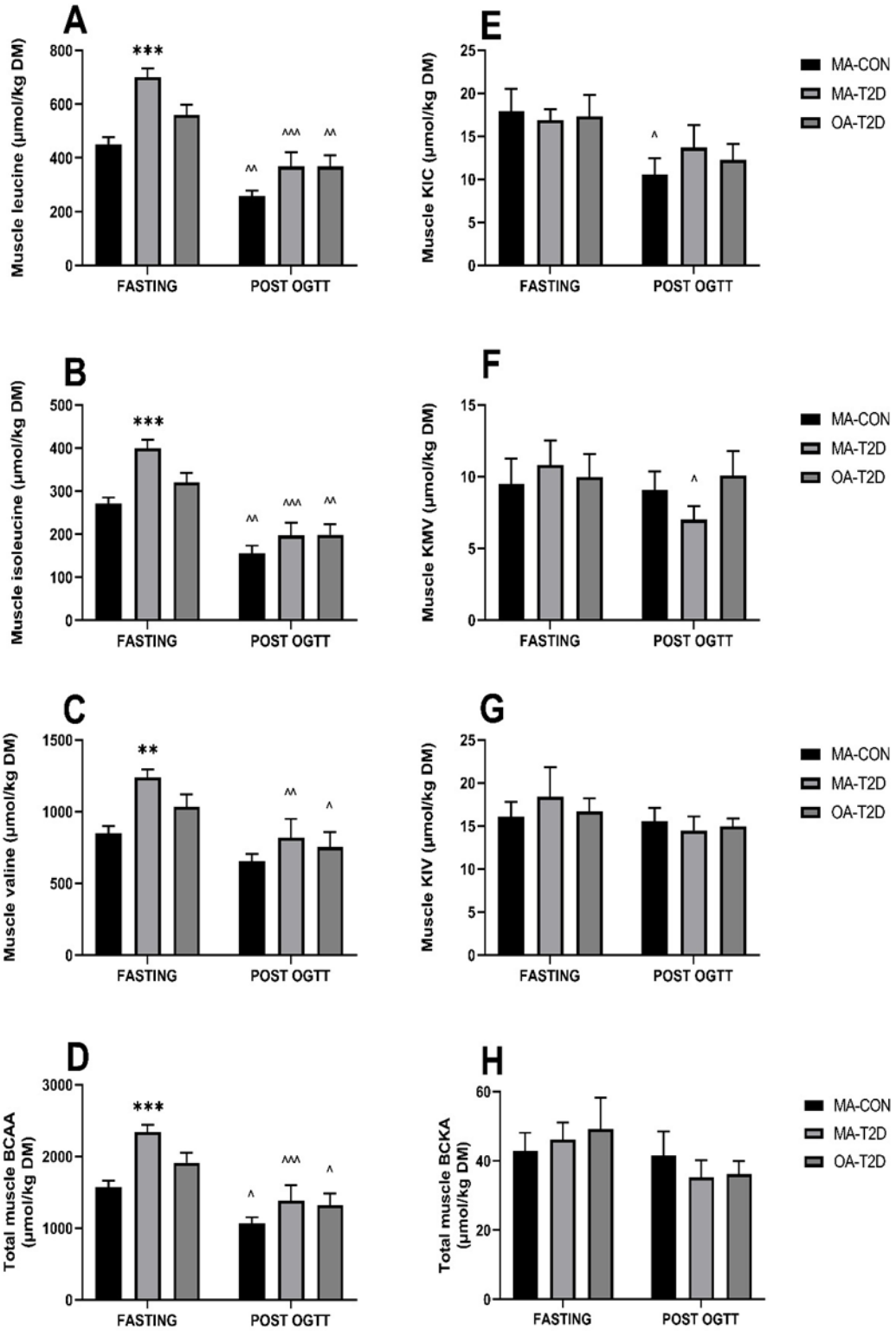

Figure 6.5 Skeletal muscle leucine (A) isoleucine (B) valine (C) and total branched chain amino acid (BCAA) (D), KIC (D) KMV (E) and KIC (F) and total branched chain keto acid (BCKA) in the fasted stated and post OGTT. ** $p < 0.01$ and *** $p < 0.001$ MA-T2D compared to MA-CON. ^ $p < 0.05$, ^^ $p < 0.01$ and ^^^ $p < 0.001$ post OGTT different from corresponding fasting value. Data are mean ± SEM n =7

6.3.4.2 Carnitine and acylcarnitines

MA-T2D vs OA-T2D

There were no differences between groups in free carnitine at either time point (Table 6.4). Muscle acteylcarntine content did not differ in the fasted state but there was a main effect of OGTT ($p = 0.006$). (Table 6.4). Independent t tests revealed no significant difference in any single acylcarnitine species between the two groups in the fasted state or after OGTT. When short, medium, and long chain-chain acylcarnitines were summed there was no differences between groups in the fasted state or post OGTT (Table 6.4). OGTT significantly reduced total short ($p=0.01$) and long ($p=0.04$) but not medium chain acylcarnitines in both groups.

MA-T2D vs OA- T2D

There were no differences between groups in fasting free carnitine content and there was no effect of OGTT (Table 6.4). Fasting muscle acetylcarnitine content was not different between groups in the fasting or post OGTT state. Total fasting short, medium and long chain acylcarnitines did not differ between groups and ANOVA revealed a significant main effect of OGTT for short ($p=0.001$) and long ($p=0.04$) chain acylcarnitines and a trend ($p=0.06$) for medium chains but specific post hoc comparisons were not significant.

A full profile of muscle acylcarnitine concentrations in the fasted and post OGTT state can be seen in Appendix D

Table 6.4 Skeletal muscle free carnitine, acetylcarnitine and the sum of short, medium, and long chain acylcarnitines in the fasted state and in response to oral glucose tolerance test (OGTT).

	MA -CON		**MA -T2D**		**OA -T2D**	
	Fasting	Post OGTT	Fasting	Post OGTT	Fasting	Post OGTT
Free carnitine (mmol/kg dm)	11.4 ± 0.7	12.7 ± 1.6	13.4 ± 2	11.9 ± 1.9	13.9 ± 0.8	13.8 ± 1.8
Acetylcarnitine (mmol/kg dm)	1.8 ± 0.5	0.6 ± 0.1	1.7 ± 0.3	0.9 ± 0.2	1.7 ± 0.6	0.6 ± 0.1
Short chain acylcarnitine (C_3-C_5) (µmol/kg dm)	47 ± 4.7	39.8 ± 4.2	54 ± 6.0	38 ± 6.9	60 ± 8.1	43 ± 8.6
Medium chain acylcarnitine (C_6-C_{10}) (µmol/kg dm)	10.3 ± 1.9	9.7 ± 2.4	13.0 ± 4.8	8.7 ± 2.0	16.9 ± 4.0	5.7 ± 0.7
Long chain acylcarnitine (C_{12}-C_{18}) (µmol/kg dm)	67.7 ± 24.5	22.5 ± 6.1	66.7 ± 28.3	23.3 ± 8.5	78.7 ± 40.8	10.5 ± 1.6

Data are mean ± SEM. n = 7. OGTT significantly reduced total short- ($p<0.02$) and long- ($p<0.05$) acylcarnitines and acetylcarnitine ($p<0.02$) regardless of group

6.3.5 Plasma metabolites

6.3.5.1 BCAA and BCKAs

MA-CON vs MA-T2D

There was a main effect of T2D and OGTT ($p < 0.001$ for both main effects) on total and each individual BCAA. Fasting plasma leucine was (19%) higher in MA-T2D ($p= 0.02$) and concentrations reduced in both groups ($p < 0.001$ and $p = 0.003$ for MA-CON and MA-T2D, respectively) but post OGTT concentration was 43% higher in MA-T2D ($p = 0.002$) (Figure 6.6A). Fasting plasma isoleucine was also (19%) higher in MA-T2D ($p = 0.02$) and concentrations reduced in both groups ($p < 0.001$ and $p = 0.001$ for MA-CON and MA-T2D, respectively) but post OGTT concentration was 36 % higher in MA-T2D ($p = 0.01$) (Figure 6.6B). Fasting plasma valine was (19%) higher in MA-T2D ($p = 0.01$) and concentrations reduced in both groups ($p < 0.001$ and $p = 0.001$ for MA-CON and MA-T2D, respectively) but post OGTT concentration was 28% higher in MA-T2D ($p = 0.01$) (Figure 6.6C).

There were main effects of T2D ($p = 0.04$) and OGTT ($p < 0.001$) on both plasma KIC and KMV. Fasting plasma KIC and KMV and KIV concentrations (Figure 6.6E-G) were not different between the two groups. However, OGTT reduced plasma KIC ($p = 0.002$) and KMV ($p = 0.003$) in MA-CON resulting in lower post OGTT KIC ($p = 0.01$) and KMV ($p = 0.03$) concentrations when compared to MA-T2D. Plasma KIV was not different between groups in the fasted or post OGTT state.

MA-T2D vs OA- T2D

There were no differences in fasting or post OGTT concentrations of each BCAA between groups (Figure 6.6A-C). Post OGTT concentrations for all 3 BCAAs were significantly reduced relative to fasting concentrations (OGTT effect $p < 0.001$ for both groups for each BCAA). There were no differences in fasting or post OGTT plasma BCKAs concentrations between groups (Figure 6.6E-G). Only KIC and KMV concentrations reduced ($p<0.0001$ for both) following OGTT ($p = 0.005$ and $p = 0.002$ for MA-T2D and OA- T2D, respectively for KIC, and $p = 0.008$ and $p = 0.002$ for MA-T2D and OA- T2D, respectively for KMV).

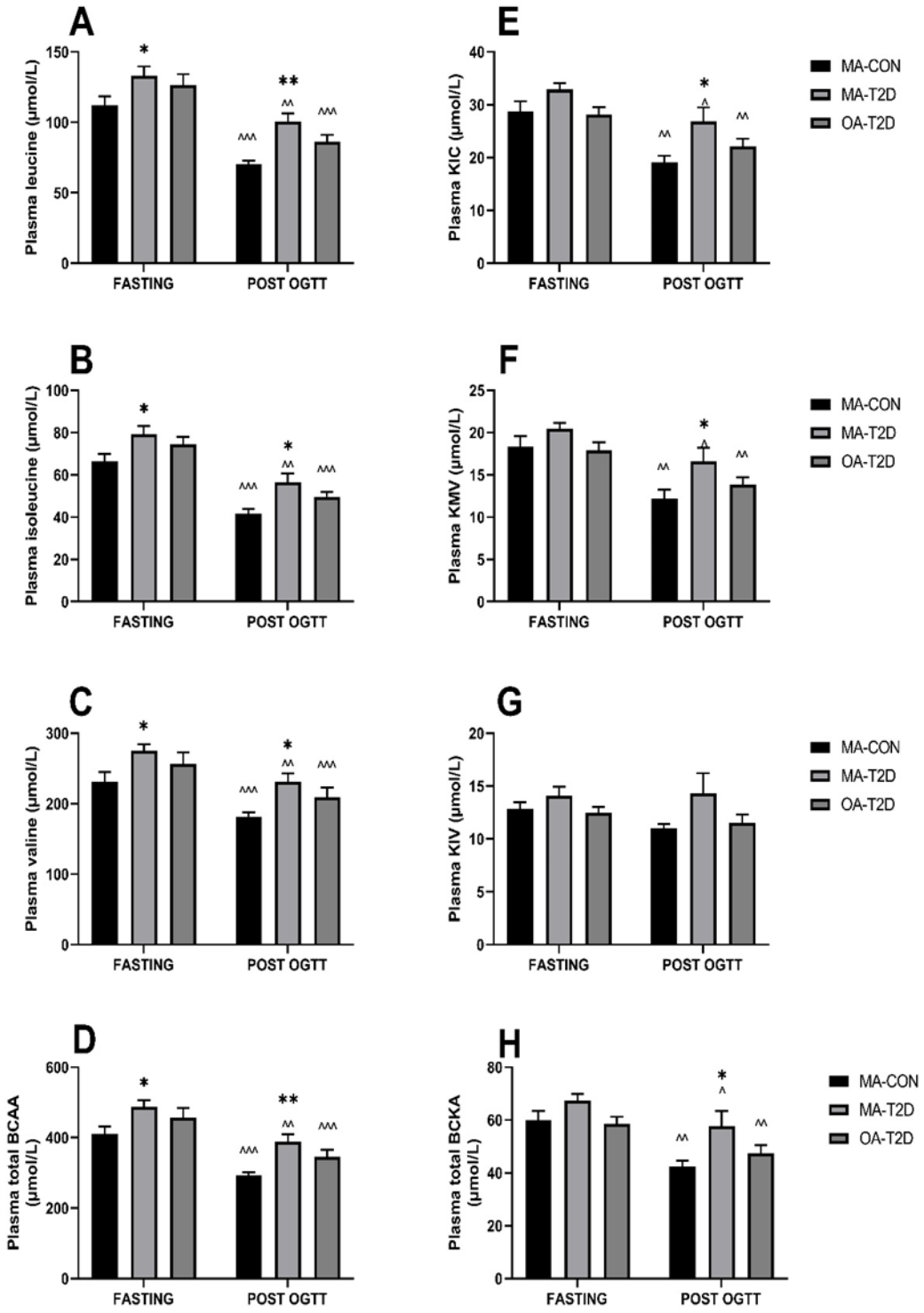

Figure 6.6 Plasma leucine (A) isoleucine (B) valine (C) and total branched chain amino acid (BCAA) (D), KIC (D) KMV (E) and KIC (F) and total branched chain keto acid (BCKA) in the fasted stated and post OGTT * $p < 0.05$ and ** $p < 0.01$, MA-T2D compared to MA-CON. ^ $p < 0.05$, ^^ $p < 0.01$ and ^^^ $p < 0.001$ post OGTT different from fasting value. Data are mean ± SEM n =7

6.3.5.2 Free carnitine and acylcarnitines

MA-CON vs MA-T2D

There were no differences in fasting or post OGTT values between groups for free carnitine and acetylcarnitine concentrations (Table 6.5). Acetylcarnitine declined from fasting values in both groups after OGTT ($p < 0.001$). Fasting plasma total short chain acylcarnitines were higher in MA-T2D ($p = 0.003$) and remained elevated post OGTT ($p =0.02$) compared to MA-CON (main effect of T2D $p=0.005$; Table 6.5). This elevation was driven by significantly higher fasting ($p < 0.001$) and post OGTT ($p=0.001$) C_3 acylcarnitine and elevated fasting ($p=0.03$) and post OGTT ($p=0.03$) iso C_5 in the former group (T2D effect p = 0002 and interaction effect $p=0.02$; Figure 6.7). In addition, elevated fasting C_4 ($p = 0.01$) and $C_{5:1}$ (T2D effect $p = 0.03$ and interaction effect $p=0.004$) were also observed in MA-T2D when compared to MA-CON. Plasma iso C_4 was higher in MA-T2D post OGTT ($p = 0.04$ and main T2D effect $p= 0.03$) (Figure 6.7). There were no significant T2D or interaction effects for plasma total medium and long chain acylcarnitines (Table 6.5). OGTT reduced the concentrations of total medium and long chain acylcarnitines to a similar extent in both groups (OGTT effect $p=0.003$ and $p<0.001$, respectively).

MA-T2D vs OA-T2D

There were no differences in fasting or post OGTT values between groups for free carnitine and acetylcarnitine concentrations (Table 6.5). Plasma acetylcarnitine declined from similar fasting values in both groups (OGTT effect $p < 0.001$). Total short chain acylcarnitines were lower in OA-T2D than MA-T2D in the fasted state and post OGTT (age effect $p=0.008$; Table 6.5).

This was driven by lower fasting ($p < 0.001$) and post OGTT ($p = 0.004$) C_3 (age effect $p < 0.001$), lower fasting iso C_5 (group effect $p = 0.02$) levels (Figure 6.7). OGTT reduced plasma total medium and long chain acylcarnitines to a similar extent in both groups (OGTT effect $p < 0.001$ for both groups).

A full profile of plasma acylcarnitine concentrations in the fasted and post OGTT state can be seen in Appendix E

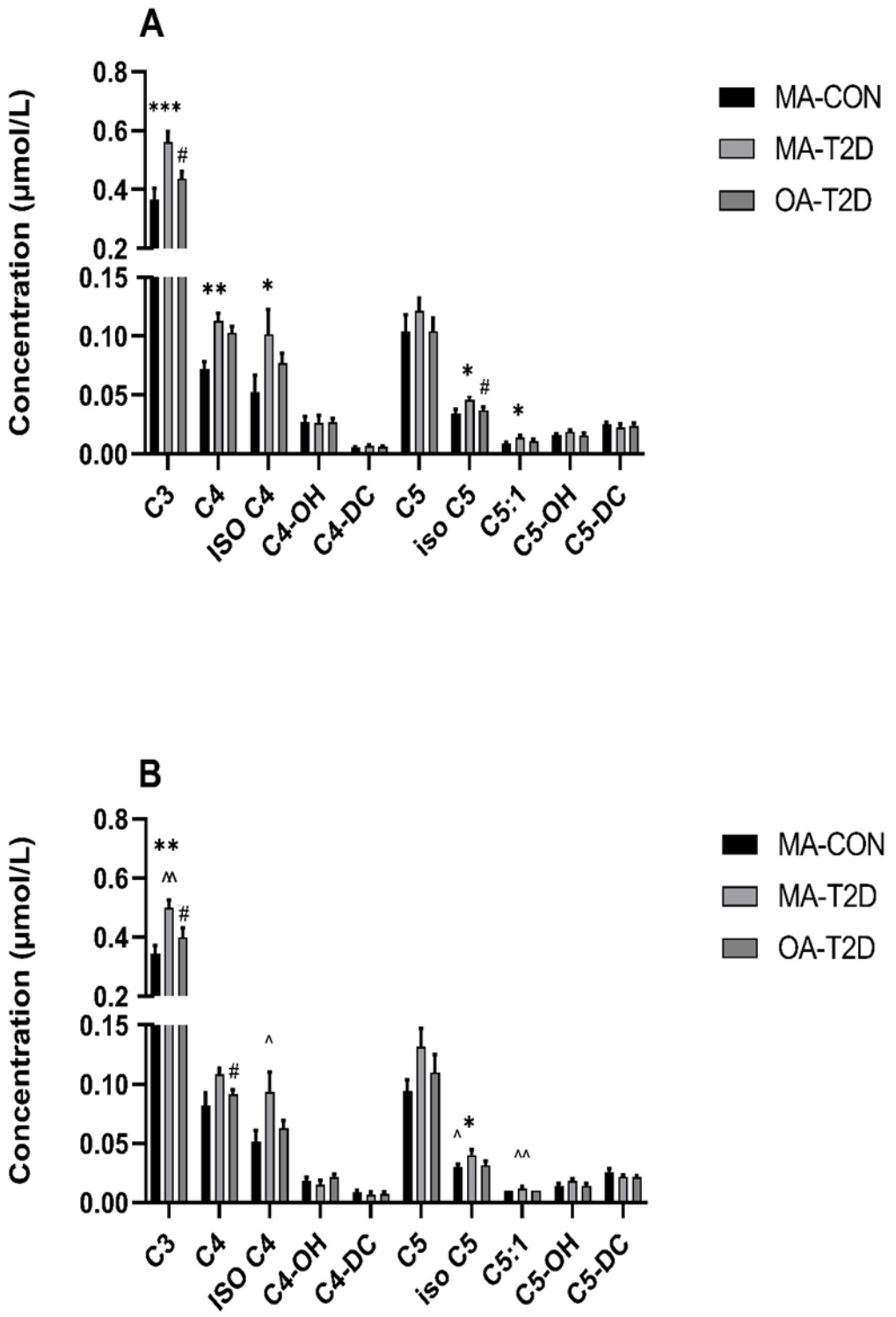

Figure 6.7. Plasma short chain acylcarnitine profile in the fasted state (A) and post OGTT (B). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ MA-T2D compared to MA-CON. # $p < 0.05$, OA-T2D different to MA-T2D. ^ $p < 0.05$, ^^ $p < 0.01$ and ^^^ $p < 0.001$ post OGTT different from fasting value. Data are mean ± SEM n =7

Table 6.5 Plasma free, acetylcarnitine and the sum of short, medium and long chain acylcarnitines in the fasted state and in response to oral glucose tolerance test (OGTT).

	MA -CON		**MA -T2D**		**OA -T2D**	
	Fasting	Post OGTT	Fasting	Post OGTT	Fasting	Post OGTT
Free carnitine (µmol/L)	41.0 ± 3.9	43.5 ± 2.4	41.9 ± 1.5	42.6 ± 1.7	41.9 ± 2.9	41.5 ± 2.4
Acetylcarnitine (µmol/L)	8.1 ± 0.5	4.4 ± 0.4	7.8 ± 1.1	4.5 ± 0.6	7.0 ± 0.5	4.1 ± 0.3
Short chain acylcarnitine (C_3-C_5) (µmol/L)	0.7 ± 0.1	0.7 ± 0.04	1.0 ± 0.1 ****	1.0 ± 0.04*****	0.8 ± 0.02 ##	0.7 ± 0.02#
Medium chain acylcarnitine (C_6-C_{10}) (µmol/L)	0.5 ± 0.1	0.3 ± 0.03	0.4 ± 0.1	0.32 ± 0.04	0.6 ± 0.1	0.4 ± 0.03
Long chain acylcarnitine (C_{12}-C_{18}) (µmol/L)	0.6 ± 0.02	0.4 ± 0.03	0.46 ± 0.01	0.32 ± 0.04	0.5 ± 0.03	0.3 ± 0.02

Data are mean ± SEM. n = 7. * $p < 0.05$ and ** $p < 0.01$ MA-T2D different to the corresponding MA-CON value. # $p < 0.05$ and ## $p < 0.01$ OA-T2D different to corresponding MA-T2D value. OGTT reduced plasma acetylcarnitine ($p < 0.001$), total short , medium and long chain acylcarnitine in all groups ($p < 0.05$).

6.3.6 BCKDH protein expression

There were no differences in the ratio of phosphorylated amount of the E1α subunit of the BCKDH complex between MA-CON and MA-T2D or MA-T2D and OA-T2D in the fasted state. ANOVA for MA-CON vs MA-T2D revealed a main effect of OGTT (p=0.02) indicating an increase regardless of treatment.

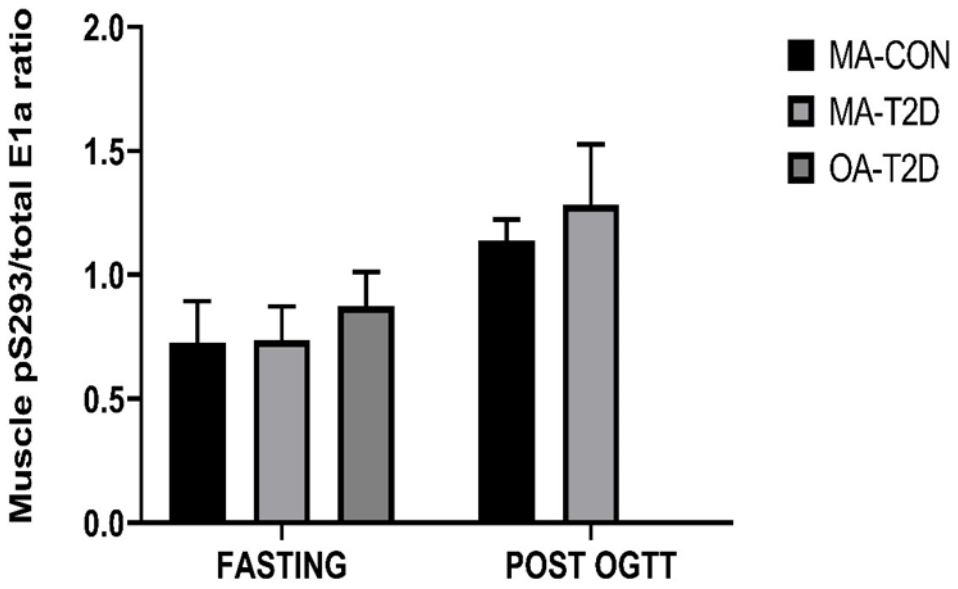

Figure 6.8 Muscle pS293 phosphorylated E1α component of the branched chain keto acid dehydrogenase complex. Post OGTT values for OA-T2D are missing due to insufficient sample number. Data are mean ± SEM. n = 6.

6.4 Discussion

T2D is characterised by derangements in glucose, lipid and protein metabolism. The use of an isotopically labelled fat drink and the application of the analytical method developed and validated in the previous experimental chapters in this thesis enabled an integrative assessment of multiple substrates at the whole-body level and in skeletal muscle. The findings from the current study reveal that compared to their middle aged, obese control subjects, patients with T2D exhibited an increased meal derived fat oxidation and an attenuated ability to suppress plasma FFA with OGTT. This was accompanied by marked elevations in plasma and muscle BCAA content and plasma (but not muscle) short chain acylcarnitines providing further evidence that excessive muscle fatty acid oxidation may contribute to increased BCAA catabolism in humans. Interestingly, these responses appear to be reversed in the older aged T2D group.

The T2D patients in the current study clearly demonstrated the twin defects that define the condition (Kahn, Hull, and Utzschneider 2006). Compared to a well-matched group for body composition, the middle-aged diabetic group displayed hyperglycaemia and a lower insulin response to OGTT, reflecting an impairment in β-cell function and insulin release. Aging *per se* did not appear to exacerbate these defects but the differences in fatty acid and amino acid metabolism highlight that despite being matched for body composition and disease duration, T2D in older age appears to represent a different metabolic phenotype compared to middle aged patients suggesting that careful considerations should be made when assessing T2D in large cohorts across a large age range.

In response to dietary fat intake, adipose tissue provides an appropriate site of fat storage (Frayn 2002). Reduced adipose tissue storage of fatty acids from chylomicron TAG (i.e. dietary fat) has been observed in obese individuals (McQuaid et al. 2011) and is proposed to be the pathophysiological basis of diversion of fatty acid to other organs such as liver and skeletal muscle and even the pancreatic β-cell (R Taylor 2013), resulting in tissue insulin resistance. Indeed, increased accumulation of liver and intramuscular lipid content differentiates insulin resistant and insulin sensitive obese phenotypes (Stefan et al. 2009). The finding from the current study that dietary derived fatty acid oxidation was elevated in middle aged diabetics could reflect an impairment of adipose tissue function resulting in inappropriate fat 'spill over' to ectopic organs. Increased postprandial fat delivery to, and extraction by muscle of postprandial TAG has been observed in insulin resistant men (van Hees et al. 2011). Uptake and utilisation of this elevated circulating fatty acid may consequently compete with glucose for oxidation in the classic concept of the glucose-fatty acid cycle proposed by Randle and colleagues (Randle et al. 1963). Therefore, in times where fatty acids should be routed towards esterification and storage, they are oxidised ectopically as fuel in the presence of concomitantly available glucose. This notion is supported by recent findings in rodents with lipodystrophy (a state defined by a lack of adipose tissue and early onset of diabetes), which provides insight into the immediate consequences of surplus fat ingestion. In response to a high formulation fat ingestion, lipodystrophic mice displayed marked increases in fatty acid oxidation to match fat intake which was accompanied by worsening of hyperglycaemia and hyperinsulinemia and liver TAG accumulation (Girousse

et al. 2018). These findings highlight the deleterious consequences of the inability to appropriately store meal derived fatty acid and support the findings of increased meal fatty acid oxidation seen in the current study. When combined with the observation of an attenuated suppression of endogenous FFA mobilisation during OGTT in the middle-aged T2D group, these findings suggest excessive postprandial fat delivery to ectopic sites such as muscle, liver and pancreas, impairing insulin action in the middle-aged but not older group with T2D. The finding that muscle acetylcarnitine (which could be formed from excess acetyl CoA produced from continued fat delivery to, and oxidation by, the muscle) was less suppressed with OGTT in the MA-T2D group further supports this notion. Taken together it is clear that the MA-T2D group is burdened by an inappropriate increase in both exogenous and endogenous delivery of fatty acids to skeletal muscle in the postprandial (insulin stimulated) state.

The absence of a middle-aged lean group precluded the ability to assess the effect of obesity *per se* on metabolism. However, when compared to age and body composition matched obese controls, T2D *per se* is characterised by marked increases in fasting muscle and circulating BCAA concentrations as well circulating BCAA derived short-chain acylcarnitines This is consistent with findings from chapter 4, in which a larger cohort of subjects with a wider age range were studied. In the current work, the MA-CON group were also obese (BMI 31.2) yet muscle BCAAs were ~50% higher in MA-T2D, suggesting that elevations in BCAA in humans may be a consequence rather than a cause of insulin resistance and diabetes. Although plasma BCAAs were also elevated with diabetes, the lack of arterial-venous differences measurements across

the muscle precludes the ability to judge any direction of flux to/from the tissue. However, the increase in plasma BCAAs was modest (~20%) and it is unlikely that muscle uptake of circulating BCAAs (resulting from reduced catabolism in other tissues) would be the sole cause of such markedly elevated muscle BCAA content.

These findings are important as longitudinal and cross sectional studies have suggested that BCAA and related catabolites could be implicated in the progression of insulin resistance (T. J. Wang et al. 2011) but have often compared only lean and obese individuals in the fasted state (Newgard et al. 2009). Therefore, if elevated BCAAs caused insulin resistance in obese individuals, it would be expected an increase in their circulating or tissue concentration would precede the development of insulin resistance and T2D. In this study, all groups were well matched for body composition, dietary intake, and physical activity. Thus, the increase in BCAA in muscle and plasma is likely to reflect the insulin resistant state. The findings from this study extend upon much of previous work measuring fasting metabolite profiles (Newgard et al. 2009; Lerin et al. 2016).

In response to OGTT there was a clear impairment of the suppression of plasma BCAA and BCKAs (KIC AND KMV) in the MA-T2D compared to the MA-CON group, which could be indicative of on-going whole body proteolysis and possibly oxidation and this response did not appear to be exacerbated by age. This is line with the blunted insulin response to OGTT in MA-T2D and OA-T2D and is reflective of the lower levels of circulating insulin and its potent inhibition of muscle protein breakdown (Tessari et al. 1986). However, BCAA content in muscle declined to similar concentrations in all groups and without

the calculation of flux across the muscle it is not possible to make any inferences on the extent to which muscle may have contributed to persistent elevations of intramuscular BCAA post OGTT. Therefore, a role of impaired insulin-stimulated uptake of BCAA during OGTT cannot be ruled out. It is likely that multiple organs are contributing to plasma metabolite concentrations and there is likely to be exchange of these metabolites between multiple tissues. Emerging evidence from human studies have demonstrated that obesity and insulin resistance is associated with increased whole body (Tan et al. 2020) and visceral adipose tissue BCAA oxidation (Piro et al. 2020) rather than impaired BCAA catabolism as seen in rodent studies (Zhou et al. 2019). Given that in the MA-T2D group there was a significant reduction in plasma BCAA derived acylcarnitines yet still remained significantly elevated compared to the MA-CON post OGTT suggests a degree of ongoing catabolism. These findings question the validity of previous rodent studies suggesting reduced BCAA catabolism with insulin resistance and their application to human metabolism. One reason for this discrepancy of findings may be due to the fact that skeletal muscle comprises a much smaller percentage of the total body mass in rats (Wolfe 2017). Therefore, proteolysis of skeletal muscle and its contribution to circulating pools may not be significant in that model.

The ability to quantitively assess fat oxidation and BCAA content in the present study enabled exploration of possible interactions between the two respective pathways. The finding that the elevations in meal derived fat oxidation in MA-T2D vs MA-CON is mirrored by elevated muscle and plasma BCAA content suggests a potential cause and effect relationship. Previous work from our group has shown that muscle BCAA derived short chain acylcarnitines were

negatively correlated with insulin-stimulated glucose disposal and muscle glycogen storage during lipid infusion in combination with a hyperinsulinaemic-euglycaemic clamp, an acute experimental model that drove high muscle fat oxidation even in the presence of high levels of circulating insulin (Stephens et al. 2014). Furthermore, plasma short chain acylcarnitine and muscle C_5:1 positively correlated with fasting blood glucose in chapter 4. The fact that short chain acylcarnitines were not elevated in muscle of MA-T2D but were in chapter 4 (where a greater number of subjects were studied n = 10) may be due a lack of statistical power in the present study. Taken together with the former report, these findings point to a novel locus of interaction between excessive fatty acids and perturbed BCAA metabolism. Therefore, it could be suggested that the findings from the longitudinal studies implicated BCAA catabolites with the progression of insulin resistance (Wang et al. 2011; Menni et al. 2013) could simply be the reflection of deranged muscle metabolism. It could be proposed that obesity (with a possible contribution of inactivity) related alterations in BCAA metabolism leads to an accumulation of BCAA in muscle and possible spill over into the circulation. Overtime, as insulin resistance is exacerbated and clinically overt T2D develops BCAA catabolism is increased due to the complete loss of insulin sensitivity/release as observed in this study. Coupled to an aggravating effect of increased muscle lipid availability due to impaired adipose tissue lipid trafficking (McQuaid et al. 2011; Brøns and Grunnet 2017), a positive feedback cycle may be set in motion, whereby lipid induced insulin resistance promotes BCAA catabolism which worsens insulin resistance. Under these circumstance short chain acylcarnitines may too, spill over into the circulation over time. Although organ

flux studies have shown liver is the main contributor to fasting acylcarnitine profiles (Schooneman et al. 2015; G. Xu et al. 2016), no studies have determined how muscle flux changes in insulin resistance states, particularly when muscle is burdened with excess lipids as described in this work. Studies in larger cohorts are required to confirm these assumptions. It is also important to highlight that both T2D groups in this study had a deficiency in glucose-stimulated insulin release, which is different to the experimental model used in the previous section in which exogenous insulin was administered under clamp conditions without a concomitant elevation of circulating fatty acids through infusion.

BCAA and fatty acids both require esterification to form acylcarnitines to transport their respective CoA adducts across mitochondrial membranes. It has been suggested that excessive BCAA catabolism may compete with fatty acid oxidation in the mitochondria leading to incomplete fatty acid oxidation and the accumulation of medium- and long-chain acylcarnitines (Newgard 2012; White et al. 2016). However, more recent studies using stable isotope tracers to measure the fate of BCAA in rodents have shown that the fractional contribution of BCAA to the TCA in skeletal muscle is smaller (5%–6%) than that of fatty acids so that even a large increase in BCAA oxidation is not likely to significantly compete with fatty acids (Neinast et al. 2018). Alternatively, impairments in BCAA catabolism in muscle may impair fatty acid oxidation by reduced anaplerosis and replenishment of intermediates required for fatty acid oxidation, leading to the accumulation of medium and long chain acylcarnitines (Lerin et al. 2016). However, in the current study there were no observed differences in muscle or plasma free carnitine content between any groups

and no accumulation in muscle of medium or long chain acylcarnitines (even in chapter 4 where more subjects were studied). Together with the finding that fatty acid oxidation was increased in MA-T2D, these findings suggest that in humans, increased and not incomplete oxidation of fatty acids (in the insulin stimulated state) is associated with insulin resistance and T2D.

The experimental design of the current study also allowed for a comparison of age *per se* on T2D. Strikingly, despite a similar attenuation of insulin secretion and resultant hyperglycaemia during the OGTT, the older aged T2D patients did not exhibit many of the adverse effects seen with T2D *per se* in middle aged group. For example, given an attenuate insulin response to OGTT in MA-T2D compared MA-CON it could have been argued that the higher meal derived fat oxidation (in MA-T2D vs MA-CON) was due simply to a reduced insulin response and not adipose tissue dysfunction. However, the finding that OA-T2D could supress plasma FFA for same insulin response as MA-T2D would suggest a dysfunction in MA-T2D and a different phenotype of disease. Consequently OA-T2D are not burdened by post prandial fatty acid delivery and the mechanism underlying the T2D is different to the mechanisms in MA-T2D. Indeed, age *per se* is associated with an increased risk of T2D, even when controlling for body composition, by some level of age related decline in pancreatic β- cell function possibly mediated by a degree of age specific inflammation (De Tata 2014; Löhr et al. 2018) which could explain the differences between the T2D groups (i.e there may be an age related decline in pancreatic function rather than impaired adipose tissue function in the OA-T2D group).

Consistent with the findings from the previous chapter, intramuscular BCAA content appears to decline with age in the diabetic populations, regardless of the insulinaemic and glycaemic responses to OGTT and therefore postprandial insulin resistance. In chapter 5, the young lean group had higher total and leg lean mass so it could be argued that the reduced BCAA content simply reflected lower lean leg mass. However, in the current study, subjects were closely matched for lean mass yet an age-related decline in muscle BCAA content was still present in the fasted state. A novel finding of this study was that plasma short chain acylcarnitines of BCAA catabolism were also lower in the OA-T2D group compared to MA-T2D. This could reflect an age related reduction of whole body protein/BCAA turnover (Morais et al. 1997) despite the presence of insulin resistance. However, it could also provide further support for the notion mentioned above, with regards to an aggravating effect of excessive fatty acid oxidation on muscle BCAA metabolism in the MA-T2D group. It is tempting to speculate that the absence of excessive lipid supply in the OA-T2D group prevented perturbations of BCAA metabolism. This was the first study to assess fat oxidation and BCAA flux and provides the framework for future studies to tease out the effect of age and fatty acid availability on BCAA metabolism.

Given that there were no differences in plasma BCAA concentrations between the two T2D groups despite lower muscle BCAA content in the older T2D group suggests that plasma BCAA concentrations may not accurately reflect muscle tissue metabolism in ageing. Furthermore, older age was associated with reduced plasma BCAA-derived short chain acylcarnitines suggesting that these species are not accurate biomarkers of insulin resistance and T2D in the

context of ageing. In response to the OGTT however, the OA-T2D did not display a significant reduction in muscle BCKAs, suggesting some ongoing muscle BCAA catabolism which is consistent with the findings in chapter 5. Furthermore, there were no differences between MA-T2D and OA-T2D in plasma BCAAs or BCKAs reduction in response to OGTT. These findings are difficult to reconcile but may in part be explained by the notion that there are differential sensitivities of insulin on glucose, fatty acid and BCAA metabolism (Boirie et al. 2001) and each aspect should be considered as a separate entity with regards to insulin resistance, particularly in the face of perturbed/differential fatty acid availability.

The finding that neither elevated whole body meal derived fat oxidation nor attenuated suppression of endogenous fat delivery during the OGTT was reflected in differences in plasma fatty acid derived (medium and long chain) acylcarnitine profiles between groups may suggest that the measurement of these species lack sufficient sensitivity to accurately determine tissue level substrate oxidation. These findings argue against previous reports that have measured plasma acylcarnitines and attempted to draw conclusions about muscle metabolism (Mihalik et al. 2010). Furthermore, it has been demonstrated in rodents, that in response to an OGTT the only tissue that appeared to reflect plasma acylcarnitine profiles was the heart (Makrecka-Kuka et al. 2017) providing further evidence that plasma acylcarnitines may not be an appropriate biomarker for skeletal muscle insulin resistance.

No differences in phosphorlayion status of the BCKDH complex was observed in the current study. The BCKDH complex is also regulated by intracellular energy status; a high NADH/NAD ratio as would occur when fatty acid

oxidation is high could inhibit BCAA oxidative flux in muscle leading to efflux into the plasma as an alternative fate. High lipid availability leading to insulin resistance has also been shown to impair muscle protein synthesis in humans (Stephens et al. 2015; Smiles et al. 2019) which could in part be contributing to accumulation of intramuscular BCAA. How strategies to improve insulin sensitivity through the modulation of muscle fatty acid metabolism impact upon BCAA levels requires investigation.

6.5 Conclusion

In conclusion, excessive lipid availability and oxidation in the postprandial (insulin stimulated) state appears to differentiate middle aged obese individuals from T2D patients. This is accompanied by marked elevations in circulating and muscle BCAA content and may in part being contributing to perturbed BCAA metabolism. Remarkably, age *per se* does not appear to exacerbate any of these derangements in metabolism in T2D, rather it appears to reverse some of the effects seen in the middle aged T2D group. These discrepancies cannot be explained by differences in disease duration, body composition, physical activity, or energy intake. Taken together these findings appear to dissociate age, T2D-related insulin resistance and perturbed BCAA metabolism.

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7 Chapter 7 General Discussion

7.1 Overview of findings

A primary aim of this thesis was to develop a targeted analytical method for the absolute quantification of a number of circulating metabolites strongly associated with insulin resistance and type 2 diabetes (T2D). In recent years, BCAAs, fatty acids and associated catabolites have been implicated in the development of insulin resistance. However, much of the mechanistic investigation has been performed in animal or cell-based models which may not encapsulate various physiological aspects of human metabolism. Furthermore, investigations in humans have been limited to either the fasted state or analysis of plasma profiles, providing little insight into tissue metabolism.

In chapter 3, a liquid chromatography coupled to high resolution mass spectrometry (LC-MS) method was developed, optimised and validated in accordance with the FDA analytical guidelines for method development (FDA and CDER 2018). The method met the major validation criteria required for the accurate assessment of metabolite concentrations from biological tissues. Application of the developed LC-MS method to healthy human samples demonstrated that calculated concentrations of metabolites were strongly consistent with numerous well-established methods for individual metabolites. In chapter 4, the method was validated in plasma and skeletal muscle of patients with T2D. The method confirmed previous findings that a circulating BCAA related signature is more closely associated with insulin resistance and T2D than markers of fatty acid metabolism (i.e. medium and long chain acylcarnitines) (Newgard 2012). A novel finding was that skeletal muscle BCAA and short chain BCAA-derived acylcarnitines were also elevated in

muscle and plasma of patients with T2D providing evidence for a possible association between muscle and plasma profiles.

In chapter 5, it was revealed that ageing, despite being accompanied with a slight degree of insulin resistance, appeared to reduce intramuscular BCAA content, whereas the presence of increased adiposity and worsening of insulin resistance appeared to increase fasting muscle BCAA content. Thus, against a background of ageing and adiposity there appears to be a dissociation between BCAA content and insulin resistance. These findings were confirmed in chapter 6 where groups of individuals with and without T2D were well matched for body composition, energy intake and physical activity. In addition, the work in chapter 6 revealed that the perturbed BCAA metabolism in T2D was mirrored by increased meal derived fatty acid oxidation in middle aged but not older aged T2D patients. These findings revealed that regardless of insulin resistance age *per se* may represent a distinct phenotype of BCAA perturbation.

7.2 Development of a targeted method

In chapter 3, the commonly cited challenges associated with quantifying metabolites from biological samples using LC-MS based methodologies were systematically addressed. These challenges included chromatographic and mass spectrometry optimisation, ensuring accurate and precise measured concentrations of the analytes in the complex biological matrices and the ability to measure all the analytes in a single biological sample. Utilising isotopically labelled analytes and the standard addition method, analytical parameters of linearity (R^2 >0.99), precision, accuracy, recovery, stability, and

reproducibility were all confirmed in both human plasma and skeletal muscle tissue. These results confirmed the appropriateness of the extraction protocol used throughout this thesis. Due to laborious nature of the standard addition method, the internal standard method in an appropriate proxy matrix substitute of muscle and plasma (7.5% BSA in PBS) was also optimised and validated against the same analytical criteria for industry (Fda and Cder 2018). Assessment of metabolite concentrations from both methods had very close agreement (within 5 % for most metabolites). Collectively, these results demonstrate the method developed in chapter 3 provides a robust and accurate assessment of metabolite concentrations in human biological samples.

The advantage of the developed method was that it provided accurate concentrations of a large range of metabolites (BCAAs, BCKA, free carnitine and >40 acylcarnitines) from one single extraction protocol. The use of both reversed phase and HILIC chromatography enabled the detection of multiple metabolites that span different orders of magnitude in abundance. For example, free carnitine is in the mmol/kg (dry mass) range whereas acylcarnitines are in the nmol/ kg (dry mass) range. Thus, from one extraction protocol and two analytical acquisitions, an accurate assessment of carbohydrate, fatty and BCAA metabolites in skeletal muscle can be ascertained.

7.3 The effect of T2D per se on BCAA metabolism

The developed method revealed striking differences in a BCAA related signature between a middle-aged group with T2D and an age matched obese

group without T2D. Circulating BCAA and short chain acylcarnitines derived from BCAA catabolism were all significantly elevated with T2D, consistent with earlier reports (Newgard et al. 2009). Moreover, they correlated significantly with fasting blood glucose concentrations. Furthermore, BCKAs, despite not being statistically elevated in the T2D group also correlated with blood glucose. Collectively, these findings demonstrated marked alterations in BCAA catabolism which are likely a consequence of T2D. When investigating skeletal muscle, T2D was characterised by marked increases in muscle BCAA content and BCAA derived acylcarnitines. Interestingly muscle and plasma BCAA and $C_{5:1}$ (derived from leucine) was positively correlated, suggesting that muscle may also be contributing to circulating profiles. This is in contrast to speculative rodent literature which have implied that muscle simply acts as a 'sink' for BCAAs which accumulate secondary to reduced adipose tissue and liver BCAA catabolism (She, Van Horn, et al. 2007; Herman et al. 2010; White et al. 2016; Zhou et al. 2019). However, evidence in humans has shown that BCAA catabolism is not impaired but is increased, both in adipose tissue (Piro et al. 2020) and at the whole body level (Tan et al. 2020). Together with the findings within this thesis, this questions the validity of rodent models and questions the proposed notion that increasing BCAA catabolism is a viable treatment for insulin resistant states (Zhou et al. 2019).

7.4 The effect of age on BCAA metabolism

One consistent finding in this thesis was that age is negatively correlated with fasting BCAA content, regardless of increased insulin resistance. In chapter 5, total BCAA content was ~ 30 % lower in older lean subjects compared to a younger lean but more insulin sensitive group. In chapter 6, despite being

equally insulin resistant an older aged T2D group had 20 % lower BCAA content compared to middle aged T2D patients (Figure 7.1). Interestingly, ageing also appears to be associated with attenuated suppression of muscle BCAA catabolism regardless of insulin sensitivity. The impact, if any, of this reduced attenuation of BCAA catabolism on total skeletal muscle protein content and the development of insulin resistance requires further investigation.

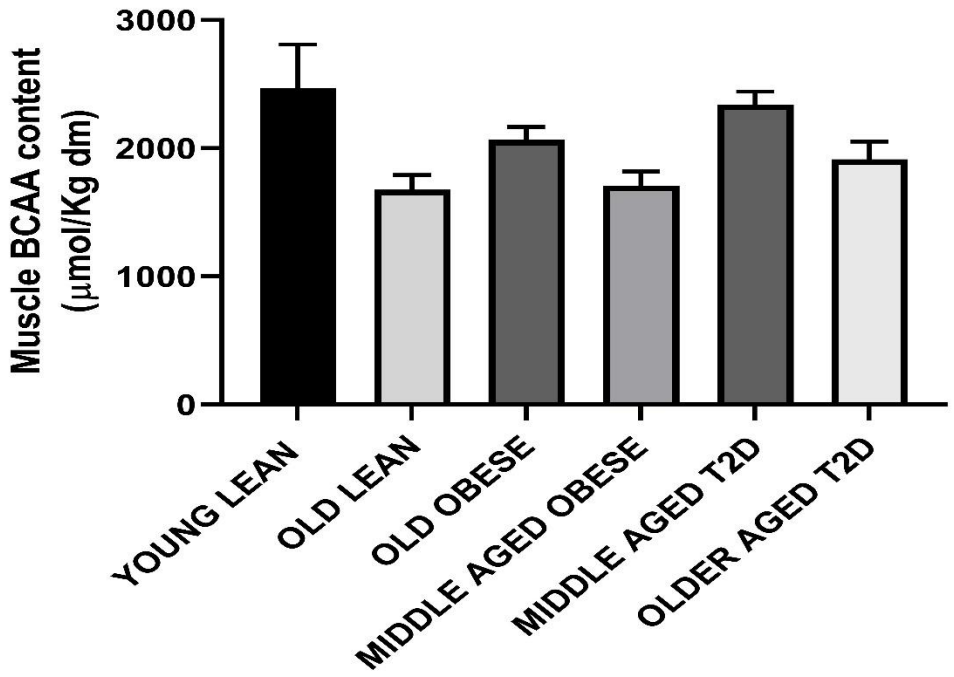

Figure 7.1 Fasting muscle total branched chain amino acid (BCAA) content measured using targeted metabolomics across increasing age and degrees of insulin resistance from all the groups measured throughout this thesis. Age per se appears to reduce muscle BCAA content whereas insulin resistance per se appears to increase muscle BCAA content. The young lean (n=7) and old lean (n=9) group were matched for BMI, but the old lean had less overall lean mass and were slightly less insulin sensitive. The old obese (n=9) and old lean group were matched for age, but the obese group were markedly less insulin sensitive as assessed by glucose disposal under clamp conditions. The middle-aged obese group (n=7) were matched for body composition with the middle-aged type 2 diabetic (T2D) group (n=7) but were more insulin sensitive. The older aged T2D group (n=7) were matched for body composition and equally insulin resistant as the middle aged T2D group. Data are mean ± SEM.

7.5 Acylcarnitines and insulin resistance

Fatty acid derived acylcarnitines have been implicated as potential causative markers of insulin resistance (Koves et al. 2008). Numerous studies have profiled circulating plasma acylcarnitines and found elevations in obese and insulin resistant individuals (Adams et al. 2009; Mihalik et al. 2010). Whilst *in vitro* evidence has shown that acylcarnitines can cause muscle insulin resistance (Céline Aguer et al. 2015b), there is a dearth of evidence linking fatty acid derived acylcarnitines and insulin resistance in humans. For any acylcarnitine species to cause insulin resistance in muscle, it would be expected that their accumulation will precede the development of insulin resistance. Throughout the experimental chapters in this thesis, it has been shown that medium and long chain acylcarnitines are suppressed in the insulin stimulated state despite markedly lower glucose disposal, dissociating them from the development of insulin resistance. In chapter 5, a young lean and highly insulin sensitive group had higher fasting muscle medium and long chain acylcarnitine content compared to older lean (matched for body fatness) albeit slightly less insulin sensitive group. Furthermore, no differences in fasted fatty acid derived acylcarnitines in plasma was observed between insulin sensitive and insulin resistant obese individuals. Moreover, medium and long chain acylcarnitines did not reflect the differences in meal derived fat oxidation and circulating FFA during OGTT between the middle-aged individuals with and without T2D in chapter 6. Therefore, unless tissue acylcarnitine flux is calculated using stable isotope tracers (which will be the basis of future work), assumptions on changes in fatty acid oxidation and insulin sensitivity based

on static acylcarnitine concentrations should be avoided and are not appropriate.

Conversely, short chain, BCAA derived acylcarnitines were elevated in muscle of patients with T2D and obese insulin resistant subjects (chapter 5). These findings confirm previous work from this group which has shown muscle short chain acylcarnitines appear to be more closely associated with insulin resistance (Stephens et al. 2014). Striking elevations of these species were evident in plasma which persisted in the insulin stimulated state. Again, age per se appeared to cause a decline in these species in the circulation despite a similar insulin release in response to OGTT. What role these species have in the development of insulin resistance remains unknown.

7.6 Potential impact of fat oxidation on BCAA metabolism

In chapter 6, the fate of meal derived fat was quantitatively assessed in middle aged obese individuals with and without T2D as well as an older aged T2D group, matched for disease duration. The middle-aged T2D group displayed higher meal derived fatty acid oxidation and a reduced ability to suppress circulating FFA flux during an OGTT, as well as markedly higher muscle BCAA content compared to the other groups. These findings extend upon previous work from this group which showed elevated fatty acid availability and oxidation in muscle resulted in the accumulation of BCAA derived short chain acylcarnitines which were negatively correlated with insulin-stimulated glucose disposal and glycogen storage (Stephens et al. 2014). In support of this, short chain acylcarnitines correlated with fasting blood glucose in chapter 4.

Therefore, a proposed model from this thesis would be, ectopic fat spill over into skeletal muscle (and other insulin sensitive tissues) can lead to the development of insulin resistance and an increased muscle BCAA catabolic flux which may exacerbate insulin resistance. Indeed, it has been demonstrated that catabolites of valine can leave the muscle cell and further drive fatty acids into the cell in a paracrine fashion (Jang et al. 2016). Thus, a vicious cycle is set in motion which results in further perturbations of BCAA metabolism and increased insulin resistance. Overtime, catabolites of BCAA degradation may also spill over into the circulation, supported by the finding that C_5:1 acylcarnitine concentrations were positively correlated between muscle and plasma in chapter 4. Further time course work is required to investigate these proposed mechanisms. The differences in fatty acid trafficking (i.e. postprandial delivery) may in part explain the reductions in muscle BCAA and circulating short chain acylcarnitines seen in the older aged T2D group in chapter 6.

7.7 Limitations of the current work

One limitation of the analytical method developed in chapter 3 is that it was unable to distinguish between structural isomers of certain species that ultimately surfaced as key sites of interaction between BCAA and lipid metabolism. Therefore, despite high levels of confidence in metabolite concentrations in tissue, the results from muscle should still be interpreted with caution. For example, the intramuscular concentrations of the species C_4-OH can be derived from both ketone bodies and valine catabolism and the species C_4-DC can be produced from both methylmalonyl-CoA and succinyl-CoA, which can be generated in valine and isoleucine catabolism, respectively.

Furthermore, the distinction of C_4 acylcarnitine in muscle was not possible, as this species can be derived from lipid and valine metabolism. Future work should aim to refine this method further to enable chromatographic separation. Nevertheless, the current analytical method was able to extract and quantify a wide range of metabolites with one simple extraction protocol from multiple biological matrices. It is likely that modifications in the extraction protocol, which may include derivatisation of acylcarnitines could improve chromatographic performance but compromise the range of analyte coverage.

Another limitation of this work is that all comparisons in the experimental chapters are cross sectional in nature and therefore it is difficult to assess how the significant elevations in metabolites observed throughout the experimental chapters contribute to, or simply reflect metabolic changes associated with, age and insulin resistance. Nevertheless, prior to the work in chapter 3, a simple yet accurate and quantitative method to measure these metabolites in human samples was lacking. This analytical method can now be used in interventional studies to assess how these metabolites may change over time.

The finding that short chain acylcarnitines increased in muscle of T2D patients in chapter 4 (with 10 subjects) but not in chapter 6 (with 7 subjects) could point to a lack of statistical power to detect differences in muscle content. Indeed, when assessing the absolute concentrations, acylcarnitines are several fold and sometimes an order of magnitude lower in abundance in muscle compared to BCAAs which were observed to be different in both chapters. Interestingly, differences in short chain acylcarnitines were observed in plasma regardless of subject number. This could be explained by the larger heterogeneity in tissue samples compared to plasma. Future work should aim

to address this by using larger cohorts. The work in this thesis is the first to quantify these metabolites simultaneously in human skeletal muscle and plasma and can be used for power calculations for future studies.

7.8 Future directions

The metabolite data presented throughout this thesis represent only ‘snapshots’ of dynamic processes. However, the findings from the present thesis do extend upon much of previous work that has only measured BCAAs and acylcarnitines in the fasted state and mostly in plasma. Future work should investigate tissue level flux of these metabolites to ascertain how impairments in tissue metabolism are related to their plasma profile. The finding that muscle and plasma C_5:1 was strongly correlated between both pools, and this species was also significantly corelated with fasting blood glucose, provides novel evidence of a potential cross talk between muscle and the circulating pool. This is in contrast to medium and long chain acylcarnitines derived from fatty acid metabolism which do not appear to accurately reflect different states of insulin sensitivity.

In chapter 6, meal derived fatty acid oxidation was elevated when assessed using stable isotope tracers, but this was not reflected in differences of any of the measured acylcarnitines. It is largely unknown how fatty acid flux relates to acylcarnitine flux, but it is likely that the former is perhaps orders of magnitude greater than the later. The analytical method developed in chapter 3 has limits of detection and quantification well below the biological range of BCAA and acylcarnitine abundance. Therefore, the current method can be applied to samples to not only measure concentration but also tracer

enrichments of acylcarnitines. This would provide novel insights into human fatty acid flux under diverse metabolic circumstances. The method is at present being developed further to measure tracer enrichment in muscle samples.

With the analytical method developed in the current thesis, it was not possible to distinguish subcellular localisation of metabolites. This may be of relevance as acylcarnitines are produced in the mitochondria and then exported into the cytoplasm and finally can be exported into the circulation. Given that acylcarnitnes have been shown to induce insulin resistance *in vitro* but not *in vivo* suggests that factors beyond simply their abundance may underlie their propensity to cause insulin resistance. For example, the accumulation of other lipid metabolites such as DAGs and ceramides have been associated with insulin resistance but the findings have been inconsistent across multiple different methodologies and experimental models (Bosma et al. 2012), and even augmenting their total abundance in muscle does not always result in insulin resistance (Timmers et al. 2012). These inconsistencies may be reconciled by determining the subcellular and membrane localisation of these metabolites (Kahn et al. 2021). A new emerging technology, ORBI-SIMS has since been able to combine the high spatial resolution of secondary ion mass spectrometry (sims; under 200 nm for inorganic species and under 2 mm for biomolecules) with the high mass-resolving power of an orbitrap (>240,000 at m/z 200). This allows exogenous and endogenous metabolites to be visualized in 3D with subcellular resolution (Passarelli et al. 2017). Application of such methods to skeletal muscle may provide the answers to the

inconsistent findings across different experimental models of insulin resistance and diabetes when assessing the role of acylcarnitines.

A consistent finding from metabolite profiling in human skeletal muscle was that the acylcarnitine species C_4-DC was elevated in both T2D and overweight/obese individuals. Given that this metabolite has also been identified in plasma samples of T2D patients (Mihalik et al. 2010) may suggest this species could be considered as a novel biomarker of tissue insulin resistance. It is likely that highly sensitive measurements such as magnetic resonance spectroscopy would need to be developed to tracer the fate of BCAA derived metabolites into the mitochondria.

7.9 Conclusions

Largely association-based evidence renewed research interest in the potential role of altered BCAA metabolism in the development of insulin resistance and T2D. However, research into the cellular mechanisms to substantiate this hypothesis is lacking. The work in this thesis investigated muscle BCAA metabolism using quantitative metabolomics in both the fasting and postprandial/insulin stimulated state in ageing, obesity and T2D. Collectively, the results demonstrate that perturbed BCAA metabolism is likely a consequence of altered carbohydrate and fat metabolism that underlies the development of insulin resistance rather than directly contributing to these metabolic changes and not a cause of insulin resistance in humans. Moreover, combined with the use of stable isotope tracer assessment of dietary fatty acid oxidation, these findings provide further human *in vivo* evidence that insulin resistance is a state characterised by increased fatty acid oxidation and BCAA

catabolism, as opposed to incomplete and/or impaired catabolism of these metabolic fuels as suggested by previous rodent studies and human association-based investigations.

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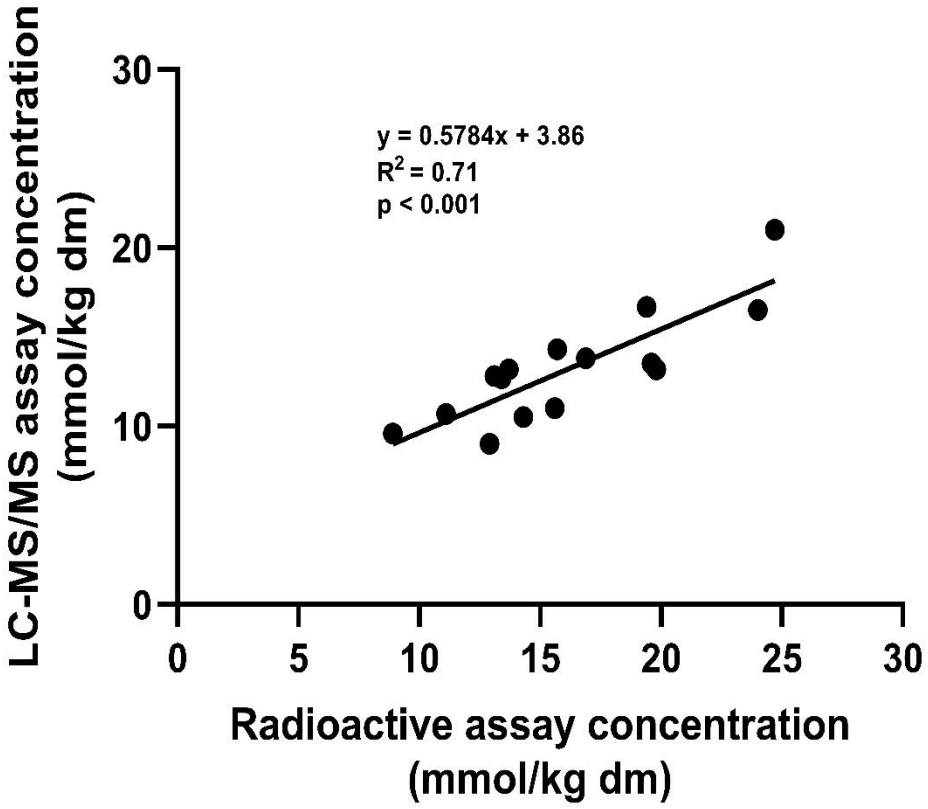
Appendices

Appendix A MS inclusion list

Mass [m/z]	Polarity	Start [min]	End [min]	(N)CE type	Comment
115.0400	Negative	2.5	3.8	NCE	KIV
120.0568	Negative	2.5	3.8	NCE	13CKIV
129.0557	Negative	4.5	6	NCE	KIC/KMV
135.0758	Negative	4.5	6	NCE	13CKIC/KMV
162.1124	Positive			NCE	CARN
171.1689	Positive			NCE	D9-CARN
204.123	Positive	1	4	NCE	C2
207.1418	Positive	1	3	NCE	D-C2
218.1387	Positive	3.4	4.4	NCE	C3
221.1575	Positive	3.4	4.4	NCE	D-C3
230.1387	Positive	4	5	NCE	C4:1
232.1543	Positive	4.4	5.4	NCE	C4
235.1732	Positive	4.4	5.4	NCE	D-C4
244.1538	Positive	5	6	NCE	C5:1
246.1700	Positive	5.3	6.2	NCE	C5
248.1125	Positive	2	7	NCE	C3-DC
248.1488	Positive	3	6	NCE	C4-OH
255.2265	Positive	5.3	6.2	NCE	D-C5
258.1700	Positive	5	6	NCE	C6:1
260.1856	Positive	6.4	7.4	NCE	C6
262.1649	Positive	3.5	4.5	NCE	C5-OH
266.1385	Positive	5	6	NCE	C1-PHE
274.2013	Positive	7	8	NCE	C7
276.1438	Positive	3.5	4.5	NCE	C5-DC
276.1806	Positive	4.5	5.5	NCE	C6-0H
286.2014	Positive	7	8	NCE	C8:1

288.2169	Positive	8	9	NCE	C8
291.2358	Positive	8	9	NCE	D-C8
304.211	Positive	6.5	7.5	NCE	C8-OH
310.2013	Positive	8	9	NCE	C10:3
314.2326	Positive	9	10	NCE	C10:1
316.2482	Positive	9	10	NCE	C10
332.2431	Positive	8	11	NCE	C10-OH
344.2795	Positive	10	11	NCE	C12
368.2795	Positive	10	11	NCE	C14:2
370.2952	Positive	10	11	NCE	C14:1
372.3108	Positive	10.5	11.5	NCE	C14
381.3673	Positive	10.5	11.5	NCE	D-C14
396.3108	Positive	10	11	NCE	C16:2
398.3262	Positive	10.5	11.5	NCE	C16:1
400.3421	Positive	11.4	12.4	NCE	C16
403.361	Positive	11.4	12.4	NCE	D-C16
414.3578	Positive			NCE	C17
416.3376	Positive	10.5	11.5	NCE	C16-0H
422.3260	Positive	10.5	11.5	NCE	C18:3
424.3421	Positive	11	12	NCE	C18:2
426.3578	Positive	11	12.3	NCE	C18:1
428.3734	Positive	12	13	NCE	C18
444.3684	Positive	11	13	NCE	C18:0H
448.3421	Positive	11	12	NCE	C20:4
456.4047	Positive	13.2	14	NCE	C20
342.263	Positive	9	10	NCE	C12:1
388.2628	Positive	9.5	10.5	NCE	C14:3
394.2947	Positive	9.8	11	NCE	C16:3

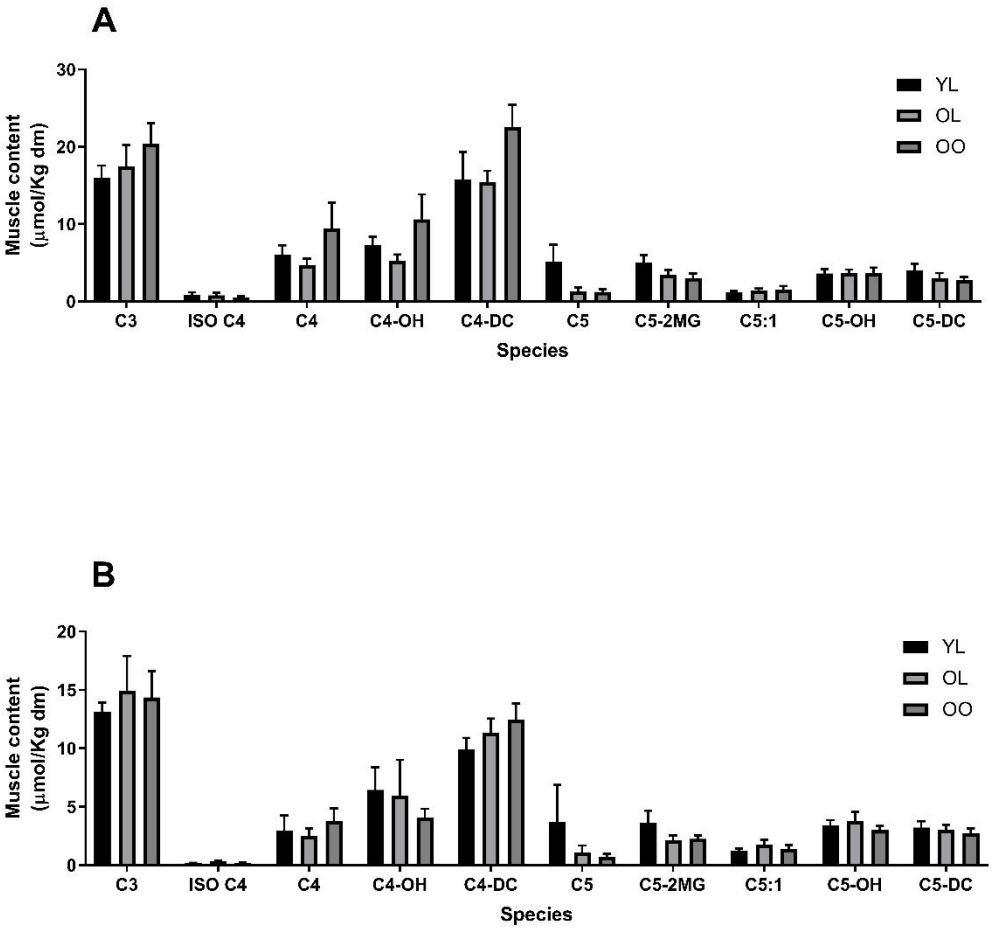
Appendix B Free carnitine concentration



Association between the calculated concentration of free carnitine in human skeletal muscle from the established radioactive assay and the newly developed LC-MS/MS method in chapter 3. Data for concentrations from the radioactive assay were provided by Professor Francis Stephens. (n=15).

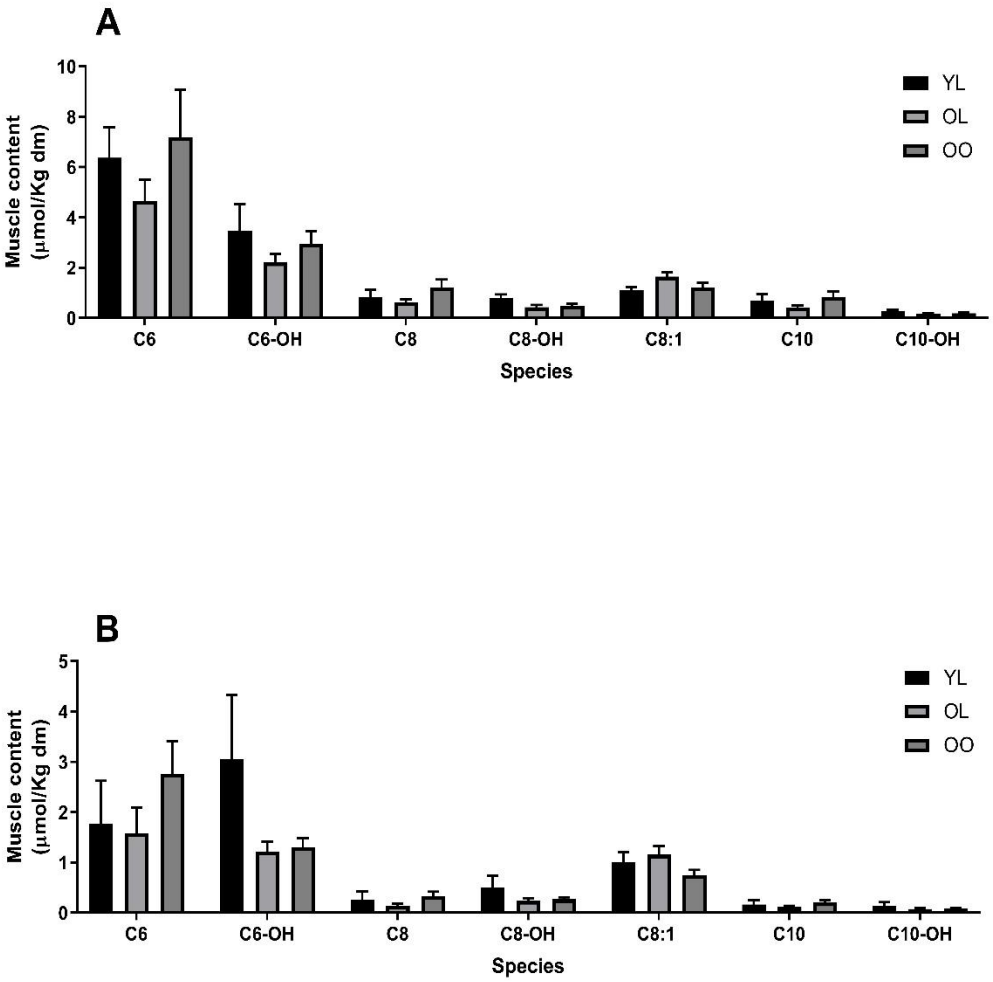
Appendix C Muscle acylcarnitine profiles

Muscle short chain acylcarnitines



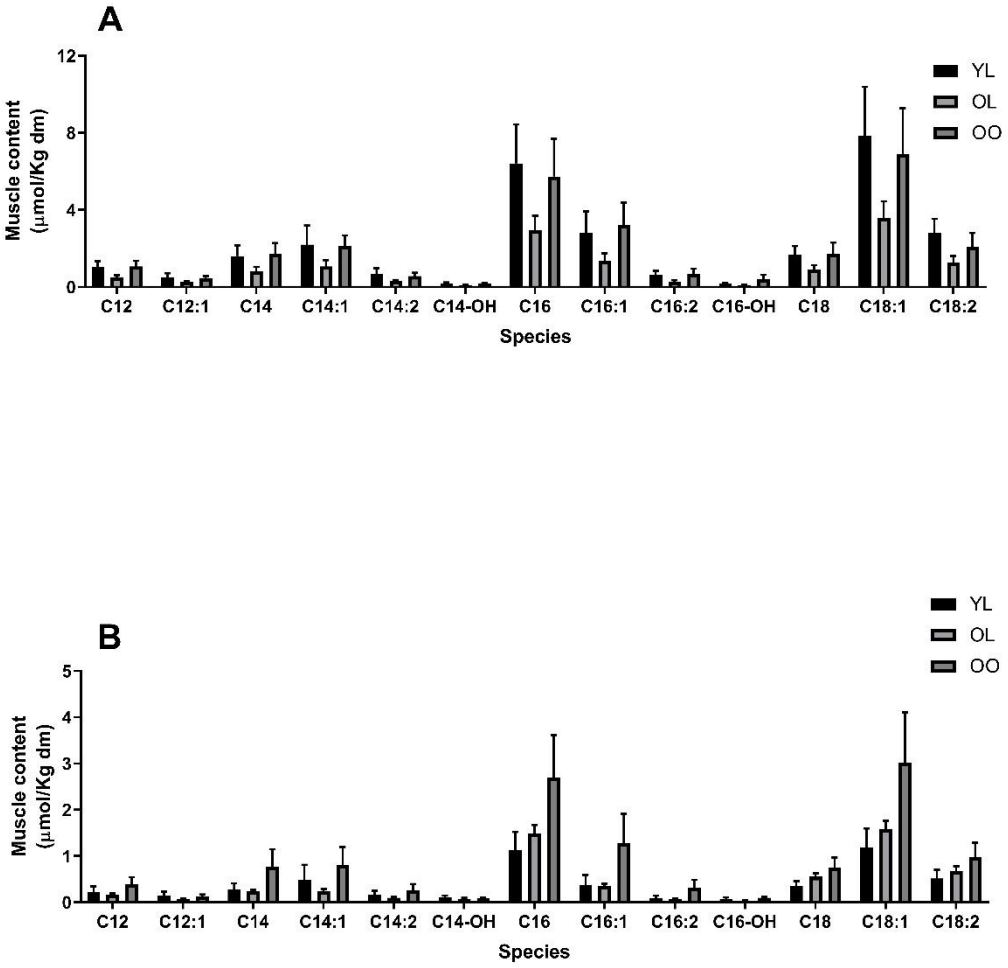
Full profile of skeletal muscle short chain (C3-C5) acylcarnitines during the fasted state (A) and following the 3-hour clamp (B). Data are mean ± SEM. n = 7, YL. n = 9, OL n= 9, OO

Muscle medium chain acylcarnitines



Full profile of skeletal muscle medium chain (C6-C10) acylcarnitines during the fasted state (A) and following the 3-hour clamp (B). Data are mean ± SEM. n = 7, YL. n = 9, OL n= 9, OO

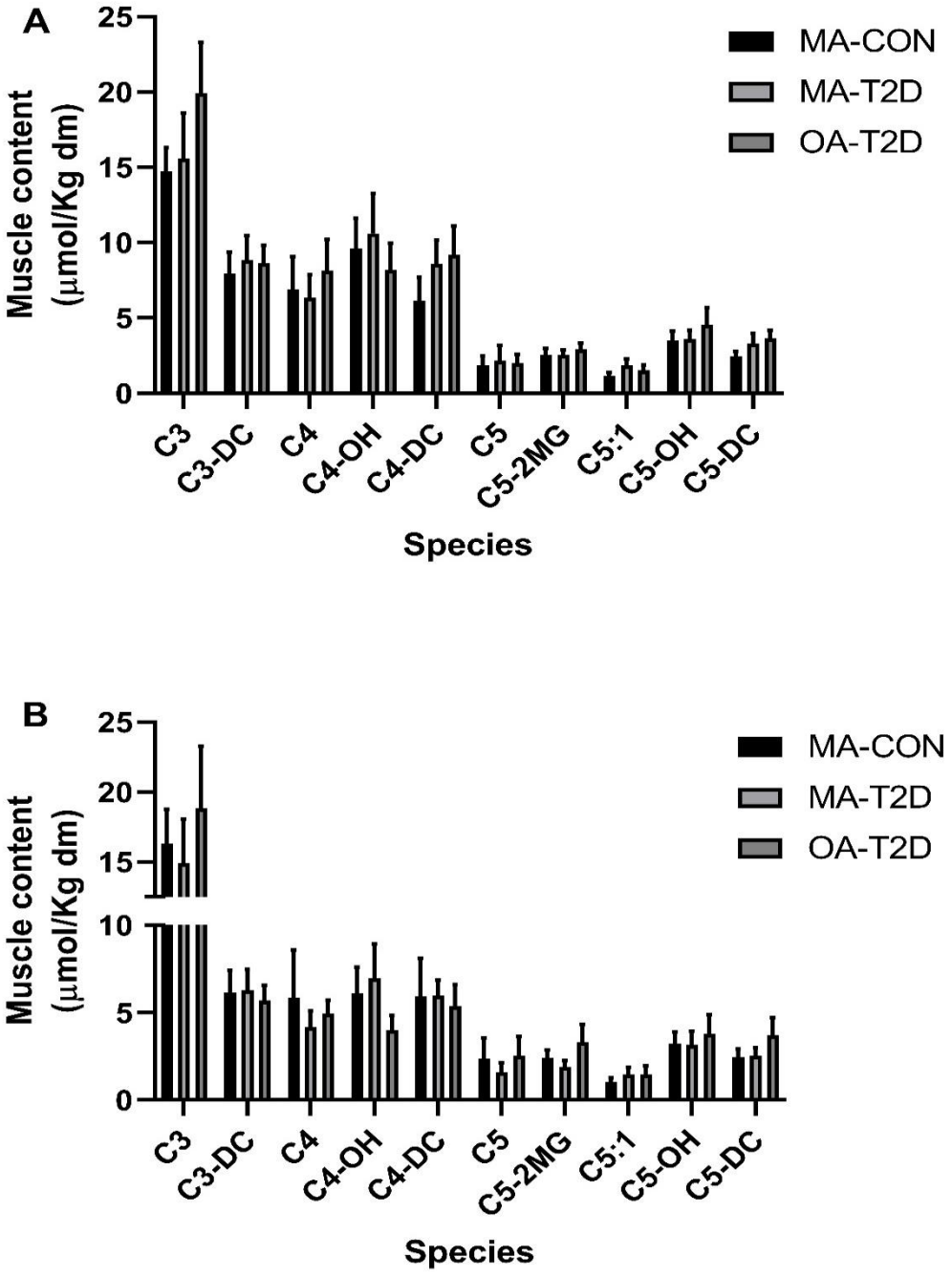
Muscle long chain acylcarnitines



Full profile of skeletal muscle long chain (C12-C18) acylcarnitines during the fasted state (A) and following the 3-hour clamp (B). Data are mean ± SEM. n = 7, YL. n = 9, OL n= 9, OO

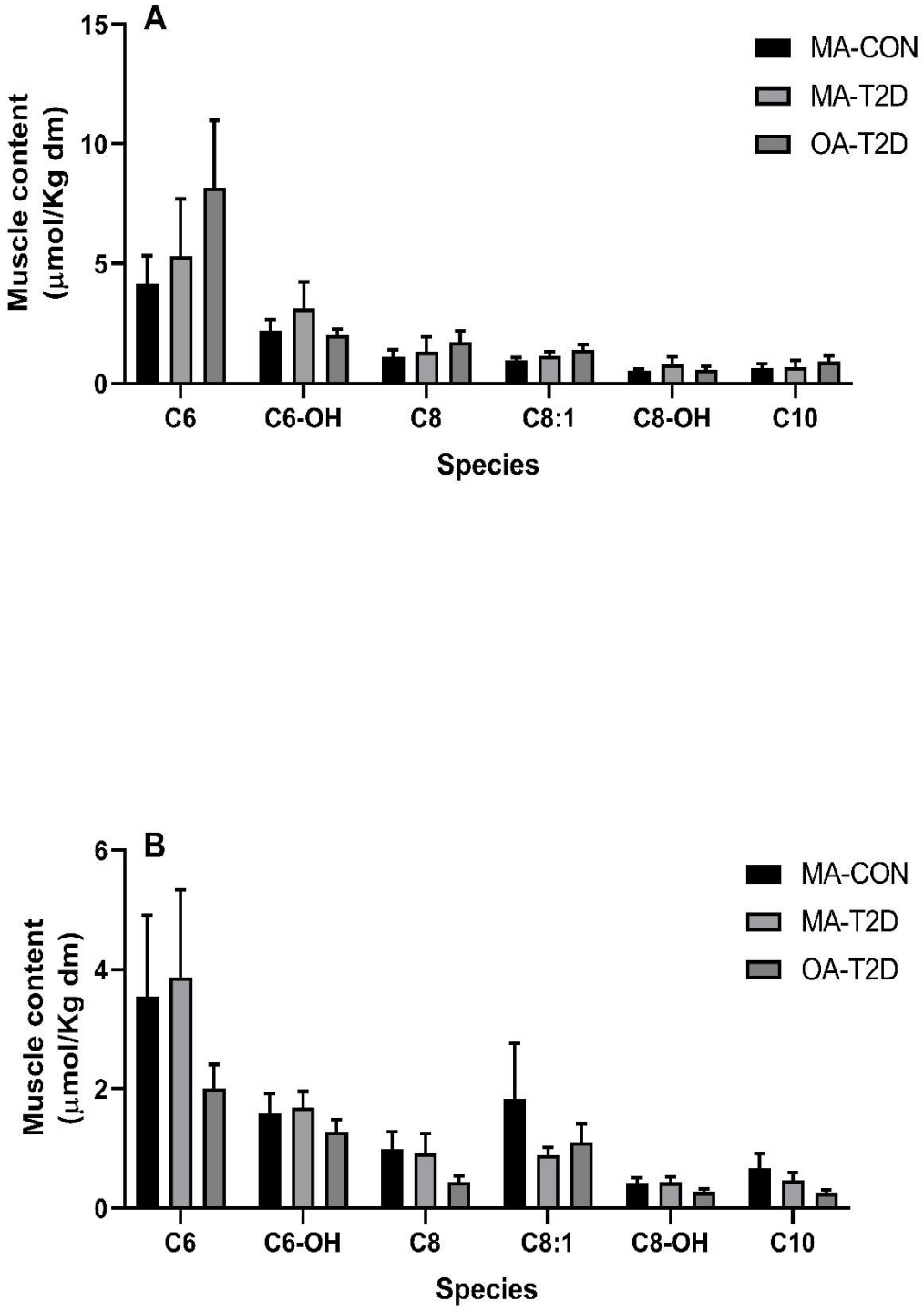
Appendix D Muscle acylcarnitine profiles

Muscle short chain acylcarnitines



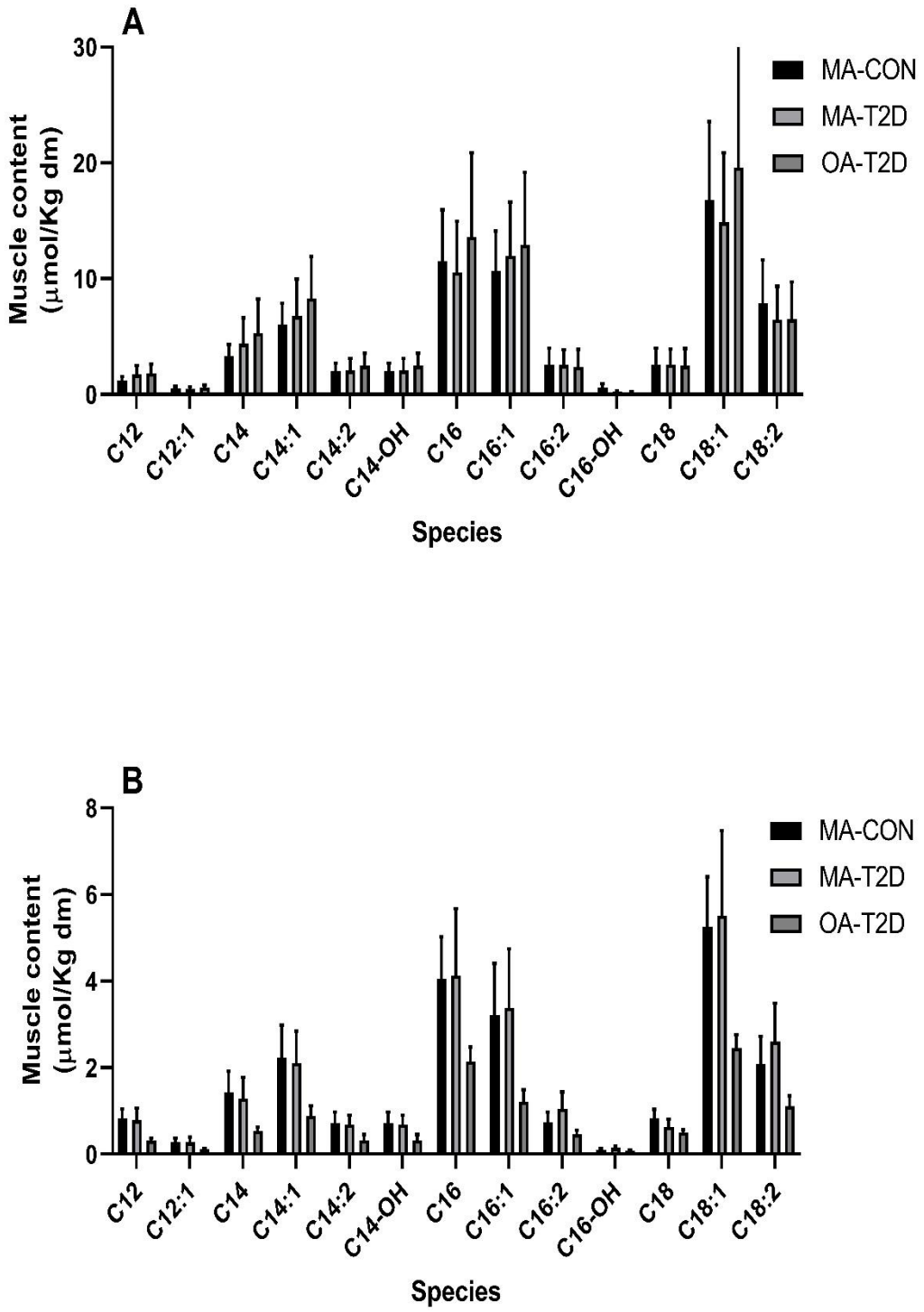
Full profile of skeletal muscle short chain (C3-C5) acylcarnitines during the fasted state (A) and following a 2-hour oral glucose tolerance test (B). Data are mean ± SEM. n = 7

Muscle medium chain acylcarnitines



Full profile of skeletal muscle medium chain (C6-C10) acylcarnitines during the fasted state (A) and following a 2-hour oral glucose tolerance test (B). Data are mean ± SEM. n = 7

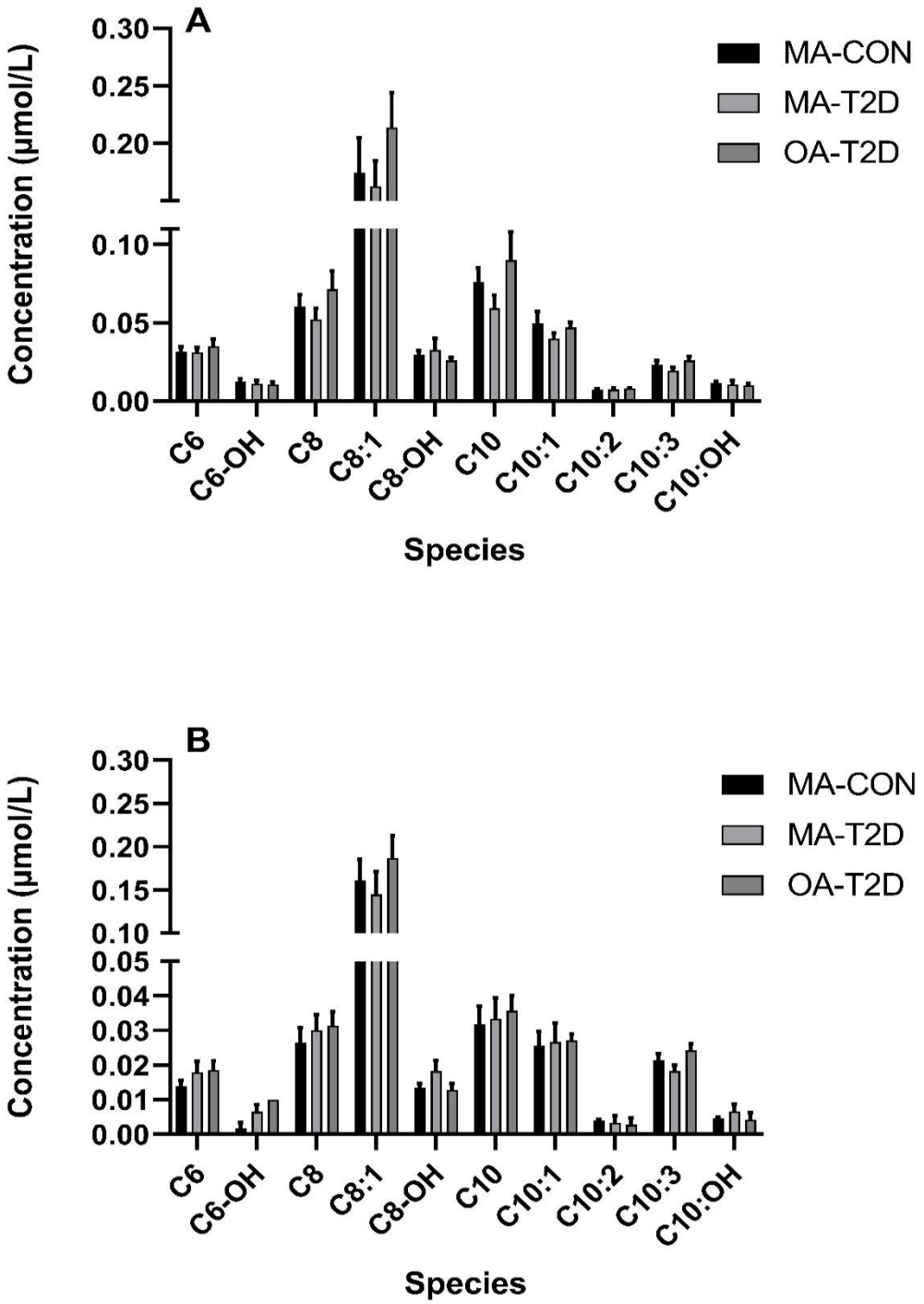
Muscle Long chain acylcarnitines



Full profile of skeletal muscle long chain (C12-C18) acylcarnitines during the fasted state (A) and following a 2-hour oral glucose tolerance test (B). Data are mean ± SEM. n = 7

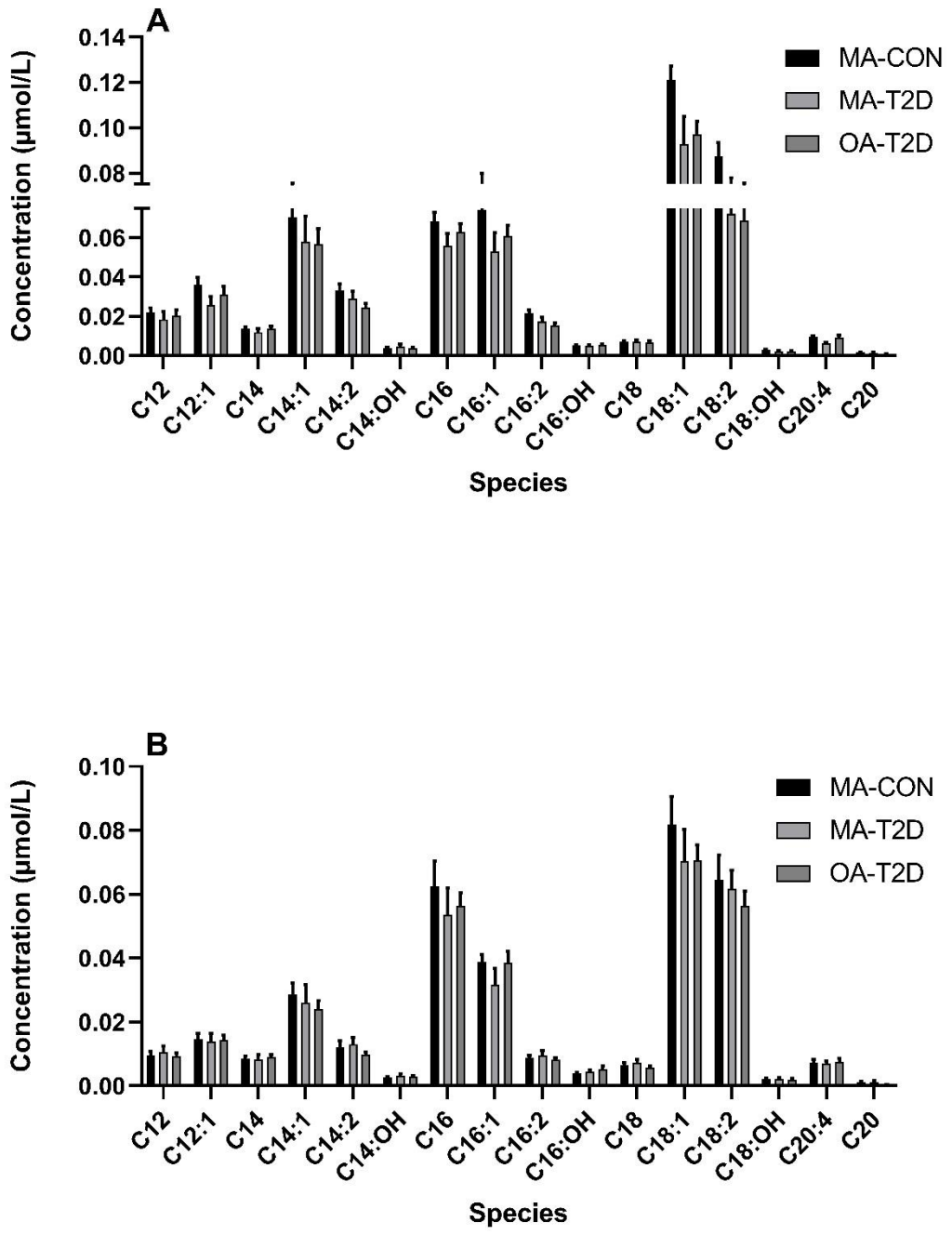
Appendix E Plasma acylcarnitine profiles

Plasma medium chain acylcarnitines



Full profile of plasma medium chain (C6-C10) acylcarnitines during the fasted state (A) and following a 2-hour oral glucose tolerance test (B). Data are mean ± SEM. n = 7

Plasma long chain acylcarnitines



Full profile of plasma long chain (C12-C18) acylcarnitines during the fasted state (A) and following a 2-hour oral glucose tolerance test (B). Data are mean ± SEM. n = 7

Appendix F PIP reflective statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

I completed my PIP at Maastricht University in the Netherlands under the supervision of Professor Luc Van Loon and Dr Jorn Trommelen. I chose to travel to Maastricht University because of its reputation and expertise in utilising stable isotope tracers to investigate human metabolism. I wished to gain insight into how such studies were designed and conducted. In addition, I was able to expand upon my analytical skillset by spending time in stable isotope research centre (SIRC) and learned many new laboratory techniques.

My primary responsibilities involved the set up and running of two human studies which utilised stable isotope tracers. The first investigated how different types of protein (whey and casein) consumed prior to sleep impacted upon muscle protein synthesis during recovery from endurance type exercise. This study involved exercising participants in the evening and collecting blood samples throughout the night. This study had obvious

challenges (not least staying awake) but most importantly I gained a thorough appreciation of costs involved in conducting studies using stable isotopes and the diligence required at every step so that any risk of wasting expensive tracer is mitigated. The second study involved investigating the effect of consuming different amounts (moderate or large) of proteins and measuring muscle protein synthesis over a 12-hour recovery period following resistance exercise. This was my first experience of conducting resistance training in a research setting and I learned how to accurately assess maximal strength.

I also spent time in the SIRC and was introduced to the technique of gas chromatography linked isotope ratio mass spectrometry (GC-IRMS). This was particularly exciting as I had only read about this technique and did not have any working appreciation of how to operate the instrument. I got to speak at length with mass spec technicians and provided my own input into how to overcome a number of analytical challenges often faced when using mass spec. I also spent time in lab learning the protein extraction protocol used to prepare samples for GC-IRMS analysis.

Upon completion of the studies, my supervisor gave me thorough explanation of how the numerous calculations are performed from the stable isotope infusions studies we ran, and I was able to understand how numerical data generated from lab analysis in the SIRC is used to compute muscle and whole-body synthetic, breakdown and oxidation rates. This was my primary objective, and I was delighted to achieve it.

Indirectly, I also learned a lot from other PhD students in the department who I shadowed during lab days. For example, I did not have any previous

experience of using deuterated water to assess energy expenditure and protein synthesis. Although I had read literature about this technique, I had no real understanding of how it was applied and how the analysis is performed. Luckily, I shared an office with 3 incredibly bright students who explained the basis of this technique and calculations.

Though I had not anticipated it, I also had the opportunity to supervise a number of under and post graduate students through their final year projects. I thoroughly enjoyed this experience and learned a lot as a result.

Another unanticipated skill I developed during my time working with Jorn was how to communicate science succinctly to lay audiences. Jorn has a large social media (something I generally avoid) presence and has understood how to exploit it to its full potential to disseminate his research to the public. In the Netherlands, this is becoming a crucial question that researchers grant providers ask, what is the wider impact this research will have on the public? Admittingly, I had no appreciation of this before working with Jorn, but I realised that the way in which the lay public interact and understand the type of science I am interested in, is drastically different to how I do.

From a personal perspective I learned a lot from living in a new country for 3 months. I met a vast range of people and was able to put my conversational German skills to good use, whilst trying my best to pick up some Dutch. Most importantly, it gave me a new perspective on research and how other groups in this area of research operate.