An Investigation into the Importance of Intramuscular Triglyceride Turnover in Skeletal Muscle Metabolism

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy

August 2022

General Abstract

High intramuscular triglyceride (IMTG) stores in sedentary, obese and elderly individuals have been linked to insulin resistance, yet elite athletes often have higher amounts of IMTG whilst maintaining a high muscle and whole-body insulin sensitivity. It is now known that a high IMTG content per se does not result in insulin resistance, but is caused, at least in part, by the presence of high muscle concentrations of harmful lipid metabolites, such as diacylglycerols (DAGs) and ceramides. The latter are elevated in obesity and type 2 diabetes (T2D) (Goodpaster *et al.,* 1997; Pan et al., 1997; Forouhi *et al.,* 1999) in comparison to healthy lean controls. Individuals with T2D demonstrate reduced IMTG turnover rates compared to those that are insulin sensitive and/or well trained. This thesis focuses first on the adaptations that affect IMTG turnover and explores methods in which IMTG utilisation can be targeted in obese individuals to reduce the risk of developing T2D, specifically focussing on the ingestion of an antilipolytic drug combined with exercise, as well as fasted exercise training. Chapter 4 determined that following exposure to high plasma free fatty acids (FFA), GLUT4 translocation to the plasma membrane is unaltered in both trained and sedentary individuals. Interestingly, we report that in both trained and sedentary individuals GLUT4 expression is higher in both type 1 and type 2 fibres in trained individuals, who have more small GLUT4 spots making up ~97% of total GLUT4 spots. Chapter 5 demonstrated that exercising with reduced plasma FFA via lipolytic inhibition leads to reduced ceramide 20:0 accumulation and an increase in several lactosylceramide species. Chapter 6 demonstrated that targeting IMTG turnover in a 12-week home-based walking programme by exercising in the fasted state (known to lead to greater IMTG utilisation) compared to the fed state made little difference to the effectiveness of the intervention to improve glucose tolerance and reduce HbA1c (~7% FAST, ~2% FED). However, exercising in the fasted state during the programme also reduced alanine transferase concentrations compared to fed state exercise (~16% FAST, ~2% FED). In conclusion, this thesis presents novel data on the differences in insulin signalling in both trained and sedentary individuals. Furthermore, data is presented on the health benefits resulting from interventions in obese individuals that target the use of IMTG stores during exercise, and therefore, result in greater turnover of IMTG stores.

Acknowledgements

There are many people I wish to thank for their support and involvement in the completion of this PhD thesis.

Firstly, I would like to thank every member of Team Anton. I have been very fortunate to work within such a supportive and knowledgeable research group. To Dr Sam Shepherd and Dr Juliette Strauss, I cannot thank you enough for your continued encouragement and support during my research, and the opportunities you have provided me. I look forward to continuing to work together in the future. To Prof James Morton, thank you for your trust in me and providing me with an incredible early career's opportunity.

Importantly, I would like to thank the collaborators of this work including Prof Dan Cuthbertson and Dr Emily Brown for your valuable knowledge. I would also like to thank Marcus Henricsson and Per-Olof Bergh for your involvement in our Lipidomics data reported in chapter 5.

To the support staff in the LJMU sport science department, and in particular, Dean and Gemma, thank you for tolerating endless equipment requests and problems to solve. These projects would not have been completed without you both. A big thank you also to Louise, Zoe, and Rachel for their organizational support throughout my research projects.

To all my fellow researchers that I have crossed paths within the PGR and post-doc office's including Sam, Harvey, Connor, Hannah M, Andy, Harry, Chloe, Rob, Miffy, Hannah C, Katie H, Katie W, Lucy, Mark, Jamie and many more… thank you for plenty of laughs, coffee breaks and a lot of procrastination.

For my friends, to Sam and Heather (and Rudy), thank you for being my family here in Liverpool. Thank you to Fran, Evie, Juliet and Emily for your endless patients and support over these past 4 years. And to Hannah and Eve, I would never have been able to finish this without your constant encouragement. To Harvey, thank you for adding happiness and laughter into every day.

Most importantly, thank you to my mum, dad, and Thomas for everything you have done for me. Your unwavering encouragement and support have been fundamental to my achievements. I hope I can continue to make you proud.

Conference Communications and Publications

During the period of postgraduate study at Liverpool John Moores University, data from the current thesis resulted in the following conference communications:

Physiological society Future Physiology meeting, Liverpool, UK, December 2019 – Poster communication - Can the health benefits of a walking-based exercise programme be enhanced by co-ingestion of a lipid lowering drug?

American Diabetes Association 82nd Scientific Session, New Orleans, USA, June 2022 – Poster Communication and Oral Theatre Communication - A free-living walking-based exercise programme improves HbA1c in individuals with obesity, with additional benefits of nutritional status on metabolic health.

During the period of postgraduate study at Liverpool John Moores University, data from the current thesis resulted in the following publications:

J.S. Barrett, K.L. Whytock, J.A. Strauss, A.J.M. Wagenmakers, and S.O. Shepherd. High intramuscular triglyceride turnover rates and the link to insulin sensitivity: influence of obesity, type 2 diabetes and physical activity. Applied Physiology, Nutrition, and Metabolism. 47(4): 343-356. https://doi.org/10.1139/apnm-2021-0631

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Chapter 1: General Introduction

1.1 The nutritional needs of homo sapiens

Through a multimillion-year evolutionary process came the development of the nutritional needs of humans today, where genetic change reflected the life circumstances of our ancestral species (Eaton, Konner and Shostak, 1988). The human genetic make-up has changed very little since the appearance of 'modern human beings', or *homo sapiens*, around 40,000 years ago, and significant milestones in history such as the industrial revolution, agribusiness and modern food processing techniques have occurred too recently to have had any evolutionary effect (Eaton, Konner and Shostak, 1988). As such, the foods available to pre-agricultural humans remains the nutritional needs for humans today in the 21st century and could be considered a possible paradigm or standard for human nutrition (O'Dea, 1983; Eaton, Konner and Shostak, 1988; Burkitt and Eaton, 1989). The early environmental pressures of low food abundance have resulted in humans becoming efficient at storing and utilising fuel sources. In particular, humans have the capacity to utilise both carbohydrates (glycogen) and fats (triglycerides), and switch between these fuel sources depending on availability and energy demand (Chakravarthy and Booth, 2004).

In the 21st century, humans in westernised civilisation are challenged with the accessibility of readily available, poor quality and energy dense food sources. These options, coupled with ever increasing sedentary living has resulted in disastrous consequences to vital metabolic processes, and ultimately lead to disease. Currently, only 38% of the UK population meet the recommended 150 minutes of weekly moderate-intensity activity or 75 minutes of vigorous exercise (British Heart Foundation, 2017), and around 67% of men and 60% of women are overweight or obese (Statistics on obesity, 2020). Although sedentary behaviour is not the sole cause of obesity, exercise contributes significantly to improved cardio metabolic health markers. Energy intake should be matched to energy expenditure to maintain health. However, when energy intake outweighs energy expenditure individuals are in a positive energy balance, which subsequently leads to an increase in body mass if maintained over a period of time.

Living with a westernised lifestyle yet having the nutritional genotypic boundaries of ~40,000 years ago has led to an increase in the prevalence of obesity and associated metabolic

disorders such as heart disease and type 2 diabetes (T2D). In 2017, approximately 462 million individuals were affected by T2D corresponding to 6.3% of the world's population (Khan et al., 2020). In the UK alone, it is estimated that 3.9 million individuals are living with diabetes with 13.6 million people estimated to be at increased risk of developing T2D (DiabetesUK, 2019). This comes at an estimated cost of £6.1 billion to the NHS and is estimated to rise to £9.7 billion by 2050. Over 1 million deaths per year can be attributed to diabetes alone, making it the ninth leading cause of mortality.

T2D is characterised by insulin resistance and a loss of glucose homeostasis, which is linked to impairments in fat storage and metabolism. In order to understand the metabolic impairments associated with T2D, it is pertinent to first provide an overview of the regulation of glucose homeostasis in healthy individuals. After outlining the mechanisms regulating glucose homeostasis, this chapter will then introduce the metabolic disturbances that contribute to the development of insulin resistance in obesity and T2D. Importantly, how changes in insulin sensitivity and glucose control can be measured and monitored will also be considered.

1.2 The development of homeostatic glucose regulation

Carbohydrates are ingested as part of a mixed meal and are broken down into their most basic components during the digestive process. Salivary amylase breaks complex carbohydrates down into polysaccharides, which the stomach degrades further into disaccharides and in the intestine, these are fractionated into monosaccharides and absorbed into the circulation as glucose. This post-prandial rise in plasma glucose concentrations triggers the secretion of insulin from the pancreas into the circulation. Circulatory insulin binds to the insulin receptor on the plasma membrane of skeletal muscle, liver and adipose tissue and promotes glucose clearance from the circulation into these tissues. The post-prandial glucose uptake into these three tissues ensures that plasma glucose concentrations are maintained at approximately 5 mmol.L and avoids a situation where chronic hyperglycaemia may develop. Upon entering skeletal muscle, liver or adipose tissue, glucose can either be used for energy through the glycolytic processes or be directed towards storage

as glycogen in skeletal muscle and liver. In adipose tissue glucose is converted to palmitate through *de novo* lipogenesis or used as the glycerol backbone for triacylglycerol (TAG). Skeletal muscle is the major site of glucose clearance from the circulation and is responsible for ~80% of glucose clearance during a hyperinsulinemic euglycemic clamp (DeFronzo et al., 1981; Thiebaud et al., 1982). Under more physiological conditions such as an overnight fast followed by a mixed meal, skeletal muscle uptake remains high and is responsible for ~50% of glucose disposal (splanchnic glucose clearance accounts for the other ~50%) (Capaldo et al., 1999). Insulin binds to the insulin receptor on the plasma membrane of skeletal muscle and stimulates glucose uptake, and therefore the action of insulin directly contributes to the maintenance of glucose homeostasis. Importantly, insulin also stimulates recruitment of the microvascular system to promote greater delivery of insulin and other substrates to skeletal muscle (reviewed in detail by (Muniyappa et al., 2007; Clark, 2008; Barrett et al., 2009; Barrett et al., 2011). It is important to acknowledge the importance of insulin action on the microvascular system and its contribution to glucose homeostasis, however, this thesis will predominantly focus on intramuscular metabolic factors determining insulin action on skeletal muscle fibres and glycemic control.

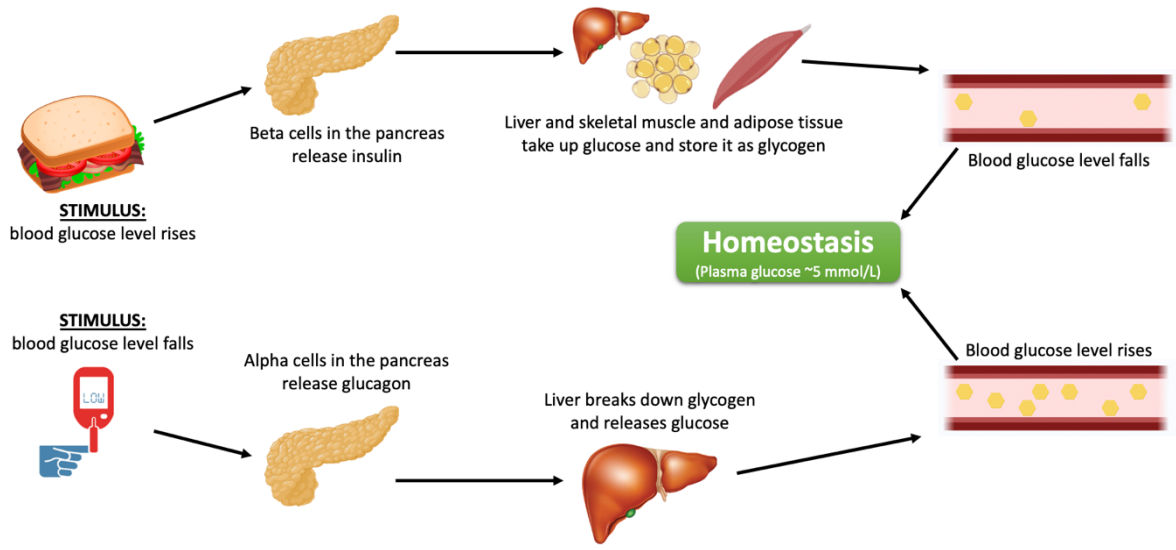


Figure 1.1. **Glucose Homeostasis**. Maintenance of blood glucose levels by glucagon and insulin. When plasma glucose levels are low, the pancreas secretes glucagon which increases endogenous blood glucose levels through glycogenolysis. After a meal, when plasma blood glucose levels are high, insulin is released to stimulate glucose uptake into the liver, skeletal muscle and adipose tissues as well as to promote glycogenesis.

1.2.1 Insulin-mediated glucose uptake

Insulin-dependent glucose uptake into skeletal muscle occurs first via glucose transporter-1 (GLUT-1) meditated transendothelial transport of glucose from the lumen of capillaries into the interstitial fluid which surround the skeletal muscle fibres. This process occurs primarily by facilitated diffusion in which glucose transporter proteins work together playing a fundamental role and requires a descending concentration gradient from the capillary lumen to the interstitial fluid (Wagenmakers et al., 2016). Collectively, 14 glucose transporter isoforms have been identified (Uldry and Thorens, 2004), with glucose transporter 4 (GLUT-4) being the predominant insulin sensitive isoform and is required for glucose uptake into skeletal muscle (Watson and Pessin, 2001). In the fasted state, GLUT4 resides within intracellular cytosolic microvesicles, but in the fed state when plasma insulin concentrations are elevated, these GLUT4 vesicles translocate to the plasma membrane where GLUT4 is deposited and ultimately facilitates the uptake of glucose into skeletal muscle. The mechanism by which insulin promotes GLUT4 translocation to the plasma membrane have been studied in depth, and it is evident that the redistribution of GLUT4 is dependent on multiple signalling events. In healthy individuals, insulin-stimulated glucose uptake into skeletal muscle transpires through the activation of the insulin signalling cascade. The end of this signalling event results in the translocation of glucose transporter-4 (GLUT-4) from intracellular storage vesicles in muscle fibres to the plasma membrane (Watson, Kanzaki and Pessin, 2004; Bradley et al., 2015).

1.2.2 IRS-PI3-K insulin signalling pathway

The insulin signalling cascade is initiated upon the binding of insulin to the extracellular α-subunit of the insulin receptor (IR). This results in a structural re-arrangement of the IR and the auto-phosphorylation of the transmembrane β-subunit. Phosphorylation of the IR stimulates recruitment of the insulin receptor substrate proteins one to four (IRS1 – 4), activating the IRS-PI3-K pathway. The specific phosphorylation of Tyr612 and Tyr632 sites on IRS-1 are considered vital for downstream activation of PI3-K and GLUT4 translocation (Esposito et al., 2001). Upon phosphorylation, key sites on IRS-1 are revealed for PI3-K to bind to (Myers et al., 1992; White and Kahn, 1994), activating PI3-K. As a result, the active PI3-K goes on to phosphorylate PIP2 to PIP3. Downstream of PI3K, there is at least 2 parallel

signalling pathways required for GLUT4 translocation, both of which end with GTP-loading of small GTPases (Ishikura, Koshkina and Klip, 2008). This first involves the phosphorylation and activation of Akt via phosphoinositide-dependant kinase (PDK1), which are both recruited to the PIP3-containing membranes. PDK1 phosphorylates Akt (Alessi et al., 1996), triggering the phosphorylation, and therefore activation, of the downstream target Akt substrate of 160 kDa (AS160/TBC1D4) and inactivation of glycogen synthase kinase-3 (GSK-3) via phosphorylation. Phosphorylation of GSK-3 inhibits its activity of inhibiting glycogen synthase to promote glycogen synthesis. GLUT4 storage vesicles are loaded with Rab proteins, which under basal conditions are rendered inactive due to being GDP-bound, preventing GLUT4 translocation. When TBC1D4 is stimulated via activation of the insulin signalling pathway, Rab proteins are GTP-loaded, reorganising the cytoskeleton, allowing GLUT4 translocation to occur.

The second arm of signalling downstream of PIP3 involves the activation of Rho family GTPase Rac1, where insulin stimulates the GTP-loading of Rac1. Once activated, Rac1 mediates the dynamic process of cortical actin reorganisation beneath the muscle cell membrane via Arp2/3 for actin polymerisation and cofilin for depolymerisation. The cortical F-actin network facilitates insulin-stimulated GLUT4 translocation likely by recruiting signalling molecules, guiding vesicle movements, and tethering GLUT4 vesicles (Symons and Rusk, 2003; Raftopoulou and Hall, 2004).

1.2.3 APS/CAP/Cbl complex pathway

Several studies have shown that pharmacological inhibition of PI3-K does not completely abolish glucose uptake, and equally, pharmacological activation of PI3-K does not necessarily promote insulin-mediated glucose uptake (see review by (Kanzaki, 2006). Evidently, there is a separate pathway to stimulate GLUT4 translocation, known as the APS/CAP/Cbl pathway, which originates from lipid raft microdomains. Lipid raft microdomains reside in the PM and are enriched in cholesterol. Caveolin and cholesterol within these microdomains bind to form caveolae; small cavities in the PM that are enriched with signalling proteins, including insulin receptors (Gustavsson et al., 1999; Parpal et al., 2001). Activation of these insulin receptors located in plasma membrane subdomains stimulate tyrosine phosphorylation of the proto-oncogenes c-Cbl and Cbl-b via recruitment of Cbl to adaptor protein APS which has SH2 and

PH domains (Baumann et al., 2000; Kimura et al., 2002; Liu et al., 2002). The SH2 domain of APS binds to the ß-subunit of the IR, meaning that one IR recruits two APS molecules (Hu et al., 2003). The binding of APS results in its phosphorylation at the C-terminal tyrosine, stimulating recruitment of Cbl via SH2 domain, causing phosphorylation on 3 tyrosine's of Cbl (Liu et al., 2002).

Cbl associated protein (CAP); a bifunctional adaptor protein, binds to the insulin: APS complex along with Cbl. Protein CAP is predominantly found in insulin-sensitive tissues and its expression increases following activation of PPARy. CAP is a bifunctional adaptor protein with 3 SH3 domains in its COOH-terminus, and an NH2-terminal region known as the sorbin homology (SoHo) domain (Kimura et al., 2001). This SH3 domain in the c-terminus of CAP binds to the proline rich domain of Cbl, whereas the Sorbin homology (SoHo) domain in the N-terminus of CAP binds to flotillin which incorporates CAP/Cbl complex into the lipid raft.

1.2.4 Contraction-induced glucose uptake

Glucose uptake into skeletal muscle is also increased during exercise by an insulin-independent mechanism. In a similar manner to the physiological effects of insulin, muscle contraction stimulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane and t-tubules to enable glucose uptake into skeletal muscle. However, incubation of rat soleus muscle with wortmannin, a PI3-K inhibitor, has no impact on contraction-mediated GLUT4 translocation (Lund et al., 1995), demonstrating that contraction-induced GLUT4 translocation is independent of the PI3-K signalling pathway. Moreover, using electrical stimulation to induce muscle contraction of isolated rodent skeletal muscle fibres has no effect on IRS-1, PI3-K or Akt/PKB activity (Goodyear et al., 1995; Wojtaszewski, 1996).

Muscle contraction causes an increase in the AMP to ATP ratio which leads to activation of AMP-activated protein kinase (AMPK) (Wojtaszewski et al., 2000b). Importantly, GLUT4 translocation to the plasma membrane and subsequent glucose uptake is enhanced by AMPK activation by 5- aminoimidazole-4-carboxamide-1- -D-ribonucleoside (AICAR; an AMP analogue) (Merrill et al., 1997; Kurth-Kraczek et al., 1999). AICAR-stimulated glucose uptake is not affected by incubation with wortmannin (Hayashi et al., 1998), indicating that AMPK

activation, like muscle contraction, leads to GLUT4 translocation independent of the PI3-K signalling cascade. It is important to note that knockout of AMPK does not completely blunt contraction induced GLUT4 translocation, with 60% of GLUT4 translocation maintained, suggesting that other intracellular factors besides AMPK influence glucose uptake. Interestingly, blocking calmodulin-dependant protein kinase (CAMK) activation reduces glucose uptake (Wright et al., 2004; Wright et al., 2005). When calcium ions (Ca^{2+}) are released from the sarcoplasmic reticulum during muscle contraction, they form a complex with calmodulin (CaM) that subsequently binds to and activates Ca2+/CaM-activated kinase (CAMK). When Ca^{2+} concentrations are increased in response to muscle contraction, there is increased PKC activation which may have an important role in contraction induced GLUT4 translocation. This is because pharmacological inhibition of PKC with calphostin C reduces contraction-induced glucose uptake (Ihlemann, Galbo and Ploug, 1999). However, calphostin C is not specific to certain isoforms of PKC and therefore makes it difficult to identify the PKC isoforms that play a role in GLUT4 translocation. Studies after this demonstrated that electrical stimulation of skeletal muscle in rodents increased PKC activity and concurrently increased conventional and novel PKC isoforms (Richter et al., 2003). Yet, none of these isoforms were activated during muscle contraction in human skeletal muscle (Rose et al., 2004). As a result, it is thought that the increased PKC activity due to exercise is due to activation of atypical PKC isoforms (Perrini et al., 2004; Rose et al., 2004), yet this may not be crucial for contraction-mediated glucose uptake (Sajan et al., 2010). It has been suggested that increased aPKC activation during exercise may potentially enhance insulin action (Maarbjerg, Sylow and Richter, 2011). Notably, numerous MAPK isoforms, P38 and c-Jun N-terminal kinase are also activated in response to muscle contraction (Sakamoto and Goodyear, 2002). The increase in aPKC following exercise is actually paralleled by increased ERK activity (Nielsen et al., 2003), but blocking ERK does not completely inhibit contraction induced glucose uptake (Wojtaszewski et al., 1999). The phosphorylation of p38 MAPK is increased in response to exercise alongside glucose uptake, and p38 MAPK inhibition blocks this increase in glucose uptake(Chambers et al., 2009). However, the inhibitor used for p38 has more recently been shown to directly interact with GLUT4, and so using this inhibitor cannot provide clarity on the role of p38 in contraction-mediated glucose uptake (Jensen and Richter, 2012).

1.2.5 GLUT-4 Translocation

The translocation of GLUT4 to the plasma membrane occurs in multiple stages. When insulin is absent, GLUT4 slowly cycles between the PM and the intracellular storage vesicles (Foster et al., 2001). Cell culture research suggests that there is approximately 2-5% of GLUT4 located at the plasma membrane in basal conditions (Kanzaki, 2006). When insulin is present, a large proportion of the cellular GLUT4 pool is stimulated via the insulin signalling cascade and is redirected to the plasma membrane (Birnbaum, 1989; James, Strube and Mueckler, 1989) through targeted exocytosis from storage vesicles (Bryant, Govers and James, 2002). This process of GLUT4 making its journey to the plasma membrane occurs by two methods; polymerized microtubules or actin cytoskeleton pathways (Fletcher et al., 2000; Lopez et al., 2009). Concurrently, endocytosis of GLUT4 is reduced. As a result, the steady recycling of GLUT4 in 'basal conditions' to the PM is altered to ensure GLUT4 remains at the PM and the amount of GLUT4 cycling back to the vesicle is reduced to increase glucose uptake. The rate of glucose uptake into skeletal muscle is managed by the amount of GLUT4 at the PM and the duration of which this transporter protein is stationed there.

As well as insulin stimulation, exercise can mediate the quantity of glucose which is taken up into skeletal muscle. The mechanism by which GLUT4 transport is increased in response to exercise has been heavily researched yet is still not fully understood. Recent work in rodents has established that, whilst necessary for insulin-stimulated GLUT4 translocation, muscle contraction also activates signalling protein Rac 1 (Sylow et al., 2013; Sylow et al., 2016). Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small signalling protein belonging to the Rac subfamily of the Rho family GTPases which are responsible for many cellular functions including cell growth and activation of protein kinases (Laine et al., 2020). In particular, the Rho and Rac GTPases are well known as regulators for cytoskeletal reorganisation and vesicle trafficking, where they have been shown to govern exocytosis vesicle movement and fusion to the plasma membrane (Takai, Sasaki and Matozaki, 2001). To do so, these GTPases swap between the active guanosine triphosphate (GTP) and the inactive guanosine diphosphate (GDP) form. When in the active form, GTP, these proteins bind to effector proteins and regulate cell response/function. Sylow *et al.,* (2016) have shown in rodents that Rac1 regulates the translocation of the glucose transporter GLUT4 to the plasma membrane in

skeletal muscle during exercise, and knockout of Rac 1 significantly reduces glucose uptake in response to exercise. The upstream and downstream targets of Rac 1, and therefore the mechanism for its role in glucose transport are not yet confirmed.

1.2.6 Visualisation of GLUT4 using immunofluorescence methods

Early studies of GLUT4 translocation utilised subcellular fractionation techniques in both rodent (Klip et al., 1987; Douen et al., 1990; Marette et al., 1992) and human skeletal muscle (Goodyear et al., 1996; Kennedy et al., 1999) by quantification of GLUT4 content in plasma membrane fractions. Importantly, myofibrillar proteins and other intracellular membrane fractions that have high GLUT4 content can contaminate the isolated PM fractions (Fazakerley et al., 2009). More so, it is not possible to discriminate between GLUT4 that is just adjacent to the PM and GLUT4 that is fully incorporated into the PM after docking and fusion of glucose storage vesicles and, therefore, able to transport glucose (Schertzer et al., 2009). Alternative methods involving ectopic expression of tagged glucose transporters provides novel opportunities for the molecular analysis of GLUT4 translocation in cultured cells (Wang et al., 1998; Dawson et al., 2001; Eguez et al., 2005) and in rodents (Fazakerley et al., 2009; Schertzer et al., 2009; Lizunov et al., 2012) for confirmation of GLUT4 insertion into the PM. However, it is not technically possible to chronically express HA-tagged GLUT4 *in vivo* in human skeletal muscle and so new methods were required to investigate GLUT4 localisation and trafficking in human skeletal muscle.

Bradley *et al.,* (Bradley et al., 2014) were the first to utilize immunofluorescence microscopy to visualize changes in the subcellular distribution and content of GLUT4 in response to endurance and sprint interval training. GLUT4 content increased by comparable amounts after both training methods, as shown by an increase in the number of large and small GLUT4 storage vesicles. This increase in GLUT4 storage vesicles was linked to the observed increase in insulin sensitivity and insulin-stimulated glucose uptake following training. Building upon this, Bradley *et al.,* (2015) applied this novel technique to quantify GLUT4 translocation to the plasma membrane in human skeletal muscle following glucose feeding and exercise (Bradley et al., 2015). This study demonstrated GLUT4 depletion from large and small clusters as net GLUT4 translocation to the plasma membrane. Missing from this data though was whether

fibre type differences in GLUT4 localisation with the plasma membrane were apparent following exercise or insulin stimulation.

1.2.7 GLUT4 tethering and fusion

For GLUT4 to become a part of the plasma membrane and allow glucose uptake, N-ethylmaleimide sensitive factor (NSF) attachment protein receptor (SNARE) proteins are required. SNARE proteins are both on GLUT4 vesicles (vSNAREs) and on specific target sites of the plasma membrane (tSNAREs). SNARE proteins facilitate numerous processes of exocytosis, however the vSNAREs and tSNAREs are those involved in GLUT4 vesicle docking. Vesicle associated membrane protein-2 (VAMP2) dock to specific tSNAREs; synaptosomal associated protein 23 (SNAP23) and syntaxin 4, allowing for extracellular exposure of GLUT4 (Inoue et al., 2006).

For these proteins to form a SNARE complex and result in GLUT4 tethering, they need NSF and alpha SNAP to initiate a conformational change in shape (Sollner et al., 1993). A SNARE complex results in the fusion of the lipid bilayers on GLUT4 and the plasma membrane. The APS/CAP/Cbl pathway activates TC10, which in turn, triggers the translocation and activation of Exo70 to lipid raft domains where it binds to snapin. Snapin-Exo70 binding activates SNARE protein SNAP23 (Bao et al., 2008) necessary for GLUT4 tethering. When SNAP23 is inactivated *in vitro*, we see reduced translocation and docking of GLUT4 to the plasma membrane (Foster et al., 1999; Kawanishi et al., 2000) as well as reduced glucose transport (Foster et al., 1999).

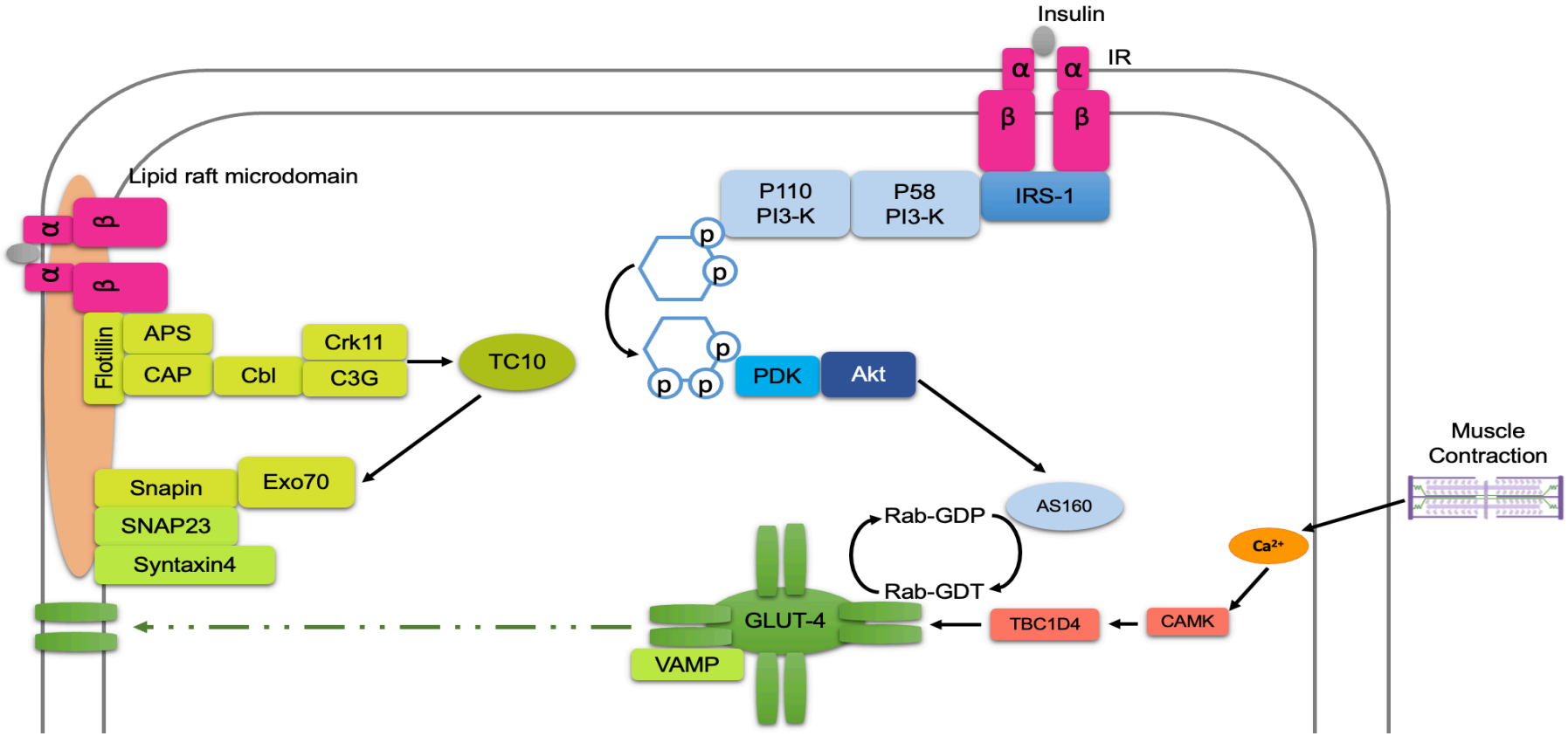


Figure 1.2 Insulin-mediated, and contraction induced, glucose uptake into skeletal muscle. APS, adaptor protein with PH and SH2 domain; AS160, Akt substrate of 160 kD; CAP, Cbl-associated protein; GLUT4, glucose transporter 4; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PDK, phosphoinositide-dependent kinase 1; PI3-K phosphoinositide 3-kinase; Rab-GDP, Rab guanosine disphosphate; Rab-GDP, Rab guanosine triphosphate; SNAP23, synaptosomal associated protein; VAMP2, v-SNARE vesicle associated membrane protein-2; CAMK, calmodulin-dependant protein kinase; TBC1D4, TBC1 domain family member

1.3 Interactions between carbohydrate and lipid metabolism

The accumulation of specific lipid metabolites in skeletal muscle is crucial to the development of insulin resistance. It was previously proposed that elevated FA availability could ultimately lead to an accumulation of Glucose-6-phosphate and a rise in intracellular glucose concentrations, causing negative feedback for glucose uptake from the circulation (Randle et al., 1963). This first proposal of an interaction between glucose and fatty acid oxidation, known as the "Randle cycle", describes the fluctuations in fuel selection focussing on the competition between oxidation of glucose and fatty acids in both skeletal muscle and adipose tissue. Fatty acids are predominantly oxidised in skeletal muscle both at rest and during exercise, and it is well known that during low-intensity exercise fatty acid metabolism is increased compared to resting conditions. Increasing exercise intensity from low to moderate results in greater fatty acid metabolism until the intensity reaches approximately 65% VO_{2max} (Achten, Gleeson and Jeukendrup, 2002). At this point, the rate of fatty acid oxidation declines, and carbohydrate becomes the main source of fuel. It is also evident that this curve will shift upward in response to endurance training, whereby trained individuals can sustain a greater rate of fat oxidation at the same relative exercise intensity. Since 1963, an interaction between these fuels has been well established, along with the discovery of additional mechanisms that control the utilisation of glucose and fatty acids. It important to note that the relationship between these two fuels is reciprocal rather than dependent, whereby the use of one fuel directly impairs the use of the other without hormonal mediation.

1.3.1 Inhibition of carbohydrate metabolism by FA oxidation

At low-to-moderate exercise intensities, the increased delivery of FA into skeletal muscle is proposed to suppress carbohydrate (CHO) oxidation. The first credible explanation for this reverse interaction between fatty acid oxidation and glucose utilization was published 14 years later by McGarry et al., (1977) (McGarry, Mannaerts and Foster, 1977). The proposed mechanism for the downregulation of carbohydrate metabolism when the availability of FA is high has three components: 1) Increases in acetyl-CoA/CoA and NADH/NAD+ ratios downregulate PDH, which leads to an accumulation of citrate; 2) Citrate accumulation is

proposed to suppress PFK activity, and result in 3) glucose-6-phosphate accumulation and ultimately the suppression of hexokinase activity (Newsholme, Randle and Manchester, 1962; Garland, Randle and Newsholme, 1963).

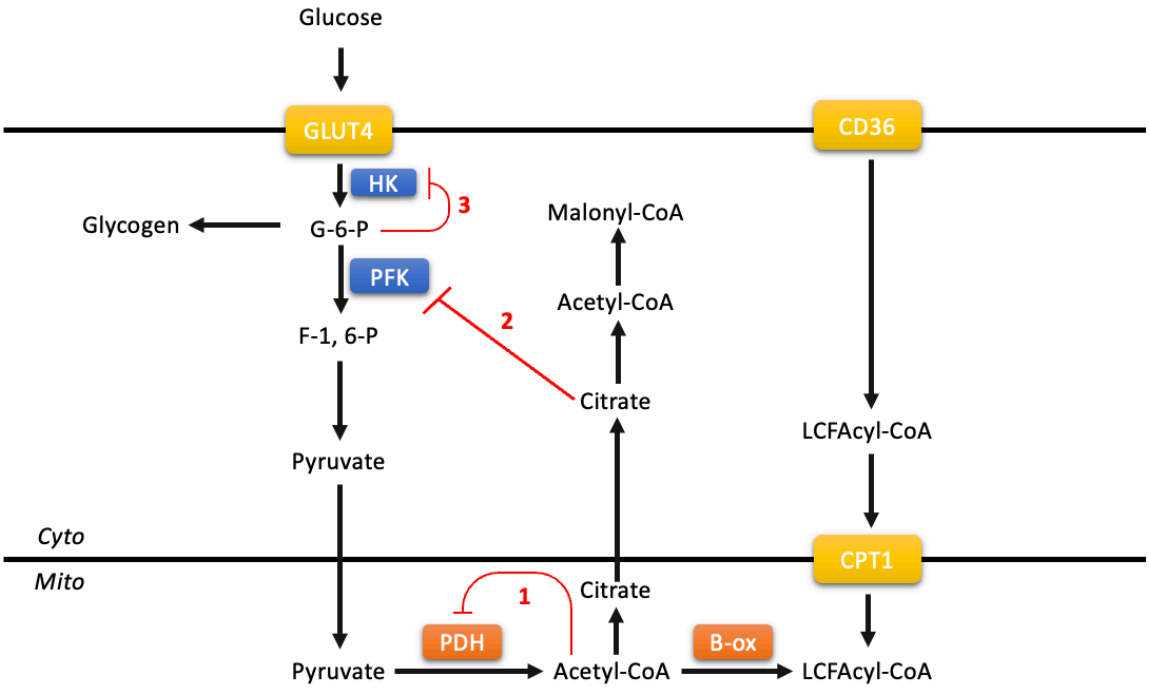


Figure 1.3. The interactions between glucose and fatty acid metabolism. Increases in acetyl-CoA/CoA and NADH/NAD+ ratios downregulate PDH, which leads to an accumulation of citrate (1); Citrate accumulation is proposed to suppress PFK activity (2), and result in glucose-6-phosphate accumulation and ultimately the suppression of hexokinase activity (3).

The mitochondrial multi-enzyme complex known as the PDH complex regulates the oxidation of pyruvate, the product of glycolysis, to produce acetyl-CoA for metabolism via TCA cycle (Randle, 1998). At the lower intensities, long chain fatty acid uptake into the mitochondria is occurring at a fast rate due to increased delivery and uptake of plasma FFA into skeletal muscle. As a result, elevated rates of beta oxidation result in increased generation of acetyl-CoA. These high concentrations of acetyl-CoA from FA metabolism increase PDK activity and concurrently inhibit PDH activity. The reversible phosphorylation of the PDH complex was identified by Reed in (1969) who demonstrated this concept that the products of fatty acid oxidation resulted in phosphorylation and therefore inactivation of PDH. PDH activity is

controlled by other mitochondrial kinases PDK and PDP. All PDK isoforms phosphorylate site 1 of PDH, but PDK2 is the most active kinase responsible for the inactivation of this enzyme. Phosphorylation of PDH at sites 2 and 3 by PDK4 and PDK1, respectively, result in dephosphorylation of site 1 and holding PDH in an inactive state. The inhibition of PDH means that pyruvate oxidation is therefore inhibited and increased conversion of pyruvate to phosphoenolpyruvate by pyruvate carboxylase occurs. Ultimately, increased availability of plasma FFA leads to the downregulation of CHO metabolism but is also a key determinant of IMTG utilisation during low-moderate intensity exercise. During the first 2 hours of exercise, IMTG utilisation is greatest, however as plasma FFA concentrations increase during hours 3-4, IMTG utilisation is minimal (Watt, Heigenhauser and Spriet, 2002; van Loon et al., 2003b). More so, when a nicotinic acid based anti-lipolytic drug (Acipimox) is used to lower plasma FFA levels, IMTG use is increased (van Loon et al., 2005a).

Hexokinase II is a key enzyme in insulin-sensitive tissues responsible for the first step in glycolysis; the conversion of glucose to glucose-6-phosphate. Importantly, glucose-6-phosphate is an inhibitor of hexokinase so when the concentration of glucose-6-phosphate is elevated, this first step of glycolysis is inhibited. Fatty acid metabolism results in citrate-mediated inactivation of phosphofructokinase, preventing the conversion of fructose-6-phosphate to fructose 1,6-bisphosphate. Consequently, fructose 1,6-bisphosphatase remains active and fructose-6-phosphate concentrations are high. As a result, this skews the equilibrium of the reversible reaction between glucose-6-phosphate and fructose-6-phosphate in favour of glucose-6-phophate, inhibiting hexokinase II and the first step in glycolysis is inhibited.

1.3.2 Glucose metabolism dominates at high intensities

It is well established that as exercise intensities increase from low-moderate to high intensity (85% VO_{2max}), skeletal muscle glycogenolysis and glucose uptake increases, and CHO metabolism dominates as the energy supply. Several lines of evidence link plasma FFA availability as a key determinant of substrate utilisation, whereby the decrease in IMTG utilisation after 2 h of exercise corresponded with an increase in plasma FFA concentrations (Watt et al., 2002) and was replicated the following year with the same observation (van Loon

et al., 2003). Reducing plasma FFA concentrations using nicotinic acid (an inhibitor of adipose tissue lipolysis), enhanced the utilisation of IMTG during 2 h of exercise, yet no changes in AMPK activation, acetyl Co-A carboxylase (ACC) phosphorylation, or HSL phosphorylation were observed between the control and nicotinic acid trials that would have explained the increased IMTG utilisation. These studies do, however, show that plasma FFA availability is a key determinant of IMTG utilisation during low-to-moderate intensity exercise. Although acipimox increased the relative contribution of IMTG oxidation to total fat oxidation during exercise, the absolute fat oxidation rates were reduced and glycogen use increased (van Loon et al., 2005). Notably, plasma FFA availability may regulate the relative contribution of both carbohydrate and fat oxidation to total energy expenditure during exercise.

Since FA oxidation takes place in the mitochondria through β-oxidation, it is feasible that FA entry into the mitochondria is what regulates fat oxidation. Long-chain FA require a transport mechanism to facilitate transport across the outer and inner mitochondrial membranes, whereas medium-chain FA can enter the mitochondria directly. Because of this, the oxidation of MCFA and LCFA have been investigated under conditions of high glycolytic flux from either pre-exercise glucose feeding (Coyle et al., 1997) or from transitioning from a low to high exercise intensity (from 40 to 80% $VO_{2\ max}$) (Sidossis et al., 1997). Utilising a tracer method of ^{13}C-labeled LCFA (oleate) and ^{14}C-labeled MCFA (octanoate) enabled the quantification of MCFA and LCFA uptake and oxidation during the exercise bout. Importantly, at the higher intensity of 80% $VO_{2\ max}$ LCFA oxidation that requires a transport mechanism was reduced, whereas MCFA oxidation remained comparable to oxidation rates at 40% $VO_{2\ max}$ (Sidossis et al., 1997). From this, it was concluded that LCFA oxidation was limited by carnitine palmitoyl transferase-1 (CPT-1) required for LCFA uptake into the mitochondria. Thus, increasing malonyl-CoA concentrations during high intensity exercise inhibits CPT-1 activity and reduces LCFA transport into mitochondria. Yet, there is no evidence to date that supports the notion that malonyl-CoA concentrations are increased in human skeletal muscle as exercise intensity increase (Odland et al., 1998). Actually, malonyl-CoA concentrations appear to decrease during exercise as intensity increases (Dean et al., 2000). While it seems that LCFA transport into the mitochondria is what limits fat oxidation during high intensity exercise, this cannot be credited to reduced CPT-1 activity from increased malonyl-CoA concentrations.

An important consideration is that high intensity, submaximal exercise is linked with an increase in the ratios of AMP:ATP and ADP:ATP, and these metabolic imbalances motivate glycolytic flux. More specifically, an increase in AMP and ADP leads to allosteric activation of the key glycolytic enzymes glycogen phosphorylase, PFK and PDHa. As a result, during high intensity exercise the flux through PDC will be more than the flux through the TCA cycle and will cause an accumulation of acetyl-CoA (van Loon et al., 2001). Since a precursor to malonyl-CoA Is acetyl-CoA, an increase in the concentrations of acetyl-CoA could hypothetically lead to an increase in malonyl-CoA production. But, malonyl-CoA concentrations are not increased during high intensity exercise. Instead, acetyl-CoA has an alternative outcome by forming acylcarnitine catalysed by carnitine acetyltransferase (CAT). As such, free carnitine acts as a buffer for excess acetyl-CoA to ensure there is a sufficient pool of CoASH maintained to continue the PDC and TCA flux. This does mean, however, that the amount of free carnitine is slightly reduced. So, it is suggested that this reduction in free carnitine due to acetyl CoA buffering limits the capacity for carnitine to partake in the transport of LCFA transport into the mitochondria through CPT-1 at higher intensity exercise. This has been confirmed by increased acetyl carnitine concentrations and a concomitant reduction in free carnitine and fat oxidation during high intensity submaximal exercise (van Loon et al., 2001). Together, current evidence suggests that the contribution of fat oxidation during exercise to total energy expenditure is primarily regulated by the glycolytic and PDC flux.

1.4 Introduction to Insulin Resistance: Obesity and Lipotoxicity

Physical inactivity combined with chronic over-consumption of an energy-dense diet causes expansion of subcutaneous and visceral adipose tissue depots around the body leading to obesity and insulin resistance. Currently, one third of the global population over the age of 15 engages in insufficient levels of physical activity (Park et al., 2020). Multiple factors that make up the western lifestyle are strongly associated with physical inactivity levels, such as environmental factors including limited availability of green spaces and pedestrian walkways in cities (WHO, 2020), as well as lifestyle choices such as high television viewing time and mobile phone usage (Fennell et al., 2019). With the addition of humans living ever increasingly busy lives, an increase in working from home, and public transport more accessible, we have less reasons throughout the day to be physically active. Physical inactivity has been identified as the leading cause of mortality worldwide (WHO, 2010). Because skeletal muscle accounts

for ~50% of glucose disposal following a mixed meal (Capaldo et al., 1999), and therefore is a key determinant of whole-body insulin sensitivity, it is likely that physical inactivity and excess adiposity contributes to the development of insulin resistance through an effect at the level of skeletal muscle.

Physical inactivity is associated with a collective of metabolic diseases including obesity, insulin resistance, type 2 diabetes, cardiovascular disease that contribute to a state of metabolic syndrome. Obesity is the consequence of an imbalance between energy intake and expenditure, essentially a combination of an energy dense diet and physical inactivity. Obesity and physical inactivity are associated with an inability of tissues to respond to a physiological increase in plasma insulin concentrations, known as insulin resistance. Insulin resistance develops first with inadequate physiological insulin levels to maintain glucose homeostasis by glucose uptake into skeletal muscle and so the pancreas is required to work harder and secrete more insulin to maintain homeostasis. Over time, insulin becomes ineffective in stimulating glucose uptake into muscle and chronic hyperglycaemia and chronic hyperinsulinaemia are observed. T2D is the end point, at which the pancreas is essentially failing and prolonged periods of postprandial hyperglycaemia and hyperinsulinaemia occur.

1.4.1 Lipid overflow hypothesis

In the fasted and post-prandial states, adipose tissue is the organ responsible for controlling plasma FFA and TAG concentrations, with plasma FFA concentrations at their highest following an overnight fast (~ 0.3 – 0.6 mmol.l^{-1}). Plasma glucose concentrations are elevated following ingestion of a mixed meal and cause secretion of insulin from pancreatic β-cells. Elevated insulin concentrations subsequently suppress adipose tissue hormone sensitive lipase (HSL) activity to reduce FFA release into the circulation, whilst also activating lipoprotein lipase (LPL) in adipose tissue capillaries to stimulate hydrolysis of chylomicron-TAG to release FA to be taken up by adipose tissue for TAG storage. Lastly, insulin also suppresses LPL activity in the skeletal muscle capillary bed. Ultimately, it is the ability for adipose tissue to buffer the lipid flux that is important in protecting non-adipose tissues such as the liver, skeletal muscle, heart and pancreas from the detrimental effects of high plasma FFA and TAG concentrations (Frayn, 2002).

There is substantial evidence that obese individuals with large adipose tissue stores have a compromised ability to buffer the lipid flux, causing consistently elevated plasma FFA concentrations, and this observation is frequently associated with insulin resistance (Lewis et al., 2002; Frayn et al., 2002; Bays et al., 2004). Specifically, individuals with obesity are reported to show impaired insulin-mediated suppression of HSL, and therefore adipose tissue lipolysis is not reduced and sustained FFA liberation from adipocytes causes elevated FA appearance in the circulation (Coppack et al., 1992; Hickner et al., 1999). High plasma FFA concentrations is strongly associated with insulin resistance, since high-fat feeding for 2-3 days causes elevated plasma FFA concentrations concomitant with reduced insulin sensitivity (Johnson et al., 2006; Pehleman et al., 2005). Moreover, raising plasma FFA concentrations with an intralipid/heparin emulsion infusion reduces skeletal muscle insulin stimulated glucose uptake (Bachman et al., 2001; Boden and Chen, 1995; Itani et al., 2002; Kelley et al., 1993; Roden et al., 1999). Yet, insulin-stimulated glucose disposal rates were only reduced 2 hours into a lipid infusion highlighting that plasma FFA per se are not directly interfering with insulin signalling. Plasma FFA concentrations may not differ between lean and obese individuals (Bickerton et al., 2008; McQuaid et al., 2011; Reeds et al., 2006), since the rates of adipose tissue lipolysis in obese individuals are normal when adjusted relative to total fat mass (Bickerton et al 2008; Campbell et al., 1994; Fabbrini et al., 2009; McQuaid et al., 2011; Robinson et al., 1998), implying that plasma insulin concentrations observed in obese individuals may actually be adequate in supressing FFA release from adipose tissue. Instead, it appears that the capacity to store plasma-derived TAG in adipose tissue is what is impaired in obese individuals (McQuaid et al., 2011), potentially due to reduced rate of LPL activity in the adipose tissue capillary bed (Frayn, 2002; Karpe et al., 2011). This causes an increase in non-lipolyzed TAG in the plasma, and elevated concentrations of TAG in plasma, rather than FFA, are more closely associated with insulin resistance (Bickerton et al., 2008; Karpe et al., 2011). In this regard, TAG fragments of chylomicron-TAG are deposited in the liver to be a substrate for very low-density lipoprotein production containing TAG (VLDL-TAG), an increase in assembly and secretion of which, is a common observation in obese and insulin resistant individuals (Karpe et al., 2011; Reilly and Rader, 2003). Fittingly, elevated plasma FFA and TAG concentrations during an intralipid/heparin infusion are linked with insulin resistance (Bachmann et al., 2001), and it is suggested that FFA ‘spill over’ into non-adipose tissues

occurs to maintain plasma lipid homeostasis. Specifically, elevated TAG concentrations are commonly observed in both the liver and skeletal muscle. As a result, insulin resistant individuals are characterised by reduced suppression of glucose released from the liver and impaired glucose uptake into skeletal muscle. Understandably, a link has been highlighted between lipid accumulation in non-adipose tissues in obese individuals and the occurrence of hyperglycaemia in insulin resistance.

Evidence that lipid accumulation in non-adipose tissues is a key contributor to the development of insulin resistance has several pathways. In the first instance, in lipodystrophy; a condition characterised by partial or complete loss of adipose tissue, excess caloric intake promotes lipid storage in non-adipose tissues such as the liver and skeletal muscle in both mice and humans, paired with reduce insulin sensitivity (Gavrilova et al., 2000; Kim et al., 2000a). Furthermore, a transplant of adipose tissue from wild type mice into A-ZIP/F-1 mice that exhibit lipoatrophic diabetes reverses hyperglycaemia, reduces insulin concentrations, and improves skeletal muscle insulin sensitivity (Gavrilova et al., 2000; Kim et al., 2000a). These adaptations also occurred alongside a decrease in lipid storage in muscle and liver tissues (Kim et al., 2000a), emphasising the need of adipose tissue to maintain skeletal muscle insulin sensitivity. Secondly, activation of peroxisome proliferator Y (PPARy) receptors in adipocytes by thiazolidinedione (TZD) treatment in obese Zucker rats leads to adipocyte differentiation and lipid stored in both skeletal muscle liver tissues is redistributed to adipocytes (Hallakou et al., 1997). As expected, improved insulin sensitivity was observed (Hallakou et al., 1997). Human studies in both healthy and obese individuals provide further confirmation of this notion, whereby supplementation of Pioglitazone (a thiazolidinedione) reduces muscle lipid concentrations and improves insulin sensitivity (Rasouli et al., 2005; Teranishi et al., 2007). Collectively, these studies show that lipid accumulation in non-adipose tissues is a contributor to insulin resistance.

1.5 Skeletal muscle lipid metabolism

FA are an essential fuel source for skeletal muscle. Importantly the transport of FA into skeletal muscle tissue occurs through a highly regulated, protein-mediated process (Jain et al., 2015). This transport is facilitated by several transport proteins including fatty acid

translocase (FAT/CD36), plasma membrane associated fatty acid-binding protein ($FABP_{pm}$), and fatty acid transport proteins (FATP1 & -4) (Glatz, Luiken and Bonen, 2010). Much like how GLUT4 translocation influences glucose metabolism, changes in demand for FA utilisation by skeletal muscle can be met by changes in the translocation, and potentially content, of certain FA transporter proteins to alter the rate of FA utilisation (Glatz, Luiken and Bonen, 2010).

1.5.1 FA transport into skeletal muscle in health individuals

Due to their low solubility, FA are bound to protein albumin which acts as fatty acid binding protein in extracellular fluids, containing 7 binding sites for FA with moderate to high affinity (Vusse, 2009). Albumin, despite its high affinity for FA, aids the transport of FA into skeletal muscle likely by reaching a rapid equilibrium of FA between plasma albumin on one side, and interstitial albumin on the other (Vusse, 2009). There are two main transporter proteins involved in this trans-endothelial transport of FA from the circulation into the skeletal muscle interstitium: fatty acid translocase (CD36) and fatty acid transporter protein (FATP). First, the FA-Albumin complex in the plasma of the capillary binds to CD36 and FATP on the plasma membrane of the endothelial cells. Once FA has entered the endothelial cell, it binds to fatty acid binding protein (FABP) and albumin remains in the circulation. FA-FABP complex travels through the endothelial cells where CD36 and FATP transport FA through the plasma membrane into the skeletal muscle interstitium. FA then binds to Albumin within the interstitium to again form a FA-Albumin complex. To enter the SM fibre, the same transporter proteins are integrated into the SM fibre PM, CD36 and FATP with the addition of FABPpm. Once inside the cell, FA binds with FABPcyt.

Studies on the transport capacity of these FA transporter proteins has been of interest to determine which is the most effective. When all FA transporter proteins (CD36, FATP1, FATP4, FABPpm) are independently over expressed in Female Sprague-Dawley rats, the overexpression of each transporter protein did not affect the expression of the others (Nickerson et al., 2009). Each protein, when over expressed, increased FA transport, but FAT/CD36 and FATP4 were 2.3- and 1.7-fold more effective than FABPpm and FATP1, respectively (Nickerson et al., 2009). In contrast, CD36 and $FABP_{pm}$ increased the rates of long chain fatty acid oxidation by 3-fold compared to FATP1 and FAT4 (Nickerson et al., 2009).

From this, FAT/CD36 and FATP4 were deemed the most effective fatty acid transporters, whereas CD36 and FABPpm appeared to be the key proteins for stimulating FA oxidation.

1.5.2 FA transport in individuals with obesity/T2D

Transporter proteins within the endothelial layer of capillaries and skeletal muscle are required for long chain fatty acids to move from the circulating blood into skeletal muscle fibre. Fatty Acid Translocase (FAT/CD36) is the predominant transporter protein in the regulation of long-chain fatty acid uptake into skeletal muscle (Coburn et al., 2000; Holloway et al., 2007), with other transporter proteins being fatty acid transport protein 1-5 (FATP1-5) and plasma membrane fatty-acid binding protein (FABPm). In healthy individuals, these fatty acid transporter proteins maintain the balance between fatty acid uptake and subsequent storage/ oxidation (Aguer et al., 2011).

The identification of intracellular FAT/CD36 in addition to its presence at the cell membrane brought to light its pivotal role as a regulator of fatty acid uptake as opposed to simply a facilitator (Glatz & Luiken, 2018). Mechanisms known to induce CD36 translocation to the plasma membrane are insulin signalling pathway, AMPK signalling pathway and muscle contraction (Koonen et al., 2005; Chabowski et al., 2007). In the presence of insulin or following muscle contraction, translocation of CD36 to the cell membrane increases approximately two-fold (Luiken et al., 2002; 2003).

The abnormal lipid accumulation seen in obese individuals has been analysed in much detail, with strong links associated with increased rates of fatty acid transport into skeletal muscle (Bonen et al., 2004), suggesting increased rate of fatty acid transport into skeletal muscle is associated with skeletal muscle lipid accumulation. It has become apparent that CD36 plays a pivotal role in the molecular mechanisms whereby obesity and high fat-diet induce lipid accumulation and insulin resistance. Cellular and animal models have shown that chronic oversupply of fatty acids to skeletal muscle causes increases in the cycling of CD36 and causes the permanent relocation of this transporter protein to the endothelial membrane (Auger et al., 2010; Bonen et al., 2015). Myotubes derived from obese type 2 diabetes patients showed abnormal cell-surface localization of CD36 (Aguer et al., 2010), and increased cycling of

FAT/CD36 when compared to control myotubes that maintain an equilibrium between lipid uptake and lipid oxidation (Aguer et al., 2011). Furthermore, high fat feeding in rats demonstrated this permanent change in the location of CD36 to the cell membrane is very rapid, increasing after 2 days (Bonen et al., 2015). This permanent relocation of FAT/CD36 saw an increase of membrane-associated FAT/CD36 concurrent with a decrease in intra-cellular FAT/CD36 rather than an increase in overall expression of the transporter protein (Bonen et al., 2004; Han et al., 2007). The result of this permanent localisation of CD36 is that the rate of fatty acid uptake is no longer manipulated depending on the metabolic needs of the skeletal muscle and there is a greater influx of fatty acids. Excess fatty acids within skeletal muscle contribute to the accumulation of lipid metabolites (DAGs and ceramides) known to contribute to skeletal muscle insulin resistance.

It is yet to be determined why an increase in fatty acid supply to skeletal muscle affects the cycling of CD36 where the majority of the transporter protein permanently resides on the sarcolemma. Recent research suggests that this is the result of a modification to the properties of endosomes, where CD36 is stored within the cell. Endosomes are acidic cellular organelles, however, are shown to become alkalised as a result of high fat feeding in rats (Liu et al., 2017).

Collectively, data shows a greater delivery and uptake of FFA into skeletal muscle in individuals with obesity/T2D and so the rate of these FA entering skeletal muscle is what determines whether they induce skeletal muscle insulin resistance.

1.5.3 Lipid metabolites and their potential influence on insulin signalling

The accumulation of lipid in non-adipose tissues, such as skeletal muscle and the liver, contributes to insulin resistance. Therefore, IMTG content is inversely related to insulin sensitivity in obese individuals. IMTG content per se cannot be the cause of insulin resistance however, due to its elevation in highly trained individuals who remain highly insulin sensitive (Goodpaster et al., 2001). Rather, the relationship between IMTG and insulin resistance is more complex, and is related to maladaptations to IMTG synthesis, storage and oxidation.

Intermediates in the pathway of FA storage as TAG in skeletal muscle, known as lipid metabolites, have been implicated in lipotoxicity. More specifically, both DAGs and ceramides have been shown to have detrimental effects on insulin action through their ability to activate novel PKC isoforms, at least in cell models and rodent studies (Merrill and Jones, 1990; Nishizuka, 1995). However, cross-sectional studies of humans do not provide strong evidence that ceramides and DAGs are elevated in type 2 diabetes compared to trained individuals (Skovbro et al., 2008; Perreault et al., 2010; Coen et al., 2010; Dube et al., 2011). In fact, DAG content has been reported to be markedly higher in trained athletes compared to obese individuals (Amati et al., 2011). Because of this contradiction in the literature, studies have sought to investigate DAGs and ceramides in more detail by exploring the content of specific species of both DAGs and ceramides as well the subcellular location of these specific species.

When investigated at this level, the link to insulin resistance is stronger. The location of DAG within skeletal muscle appears significant, where DAG that resides near the membrane is shown to be more closely aligned to insulin resistance (Bergman et al., 2012). However, more recently the subcellular content of DAG seems to have no relationship with insulin resistance. In fact, whilst it is generally accepted that 1,2-DAGs activate PKC and induce insulin resistacne (Boni et al., 1985; Rando et al., 1984), 1,2-DAGs were actually positively related to insulin sensitivity when located within the mitochondria/endoplasmic reticulum fraction of muscle (Perreault et al., 2018). The first study to provide a comprehensive profile of the molecular species of DAG present in the skeletal muscle of obese, normal-weight and athletic individuals showed that particular species of DAG are more related to insulin resistance, but this was not related to saturation or chain length (Amati et al., 2011). This differed from Bergman et al., 2010 who found that the degree of saturation of DAGs within muscle in athletes was lower compared with sedentary subjects and was related to higher insulin sensitivity (Bergman et al., 2010). Importantly, Bergman et al., (2010) measured the total FA content within the DAG pool, whereas Amati (2011) quantified specific DAG species, making these difficult to compare.

The saturation of ceramides appears to be more closely linked to their influence on insulin resistance as saturated ceramide species (those with no double bonds) more readily form lipid ‘rafts’ in membranes (Pinto et al., 2011). Lipid rafts that contain saturated ceramide

species decrease membrane fluidity subsequently reducing insulin receptor function in the membrane (Storlien et al., 1991). Specifically, C18 ceramides have been implicated in insulin resistance in high fat fed rodents (Blachnio-Zabielska et al., 2016) as well as in humans (Bergman et al., 2016; Perreault et al., 2018). Although sarcolemmal DAG was found to hold no relationship with insulin resistance, sarcolemmal ceramides present an inverse relationship with insulin sensitivity with a significance for C18 (Perreault et al., 2018). Ceramides located within the mitochondria/endoplasmic reticulum fraction also appear to have an inverse relationship with insulin sensitivity (Perreault et al., 2018).

Liquid chromatography-tandem mass spectrometry has been used to measure the concentrations and enrichment of skeletal muscle ceramides in more detail in human skeletal muscle. Insulin resistance, plasma insulin, and triglyceride concentrations were positively correlated with sub-cellular C16:0 and C18:1 ceramide, but not sub-cellular C14:0-Cer, C20:0-Cer, C24:0-Cer, and C24:1-Cer concentrations (Chung et al., 2017). More so, the fractional contribution of plasma palmitate to intramyocellular sub-cellular 16:0 ceramide was positively correlated with sub-cellular C16:0 ceramide concentrations (Chung et al., 2017). Measuring these individual ceramides, alongside a palmitate infusion, has began to reveal in more detail where the concentration of these ceramides originates from. The positive relationship between the subcellular C16:0 ceramide concentrations and the fractional contribution of plasma palmitate to C16:0 ceramides may suggest the de novo synthesis of ceramides to be responsible for greater sub-cellular ceramide content (Chung et al., 2017).

It is evident that the investigation of individual DAG and ceramide species is crucial to understand insulin signalling disruption. More so, lipids reside in many subcellular compartments and are continuously being trafficked between these, so changes to compartmentation and trafficking of lipids may reveal key differences between trained individuals and T2D patients (Bergman and Goodpaster, 2020).

Chapter 2: High intramuscular triglyceride turnover rates and the link to insulin sensitivity: influence of obesity, type 2 diabetes, and physical activity.

This thesis chapter, as it is, is published in the Journal of Applied Physiology, Nutrition and Metabolism:

J.S. Barrett, K.L. Whytock, J.A. Strauss, A.J.M. Wagenmakers, and S.O. Shepherd. High intramuscular triglyceride turnover rates and the link to insulin sensitivity: influence of obesity, type 2 diabetes and physical activity. Applied Physiology, Nutrition, and Metabolism. 47(4): 343-356. https://doi.org/10.1139/apnm-2021-0631

2.1 Abstract

Large intramuscular triglyceride (IMTG) stores in sedentary, obese individuals have been linked to insulin resistance, yet well-trained athletes exhibit high IMTG levels whilst maintaining insulin sensitivity. Contrary to previous assumptions, it is now known that IMTG content *per se* does not result in insulin resistance. Rather, insulin resistance is caused, at least in part, by the presence of high concentrations of harmful lipid metabolites, such as diacylglycerols and ceramides in muscle. Several mechanistic differences between obese sedentary individuals and their highly trained counterparts have been identified, that determine the differential capacity for IMTG synthesis and breakdown in these populations. In this review, we first describe the most up-to-date mechanisms by which a low IMTG turnover rate (both breakdown and synthesis) leads to the accumulation of lipid metabolites and results in skeletal muscle insulin resistance. We then explore current and potential exercise and nutritional strategies which target IMTG turnover in sedentary obese individuals, to improve insulin sensitivity. Overall, improving IMTG turnover should be an important component of successful interventions which aim to prevent the development of insulin resistance in the ever-expanding sedentary, overweight and obese populations.

2.2 Introduction

Physical inactivity combined with chronic over-consumption of an energy-dense diet causes expansion of adipose tissue depots around the body leading to obesity. The buffering capacity of adipose tissue can become impaired in obesity resulting in spill-over of circulating fatty acids (FA) and triglycerides into non-adipose tissues, such as the liver and skeletal muscle, leading to ectopic lipid deposition (Frayn, 2002). The delivery of excess lipid to skeletal muscle leads to accumulation of intramuscular triglyceride (IMTG) (Bachmann *et al.*, 2001; Chow *et al.*, 2014), which is characteristic of the obese and T2D states. Thus, high IMTG levels in sedentary obese individuals and T2D patients are associated with insulin resistance (Pan *et al.*, 1997; Kelley and Goodpaster, 2001; van Loon *et al.,* 2004). However, it is now known that a high IMTG content *per se* does not result in insulin resistance. Rather, insulin resistance is believed to be caused, at least in part, by the presence of harmful lipid metabolites, such as diacylglycerols (DAGs) and ceramides (Goodpaster et al., 1997; Pan et al., 1997; Forouhi et al., 1999). Indeed, the accumulation of DAGs and ceramides has been shown to disrupt cell function, and specifically the capacity for insulin-stimulated glucose uptake into skeletal muscle via direct interference with the insulin signalling cascade (Yu et al., 2002; Summers and Nelson, 2005; Chaurasia and Summers, 2015). However, defects in insulin signalling are not always observed in insulin resistant individuals (Meyer et al., 2002; Hojlund et al., 2003; Ramos et al., 2021), and the precise mechanism by which lipid metabolites induce insulin resistance is far from certain. Consequently, the link between lipid metabolite accumulation and insulin resistance appears complex, and may be rooted in other factors such as lipid metabolite composition and subcellular localisation (extensively reviewed recently by (Bergman and Goodpaster, 2020). The association between IMTG accumulation and insulin resistance has also been disputed due to endurance trained athletes having a comparable or even higher IMTG content than obese individuals and T2D patients, whilst remaining highly insulin sensitive (Goodpaster et al., 2001; van Loon et al., 2003a). This phenomenon is now well known as the "athlete's paradox" (Goodpaster *et al.,* 2001). The question of how endurance trained athletes exhibit similar IMTG content compared to obese individuals but are able to combine this with high levels of insulin sensitivity has been the subject of intense research in the last 20 years.

Although the mechanistic link between IMTG accumulation and insulin resistance is not yet fully established, the fundamental difference between endurance athletes when compared to obese individuals with or without T2D is their greater ability to utilise IMTG as a source of fuel during exercise (Schrauwen *et al.*, 2002; van Loon, 2004). Regular breakdown (lipolysis) of IMTG and oxidation of FA during exercise, coupled with elevated rates of FA uptake and IMTG (re)synthesis following exercise, creates a dynamic IMTG pool with a high turnover rate (van Loon, 2004; Moro, Bajpeyi and Smith, 2008). An attractive hypothesis is that regular IMTG turnover maintains insulin sensitivity by regulating the concentration and spatial distribution of lipid metabolites thereby ameliorating their impact on insulin signalling and cell function. However, exercise training appears to have little impact on insulin signalling (Christ-Roberts et al., 2004; Frosig et al., 2007) and does not always alter the concentration of lipid metabolites in muscle (Meyer et al., 2002; Hojlund et al., 2003). Rather, a greater ability to utilise IMTG in trained individuals leads to the hypothesis that the capacity to appropriately adjust FA storage and efficiently breakdown and oxidise FA in line with metabolic demand and FA availability is fundamental to improve insulin sensitivity. Because of this, focus has shifted to identifying the mechanisms that enable a high turnover rate of the IMTG pool in trained individuals in order to be able to create the optimal intervention in obese individuals and people with T2D and subsequently improve insulin sensitivity.

With this in mind, the first aim of this review is to evaluate the differences in the storage and utilisation of IMTG between trained and more sedentary populations (i.e., obese and elderly individuals and those with T2D) to demonstrate that the dynamic nature of the IMTG pool in trained individuals is a crucial characteristic to the preservation of insulin sensitivity. Based on this information, we then aim to explore potential strategies to maximise IMTG turnover which could be implemented as interventions to improve insulin sensitivity in obese individuals and T2D patients.

2.3 The differences in IMTG storage between trained and sedentary individuals

Although IMTG content itself has no mechanistic link to insulin resistance, in this context it is important to consider the fibre-specific distribution, subcellular location, and morphology of

IMTG-containing lipid droplets (LD). In lean, healthy individuals there is a hierarchical distribution between the different fibre types with the majority of IMTG being stored in type I fibres, followed by type IIa then type IIx fibres (Daemen, van Polanen and Hesselink, 2018; Whytock e*t al.,* 2020). Within skeletal muscle, LDs are located either between the myofibrils (intermyofibrillar [IMF] LDs) or just beneath the surface membrane (subsarcolemmal [SS] LDs) (Nielsen *et al.*, 2017). Using transmission electron microscopy, it was recently shown that type I fibres of healthy males have small LD located in both the intermyofibrillar region and the subsarcolemmal region (Nielsen *et al.,* 2017), whereas type II fibres contain a similar number of LD in the intermyofibrillar region and the subsarcolemmal region but those in the subsarcolemmal region are ~20% larger in diameter (Nielsen *et al.*, 2017). Consequently, the size of subsarcolemmal LD in particular was associated with poorer insulin sensitivity, rather than LD number, at least in healthy untrained males (Nielsen *et al.*, 2017). Daemen *et al,* (2018) extended these observations when comparing differences between trained individuals and patients with T2D. To this end, the elevated IMTG content in trained individuals was explained by a greater number of LD in the intermyofibrillar region of type I fibres, whereas individuals with T2D had a greater number of larger LD in the subsarcolemmal region of type II fibres (Daemen, van Polanen and Hesselink, 2018). It is important to note here that to date the majority of research investigating differences in LD location and morphology has been conducted in males, or without distinction between sex. Thus, differences in LD location and morphology over the lifespan and between sexes should be explored in future studies. Older adults have been shown to have larger LD, fewer mitochondria, and a lower proportion of LD in contact with mitochondria (Crane et al., 2010), likely contributing to age-related decline in mitochondrial function and lipid metabolism.

Interestingly, 8 weeks of a high-calorie, high-fat diet induced insulin resistance in sedentary individuals and resulted in an increase in LD size rather than any changes in LD number (Covington *et al.,* 2017). More recently, we reported that a short-term (7-day) high-calorie, high-fat diet increased LD size and number in type I fibres in both the central and peripheral regions (Whytock *et al.*, 2020). In type IIa fibres LD size increased in both the SS and IMF region but only LD number increased in the SS region, whereas in type IIx fibres only SS-located LD increased in size with no changes in LD number observed (Whytock *et al.*, 2020). This suggests that changes in LD number and size may occur in a hierarchical manner based on both fibre

type and subcellular region, at least in response to a high-calorie, high-fat diet. In contrast, a combined weight loss and exercise training intervention in previously overweight or obese individuals resulted in a decrease in LD size concomitant with improved insulin sensitivity, even in the absence of a reduction in IMTG content (He, Goodpaster and Kelley, 2004). A large number of small LD located in the IMF region, as observed in healthy lean and trained individuals, creates a larger surface area to volume ratio, which is thought to be beneficial for the binding of proteins and lipolytic enzymes to the LD in order to liberate and release FA from the IMTG stored within. Moreover, LD are located in close proximity to mitochondria within skeletal muscle in healthy, trained individuals (Hoppeler *et al.*, 1999; Shaw, Jones and Wagenmakers, 2008), and exercise training in healthy or obese individuals increases the proportion of LD that are in contact with mitochondria (Tarnopolsky et al., 2007; Shepherd et al., 2017b). Together with a large number of small LDs, this adaptation likely creates an efficient means by which to channel FA liberated from IMTG within LD to the mitochondria for subsequent oxidation (Fig. 1). It is important to note here though that increased LD association with mitochondria does not necessarily mean the LD are utilised for oxidation, and this LD-mitochondria interaction may also support triacylglycerol synthesis and LD growth (Benador et al., 2018; Benador et al., 2019).

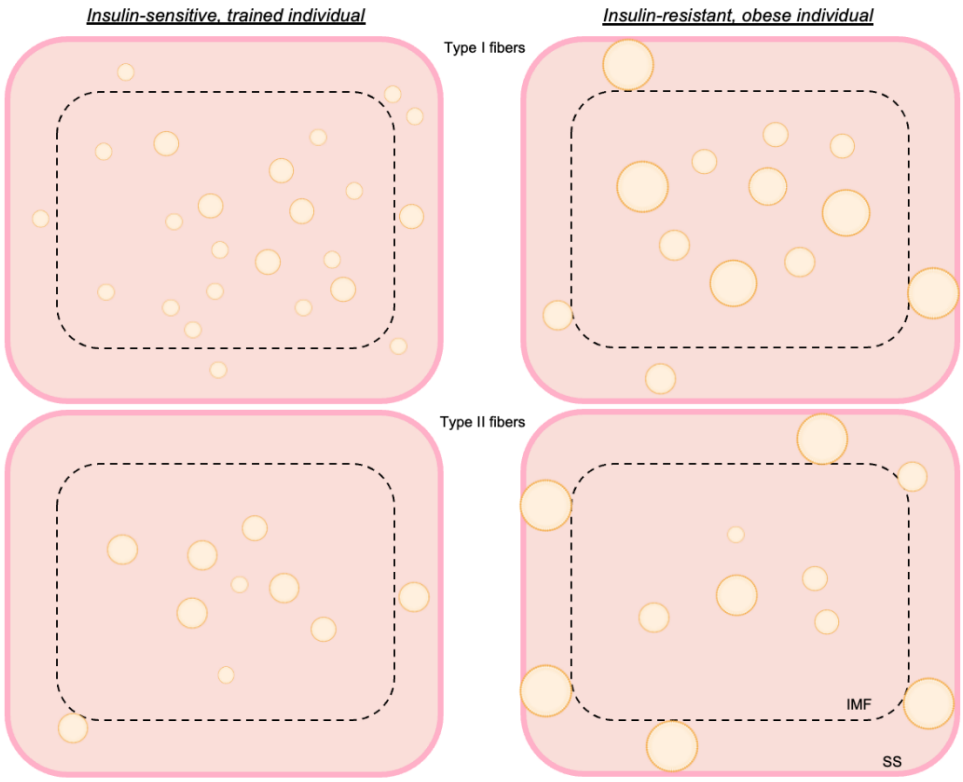


Figure 1. A representation of the subcellular location of LD in skeletal muscle in insulin-resistant, obese individuals and insulin-sensitive, trained individuals. Insulin-sensitive, trained individuals typically exhibit a large number of small LD which are primarily located in the intermyofibrillar region of type I fibres. Moreover, the number of LD is two-to-three-fold greater in type I compared to type II fibres in trained individuals. In contrast, insulin-resistant, obese individuals tend to exhibit LD that are much large in size compared to insulin-sensitive, trained individuals. Furthermore, in insulin-resistant individuals a higher proportion of LD appear to be present in the subsarcolemmal region compared to trained individuals. This is especially true in type II fibres, where the number and size of SS LD is ~two-fold greater compared to trained individuals (Daemen et al., 2018). Thus, the muscle of trained individuals is characterised by a large number of small LD located in the intermyofibrillar region of type I fibres, whereas the muscle of insulin-resistant obese individuals is characterised by large LDs stored in the subsarcolemmal region of type II fibres.

2.3.1 IMTG turnover in trained versus sedentary individuals

Cross-sectional comparisons between trained and untrained individuals confirm that endurance-trained individuals have a greater capacity to use IMTG as a substrate during exercise (Klein, Coyle and Wolfe, 1994; Coggan et al., 2000). During moderate-intensity exercise in healthy individuals, IMTG-derived fatty acids contribute ~50% to total fat oxidation, with the remaining ~50% attributable to plasma FA (van Loon *et al.,* 2001). Serial muscle biopsies combined with microscopy-based analyses enable net changes in IMTG content to be determined and using this approach it is now known that IMTG utilisation preferentially occurs in type I fibres from IMTG-containing LD (van Loon et al., 2003a; Shepherd et al., 2013) that are located in the IMF region (Koh *et al.,* 2017; Jevons *et al*., 2020). Moreover, in healthy individuals IMTG utilisation and FA oxidation during exercise is closely related to pre-exercise IMTG content (Shepherd et al., 2013) whereby those with greatest IMTG stores have the greatest IMTG utilisation. Therefore, the high rate of IMTG utilisation observed in healthy, trained individuals must be matched by a large capacity for esterification and storage of FA as IMTG following exercise. This has been illustrated in a recent study, which demonstrated that myotubes from athletic subjects have higher lipid turnover and lipid oxidation compared to those from sedentary individuals (Lund *et al.,* 2018) Specifically,

myotubes from athletes exhibit higher rates of lipolysis and re-esterification of FA into the triacylglycerol (TAG) pool, indicating greater turnover of TAG stores. Importantly, higher complete oxidation and incomplete β-oxidation of FA in myotubes from the athletic population was also observed, suggesting they are able to more effectively rely on FA as a fuel source (Lund *et al.,* 2018). A greater accumulation of FA in myotubes derived from sedentary compared to athletic individuals led the authors to question whether the capacity for IMTG synthesis is downregulated in these individuals, and/or the capacity for lipid metabolite generation is upregulated (Lund *et al.,* 2018). The latter, of course, would consequently reduce insulin sensitivity.

Measuring the fractional synthesis rates (FSR) of IMTG in healthy individuals provides *in vivo* information on the rate of turnover of the IMTG pool. In this regard, IMTG FSR at rest in healthy individuals was first reported to be as high as ~3.4%/h, suggesting that in this cohort complete turnover of the IMTG pool would occur in ~29 h (Sacchetti et al., 2004). Although Bergman *et al.* (2018) have since reported a lower resting IMTG FSR in trained individuals (~1.56%/h), this was still more than 2-fold higher when compared to sedentary, lean individuals (~0.61%/h) (Bergman *et al.,* 2018). Obese individuals have a lower IMTG FSR (~0.42 %/h) than the rates reported for lean, sedentary individuals, and the resting IMTG FSR for obese individuals with pre-diabetes is even lower (~0.21 %/h) (Perreault et al., 2010). With these data, it is no surprise that Bergman *et al.* (2018) reported a positive correlation between IMTG FSR and insulin sensitivity at rest, along with a negative correlation between IMTG synthesis rates and the concentration of key lipid metabolites associated with insulin resistance. There is evidently a strong link between an individual's ability to breakdown and resynthesise IMTG and their level of insulin sensitivity.

As well as measuring IMTG FSR at rest, studies examining IMTG FSR during exercise alongside net changes in IMTG concentration provide further insight into the dynamics of the IMTG pool in trained and sedentary obese and T2D individuals. During 1 hour of moderate-intensity exercise, IMTG FSR is elevated compared to rest and while a net reduction in IMTG content is observed in trained individuals, IMTG content remains unchanged in obese individuals and those with T2D (Bergman *et al.,* 2018). The latter finding is in line with previous studies measuring IMTG content in biopsies pre- and post-exercise which concluded that there is no

net utilisation of IMTG in obese individuals and those with T2D (Kelley and Simoneau, 1994; Blaak and Wagenmakers, 2002). IMTG FSR is elevated during exercise in obesity and T2D (Bergman *et al.,* 2018), which could be due to the high circulating FFA concentrations often observed in these individuals (Axelsen et al., 1999) supplying fatty acids for the synthesis of IMTG. In obese individuals and T2D patients though there is no net change in IMTG content during exercise, but for this to be true IMTG utilisation during exercise must be matched to IMTG FSR. Therefore, by combining pre- and post-exercise measures of IMTG content with estimates of IMTG FSR during exercise, it appears possible that obese and T2D individuals may utilise their IMTG stores, but this occurs in the absence of a net reduction in IMTG content (Bergman *et al.,* 2018), potentially due to replenishment of the IMTG stores from high circulating FFA concentrations (Axelsen et al., 1999). It has also been reported that in individuals with normal glucose tolerance, IMTG FSR during exercise may be reduced, especially compared to individuals with prediabetes (Perreault *et al.,* 2010), obese individuals, and T2D patients (Bergman *et al.,* 2018) who all exhibit only very small changes in IMTG FSR during exercise. Thus, while there is not yet a consensus on how IMTG FSR is altered during exercise in trained, glucose tolerant individuals, these cross-sectional comparisons do highlight an inability to adjust IMTG FSR relative to metabolic demand in obese individuals with pre-diabetes and T2D. Importantly, a net reduction in IMTG content during exercise in endurance trained individuals will theoretically enable a greater capacity for uptake of plasma FFA and storage as IMTG in the post-exercise period. Without a net reduction in IMTG content during exercise in obese individuals, this may limit the capacity for FA's entering skeletal muscle following exercise to be stored as IMTG, and rather these FA's may instead be directed towards the generation of lipid metabolites.

2.4 Molecular mechanisms regulating IMTG turnover

While the aim of this review is not to provide an in-depth account of the molecular mechanisms that regulate FA uptake and esterification, IMTG storage and breakdown, it is pertinent that an up-to-date overview of these regulatory mechanisms is provided. For the former, the reader is directed to two excellent reviews (Badin, Langin and Moro, 2013; Lundsgaard, Fritzen and Kiens, 2018).

IMTG Synthesis

Exogenous FA, derived from either adipose tissue or from the diet, are transported in the circulation and taken up into skeletal muscle to be stored as IMTG and/or oxidised as a fuel source. FA uptake into skeletal muscle is regulated primarily by FAT/CD36, although it is likely that this process is mediated by a series of transporter proteins reviewed in detail in (Schwenk *et al.*, 2010; Glatz and Luiken, 2018). Once in skeletal muscle, FA are converted to fatty acyl-CoA and directed to IMTG synthesis. Briefly, FA-CoA undergoes acylation catalysed by key enzymes glycerol-3-phosphate acyltransferase (GPAT), monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) (Teodoro et al., 2017), ultimately leading to the generation of TAG (or IMTG). The synthesised IMTG are then stored within LD and are a readily available fuel for healthy individuals.

Although the key enzymes that control IMTG synthesis have been identified, little is known about how they regulate this process in skeletal muscle. Following moderate-intensity exercise and a subsequent elevation of lipid availability (induced by an overnight lipid infusion), there is an increase in the protein expression of DGAT1 and GPAT1 (Schenk and Horowitz, 2007) and increased GPAT1 activity (Newsom *et al.*, 2011). Furthermore, overexpression of DGAT1 in rodents results in an increase in TAG content and a decrease in DAG (Liu *et al*., 2007). However, GPAT1 and DGAT1 do not differ in expression between obese and lean individuals (Thrush *et al.,* 2009; Li *et al.,* 2011), and no differences are observed in DGAT1 mRNA expression between endurance trained, obese individuals and those with type 2 diabetes (Bergman *et al.*, 2018), nor in DGAT protein content (Amati *et al.,* 2011). Overall, there is currently no evidence to suggest that the expression or activity of DGAT or GPAT is impaired in obese and T2D individuals. As a result, it could be speculated that the machinery for IMTG synthesis is sufficient in all individuals, and it is the (as yet unknown) activation mechanism which is impaired in obesity and T2D.

IMTG lipolysis

The reduced IMTG utilisation reported in obese individuals and those with T2D could be, at least partly, attributed to impaired rates of lipolysis. Indeed, when compared to lean individuals, obese individuals show impaired $ß_2$- adrenergic-mediated stimulation of lipolysis in skeletal muscle (Blaak *et al.,* 2004). In skeletal muscle, the majority (~98%) of total TAG

hydrolase activity (at least at rest) is regulated by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL). In this regard, it is important to note HSL protein content (Jocken *et al.*, 2007) and HSL phosphorylation (at Ser^{563}, Ser^{555} and Ser^{659}) (Jocken *et al.*, 2008) are both lower in obese compared to lean individuals. However, it was reported that individuals with T2D actually had greater ATGL protein expression compared to lean and obese individuals (Badin *et al.*, 2011). Because ATGL may have a higher affinity to TAG (Haemmerle *et al.*, 2006), and HSL a higher affinity to DAG (Fredrikson *et al.,* 1981; Haemmerle *et al.*, 2002), it has been suggested that in obesity and T2D the imbalance between ATGL and HSL protein content favours DAG accumulation, and this contributes to the disruption of insulin signalling. Indeed, overexpression of ATGL in myotubes from lean, healthy, insulin-sensitive individuals induced DAG and ceramide accumulation, which was associated with reduced insulin-stimulated glycogen synthesis and reduced activation of IRS-1 and Akt (Badin *et al.*, 2011). Although, this imbalance was reported by Jocken *et al.,* (2008) with greater ATGL content and lower HSL content in obese individuals with T2D compared to lean (Jocken *et a*l., 2008), it was not evident in non-obese T2D, questioning its role in the development of insulin resistance. Moreover, it is now known that DAG that is derived from ATGL-mediated lipolysis is unable to activate the atypical PKC isoforms known to disrupt the insulin signalling cascade (Eichmann *et al.*, 2012). Additionally, Bergman *et al.,* (2018) has more recently shown that elevated IMTG content in obese individuals is not due to an imbalance between HSL and ATGL content, but more likely due to the specific species of ceramides present in obese individuals, and the subcellular location in which they are stored (Bergman *et al*., 2018).

LD proteins and their regulation of IMTG turnover

Surrounding a core of TAG and cholesterol esters, LDs have a phospholipid monolayer that is now known to be coated with numerous proteins which likely determines the functional role of each LD. The perilipin proteins (PLIN) are the group of LD proteins most extensively investigated (Morales, Bucarey and Espinosa, 2017), with PLIN2, 3, 4 and 5 all being expressed in human skeletal muscle. Research conducted over the last decade has started to uncover a potential role for the PLIN proteins in both IMTG storage and lipolysis.

The role of PLIN in IMTG storage - It is evident from *in vitro* studies that the knockout of PLIN2 or PLIN5 in skeletal muscle compromises TAG storage (Bosma *et al.*, 2012; Gallardo-Montejano *et al.,* 2016). It makes sense then, that the overexpression of these PLIN isoforms results in quite the opposite, promoting TAG storage (Xu *et al.*, 2005; Bosma *et al.*, 2012; Gallardo-Montejano *et al.*, 2016). Similarly, the suppression of PLIN3 reduced LD maturation and TAG incorporation into IMTG stores in HeLa cells (Bulankina *et al.,* 2009), whereas in skeletal muscle myotubes augmenting PLIN3 gene expression increases IMTG content (Kleinert *et al.,* 2016). PLIN4 is purported to be the most abundant PLIN in skeletal muscle (Deshmukh *et al.*, 2015), yet its knockout in mice has no effect on skeletal muscle IMTG concentrations (Chen *et al*., 2013), questioning the importance of this protein in IMTG storage.

Human biopsy studies demonstrate that PLIN2 protein expression is greater in trained versus sedentary individuals (Amati et al., 2011; Shaw et al., 2012; Shepherd et al., 2013), females versus males (Shaw *et al.,* 2009; Peters *et al.,* 2012), and type 1 versus type 2 fibres (Shaw *et al.*, 2009), suggesting that PLIN2 is closely related to IMTG content in healthy individuals. The same observations also extend to PLIN3 (Peters *et al.*, 2012; Shepherd *et al.*, 2017b) and PLIN5 (Shepherd et al., 2013; Shepherd et al., 2017a; Shepherd et al., 2017b; Daemen, van Polanen and Hesselink, 2018). Furthermore, when exercise training augments IMTG content, increases in PLIN2 (Shaw et al., 2012; Shepherd et al., 2013), PLIN3 (Shepherd *et al.,* 2017b) and PLIN5 (Peters et al., 2012; Shepherd et al., 2013) are also observed. Much less research has been conducted on PLIN4, although we recently showed that PLIN4 protein expression is greater in trained versus untrained individuals (Shepherd *et al.,* 2017b). Despite this, endurance training fails to augment PLIN4 mRNA or protein expression in healthy individuals (Peters *et al.,* 2012; Pourteymour *et al.*, 2015). Together, these data suggest that the expression of PLIN2, PLIN3 and PLIN5 is closely related to IMTG content, at least in healthy individuals or following a period of exercise training. This may be an important adaptation in order to support greater IMTG storage, especially in the face of elevated FA availability and turnover. Indeed, in response to an acute lipid infusion (Shepherd et al., 2017a) or 48 h of fasting (Gemmink *et al.*, 2016) (both conditions increase FA availability) in trained individuals, there appears to be a redistribution of the pre-existing PLIN2, PLIN3 and PLIN5 protein pool (which could be from either LD-bound or non-LD-bound) to the expanded LD pool. Importantly, this redistribution

was not apparent in sedentary individuals (Shepherd et al., 2017a) and the capacity to redistribute PLIN5 to maintain coverage of the expanded LD pool was associated with a greater maintenance of insulin sensitivity (Gemmink *et al.*, 2016). More recently, we showed that in elite triathletes, post-exercise increases in IMTG content occurred *prior* to a redistribution of the PLIN (2,3,5) protein pool (Jevons *et al.*, 2020). Taken together, this suggests that the PLIN proteins do not play a direct role in IMTG synthesis but coating of LD with PLINs may be an important adaptation which supports IMTG storage.

The role of PLIN in IMTG breakdown - Research has also focused on the potential role of PLIN2, PLIN3 and PLIN5 in supporting IMTG breakdown and utilisation during exercise. A role for the PLIN proteins in TAG breakdown stems from evidence showing that PLIN2, PLIN3 and PLIN5 can interact with the key lipolytic enzymes ATGL and HSL (Anthonsen *et al.*, 1998; Granneman *et al.,* 2011; Macpherson *et al.*, 2013). Moreover, both PLIN2 and PLIN5 are thought to suppress lipolysis at rest by preventing the interaction between ATGL and CGI-58, whereas this inhibition is relieved permitting ATGL to interact with CGI-58 upon lipolytic stimulation (Wang *et al.,* 2011; Macpherson *et al.*, 2013). PLIN3 knockout in myotubes results in a reduction of lipid oxidation (Covington *et al.*, 2014). However, in response to an endurance exercise bout PLIN3 expression is positively correlated to whole-muscle homogenate palmitate oxidation rates as well as whole-body cumulative fat oxidation (Covington *et al.*, 2014). Recently, AMPK phosphorylation of PLIN3 was shown to bring about conformational changes to PLIN3 that expose the C-terminus and promote LD dispersion to facilitate lipolysis (Zhu *et al.*, 2019). This new data potentially underpins the relationship between PLIN3 and lipolysis.

In human studies, we initially showed that LD labelled with PLIN2 or PLIN5 are preferentially broken down in lean, sedentary individuals during 1 h of moderate-intensity exercise (Shepherd et al., 2012; Shepherd et al., 2013). However, when assessed following six weeks of endurance training or sprint interval training, only LD labelled with PLIN5 were preferentially targeted for breakdown in an equivalent bout of exercise (Shepherd et al., 2013). More recently, we have shown that during more prolonged (4 h) of moderate-intensity exercise in elite triathletes, this preferential use of PLIN labelled LD is not apparent. The use of LD not labelled with PLIN proteins could be attributed to a very high rate of IMTG turnover

compared to sedentary individuals, especially during such prolonged exercise. Nevertheless, it does appear that PLIN5 plays a key functional role regulating IMTG breakdown, since 1 h of moderate-intensity exercise led to a redistribution of HSL specifically to LD labelled with PLIN5 (Whytock *et al.*, 2018).

FA as signalling molecules

It is now beginning to be understood that FA play a crucial role in skeletal muscle adaptation to exercise, by acting as ligands for peroxisome proliferator-activated receptor (PPAR) α and δ to support transcription of genes involved in lipid metabolism (Banner *et al.,* 1993). An extensive review of this topic is beyond the scope of the current paper (readers are directed to (Funai and Semenkovich, 2011) but it is pertinent to briefly consider the importance of FA as signalling molecules in the context of adaptation. In humans, suppression of lipolysis with nicotinic acid prior to exercise resulted in reduced mRNA expression of Peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1-alpha (PGC-1α), PPARα and PPARδ, demonstrating a role for FA availability in exercise-induced gene expression (Watt *et al.,* 2004). In contrast, treatment with a PPARδ agonist resulted in a dose-dependent increase in skeletal muscle FA oxidation in mice, as well as increased expression of mRNA encoded for proteins involved in FA catabolism, such as *β*-oxidation enzymes, FA transport proteins and uncoupling proteins (Tanaka *et al.*, 2003).

The source of the FA that act as ligands and activate PPARα and PPARδ is not yet confirmed but could be linked to the activation of intramuscular lipases acting upon IMTG. In support, ATGL-mediated hydrolysis of triacylglycerol promotes activation of PGC-1α and PPARα signalling in order to upregulate mitochondrial biogenesis. Moreover, ATGL-mediated lipolysis activates SIRT-1, the protein responsible for the deacetylation, and therefore activation, of PGC-1α (Khan *et al.,* 2015). Until recently, the mechanism by which ATGL-mediated lipolysis promotes mitochondrial biogenesis was unknown. However, Najt *et al.* (2020) recently reported that monounsaturated FA are able to activate SIRT-1, thereby enhancing PGC-1α/PPARα signalling. Importantly, these monounsaturated FA were derived from intracellular LDs (Najt *et al.*, 2020), permitting speculation that FA liberated from IMTG stored in LD may play a role in promoting mitochondrial biogenesis, although evidence for this is not yet available. Najt *et al.* (2020) also identified a role for PLIN5 as a FA binding

protein, which is able to bind LD-derived monounsaturated FA and transport them to the nucleus, at least in response to cAMP/PKA-mediated lipolytic stimulation in hepatocytes. This observation is consistent with that of Gallardo-Monejano *et al.* (2016), who reported that fasting-induced lipolysis stimulates PKA-mediated phosphorylation of PLIN5 followed by its translocation to the nucleus (Gallardo-Montejano *et al.,* 2016). Here, PLIN5 interacts with SIRT-1 and PGC-1a to increase transcription of proteins involved in FA catabolism. The translocation of PLIN5 was also shown for the first time to influence the transcriptional regulation of mitochondrial respiration and mitochondrial biogenesis (Gallardo-Montejano *et al.,* 2016). Taken together, it appears that intracellular LDs are more than a source of FA for oxidation but may play a key role in the regulation of mitochondrial biogenesis and the FA catabolism programme. Theoretically then, enhancing the utilisation and turnover of the IMTG pool could be one strategy to stimulate mitochondrial biogenesis and increase the capacity for fat oxidation. We will now explore potential strategies that could be used to induce these adaptations and subsequently enhance insulin sensitivity and discuss whether these have application in clinical populations.

2.5Strategies to improve IMTG Turnover

2.5.1 Exercise Training

It is well known that endurance exercise training is a powerful stimulus to augment oxidative capacity and IMTG utilisation during exercise (Baldwin et al., 1972; Kiens et al., 1993; Phillips et al., 1996; Bergman et al., 1999; van Loon, 2004). Following endurance training there is an increase in the number of IMTG-containing LDs that are in direct contact with mitochondria (Tarnopolsky et al., 2007; Devries et al., 2013; Shepherd et al., 2017a), which together with expansion of the mitochondrial network enhances the total capacity for FA ß-oxidation (Granata, Jamnick and Bishop, 2018). In sedentary lean and obese individuals endurance training also augments the expression of proteins that regulate IMTG breakdown and LD dynamics, including ATGL (Alsted *et al.*, 2009), PLIN2 (Shaw *et al.,* 2012; Shepherd *et al*., 2013), PLIN3 (Shepherd *et al.*, 2017a) and PLIN5 (Shepherd *et al.,* 2017a). Notably, the increased expression of the PLIN proteins occurs primarily in type I fibres, which may explain why following a period of endurance training the increase in IMTG utilisation is also predominantly in type I fibres (Van Proeyen *et al.,* 2011a; Van Proeyen *et al.,* 2011b; Shepherd

et al., 2013). Importantly, the augmented use of IMTG following endurance training is related to an improvement in insulin sensitivity (Van Proeyen *et al.,* 2011a; Van Proeyen *et al.,* 2011b; Shepherd *et al.,* 2013).

Other forms of exercise training can also increase the capacity for IMTG utilisation during exercise. For example, 6 weeks of sprint interval training in sedentary individuals enhances IMTG utilisation during a single bout of moderate-intensity exercise, to a similar degree as endurance training (Shepherd *et al.,* 2013; Scribbans *et al.*, 2014) at least in lean, sedentary individuals. Underpinning this, several different forms of high intensity (or sprint) interval training have been shown to enhance skeletal muscle oxidative capacity (Little *et al.*, 2010; MacInnis and Gibala, 2017; Astorino and Schubert, 2018), and we have also reported an increased expression of PLIN2 and PLIN5 following sprint interval training (Shepherd *et al.,* 2013). Interestingly, in lean, sedentary individuals 6 weeks of whole-body resistance training also increases IMTG utilisation during a single bout of moderate-intensity exercise (Shepherd *et al.*, 2014), although the net changes in IMTG content during exercise following training are typically less than when compared to endurance or sprint interval training (Shepherd *et al.,* 2013). Given that resistance training can enhance mitochondrial content and oxidative capacity (Tang, Hartman and Phillips, 2006; Balakrishnan *et al.,* 2010; Pesta *et al.,* 2011) as well as resting IMTG content (Shepherd *et al.*, 2014), this finding is perhaps not unexpected. Moreover, because both high intensity interval training and resistance training improve insulin sensitivity in sedentary (Ishii et al., 1998) and obese individuals (Croymans et al., 2013; Ryan et al., 2020), it is tempting to speculate that an enhanced capacity for IMTG utilisation during exercise could, at least in part, contribute to this effect. Although this is yet to be investigated directly, high intensity interval training in obese individuals augments several adaptations that would support greater IMTG turnover, including increased mitochondrial content (Gibala *et al*., 2006; Burgomaster *et al.,* 2008; Larsen *et al.,* 2015; Chrois *et al.*, 2020) and mitochondrial interaction with LDs, increased HSL and CD36 content (Talanian *et al.,* 2010) and greater protein expression of PLIN2, PLIN3, and PLIN5 (Shepherd et al., 2013; Shepherd *et al.*, 2017b).

As discussed above, exercise stimulates IMTG utilisation in lean, healthy individuals (Shepherd *et al.,* 2013; Scribbans *et al.*, 2014), and to a lesser extent in those with obesity and T2D

(Shepherd *et al.,* 2017a; Bergman *et al*., 2018). However, a poor capacity to simultaneously reduce the rate of IMTG synthesis during exercise in individuals with obesity and T2D results in a minimal to zero decrease in IMTG content post-exercise (van Loon *et al.,* 2004). The result of this is a limited capacity for FA's entering skeletal muscle following exercise to be stored as IMTG and interfere with insulin signalling. Thus, in sedentary and obese individuals, additional strategies to exercise alone may be required to augment IMTG utilisation (or turnover) in order to create a net decrease in IMTG content post-exercise. In this context, the question then arises as to whether the insulin sensitising-effect of regular exercise training can be enhanced, by manipulating the conditions under which exercise is undertaken in order to maximise IMTG utilisation and obtain a post-exercise decrease in IMTG content (Fig. 2).

2.5.2 Anti-lipolytic Drug Therapy

During prolonged exercise there is a progressive decline in IMTG oxidation rate which is inversely related to the concomitant increase in plasma free fatty acid (FFA) concentrations (Romijn et al., 1993; Romijn et al., 1995; van Loon et al., 2003a). Thus, it is purported that elevated plasma FFA concentrations may suppress IMTG utilisation during exercise. Pharmacological inhibition of adipose tissue lipolysis, via the anti-lipolytic agent Acipimox, before and during exercise abolishes the progressive rise in plasma FFA during exercise and results in enhanced IMTG oxidation in lean, healthy individuals (van Loon et al., 2005a). Individuals with obesity and T2D exhibit elevated circulating plasma FFA and triglyceride concentrations, which is linked to the development of insulin resistance (Boden, 2003), and therefore could also be part of the mechanism by which exercise-induced IMTG utilisation is suppressed in these individuals. In support, inhibition of adipose tissue lipolysis before and during exercise in obese T2D patients increases IMTG oxidation at rest, during 60 minutes of moderate-intensity exercise, and for several hours' post-exercise (van Loon et al., 2005b). Moreover, this was accompanied by a superior rate of glycogen oxidation, and greater post-exercise insulin sensitivity (van Loon et al., 2005b).

Several studies show that short-term Acipimox treatment (250 mg, two-to-three times per day) for up to 2 weeks can reduce fasting plasma free fatty acids and increase insulin sensitivity and glucose control in obese and T2D individuals (Bajaj *et al.,* 2005; Daniele *et al.,*

2014; Phielix *et al.,* 2014; van de Weijer *et al.*, 2015). Furthermore, 8 weeks of Acipimox treatment lowers plasma free fatty acids, cholesterol and triglyceride concentrations in obese individuals and T2D patients (Crepaldi *et al*., 1988; Stuyt, Kleinjans and Stalenhoef, 1998). Lower plasma free fatty acid levels reduce the availability of FA for uptake into skeletal muscle, potentially minimising the accumulation of lipid in this tissue. Despite these positive changes to blood lipids and insulin sensitivity, longer term Acipimox treatment results in a rebound rise in fasting plasma FFA (Fulcher *et al.*, 1992; Vaag and Beck-Nielsen, 1992; Saloranta *et al.,* 1993) and both hepatic and skeletal muscle insulin sensitivity is unchanged (Makimura *et al.*, 2016). Therefore, while chronic treatment with Acipimox does not seem feasible, combining exercise with anti-lipolytic therapy may represent an effective strategy to augment insulin sensitivity in individuals with obesity and T2D.

2.5.3 Fasted Exercise

As high plasma FFA concentrations suppress IMTG utilisation during exercise, a simple strategy to reduce FFA availability would be to feed carbohydrate, since carbohydrate ingestion increases plasma insulin levels and subsequently suppresses circulating FFA through insulin-mediated inhibition of HSL in adipose tissue (Watt *et al., 2004)*. However, insulin also suppresses HSL activity in skeletal muscle (Enoksson *et al.*, 1998), and therefore carbohydrate ingestion would theoretically lead to a decrease in IMTG utilisation. The overnight fasted state is characterised by low plasma insulin concentrations, and therefore skeletal muscle lipolytic enzyme activity remains functional (Horowitz et al., 1997; Arkinstall et al., 2001). Indeed, in healthy individuals, two hours of moderate-intensity cycling in the overnight-fasted compared to carbohydrate-fed state led to greater IMTG utilisation in type 1 fibres (De Bock *et al.,* 2005). Similar results were obtained in overweight/obese males, although in this population IMTG utilisation was more pronounced in both type 1 and type 2 fibres following fasted compared to postprandial exercise (Edinburgh *et al.,* 2020). It should be noted though that when the exercise duration is extended to 3 h, there appears to be no effect of carbohydrate feeding before (Fell et al., 2021) and/or during (Stellingwerff et al., 2007; Fell et al., 2021) exercise on IMTG utilisation. In this case, it is possible that the duration of exercise overrides the inhibitory effect of carbohydrate feeding on IMTG utilisation. Nevertheless, it is worth noting that limiting glucose availability by exercising in the fasted state encourages

skeletal muscle cells to increase transcriptional activities of factors that upregulate the fatty acid oxidation programme and thereby induce metabolic adaptations for efficient lipid oxidation (Canto et al., 2010). Six weeks of endurance training in lean, healthy individuals in a fasted state was more effective for increasing skeletal muscle oxidative capacity, CD36 and $FATBP_m$ content, and net IMTG breakdown during a single exercise bout compared to undertaking the same training in a fed condition (Van Proeyen *et al*., 2011b). Moreover, 6 weeks of endurance training in a fasted state in overweight/obese individuals augmented skeletal muscle remodelling of phospholipids (Edinburgh *et al.,* 2020). Thus, fasted exercise appears to augment changes in skeletal muscle phospholipids, by reducing saturated FFA, that correlate with improved post-prandial insulinemia.

Whether these beneficial adaptations to fasted exercise can enhance the insulin-sensitising effect of exercise training for obese individuals and individuals with T2D has been investigated in two studies to date. Edinburgh *et al.,* (2020) reported increased oral glucose sensitivity in overweight/obese individuals completing 6 weeks of training in the fasted state. Additionally, 12-weeks of endurance training in males diagnosed with T2D and randomised to exercising in an overnight fasted state or after breakfast, saw greater improvements in HbA1c when an exercise programme Is completed in the fed state compared to the fasted state. There was no difference in the ability to reduce fat mass, increase fat oxidation or improve HDL concentrations between nutritional strategies (Verboven *et al.*, 2020). While fasted exercise typically enhances IMTG utilisation, it is not yet clear whether this translates into greater benefits to insulin sensitivity and other markers of cardiometabolic health.

2.5.4 Training with Low Muscle Glycogen

The “train-low” paradigm has gained interest over the last decade, and typically consists of performing an initial bout of high intensity exercise (to reduce muscle glycogen concentrations) before a second exercise bout is performed several hours later or the following morning. Importantly, the ingestion of carbohydrate is restricted between sessions to prevent muscle glycogen resynthesis, such that the second exercise bout is commenced with low muscle glycogen concentrations. Under these conditions, rates of whole-body fat oxidation are augmented (Hansen *et al.*, 2005; Yeo *et al.,* 2008b; Morton *et al.,* 2009; Hulston

et al., 2010) and while there is no direct evidence for an increased IMTG utilisation when exercising with low muscle glycogen, one would speculate that this does occur. If true, this strategy could prove to be applicable for individuals with metabolic disease. Note though that research in this area to date is limited to highly trained males.

Importantly, in trained males systematically commencing exercise with reduced muscle glycogen availability augments the activation of signalling proteins (Baar and McGee, 2008) leading to elevated gene expression of mitochondrial proteins (Bartlett, Hawley and Morton, 2015). The signalling pathways stimulated by a reduced muscle glycogen availability have been explained in detail elsewhere (Hawley *et al.*, 2018; Hearris *et al*., 2018; Impey *et al.*, 2018). Briefly, low muscle glycogen availability stimulates greater activity of AMPK and p38MAPK (Wojtaszewski et al., 2003b; Chan et al., 2004), which in turn leads to activation and translocation of p53 and PGC-1α to the nucleus and mitochondria (Bartlett *et al.,* 2013; Andrade-Souza *et al.,* 2019). Here, these proteins help regulate the transcription of key mitochondrial proteins and those involved in mitochondrial fusion and fission. Exercising under conditions of reduced muscle glycogen availability also enhances circulating FFA concentrations, which in turn activates the nuclear transcription factor PPARδ (Philp *et al.,* 2013), to upregulate the expression of proteins linked to lipid metabolism, including ß-HAD (Yeo *et al.*, 2008b; Hulston *et al.,* 2010), HSL (Arkinstall *et al.*, 2004), and the FA transport proteins, FATBP and CD36 (Arkinstall *et al.*, 2004; De Bock *et al.,* 2008; Lane *et al.*, 2015). With reduced CHO oxidation, there is a concomitant increase in total lipid oxidation (Hearris *et al*., 2019) and step wise increases in AMPK activation showing that "train-low" provides a potent stimulus for promoting endurance adaptation.

Studies which incorporate training sessions that are commenced with low muscle glycogen availability over several weeks report increased activity and content of the mitochondrial proteins citrate synthase, β-HAD, and SDH (Hansen et al., 2005; Yeo et al., 2008a; Morton et al., 2009), alongside elevations in whole-body fat oxidation (Yeo et al., 2008a), and result in a greater contribution of IMTG to total energy expenditure during moderate-intensity exercise in well-trained cyclists (Hulston *et al.,* 2010). Increasing oxidative enzyme capacity supports greater fat oxidation, which in athletic populations, is a key consideration for improving substrate utilisation and promoting glycogen sparing, thereby enhancing performance. From

a clinical perspective, manipulating carbohydrate availability around exercise is also of relevance, since the adaptations outlined above would likely contribute to an improved IMTG turnover and underpin an increase in insulin sensitivity. Of course, using a prior exercise session to create a stimulus of low muscle glycogen availability (i.e., the 'traditional' train-low model) is challenging in sedentary, overweight/obese individuals, who likely will not perform exercise of sufficient intensity and/or duration to reduce glycogen below the 'threshold' required to stimulate the signalling responses mentioned above. Therefore, whilst effective, the traditional train-low model may only be adopted in overweight/obese individuals with high motivation and who have access to a specialist support network.

2.5.5 Low Carbohydrate High Fat Diet

Using diet may be a more appropriate strategy to generate a state of low muscle glycogen availability in sedentary overweight/obese individuals with or without T2D. To target fat oxidation, macronutrient intake can be manipulated by either a non-ketogenic low-carbohydrate high-fat diet (where fat supplies 60-65% fat), or by a ketogenic low-carbohydrate high-fat diet (where fat supplies 75-80% of daily intake) (Burke *et al.,* 2020). The purpose of a ketogenic diet is to induce fasting-like effects and leads to the production of ketone bodies, which can provide an additional substrate for oxidative energy production. Moreover, a ketogenic diet therefore leads to increases in whole-body fat oxidation rates, and a subsequent reduction in whole-body carbohydrate oxidation, which can be explained entirely by a decrease in muscle glycogen utilization (Starling *et al.,* 1997; Burke *et al.*, 2000; Helge *et al.,* 2001). Unlike the above methods of glycogen manipulation, a ketogenic diet is typically a chronic strategy to maximise fat as a source of fuel (Burke, 2021).

The specific adaptations to a ketogenic diet, and the timeline of these adaptive responses, are controversial (Lindseth, 2017; Burke *et al.*, 2020), but in terms of exercise capacity, they seem to be related to increased delivery, uptake and oxidation of free fatty acids in skeletal muscle (see detailed review by (Burke, 2021). Adaptations to a ketogenic diet in trained athletes can occur as quickly as within 5 days but are often accompanied by feelings of fatigue due to exposure to this extreme change in diet (Burke *et al.,* 2017). Over a longer period (12 weeks) though, it was recently reported that a LCHF ketogenic diet combined with exercise

increased mitochondrial respiratory control ratio, ATP production and muscle triglyceride content (Miller *et al.*, 2020). Whilst there is still little data on changes in mitochondrial proteins, LD proteins or IMTG content in human skeletal muscle in response to a ketogenic diet, there is a robust increase in whole-body fat oxidation.

A non-ketogenic LCHF diet also enhances whole-body fat oxidation studies investigating the mechanisms for greater oxidative capacity due to high fat diet typically investigate this short-term using a non-ketogenic LCHF diet. Following 5 days of a high fat diet, increases in whole-body fat oxidation occurred, despite reduced skeletal muscle mitochondrial respiration (Leckey *et al.*, 2018). Importantly, a 12% increase in FAT/CD36 protein was observed, which suggests an increased capacity for FA uptake (Leckey *et al.*, 2018). Therefore, it is likely that increased whole-body fat oxidation following a non-ketogenic LCHF diet is the result of increased transport and delivery of FA to skeletal muscle. To date, no study has detailed the mechanisms underpinning the increase in fat utilisation in human skeletal muscle, yet previous studies has shown that short-term exposure (~5 days) to a high fat diet increases IMTG content (Yeo et al., 2008a), HSL content (Stellingwerff *et al.,* 2006), and the protein abundance of FAT/CD36 (Cameron-Smith *et al.*, 2003) and carnitine palmitoyl transferase (Goedecke *et al.,* 1999). These adaptations demonstrate an adaptive response which could improve the capacity of the exercising muscle to increase the breakdown and resynthesis of IMTG stores in response to long-term ingestion of a LCHF diet. Future studies should then investigate whether an improved turnover of fat at a whole-body and muscle level contribute to the improved glycaemic control reported following a LCHF diet (Ahmed et al., 2020).

Whilst the above addresses the potential impact of chronic changes in diet on IMTG turnover, it should also be noted that macronutrient intake in the hours and days following an exercise bout can impact IMTG stores. For example, traditional sports nutrition guidelines suggest the consumption of a high carbohydrate diet following exercise in order to support glycogen resynthesis (Ivy, 1991). However, when diets high in carbohydrate, and therefore low in fat (<15% energy from fat), are consumed post-exercise, IMTG resynthesis is shown to be substantially impaired (Decombaz et al., 2000; Decombaz et al., 2001; Larson-Meyer, Newcomer and Hunter, 2002). Even when the diet is made up of ~24% energy from fat (which could be considered quite typical of a high carbohydrate diet), IMTG repletion over the

subsequent 48 h following exercise is still impaired (van Loon et al., 2003b). To date, work in this area has only used healthy, trained individuals who have high rates of IMTG utilisation during exercise resulting in a net decrease in IMTG content post-exercise. Given that a net decrease in IMTG content following exercise appears to be absent in obese individuals or T2D patients, a high-fat diet in the period following exercise could theoretically contribute to aberrant storage of IMTG and the generation of lipid metabolites. Future research is required to examine this though.

2.5.6 Weight loss and Calorie Restriction

Weight loss achieved via a restriction in caloric intake (but where the relative macronutrient contribution remains the same) is a powerful strategy to improve insulin sensitivity in overweight and obese individuals with or without T2D (Moore *et al., 2000)*. Notably, diet-induced weight loss often results in a decrease in IMTG content (Goodpaster *et al.,* 2000; Dube *et al.,* 2011), and this occurs alongside reductions in the concentrations DAGs and ceramides in muscle (Dube *et al.,* 2011), suggesting a remodelling of the intramuscular lipid pool.

Six months of CR increased markers of mitochondrial biogenesis and mitochondrial DNA and reduced oxidative stress markers in overweight individuals (Civitarese *et al.,* 2007), but key enzymes of the TCA cycle, β-oxidation and electron transport chain were unchanged. Over 16-weeks, CR has shown to increase citrate synthase activity (Menshikova *et al.*, 2017) and reduce skeletal muscle lipid content (Goodpaster *et al.,* 2000), but does not alter mitochondria volume or enzymes from the beta oxidation pathway (Menshikova *et al.*, 2017). Even when similar weight loss is achieved, a CR intervention alone does not achieve improvements in mitochondrial content or electron transport chain enzyme activity, whereas a CR plus exercise intervention does (Toledo *et al.,* 2008). CR has also been shown to reduce IMTG in skeletal muscle, as well as mRNA of genes involved in lipogenesis and FA transport yet showed no change in mitochondrial content or mRNA genes involved in mitochondrial biogenesis (Sparks *et al.,* 2017).

A combined intervention of exercise and CR in an athletic population increases IMTG content (Nadeau *et al.,* 2006), and skeletal muscle oxidative capacity (Pruchnic *et al.*, 2004; Nadeau *et al.,* 2006). Conversely, weight loss induced by a combination of exercise and CR in obese individuals either decreases (Rabol *et al.,* 2009), or does not change IMTG content, but leads to reductions in LD size and increases mitochondrial content (He, Goodpaster and Kelley, 2004). This suggests that a combined exercise and weight loss intervention remodels the LD pool such that the ability to utilise fat as a fuel source and regularly turnover IMTG would be enhanced, rather than simply reducing the amount of IMTG in skeletal muscle. Considering long-term CR interventions fail to improve oxidative capacity, a combined intervention of CR and exercise to reduce lipid metabolites and improve mitochondrial oxidative capacity, is likely the most powerful strategy to increase IMTG utilisation and improve skeletal muscle lipid turnover in individuals at risk of T2D.

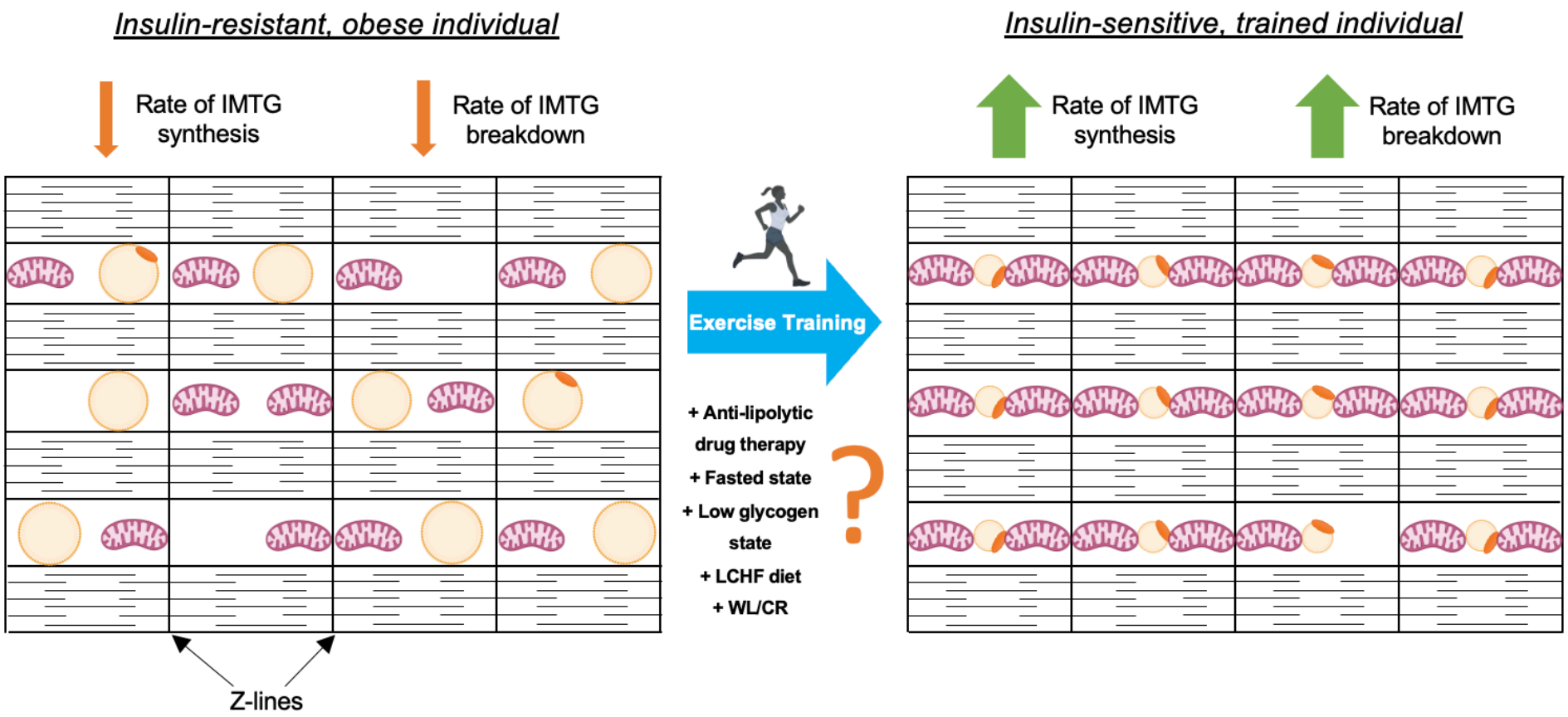

Insulin-resistant, obese individual
Rate of IMTG synthesis
Rate of IMTG breakdown
Z-lines
Exercise Training
+ Anti-lipolytic drug therapy
+ Fasted state
+ Low glycogen state
+ LCHF diet
+ WL/CR
?
Insulin-sensitive, trained individual
Rate of IMTG synthesis
Rate of IMTG breakdown

Figure 2.5 Schematic overview of the distribution of LD and mitochondria in skeletal muscle in insulin-resistant, obese individuals and insulin-sensitive, trained individuals. Endurance, resistance, or high intensity interval exercise training promotes a shift towards a more structured network of LD and mitochondria; that is, more LD are labelled with PLIN proteins (PLIN2, 3, 4 or 5, but indicated as one by the small orange ellipse) and are located in close proximity to the mitochondria. This collective adaptation to exercise training enables more efficient breakdown and oxidation of IMTG stored in LD. This is important in order to create a net decrease in IMTG following exercise, which subsequently increases the capacity for IMTG synthesis and storage in the hours following exercise. Thus, following training the overall turnover rate of the IMTG pool is enhanced, which reduces the risk of generating and accumulating toxic lipid metabolites that would otherwise contribute to the development of insulin resistance. In obesity and T2D there is a poor capacity to reduce the rate of IMTG synthesis during exercise and therefore generate a net decrease in IMTG content post-exercise. Additional strategies to exercise alone may therefore be required to create a post-exercise decrease in IMTG content in obese individuals. We propose several co-strategies (anti-lipolytic drug therapy, fasted exercise, training with low muscle glycogen, a LCHF diet or WL/CR) that target an improved rate of IMTG turnover, and therefore maximize the insulin-sensitising effects of exercise training alone in obese individuals. Future work is now required to test these interventions in the longer term in clinical populations, with consideration for how these interventions can be successfully adopted in the real world.

2.6 Practicality and barriers to implementation

Although all the interventions above have been shown to enhance fatty acid utilisation, increase weight loss and improve insulin sensitivity, but the for the general population needs to be considered. We present data first on the use of exercise to enhance IMTG utilisation, and it is clear that with exercise training, oxidative capacity Is enhanced and the ability to use IMTG stores as a source of fuel is improved. However, a poor capacity to simultaneously reduce the rate of IMTG synthesis during exercise in individuals with obesity and T2D results in a minimal decrease in IMTG content post-exercise (van Loon *et al.,* 2004) and therefore minimal IMTG turnover. Furthermore, the adherence and compliance to moderate intensity endurance or high intensity interval exercise programs in these populations is often low due to lacking in confidence and fear of exercise.

Exercise combined with several different nutritional manipulations to further enhance IMTG turnover are considered, some of which are common practice in elite endurance sport such as 'train low' and training fasted. Both methods do in fact increased fatty acid oxidation under these conditions and so arguably would be an effective programme to increase IMTG turnover. However, an exercise intervention where individuals are required to exercise twice a day to ensure the second session is carried out with low muscle glycogen is not suitable for individuals with obesity. Adherence and compliance to this type of exercise would be very low due to the demanding nature.

Consequently, different ways to further enhance the effects of exercise where a lower intensity exercise programme could be utilised, either with the addition of an anti-lipolytic drug or by exercising fasted, may be more feasible. The short-term increases in IMTG utilisation during a single bout of exercise performed fasted or with the ingestion of acipimox (van Loon et al., 2005a) indicate that these may be two suitable interventions to be prescribed to individuals at risk of developing T2D.

2.7 Summary

It is now widely agreed that the absolute level of IMTG is unrelated to insulin resistance, but rather a high rate of turnover of the IMTG pool appears to be fundamental to the preservation of insulin sensitivity. Thus, in the trained state IMTG represents a highly dynamic lipid pool within muscle that can be adjusted relative to metabolic demand. Chronic exercise training creates a stimulus of regular breakdown and resynthesis of the IMTG pool, reducing the potential for accumulation of toxic lipid metabolites and therefore the risk of skeletal muscle insulin resistance. In obese individuals and people with T2D there is a collective imbalance between the rate of FA uptake into muscle, esterification, and storage, IMTG breakdown and oxidation. Despite many years of research, the molecular mechanisms underlying this imbalance are yet to be fully understood. The discovery that the PLIN proteins may play a role regulating LD dynamics has provided additional understanding over the last decade, but efforts to identify the mechanisms responsible for the low turnover of the IMTG pool in obesity and T2D should continue to form the basis of future work. Exercise training studies demonstrate that improved IMTG turnover is a key adaptation contributing to improved insulin sensitivity at both the skeletal muscle and whole-body level. Importantly, there appears to be potential for the use of pharmacological or nutritional strategies to maximise the insulin-sensitising effect of exercise interventions. Future work is now required to test these interventions in the longer term in clinical populations, with consideration for how these interventions can be successfully adopted in the real world.

Chapter 3: Thesis Overview

3.1 Thesis Overview

Chapter 1 has provided an overview of the mechanisms regulating insulin-dependent glucose uptake and how this underpins normal glucose homeostasis in healthy, insulin sensitive individuals. Subsequently, it was also highlighted how glucose homeostasis and metabolism is impaired in obese, insulin resistant individuals, which manifests as a result of disrupted insulin-dependent glucose uptake. The poor insulin sensitivity exhibited by obese individuals is a consequence of low physical activity levels combined with excess energy intake, leading to disruption to the function of adipose tissue and lipid spill over into skeletal muscle. Thus, as a result elevated lipid availability reduces insulin-dependent glucose uptake in skeletal muscle which is mediated by GLUT4. The aim of **Chapter 4** was to investigate whether GLUT4 translocation was impaired under conditions of high plasma FFA from a lipid infusion, and whether this differed between trained and untrained individuals. It is hypothesised that GLUT4 translocation will be reduced at 6 hours of the lipid infusion compared to the glycerol control and this would be comparable between trained and sedentary individuals. Secondly, it was hypothesised that we would see differences in GLUT4 spot size and number at baseline and between trained and sedentary individuals.

The aim of **Chapter 2** was to synthesise the current evidence to demonstrate that the dynamic nature of the IMTG pool in trained individuals is a crucial characteristic to the preservation of insulin sensitivity, and secondly to explore potential interventions to enhance IMTG turnover. Here, the importance of sufficient IMTG turnover was emphasised, and how dysregulation of IMTG synthesis, storage, and breakdown, leads to the accumulation of lipid metabolites that ultimately lead to disruption of the insulin signalling cascade. Key interventions were discussed that target the improvement in IMTG use during exercise and may be applicable to those at risk of developing type 2 diabetes. Throughout this chapter, several gaps within our knowledge were identified regarding multiple interventions that may have a positive impact on IMTG turnover, and whether they are effective and feasible in obese individuals. The studies that follow in this thesis are designed to begin to examine which interventions known to improve IMTG use during exercise are suitable in obese individuals.

First, **Chapter 2** discussed the use of an anti-lipolytic drug to reduce plasma FFA and improve the use of IMTG during exercise. However, whether this strategy was effective during exercise of a low intensity (which was manageable for obese individuals) was not known, and no previous study had measured changes in lipid metabolites in skeletal muscle when exercise was combined with the use of an anti-lipolytic drug. Therefore, the aim of **Chapter 5** was to investigate whether the suppression of plasma free fatty acids alters substrate use and reduces the presence of lipid metabolites in skeletal muscle. An acute intervention was designed to report the effects of a single exercise bout with and without the ingestion of Acipimox, on plasma FFA concentrations and the impact on lipid metabolite content during and in the hours following exercise. It was hypothesised that there would be an increase in overall carbohydrate oxidation in the trials with acipimox ingestion, and an increase turnover of the intramuscular lipid pool reducing the prescence of lipid metabolites.

Chapter 2 also discussed how exercising before breakfast (i.e., following an overnight fast) may result in a greater contribution of IMTG to total fat oxidation. However, evidence for whether this strategy could improve insulin sensitivity and glycaemic control in the long-term was equivocal, and furthermore no study had compared the feasibility and effectiveness of this type of intervention outside of the laboratory (i.e., in a field-based study). Therefore, the aim of **Chapter 6** was to measure the adherence and compliance to a 'real world' intervention where exercise was undertaken before or after breakfast (i.e., fasted or fed), and effects on key health markers, including the acute and chronic postprandial glucose response to continuous and interval walking sessions in overweight and obese individuals were assessed. It was hypothesised that there would be a greater improvement in key health markers in the individuals in the fasted exercise group.

3.1.1 Impact of COVID-19

As a result of the 2020/2021 COVID-19 pandemic, my planned final research study had to be changed to ensure research could continue effectively. Although what is now chapter 6 of this thesis was not in the original plan, it adds nicely to the objectives of this thesis whilst also answering some key questions raised within the literature.

The original study that would form chapter 6 of my thesis was designed to investigate the hypothesis that an exercise programme of steady walking will have larger effects on insulin sensitivity and glycaemic control when combined with Acipimox intake prior to each exercise session in people with obesity and a diagnosis of prediabetes (HbA1c >47 mmol/mol). The design of this study included extensive pre- and post-testing measures to generate high quality, gold standard measures to assess our primary outcomes. For this, an MRI scan of hepatic fat was included, as well as a hyperinsulinemic euglycemic clamp to measure insulin sensitivity. As a result, whilst working with Astra Zeneca to design the analysis protocol Chapter 4 in the first year of funding for my PhD (Nov 2018 – Nov 2019), I also spent a lot of time at the Liverpool MRI centre shadowing the scanning process for patients undergoing an MRI scan. I was also frequently attending Aintree University Hospital to learn the complex protocol of the hyperinsulinemic euglycemic clamp, as well as practicing this method on 3 individuals.

Whilst these considerations for the COVID-19 global pandemic did alter the original thesis plan, the studies presented in this thesis collectively challenge the original hypotheses outlined above.

Chapter 4: GLUT4 localisation with the plasma membrane is unaffected by an increase in plasma FFA availability.

4.1 Abstract

Insulin stimulated glucose uptake into skeletal muscle occurs due to translocation of GLUT4 from intracellular storage vesicles to the plasma membrane. Typically, lipid-induced whole body insulin resistance was associated with inhibition of the insulin signalling cascade, however previous research shows no change in the tyrosine phosphorylation or activation of insulin receptor substrate (IRS)-1, IRS-1–associated phosphatidylinositol (PI) 3-kinase, Akt, and AS160. This study aimed to investigate the changes in GLUT4 colocalization to the plasma membrane and the changes in location and number of both large and small GLUT4 spots that occurs following 2h and 6h of either a glycerol or an intralipid infusion. Percutaneous muscle biopsy samples were taken from the *m. vastus lateralis* from trained (*n* = 11) and sedentary (*n* = 10) individuals in the basal state and following 120 and 360 minutes of either a lipid or a glycerol infusion. At baseline, trained individuals had a greater number of small GLUT4 spots in the plasma membrane where sedentary individuals had larger GLUT4 spots. Following infusion, GLUT4 localisation to dystrophin, measured using the Pearson's correlation coefficient, was increased in both infusion groups at 120 minutes, with no change in GLUT4 co-localization with the plasma membrane from 2h to 6h of the lipid infusion. The number of GLUT4 spots at the plasma membrane or the 1 µm below the membrane did not change following 2h of either the lipid or glycerol infusion compared to baseline. However, from 2h to 6h of either infusion there was a significant decrease in the number of GLUT4 spots at the plasma membrane and layer 1. More specifically, the reduction in total GLUT4 spots in the plasma membrane and layer 1 from 2h to 6h of infusion could be entirely accounted for by a decrease in small GLUT4 spots.

4.2 Introduction

Insulin-mediated glucose uptake into muscle

In healthy individuals, the physiological increase in insulin following a meal is a potent stimulus for plasma glucose uptake into skeletal muscle (Capaldo et al., 1999). Four hours following oral ingestion of 92g of glucose, leg glucose uptake is reported to increase three-fold (Katz et al., 1983), and during a hyperinsulinaemic-euglycaemic clamp leg glucose uptake is elevated approximately five-fold (DeFronzo et al., 1985). The fact that ~80% of glucose removed from the circulation during a hyperinsulinaemic-euglycaemic clamp in insulin sensitive individuals enters skeletal muscle (DeFronzo et al., 1981; Thiebaud et al., 1982), makes this tissue into the most important determinant of glucose homeostasis, with dysregulation of glucose uptake into muscle having implications for the development of insulin resistance.

Glucose uptake into skeletal muscle occurs predominantly through facilitated diffusion, where glucose transporter proteins hold a fundamental role. In total, there are 14 identified glucose transporter isoforms (Uldry and Thorens, 2004), of which, glucose transporter 4 (GLUT4) is the predominant insulin-responsive isoform required for glucose uptake into skeletal muscle (Watson and Pessin, 2001). At rest, GLUT4 resides in intracellular cytosolic micro-vesicles (GLUT4 storage vesicles; GSV), but in response to increases in plasma insulin concentrations or muscle contraction an increased number of subsarcolemmal GSV's merge with the plasma membrane. Early studies used density gradient centrifugation methods to isolate pure plasma membrane fractions and subsequently measure the protein expression of GLUT4. Using this approach, Guma et al (Guma et al., 1995) reported a 60% increase in plasma membrane GLUT4 content 30-40 min after the start of a hyperinsulinaemic-euglycaemic clamp.

However, fractionation methods (aside from the potential cross-contamination issues) preclude the ability to understand the cellular distribution of GLUT4. To overcome these issues, electron microscopy or fluorescence microscopy of immuno-stained GLUT4 in muscle fibres have provided both quantitative and spatial information regarding the location of GLUT4 vesicles during basal, insulin-stimulated, and contraction-stimulated states (Ploug et

al., 1998). Studies using both confocal immunofluorescence microscopy and electron microscopy combined with immuno-gold labelling in whole single fibres of rat soleus muscle have shown that GLUT4 is present in the trans-Golgi network (TGN) membranes, endosomal membranes and GSV's (Rodnick et al., 1992; Ploug et al., 1998; Lauritzen et al., 2008; Lizunov et al., 2012). Studies using immunofluorescence microscopy in muscle fibres of rodents have defined GSV's in the TGN as having a diameter >1 μm (Lauritzen et al., 2008). Interestingly, electron microscopy images have shown that endosomes appear smaller than TGN stores (Ploug et al., 1998), but larger than GSV's that are reportedly as small as 40 nm (Lizunov et al., 2012). In mice, *in vivo* methods have been developed where mice express GLUT4-HA that can confirm GLUT4 insertion into the plasma membrane (Fazakerley et al., 2009; Schertzer et al., 2009), however the chronic expression of tagged-GLUT4 in human models is not possible. Bradley *et al.,* (Bradley et al., 2015) developed an immunofluorescence microscopy method to visualize changes in the subcellular distribution and content of GLUT4 in response to oral glucose ingestion and exercise. This study demonstrated a measurable increase in GLUT4 co-localisation with the plasma membrane in response to both glucose ingestion and exercise, alongside depletion of GLUT4 from large and small clusters (Bradley et al., 2015). Thus, using this immunofluorescence microscopy method it appears that both exercise and glucose ingestion (separately) stimulate net GLUT4 translocation to the plasma membrane. Missing from this work, however, was whether fibre type differences in GLUT4 distribution and translocation exist.

Elevated plasma free fatty acid (FFA) and triglyceride concentrations give rise to lipid accumulation in skeletal muscle (intramuscular triglyceride; IMTG) in obesity and type 2 diabetes, which is subsequently linked to the development of insulin resistance (Barrett et al., 2022). More specifically, it is the accumulation of lipid metabolites, such as diacylglycerols and ceramides, which directly impact insulin signalling leading to impaired insulin-stimulated glucose uptake in obese individuals and type 2 diabetes patients (Goodpaster et al., 1997; Forouhi et al., 1999). Lipid infusion is a well-established model of lipid-induced insulin resistance, and specifically causes a reduction in peripheral glucose uptake when lipid is infused alongside a hyperinsulinaemic-euglycaemic clamp (Boden et al., 1994; Shah et al., 2002). Importantly, 2h of lipid infusion does not reveal any decrease in glucose disposal, but this does become apparent later on (Boden et al., 1994). In response to lipid infusion, IMTG

content increases independent of training status (Chow et al., 2014). Importantly though, there is a differential increase in diacylglycerols and ceramides. For example, Chow *et al.,* (2012) demonstrated that acute FFA elevation induced comparable reductions in insulin sensitivity between trained and sedentary individuals (Chow et al., 2012), but that trained individuals see preferential accumulation of IMTG whereas sedentary individuals accumulate both IMTG and DAG (Chow et al., 2014). Interestingly, despite reduced glucose disposal, Chow et al (2014) report no change in the phosphorylation or activation of key insulin signalling components, including insulin receptor substrate (IRS)-1 tyrosine, IRS-1–associated phosphatidylinositol (PI) 3-kinase, Akt, and AS160 (Chow et al., 2014). This leads us to question whether the suppression of glucose uptake from a lipid infusion impacts the availability of GLUT4 at the plasma membrane.

The aim of the present study was to visualize the co-localisation of GLUT4 to the plasma membrane in human skeletal muscle in response to an Intralipid™ infusion that has previously been shown to reduce glucose uptake (Chow et al., 2012; Eldor et al., 2017). Specifically, we aimed to investigate the changes in GLUT4 colocalization to the plasma membrane marker dystrophin and the changes in location and number of both large and small GLUT4 spots that occurs following 2h and 6h of either a glycerol or an Intralipid™ infusion alongside a concomitant hyperinsulinaemic-euglycaemic clamp. We tested the hypothesis that GLUT4 colocalization to the plasma membrane would increase at 2 h of either a glycerol or Intralipid™ infusion, but would be reduced after 6 h of the Intralipid™ infusion only.

4.3 Methods

4.3.1 Participants and ethical approval

The muscle samples used in the present study were collected as part of a previous study and therefore the process of recruitment and study protocol have already been described in detail (Chow et al., 2012; Chow et al., 2014). The current study included 11 healthy lean trained individuals and 10 sedentary individuals that were recruited and matched for sex, age (±5 years) and BMI (±1.5 kg/m^2). Subject characteristics for each group are presented in Table 1. The viability of the samples from two participants (both trained individuals; one from the lipid infusion group and one from the glycerol group) were compromised (due to frost damage)

and were not included in the final analysis (9 trained, 10 untrained). Glucose infusion rates for all the trained individuals was 66.1 ± 4.7 (μmol glucose infused.kg .FFM^{-1} .min^{-1}) and for sedentary was 48.3 ± 5.7 (μmol glucose infused.kg .FFM^{-1} .min^{-1}). Plasma FFA at the end of 6 h lipid infusions were 600 ± 86 μmol. l^{-1} and 932 ± 105 μmol. l^{-1} f or trained and sedentary individuals, respectively. Sedentary individuals participated in 30 minutes or less of active exercise per week, whereas trained individuals participated in a regular running program (≥45 min/day, ≥5 days/wk). Training was self-reported using the short form International Physical Activity Questionnaire, a validated physical activity questionnaire (Craig et al., 2003). The study protocol was approved by the University of Minnesota Institutional Review Board and informed consent was obtained from all participants.

Table 4.1. Baseline characteristics of trained and sedentary participants.

	Trained Lipid (*n* =5)	**Trained Glycerol (n=4)**	**Sedentary Lipid (n=5)**	**Sedentary Glycerol (n=5)**	***P* value**
Sex (males/females)	3/2	2/2	2/3	3/2	0.98
Age (years)	23 ± 1	24 ± 1	21 ± 1	21 ± 1	0.26
BMI (kg. m^{-2})	22.2 ± 0.6	22.9 ± 0.8	21.3 ± 0.6	21.9 ± 0.6	0.31
FFM (kg)	54.8 ± 3.7	52.4 ± 4.0	45.9 ± 2.3	43.2 ±	0.04
Body fat (%)	19.9 ± 2.0		27.4 ± 3.5		0.07
VO_2 $_{max}$ (ml. kg^{-1} .min^{-1})	48 ± 5	48 ± 4	40 ± 6	39 ± 5	<0.01

Data are the mean ± SEM. FFM, free fat mass; GIR, glucose infusion rate.

4.3.2 Study protocol

The study protocol has been described in detail previously (Chow et al., 2012; Chow et al., 2014). In an independent groups design, participants were allocated to either a lipid or glycerol infusion to ensure that age, gender, BMI and activity level between groups is matched (Chow et al., 2012). Briefly, pre-screening assessments were carried out of body composition (dual-energy X-ray absorptiometry) maximal aerobic fitness (VO_{2max}) and insulin sensitivity (3 h hyperinsulinaemic euglycemic clamp). On a separate day, participants attended the

Masonic Clinical Research Unit (MCRU) at the University of Minnesota and consumed a standard evening meal (41% carbohydrate, 32% fat and 27% protein) before remaining on bed rest at the unit overnight until study completion the following day. After an overnight fast, participants underwent either a 6 h lipid infusion (20% Intralipid® at 90 ml h^{-1} [Baxter, Deerfield, IL, USA]) or 6 h glycerol infusion (2.25 g/100 ml at 90 ml/h) concurrent with a hyperinsulinemic euglycemic clamp. The glycerol infusion matched the glycerol content of the lipid infusion to limit the effect of the lipid infusion on FFA elevation (Chow et al., 2014). Muscle biopsies were obtained prior to the initiation of the infusion (Bx1), at 120 minutes (Bx2) and at 360 minutes (Bx3) of infusion. Each muscle biopsy was dissected free of fat and connective tissue before being embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Alphen aan de Rijn, The Netherlands) and frozen in liquid nitrogen-cooled isopentane for immunohistochemical analyses.

4.3.3 Muscle analysis

Immunohistochemistry

The immunohistochemistry staining protocol has been previously reported by Bradley *et al.* (2014). Briefly, serial 5 µm cryosections were cut at -30°C and transferred to ethanol-cleaned glass slides and fixed and permeabilized in 75% acetone with 25% ethanol for 5 minutes (Bradley et al., 2014). Slides were then washed 3 times for 5 min in phosphate-buffered saline (PBS, 137 mmol/L sodium chloride, 3 mmol/L potassium chloride, 8 mmol/L sodium phosphate dibasic, 3 mmol/L potassium phosphate monobasic). The primary antibody targeting GLUT4 (rabbit IgG, Abcam, Cambridge, UK) was applied to the sections at a dilution of 1:200 in 5% normal goat serum (ThermoFisher) and was incubated at room temperature for 2 h. The GLUT4 antibody was combined with an antibody targeting dystrophin (Sigma Aldrich, Dorset UK), to visualise the plasma membrane, and an antibody targeting myosin heavy chain for slow twitch fibres to visualise type I fibres (MHC1; mouse IgM,). Following primary antibody incubation, slides were then washed 3 times for 5 min in PBS. Secondary antibodies were applied to the slides for 30 mins at room temperature. The GLUT4 antibody was targeted with goat anti-rabbit IgG 488, dystrophin with goat anti-mouse IgG_{2b} 546 and MHC1 with goat anti-mouse IgM 633 (Invitrogen, Paisley, UK). Following secondary antibody incubation, slides were washed 3 times for 5 min in PBS and coverslips were mounted with

20 µL mowiol mounting medium [6 g glycerol, 2.4 g mowiol 4–88, and 0.026 g 1,4-Diazabicyclo [2.2.2] octane (DABCO) dissolved in 18 mL 0.2 M Tris buffer (pH 8.5) (All reagents were purchased from Sigma Aldrich, St Louis, MO)] and sealed with nail varnish.

4.3.4 Image capture, processing, and analysis

Cross-sectional orientated images were captured using an inverted confocal microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany) with a 63x 1.4 NA oil immersion objective at 1.1 digital zoom. The Alexa Fluor 488 fluorophore was excited with an argon laser, whereas the Alexa Fluor 546 and 633 fluorophores were excited with a helium–neon laser. The objective and magnification used ensured that a single fibre was captured per image, and each imaged fibre was chosen at random only considering the fibre type and not the GLUT4 stain.

Image analysis of GLUT4 content was undertaken using Image-Pro Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA). For each participant, at least 30 images per time-point were taken. Five participants out of the 19 individuals only had samples for two out of the three time points. Therefore, in total there was 204 type I fibres and 201 type II fibres analysed for the lipid infusion group, and 279 type I fibres and 307 type II fibres analysed for the glycerol lipid infusion group.

Fibre type specific GLUT4 content was determined by measuring the fluorescence intensity of the GLUT4 stain. When assessing fibre specific GLUT4 content, fibres stained positively for myosin heavy chain type I were classified as type I fibres, whereas those with no staining were classified as type II fibres. For image analysis of GLUT4 co-localisation to the plasma membrane, Pearson's correlation coefficient was carried out between the GLUT4 stain and dystrophin border. For quantitation of GLUT4 in the plasma membrane layer (dystrophin-stained region) and in the five 1 µm intracellular layers below the plasma membrane, image processing and analysis was carried out in MAT- LAB (v. 2012b, The MathWorks Inc., Natick, MA, 2012) using an image analysis algorithm, as previously used by Bradley *et al.* (2014). Briefly, the analysis algorithm separated the fibres in the dystrophin image using the active contour, or snake, approach (Kass, Witkin and Terzopoulos, 1987) to approximately find the mid-point of the plasma membrane. A distance map from the contour then generated a 3-

pixel thick region to cover the dystrophin-stained region and was designated the plasma membrane layer. Subsequently, five 1 µm thick layers were generated inside the fibre, again using the distance map. To identify spots from background staining an intensity threshold was set for each participant. To then distinguish between large and small spots, threshold limits were set for the spot sizes detected (large spots: >1 µm or small spots: <1 µm diameter, as in (Rodnick et al., 1992; Ploug et al., 1998; Lauritzen et al., 2008; Lizunov et al., 2012; Bradley et al., 2014; Bradley et al., 2015).

4.3.5 Statistical analysis

Statistical analysis was carried out in SPSS All analyses were performed using statistical analysis software (SPSS for Mac version 26.0; SPSS, Chicago, IL, USA). Multiple group comparisons to assess GLUT4 protein expression, co-localization and clusters were performed: between: (i) lipid and glycerol infusion groups, (ii) trained and sedentary individuals, (iii) type 1 and type 2 fibres, (iv) time points, and (v) layers for cluster analysis. Linear mixed effects models, with random intercepts to account for repeated measurements within subjects, were used to examine group differences, as well as differences over time of the infusion and between fibre types. Pairwise differences between biopsies were performed using post hoc tests. $P < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Content and location of GLUT4 in human skeletal muscle at baseline

Concurrent with previous findings (Bradley et al., 2014; Bradley et al., 2015), GLUT4 staining in human skeletal muscle revealed both large clusters and small spots throughout the cell (see figure 1). Both large GLUT4 clusters and small GLUT4 spots can be seen close to and incorporated within the plasma membrane (stained in red with dystrophin). However, in contrast to Bradley *et al.,* (2014; 2015), we observed noticeably less spots within all images.

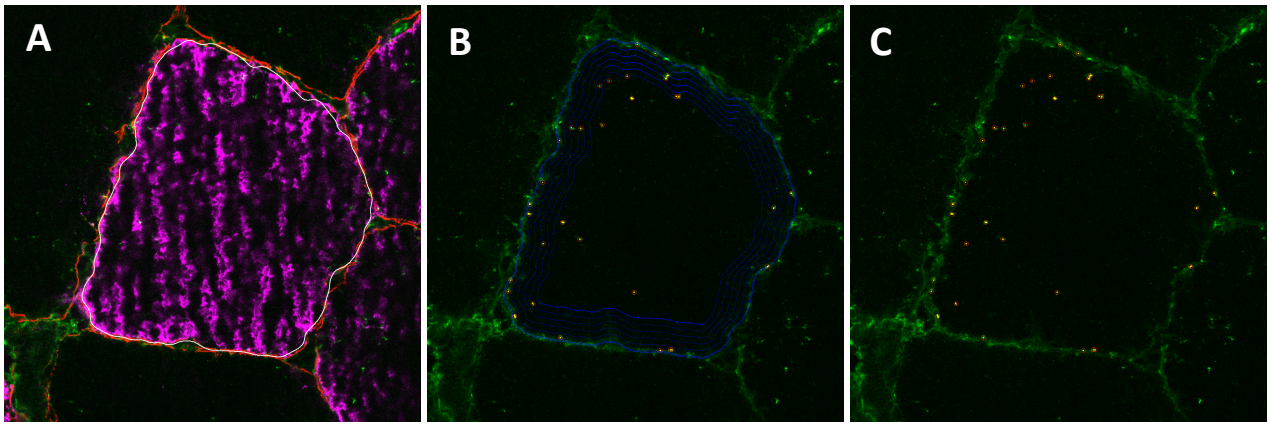


Figure 4.1. Representative immunofluorescence microscopy image detailing the dystrophin boarder identification (A), the addition of the 5 x 1 μm intracellular layers (B), and the GLUT4 spot identification (C).

Using immunofluorescence microscopy, it was observed that at baseline total protein expression of GLUT4 was greater in type 2 fibres compared to type 1 fibres (P = 0.011) but was not different between trained and sedentary individuals (P = 0.477). Pearson's correlation coefficient was used to determine the relative localisation of GLUT4 with the dystrophin stain (i.e., the plasma membrane), and was greater in trained individuals compared to the sedentary group at baseline (main training status effect; P = 0.020; figure 3). Importantly, co-localisation was not different between infusion groups (P = 0.909). GLUT4 fluorescence intensity was used as a marker of GLUT4 protein expression in the plasma membrane and the 5 intracellular layers, and at baseline GLUT4 fluorescence intensity was greatest in the plasma membrane compared to all intracellular layers (main effect of layer; P<0.001).

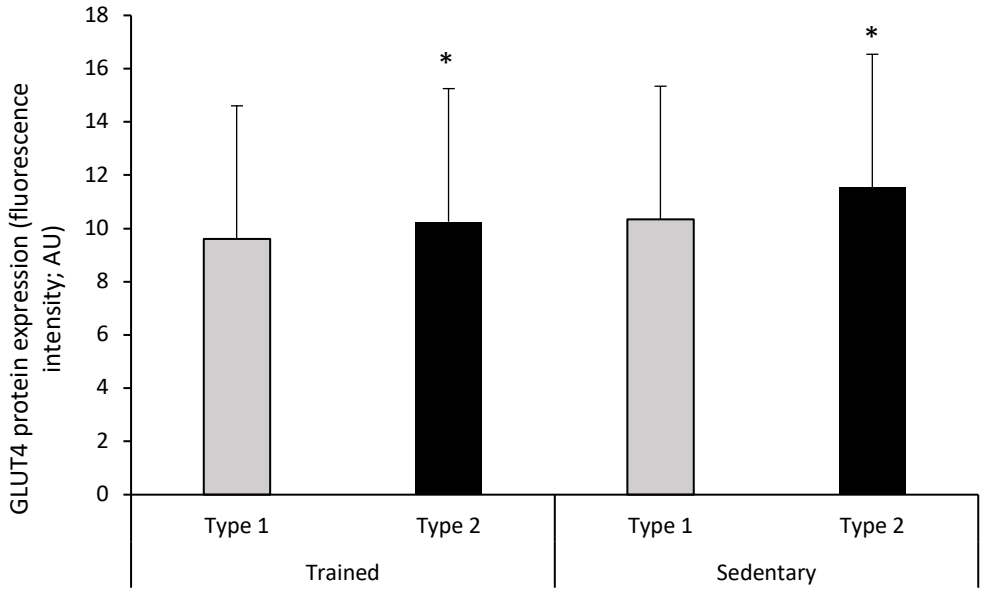


Figure 4.2. Total GLUT4 fluorescence intensity in type 1 (grey) and type 2 fibres (black) in the basal state. Data are mean ± SD. **Main fibre type effect; P=0.001.*

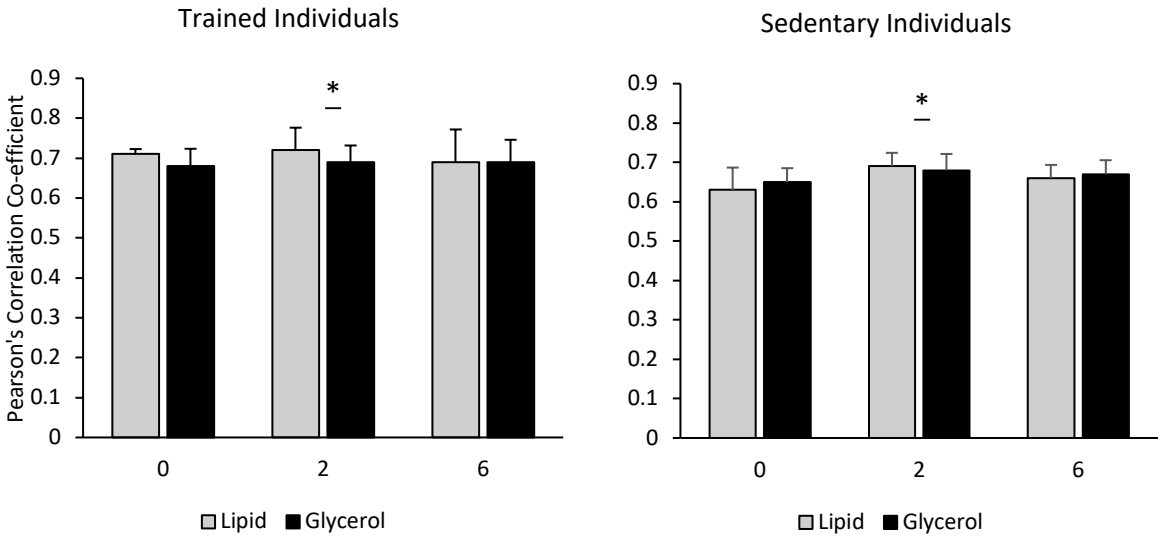


Figure 4.3. Colocalization of GLUT4 with PM marker dystrophin, measured using the Pearson's correlation coefficient using linear mixed model. Main time effect *P* = 0.039, with greater PCC at 2h vs 0h of infusion*. There was no difference between 2 and 6h (*P* = 0.557) or 0 and 6h (*P* = 0.522).

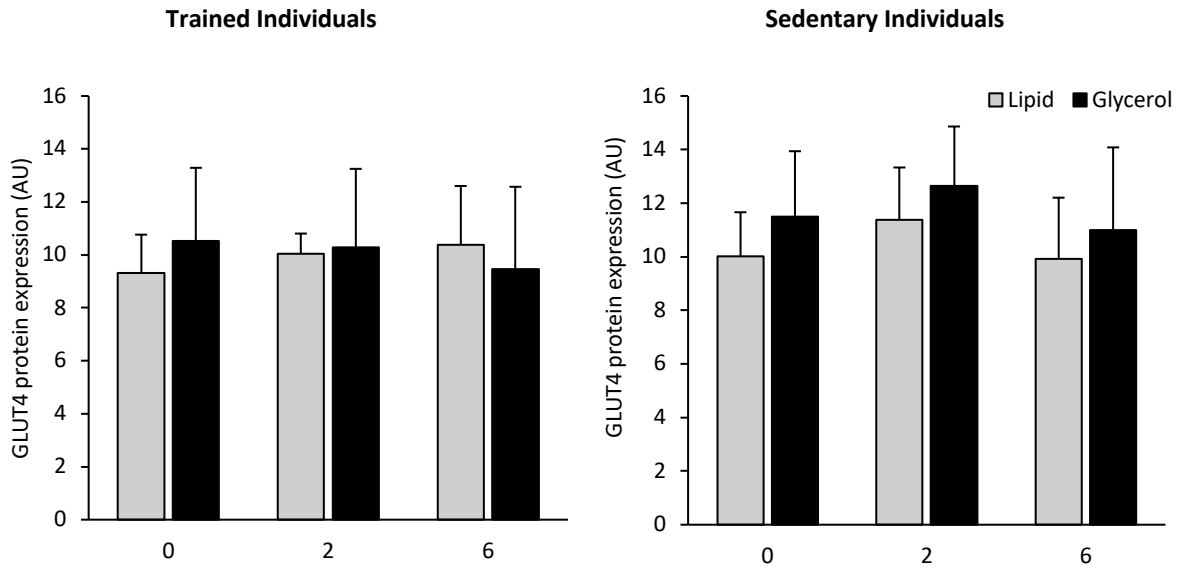


Figure 4.4. The change in total GLUT4 fluorescence intensity following a lipid (grey) and glycerol infusion (black) in trained and sedentary individuals.

4.4.2 Visualisation of large and small GLUT4 spots at baseline

Using immunofluorescence staining we identified both larger spots of GLUT4 as well as small stores which are dispersed throughout the cell. To distinguish between large and small spots, threshold limits were used on all detected spots such that they were separated into large clusters (defined as having a diameter of >1 µm) and small spots (defined as having a diameter <1 µm). These limits have been used previously in a number of studies by both ourselves and other research groups (Lauritzen et al., 2008; Bradley et al., 2014; Bradley et al., 2015). At baseline, the total number of GLUT4 spots in the plasma membrane was greater compared to all other intracellular layers ($P < 0.001$), and this was also true for both large GLUT4 clusters ($P < 0.001$) and small GLUT4 spots ($P < 0.001$). Overall, the total number of GLUT4 spots was significantly greater at the plasma membrane in trained compared to sedentary ($P < 0.001$). Interestingly though, sedentary individuals had a greater number of large GLUT4 clusters at the plasma membrane compared to trained individuals (layer × training status; $P = 0.001$), whereas trained individuals had a greater number of small GLUT4 spots at the plasma membrane compared to sedentary individuals (training status × layer; $P < 0.001$). Small GLUT4 spots made up ~97% of all GLUT4 spots in trained individuals, and at the plasma

membrane ~99% of spots in the plasma membrane were categorised as small spots in trained individuals, whereas only ~95% of GLUT4 spots in the plasma membrane were small spots in sedentary individuals. Across both groups, more large spots were observed at the plasma membrane in type 1 fibres compared to type 2 fibres (layer × fibre type; $P < 0.001$), but there was no fibre type difference in the number of small GLUT4 spots at the plasma membrane. Infusion group had no effect on the number of large or small GLUT4 spots at baseline (small spots, $P = 0.520$; large spots, $P = 0.590$).

4.4.3 GLUT4 content and localization following a lipid or glycerol infusion.

We next investigated whether there were time-dependent changes in GLUT4 localization in response to either a lipid or glycerol infusion. First though, we checked for any time-dependent changes in protein expression (using immunofluorescence microscopy) and found that GLUT4 protein expression (measured as GLUT4 fluorescence intensity) did not change over time ($P = 0.062$) and was not different between infusion groups ($P = 0.389$) or trained and sedentary individuals ($P = 0.380$) at any time point.

Using Pearson's correlation coefficient, we observed a small but significant increase in colocalization of GLUT4 with the plasma membrane from 0 to 2h of infusion (+2% in trained following both lipid and glycerol infusions, +9% in sedentary after lipid infusion and +4% after glycerol infusion, main time effect; $P = 0.039$) and this remained elevated from 2h to 6h ($P = 0.557$). Notably though, there was no significant difference in GLUT4 colocalization with the plasma membrane between 0h and 6h of infusion ($P = 0.522$). GLUT4 fluorescence intensity within the plasma membrane was greater compared to all intracellular layers (main layer effect; $p < 0.001$) at both 2h and 6h of infusion, but GLUT4 fluorescence intensity did not change significantly over time in the plasma membrane ($P = 0.071$). Importantly, there was no difference between the infusion groups or trained and sedentary individuals when examining GLUT4 localization to the plasma membrane, or GLUT4 fluorescence intensity in the plasma membrane or intracellular layers.

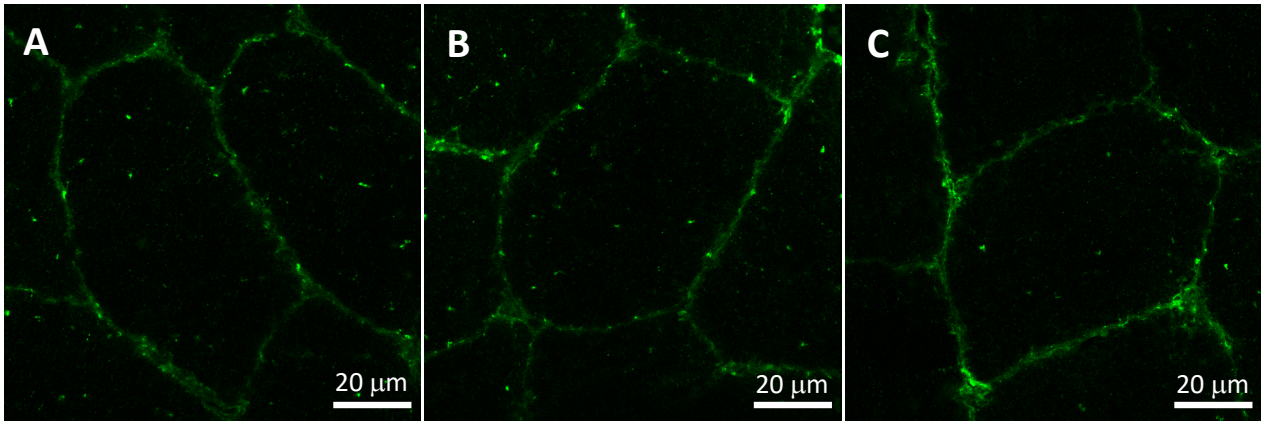


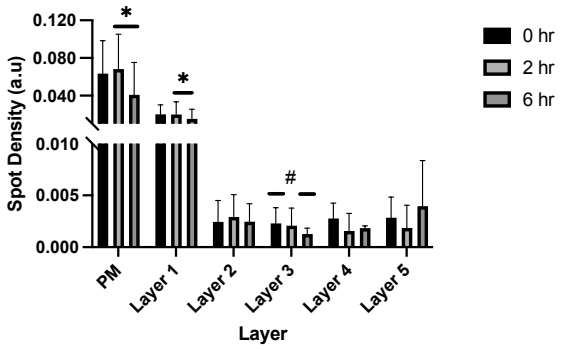
Figure 4.5. Representative immunofluorescence microscopy image demonstrating the GLUT4 stain (green) from baseline (A), 2 h (B) and 6 h (C) following an intralipid infusion.

4.4.4 Changes in GLUT4 spots following a lipid or glycerol infusion

Changes in GLUT4 spots were analysed within the plasma membrane and all 5 intracellular layers at all three timepoints.

When considering the number of small GLUT4 spots, we observed a similar pattern as described above in the plasma membrane and layer 1 (see figure 5), where there was no change in the number of small GLUT4 spots from 0h to 2h (PM; $P = 0.486$, layer 1; $P = 0.669$), but a reduction in small GLUT4 clusters from 2h to 6h (PM; $P = 0.047$: L1; $P = 0.045$). There was no difference between 0h and 6h in these two layers for number of small spots (PM; $P = 0.820$, layer 1; $P = 0.550$). Again, the same pattern was seen in layer 3 for the number of small GLUT4 spots with a decrease from 0 to 6h ($P = 0.038$), but no difference between 0 to 2h ($P = 1.000$) or 2 to 6h ($P = 0.066$). In layers 2, 4 and 5, there was no effects of time on the average number of small GLUT4 spots (layer 2; P = 0.097, layer 4; $P = 0.093$, layer 5; $P = 0.705$).

A


Lipid Trained Type 1 Small Spot Density
Spot Density (a.u)
0.120
0.080
0.040
0.010
0.005
0.000
*
*
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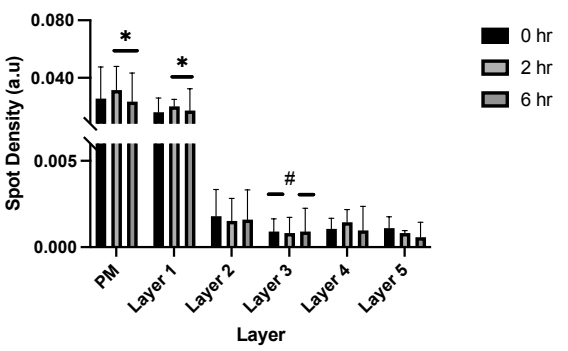
PM
Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
Layer
0 hr
2 hr
6 hr

B


Lipid Sedentary Type 1 Small Spot Density
Spot Density (a.u)
0.080
0.040
0.005
0.000
*
*
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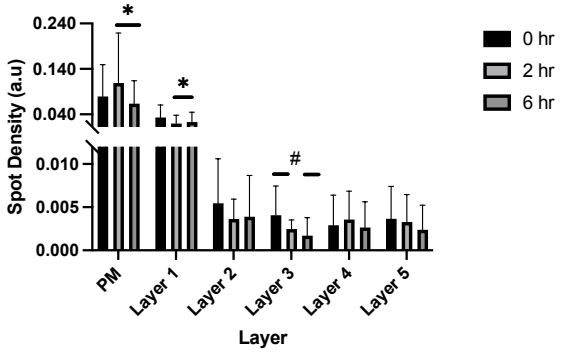
PM
Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
Layer
0 hr
2 hr
6 hr

C


Glycerol Trained Type 1 Small Spot Density
Spot Density (a.u)
0.240
0.140
0.040
0.010
0.005
0.000
*
*
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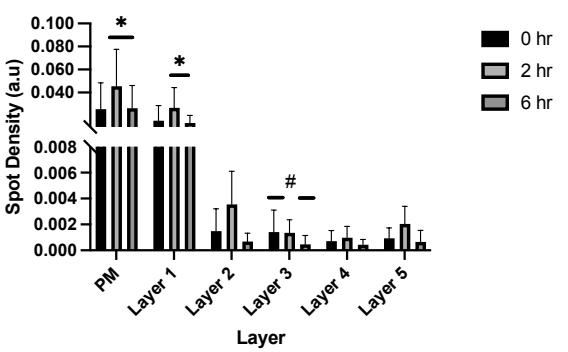
PM
Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
Layer
0 hr
2 hr
6 hr

D


Glycerol Sedentary Type 1 Small Spot Density
Spot Density (a.u)
0.100
0.080
0.060
0.040
0.008
0.006
0.004
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0.000
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Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
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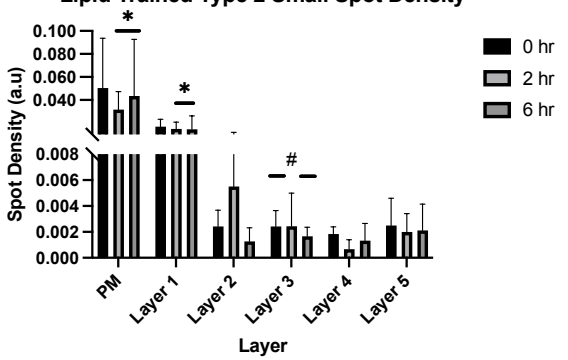
E


Lipid Trained Type 2 Small Spot Density
Spot Density (a.u)
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0.080
0.060
0.040
0.008
0.006
0.004
0.002
0.000
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Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
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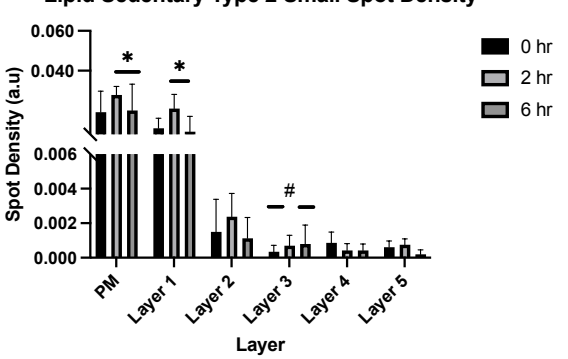
F


Lipid Sedentary Type 2 Small Spot Density
Spot Density (a.u)
0.060
0.040
0.006
0.004
0.002
0.000
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#
PM
Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
Layer
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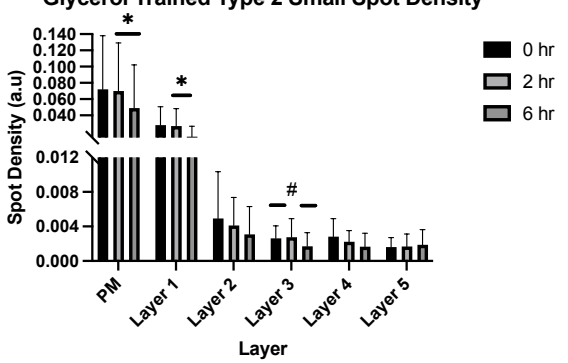
6 hr

G


Glycerol Trained Type 2 Small Spot Density
Spot Density (a.u)
0.140
0.120
0.100
0.080
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0.040
0.012
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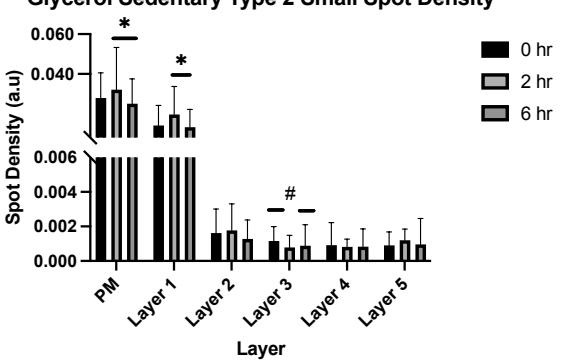
Glycerol Sedentary Type 2 Small Spot Density
Spot Density (a.u)
0.060
0.040
0.006
0.004
0.002
0.000
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PM
Layer 1
Layer 2
Layer 3
Layer 4
Layer 5
Layer
0 hr
2 hr
6 hr

***Figure* 4.6.** Average number of small GLUT4 spots in the PM and 5 intracellular layers at baseline and after 2 & 6h of a lipid (A, B, E, F) or glycerol (C, D, G, H) infusion in type 1 (A-D) and type 2 fibres (E-H) and in trained (right column) and sedentary individuals (left column). M=Linear mixed model * represents a decrease in the number of GLUT4 spots from 2h to 6h (PM; P = 0.047, layer 1; P = 0.045). # Represents greater number of GLUT4 spots at 0h compared to 6h in layer 3 (P = 0.038).

In contrast, the number of large GLUT4 clusters did not change over time in the plasma membrane (*P* = 0.809) or layer 1 (*P* = 1.000). However, layers 4 and 5 saw changes in the number of large GLUT4 spots. In layer 4 there was a significant decrease in the number of large GLUT4 spots from 2h to 6h of infusion (*P* = 0.030), with no change between 0 to 2h (*P* = 1.000) or 0 to 6h (*P* = 0.287). In layer 5, there was a reduction in the number of large GLUT4 spots from 0h to 2h of infusion (*P* = 0.004), with no changes between 2 and 6h (*P* = 0.660) or 0 and 6h (*P* = 0.126).

4.5 Discussion

The aim of present study was to examine the effects of acute FFA elevation (via infusion of intralipid®) alongside a hyperinsulinaemic euglycemic clamp on fibre type specific subcellular GLUT4 distribution in human skeletal muscle of sedentary and trained individuals. The novel findings of the study are that 1) GLUT4 localisation with the plasma membrane is unaffected by an increase in plasma FFA availability induced by lipid infusion, and 2) changes in GLUT4 spot number occurs independent of a change in GLUT4 fluorescence intensity.

4.5.1 Training status dictates the number of small and large GLUT4 clusters

At baseline, we observed greater GLUT4 protein expression in type II fibres compared to type I fibres (~7% in trained individuals, ~12% in sedentary). Using the same technique, our laboratory has previously reported no difference in GLUT4 expression between fibre types, although this was only in well-trained individuals (Bradley et al., 2014). Notably, in the current study we also observed that total GLUT4 protein expression was comparable between trained and sedentary individuals. A well-known adaptation to exercise training is an increase in total skeletal muscle GLUT4 protein content (Houmard et al., 1991; Hughes et al., 1993; Dela et al.,

1996; Phillips et al., 1996; Cox et al., 1999; Daugaard et al., 2000; Kristiansen et al., 2000; Burgomaster et al., 2007; Babraj et al., 2009; Little et al., 2010; Hood et al., 2011; Bradley et al., 2014), and therefore we expected GLUT4 protein expression to be greater in trained compared to the sedentary individuals in the present study. It may be that the training status of our trained and sedentary participants was not sufficiently distinct to result in a significant difference in GLUT4 protein expression between groups (VO_{2max} ~49 ml/kg/min in the trained individuals vs. ~39 ml/kg/min in the sedentary individuals).

Concurrent with previous data from Bradley *et al.,* (2014 & 2015), GLUT4 in muscle was primarily located at the plasma membrane compared to all intracellular layers (Figure 5.1). GLUT4 exists in clusters, which have previously been characterized as being small (<1 µm) or large (>1 µm) by ourselves (Bradley et al., 2014; Bradley et al., 2015) and others (Rodnick et al., 1992; Ploug et al., 1998; Lauritzen et al., 2008; Lizunov et al., 2012). Interestingly, trained individuals had a greater number of small GLUT4 spots in the plasma membrane, whereas sedentary individuals had more large GLUT4 spots. Small GLUT4 spots made up ~97% of all GLUT4 spots in trained individuals, and this explains why trained individuals had a greater total number of GLUT4 spots compared to sedentary individuals. Small GLUT4 spots have been identified as endosomal stores or glucose storage vesicles and are more mobile than the large GLUT4 spots (Lauritzen et al., 2008) that are present in the membrane of the Trans-Golgi network (Rodnick et al., 1992; Ploug et al., 1998). Thus, a greater number of small GLUT4 spots means that trained individuals have a larger pool of more mobile GLUT4. This difference in the number of small GLUT4 spots likely has implications for the translocation and cycling of GLUT4 at the plasma membrane, and subsequently support the greater rates of glucose uptake into muscle that are characteristic of trained individuals.

4.5.2 GLUT4 localisation following lipid or glycerol infusion

When a hyperinsulinaemic euglycemic clamp is combined with infusion of lipid-heparin, both classical and contemporary research has demonstrated that insulin-stimulated glucose uptake is normal following 2h of lipid infusion compared to a non-lipid control (Boden et al., 2001; Itani et al., 2002; Vistisen et al., 2008). By 6h of a lipid-heparin infusion though, insulin-stimulated glucose uptake is reduced compared to the control condition (Itani et al., 2002; Vistisen et al., 2008). On this basis, our hypothesis was that increased GLUT4 co-localisation

with the plasma membrane would be apparent at 2h of infusion (independent of condition), but at 6h of infusion there would be a divergence where GLUT4 co-localisation at the plasma membrane would be maintained following the glycerol infusion but reduced following the lipid infusion. Consistent with the first part of this hypothesis, we observed a significant increase in Pearson's Correlation Coefficient following 2h of both the lipid and glycerol infusion, which we interpret as increased insulin stimulated GLUT4 localisation to the plasma membrane.

The increase in GLUT4 localisation at the plasma membrane was maintained at 6h of glycerol infusion, as evidenced by the similar Pearson's correlation coefficient values. Interestingly though, and in contrast to the second part of our hypothesis, we observed no change in GLUT4 co-localization with the plasma membrane from 2h to 6h of the lipid infusion. We expected a decrease in GLUT4 at the plasma membrane at 6h of lipid infusion to explain the previously reported reduction in glucose disposal (Chow et al., 2012). Since there was no difference in GLUT4 co-localisation between 0h and 6h of infusion, we could speculate that there may have been a small but non-significant reduction in GLUT4 localisation with the plasma membrane from 2h to 6h. Nevertheless, we would only expect any reduction to occur in the lipid infusion group, but we observed the same effect in the glycerol infusion group. Taken together, our results suggest that GLUT4 localisation with the plasma membrane was unaffected by an increase in plasma FFA availability induced by lipid infusion. Insulin-stimulated glucose uptake is dependant, in part, on the insulin signalling cascade where activation of IRS-1 and Akt are critical. However, lipid infusion does not appear to decrease IRS-1 and Akt phosphorylation compared to control conditions over the same time-course (Chow et al., 2012; Chow et al., 2014). Therefore, it is perhaps not surprising that GLUT4 localisation to the plasma membrane was unchanged from 2h to 6h of a lipid infusion. This suggests that the mechanisms by which increased FFA availability leads to a reduction in glucose infusion rate are unrelated to activation of the insulin signalling cascade and GLUT4 localisation at the plasma membrane.

It is important to note that there was no fibre type difference in the co-localisation of GLUT4 to the plasma membrane. This corresponds with previous literature concluding no significant relationship between GLUT4 protein content and fibre type (Andersen et al., 1993). Very

recent data from Koh et al., (2021) further solidifies this with significant differences in glucose disposal during a hyperinsulinemic euglycemic clamp between lean and obese individuals, yet no relationship with fibre type composition (Koh et al., 2021). Even the post-exercise insulin sensitizing effect is shown to be similar between fibre types (Larsen et al., 2020).

4.5.3 Changes in GLUT4 spots following lipid or glycerol infusion

Beyond the use of co-localisation analysis to examine changes in GLUT4 localisation with the plasma membrane, confocal immunofluorescence microscopy also enables the identification and quantitation of GLUT4 clusters (spots). Our laboratory has previously shown that these GLUT4 clusters are present at both the plasma membrane and intracellular locations (Bradley et al., 2014; Bradley et al., 2015). Here, we report that the number of GLUT4 spots at the plasma membrane or the 1 μm below the membrane (layer 1) did not change following 2h of either the lipid or glycerol infusion compared to baseline. However, from 2h to 6h of either infusion there was a significant decrease in the number of GLUT4 spots at the plasma membrane and layer 1. More specifically, the reduction in total GLUT4 spots in the plasma membrane and layer 1 from 2h to 6h of infusion could be entirely accounted for by a decrease in small GLUT4 spots (<1 μm diameter). The decrease in GLUT4 spots occurred independent of a change in GLUT4 fluorescence intensity (and therefore total GLUT4 protein) within the plasma membrane, and therefore we speculate that in response to prolonged insulin infusion GLUT4 disperses from the storage vesicles (spots) in the plasma membrane. This proposed mechanism has similarities to that previously observed within adipocytes, whereby under basal conditions GLUT4 is retained in clusters at, or in close proximity to the plasma membrane, but upon insulin stimulation GLUT4 is then dispersed into the plasma membrane (Stenkula et al., 2010). Although in our study this dispersal of GLUT4 is occurring in response to continuous insulin stimulation, it has previously been shown this is not sufficient to maintain glucose uptake into skeletal muscle when lipid is infused (Chow et al., 2012). It appears that the suppression of glucose uptake in response to a lipid infusion is therefore not due to GLUT4 availability at the plasma membrane.

It is noteworthy that we also report a reduction in the number of large GLUT4 spots in layers 4 and 5. One possibility is that these reductions in large GLUT4 clusters at the more intracellular layers could suggest that this pool of GLUT4 is moving towards the membrane or

layers 1, 2 and 3. However, no change in the number of large GLUT4 spots in these layers close to, or at the plasma membrane, were observed. It may be that these large GLUT4 clusters are dispersing and becoming small GLUT4 spots, in the same or different layers and may explain why we do not see a difference in the number of small GLUT4 spots in layers 3 and 4. Alternatively, these large spots may be reducing in size and at the same time, what were identified as small spots may be reducing in size beyond the limits of our detection, explaining a reduction in the number of large spots with no change in the number of small spots.

4.5.4 Potential alternative mechanism for reduced glucose uptake

The long chain acyl-CoA's palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA have been shown to inhibit hexokinase activity in rat and human skeletal muscle (Thompson and Cooney, 2000). The impact of this inhibition is potentially a reduction in G6P concentrations, a reduction in glycogen synthesis and glycolysis, and ultimately a lower flux through hexokinase at lower G6P concentrations previously observed in insulin-resistant skeletal muscle (Rotheman *et al.,* 1992; Rothman *et al.,* 1995; Roden *et al.,* 1996; Jucker *et al.,* 1997; Petersen et al., 1998; Roden *et al.,* 1999). The well know Randle cycle demonstrates how increased lipid oxidation can result in reduced glucose uptake through pyruvate dehydrogenase and phosphofructokinase inhibition increasing G6P concentrations and inhibiting hexokinase (Randle et al., 1963). LCA-CoA inhibition of hexokinase likely occurs simultaneously to the glucose-fatty acid cycle interaction and reduces insulin-stimulated glucose uptake producing an insulin-resistant state (Thompson and Cooney, 2000), often seen following lipid infusion in previous research (Chow et al., 2012; Chow et al., 2014). Therefore, continuous insulin stimulation likely supports the increased GLUT4 co-localisation and subsequent dispersal of GLUT4 at the plasma membrane in both the lipid and glycerol infusion conditions. However, when lipid is infused, the elevated FA availability will suppress glucose uptake via the inhibition of hexokinase explaining the reduction in glucose infusion rate previously reported (Thompson and Cooney, 2000). Without the increase in FA, the glycerol infusion sees the same level of insulin stimulation, without the inhibition of hexokinase, and so GLUT4 can support glucose uptake.

4.5.5 Strengths and Limitations

In the present study, the fibres imaged were selected at random and not by inspection of GLUT4 stain. The membrane stain is used to isolate the cell, which may risk missing some of the GLUT4 that is at the plasma membrane from analysis. Previously, GLUT4 protein content had been measured in whole muscle samples and does not change over time in response to a lipid infusion in men or women (Hoeg et al., 2011). Whilst image analysis of individual muscle fibres is useful for changes in GLUT4 within the cell, using the border to isolate the cell from the rest of the image may lead to missing information at the plasma membrane.

The analysis method utilised in the present study limits our ability to make a comprehensive exploration of the different subcellular GLUT4 pools as seen in recent research (Knudsen et al., 2020). Knudsen et al utilised a technique they termed Sample Thinning Enhanced Resolution Microscopy (STERM), to visualise GLUT4 distribution throughout the endomembrane. This method required cutting ultra-thin biopsy sections to allow antibody penetration in the absence of detergent. The STERM method paired with the standard confocal microscopy workflow markedly improved the ability to resolve GLUT4 present in small vesicles from larger membrane structures. By then visualizing GLUT4 in STERM-prepared human muscle samples using transmission electron microscopy, Knudsen also confirmed GLUT4 localisation to cytosolic perinuclear, intramyofibrillar and subsarcolemmal areas, tubulovesicular structures, multivesicular endosomes and, most critically, small vesicles sized ~70 - 150 nm, some of which would presumably be detergent-sensitive (Knudsen et al., 2020).

Interestingly, it is evident that the degree of localisation of GLUT4 with the plasma membrane under resting conditions appears to be smaller than when exercise precedes insulin-stimulation (Bradley et al., 2015; Knudsen et al., 2020). Exercise appears to be a stronger stimulus to induce GLUT4 translocation in skeletal muscle than insulin (~19% vs 9% increase in localisation), also suggested by (Bradley et al., 2015).

4.5.6 Conclusions

The present data suggest that GLUT4 co-localisation is not significantly decreased following a lipid infusion when compared to a glycerol control. Decreases in GLUT4 spot number irrespective of GLUT4 intensity demonstrate that dispersal from clusters at the plasma membrane allow for glucose homeostasis.

Chapter 5: Ingestion of an anti-lipolytic drug alters substrate utilisation and ceramide content in individuals at risk of type 2 diabetes.

5.1 Abstract

Increases in plasma FFA alongside a decrease in IMTG oxidation during prolonged exercise suggests increased plasma FFA reduces IMTG utilisation. Importantly, high rates of IMTG turnover in skeletal muscle leads to reduced accumulation of lipid intermediates, such as DAGs and ceramides, and increases muscle insulin sensitivity. Acipimox is an antilipolytic drug that reduces adipose tissue lipolysis, and therefore, reduces plasma FFA concentrations. The aim of the current study was to investigate whether suppressing plasma FFA concentrations during and in the hours following exercise (using the anti-lipolytic agent, Acipimox) would lead to a reduction in muscle ceramides and DAGs in obese individuals. Ten obese sedentary individuals free of metabolic disease (BMI >30 $kg.m^{-2}$) performed two walking trials; one with acipimox ingestion and one without. Mean carbohydrate oxidation rates during exercise was greater in the Acipimox trial (1.21 $\pm$ 0.46 $g.min^{-1}$) versus the control trial (1.01 $\pm$ 0.38 $g.min^{-1}$; *P*=0.002) and mean fat oxidation rates were significantly greater in the control trial (0.38 $\pm$ 0.10 $g.min^{-1}$) compared to the Acipimox trial (0.28 $\pm$ 0.07 $g.min^{-1}$; *P*=0.009). Plasma FFA was lower in the acipimox trial compared to control. Total DAG didn't not change during exercise (P = 0.20). DAG 16:1 18:2, DAG 18:1 18:3, DAG 18:2 22:6 were significantly increased (*P*<0.05). Following the post-exercise increase, DAG 18:1 18:3 significantly reduced at 3 hours post-exercise compared to post-exercise time-point (*P*=0.043). Here, ingestion of an anti-lipolytic drug suppresses plasma FFA concentrations and alters whole-body substrate utilisation during 45 min of brisk walking in obese individuals. However, this exercise and pharmacological strategy did not differentiate from exercise alone with respect to changes in the concentration of specific species of DAGs and ceramides in skeletal muscle.

5.2 Introduction

In Chapter 2 it was explained that the accumulation of IMTG is associated with insulin resistance in obesity and T2D (Pan *et al.*, 1997; Kelley and Goodpaster, 2001; van Loon *et al.,* 2004)., which is linked to an impaired capacity to store lipid in adipose tissue. As a consequence, meal-derived fatty acids become elevated in the blood and 'spillover' and into non-adipose tissues such as liver and skeletal muscle. The increased delivery and uptake of fatty acids to skeletal muscle is not matched by esterification, storage and oxidation of fatty acids, and as a result of the imbalance in these processes lipid accumulates in skeletal muscle. Although there is an increase in IMTG in obesity and T2D, it is now appreciated that lipid metabolites, such as diacylglycerols (DAGs) and ceramides, rather than IMTG, play direct roles in the inhibition of the insulin signalling cascade (Yu et al., 2002; Summers and Nelson, 2005; Chaurasia and Summers, 2015). In addition, healthy trained individuals also exhibit a high IMTG content, comparable to that of obese and T2D individuals, yet combine this with high levels of insulin sensitivity (Goodpaster et al., 2001; van Loon et al., 2003a). Because endurance training increases muscle oxidative capacity and the utilisation of IMTG as a fuel during exercise (Schrauwen *et al.*, 2002; van Loon, 2004), it is believed that the ability to utilise IMTG during exercise is mechanistically important to preserve insulin sensitivity alongside elevated IMTG storage (van Loon and Goodpaster, 2006). High rates of IMTG utilisation in endurance trained individuals allow regular turnover of the IMTG pool (van Loon, 2004; Moro, Bajpeyi and Smith, 2008) with increased storage capacity for meal-derived lipids until 48 h after the exercise bout (van Loon et al., 2003b). This also leads to reduced accumulation of lipid intermediates, such as DAGs and ceramides, and increases muscle insulin sensitivity (van Loon and Goodpaster, 2006; Moro, Bajpeyi and Smith, 2008). Therefore, interventions should be developed that stimulate IMTG utilisation during exercise in obese individuals and T2D patients.

A single bout of exercise reduces IMTG content in healthy, trained individuals (Watt, Heigenhauser and Spriet, 2002), but a net decline in IMTG content is not observed in obese individuals or T2D patients (Watt, Heigenhauser and Spriet, 2002; van Loon et al., 2005a). During prolonged exercise in healthy individuals there is a transient increase in plasma FFA concentrations alongside a decrease in IMTG oxidation (Watt, Heigenhauser and Spriet, 2002;

van Loon et al., 2003b), suggesting that elevated availability of plasma FFA suppresses the use of IMTG. Obesity and T2D is associated with elevated circulating concentrations of fatty acids and triglycerides, and therefore likely explains the reduced capacity for IMTG utilisation in these populations. Acipimox is an anti-lipolytic agent that inhibits adipose tissue lipolysis, and ingestion of Acipimox prior to and during exercise has been shown to decrease plasma FFA concentrations and subsequently increase IMTG utilisation during exercise in both T2D patients and healthy controls (van Loon et al., 2005a; van Loon et al., 2005b).

Chronic exercise training improves insulin sensitivity alongside a reduction in muscle ceramide concentrations (Bruce et al., 2006; Dube et al., 2008; Dube et al., 2011), although the impact of exercise training on muscle DAG concentrations is less clear (Amati, 2012). Nevertheless, the effect of acute exercise on muscle ceramides and DAGs is less well understood. A single bout of exercise improves insulin sensitivity, but paradoxically 3 h of moderate intensity cycling in healthy individuals actually increased muscle ceramide concentrations (Helge 2004). This observation was also replicated when data was combined from endurance-trained athletes, obese individuals and T2D patients undertaking 90 min of moderate-intensity cycling (Bergman et al., 2016). In this latter study though, it was notable that a decrease in muscle ceramide concentrations from pre-exercise levels was only observed in T2D patients following 2 h recovery from the exercise bout (Bergman et al., 2016). Furthermore, to the best of the authors' knowledge the effect of an acute bout of exercise on the molecular species of DAG in muscle is not yet known.

On this basis, we aimed to investigate whether suppressing plasma FFA concentrations during and in the hours following exercise (using the anti-lipolytic agent, Acipimox) would lead to a reduction in muscle ceramides and DAGs in obese individuals. We tested the hypothesis that DAG and ceramide accumulation would be lower post-exercise when Acipimox was ingested. Recent advances in mass spectrometry have generated evidence that specific species of DAGs and ceramides are related to insulin resistance (Bergman et al., 2016). Therefore, we evaluated the effect of exercise, with or without Acipimox treatment, on the molecular species of DAGs and ceramides, with the intention of understanding whether anti-lipolytic treatment alongside exercise has therapeutic potential for the treatment of insulin resistance.

5.3 Methods

5.3.1 Participant information

Participant information

The current study includes 10 obese sedentary individuals free of metabolic disease (BMI >30 $kg.m^{-2}$, performing less than 2 x 30 minutes of structured exercise sessions per week) that volunteered to take part in the study, which was approved by the South Birmingham Research Ethics Committee (Midlands, UK) and conformed with the Declaration of Helsinki. Written, informed consent was obtained from all participants following a detailed explanation of what was involved in the experimental protocol and the opportunity to ask questions.

Table 5.1. Subject characteristics.

	Value
Sex (males/females)	7 / 3
Age (years)	46 ± 10
BMI ($kg\ m^{-2}$)	36 ± 5
FFM (kg)	38 ± 4
Body fat (%)	35 ± 4
$VO_{2\ max}$ ($ml\ kg^{-1}\ min^{-1}$)	33 ± 4
Systolic blood pressure (mmHg)	136 ± 9
Diastolic blood pressure (mmHg)	89 ± 5
Resting HR (bpm)	81 ± 12
Fasting glucose (mmol/l)	5.6 ± 1.1
Fasting plasma insulin (mmol/l)	20.0 ± 4.8
2 h OGTT insulin (mmol/l)	92.0 ± 66.1
2 h OGTT glucose (mmol/l)	7.2 ± 3.1

	Value
Matsuda insulin sensitivity index	2.4 ± 1.3

Data are mean ± SD.

5.3.2 Pre-experimental protocol

At least one week prior to the first experimental trial all participants attended the laboratory for baseline assessments, following an overnight fast (>10 h) and having abstained from caffeine, alcohol, and vigorous exercise 24 h prior. Participants were asked to rest for 20 minutes, before blood pressure (Dinamap; GE Pro 300V2, Tampa, Florida) and an ECG was carried out. A cannula was inserted into a vein in the antecubital fossa and venous blood samples were then taken to determine fasting glucose and blood lipids. Participants were then given a dextrose drink and remained seated whilst blood samples were collected at 30, 60, 90 and 120 minutes for an oral glucose tolerance test (OGTT). Body composition was then assessed using Dual-energy X-ray Absorptiometry (DXA Hologic QDR Series, Discovery A, Bedford, MA, United States). Following this, participants performed an incremental maximal aerobic capacity test (VO_{2peak}) to volitional exhaustion on a treadmill. Briefly, participants started walking at 3.5 $km.h^{-1}$ on a 1% gradient for 3 minutes, and the speed was increased thereafter by 1 $km.h^{-1}$ every 3 minutes until participants reached 6.5 $km.h^{-1}$, after which the gradient was increased by 2% every 3 minutes until volitional fatigue. Throughout the test heart rate was measured (Polar H10), and expired air was collected using an online gas analysis system (Metalyzer 3B, Cortex, Germany). VO_{2peak} was defined as the highest VO_2 achieved over a 15 s recording period.

5.3.3 Experimental protocol

In repeated measures protocol, participants underwent two separate identical exercise trials. No placebo was given in the non-acipimox trials and so individuals were not blinded to which trial was which. Due to noticeable side effects of the Acipimox (i.e. flushing and an increase in temperature) this would not be possible. Two days prior to the experimental trial days, participants were provided a standardised diet (50% CHO, 30% fat, 20% protein) but adjusted to each individual's habitual caloric intake, previously assessed by a 3-day diet diary. On the experimental trial days, participants arrived at the laboratory following an overnight fast (>10

h). A cannula was inserted, and a resting blood sample (~10 ml) was collected, followed by a muscle biopsy of the *vastus lateralis*. Briefly, local anaesthesia (1% Marcaine; ~2 ml) was applied to the skin and fascia of the muscle, and a small incision was made. A muscle biopsy was taken using the conchotome needle technique (~100 mg). The muscle sample was immediately dissected free of any fat and connective tissue. A small amount of tissue (~5-10 mg) was placed into a glass jar containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate at room temperature (for analysis by transmission electron microscopy), and the remaining tissue was immediately snap-frozen in liquid nitrogen. Following the muscle biopsy, participants then ingested 250 mg Acipimox (Acipimox trial), or nothing (control trial); the order in which the experimental trials were carried out was randomized. Participants rested in a supine position for 1 h while blood samples were collected every 15 min. After 1 h, participants began walking at a speed equivalent to 45% VO_{2peak} for 45 min, with blood samples collected every 15 min and expired air continuously collected throughout the exercise bout (Metalyzer 3B, Cortex, Germany). Immediately following the walk, a second muscle biopsy was obtained and then in the Acipimox trial a second dose of 250 mg was ingested (and nothing was provided during the control trial). At this point, participants remained supine for a further 3 h following the exercise bout, with blood samples taken every 15 mins for the first hour, and every 30 min thereafter. A total of 16 blood samples were collected throughout the trial. A final muscle biopsy was collected at 3 h post-exercise. Whole body rates of carbohydrate and fat oxidation were estimated from VO_2 and VCO_2 values, using equations of Jeukendrup and Wallis (Jeukendrup and Wallis, 2005).

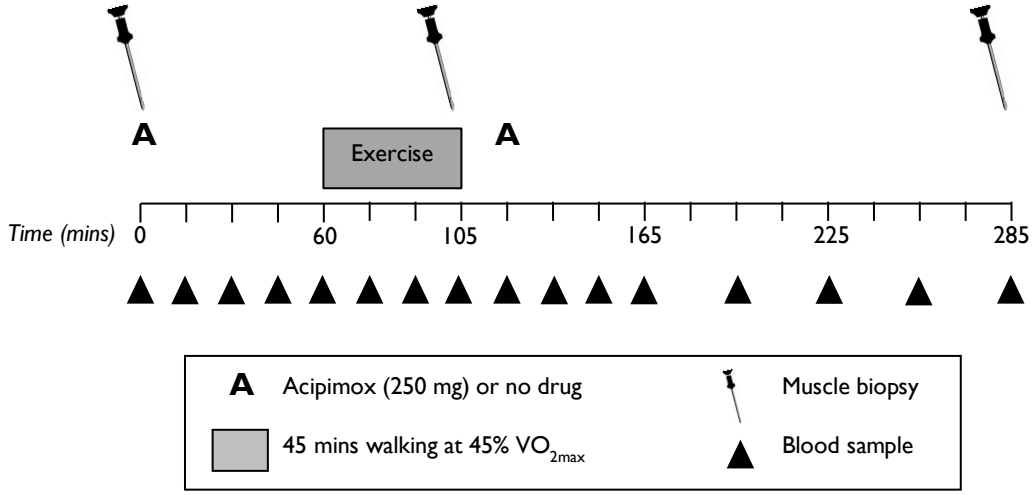


Figure 5.1. An overview of the experimental trial days consisting of a baseline biopsy with immediate ingestion of Acipimox (or nothing), one hour of rest, 45 min of walking at 45% of $VO_{2\,max}$, an immediate post-exercise biopsy and ingestion of Acipimox (or nothing), 3 h of rest and a 3 h post-exercise muscle biopsy. Blood samples were collected at baseline and at 15 min intervals until the remaining 2 h of the study protocol where they were reduced to 30 min intervals.

5.3.4 Blood sample analysis

All venous blood samples were collected into vacutainers containing EDTA or serum separating tube gel. The EDTA vacutainers were placed immediately on ice, the serum vacutainers were left at room temperature for 30 minutes then placed on ice, until centrifugation of both at 2500 g for 10 min at 4°C. Following centrifugation, aliquots of plasma and serum were stored in a freezer at −80°C for subsequent analysis. Samples were later analysed for plasma glucose, non-esterified fatty acids (NEFA) and glycerol using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) following the manufacturer's instructions.

5.3.5 Muscle sample analysis

Transmission electron microscopy

Small segments of the muscle biopsy samples (~5 mg) were prepared for enhanced glycogen and lipid droplet visualisation by transmission electron microscopy, as described previously (Nielsen et al, 2010). Briefly, muscle specimens were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 24 h at 4°C, and then rinsed three times in 0.1 M sodium cacodylate buffer. After rinsing, muscle samples were post-fixed with 1% osmium tetroxide (OsO_4) and 1.5% potassium ferrocyanide [K4Fe(CN)6] in 0.1 M sodium cacodylate buffer for 90 min at 4°C. Following post-fixation, muscle samples were rinsed twice more in 0.1 M sodium cacodylate buffer at 4°C and dehydrated through a graded series of acetone. Following two washes with 100% propylene oxide, samples were penetrated with graded mixtures of propylene oxide and epoxy resin (Agar 100 Medium, Agar Scientific, Essex, UK) at 20°C (2:1, 1:1, and 1:2 ratios of propylene oxide to epoxy resin, for 1-2 h at each dilution), and finally embedded in 100% epoxy resin for 12 h at 60°C. To maximise the number of fibres,

longitudinal ultrathin sections were obtained using a ultramicrotome (Reichert Jung Ultracut, Vienna, Austria) fitted with a diamond blade and collected on to formvar coated grids (200 copper mesh size). To obtain as many fibres as possible, sections were collected at three different depths into the tissue block separated by ~150 nm. Post-staining was then conducted to increase the contrast of subcellular structures. Prior to use, stock solutions of both the Reynold's lead citrate and uranyl acetate (UA) (3% by weight in ddH_2O) were aliquoted and microcentrifuged for 5 min. Samples were then incubated in UA for 14 min, after which excess UA was removed by manual washing of the sample in ddH_2O. Subsequently, the sample was incubated in Reynold's lead citrate for 5 min in a dark room at room temperature, followed by washing in ddH_2O. Samples were air dried at room temperature for at least 30 min prior to imaging.

Each muscle sample contained both transverse and longitudinal fibres, but only longitudinally orientated fibres were imaged using a TEM (FEI Morgagni, Field Electron and Ion Company, Oregon, USA), coupled with an Olympus Megaview III camera, which provided an average of 10 fibres per biopsy (range: 9-12 fibres). Each fibre was initially viewed at x1,000 magnification in order to locate the plasma membranes and visually assess the available myofibrillar area. Images were collected at x6800 magnification, where a total of 40 images were obtained per fibre in a randomized systematic order. Of these 40 images collected per fibre, 20 images were obtained of the subsarcolemmal region, 10 images of the superficial region of the myofibrillar space (superficial myofibrillar) and 10 images of the central region of the myofibrillar space (central myofibrillar).

5.3.6 Lipidomics

Approximately 5 mg of freeze-dried muscle tissue (20–30 mg wet weight) was used for the lipid composition analysis using the butanol–methanol(3:1;BUME) method (Lofgrenet al.2016). Briefly BUME solution was added to samples at −20°C and combined tissue homogenisation and lipid extraction was then performed using a Mixer Mill 301 instrument (Retsch GmbH,Haan, Germany). Automated liquid handling steps in the extraction procedure were performed by a Velocity11 Bravo pipetting robot (Agilent Technologies, Santa Clara, CA, USA). Total lipid extracts were stored in chloroform–methanol (2:1) at−20°C until further

analysis. Prior to mass spectrometric analysis, DAG was fractionated using straight-phase HPLC and evaporative light-scattering detection as previously described (Lofgrenet al.2016). For mass spectrometric analysis, total lipid extracts, as well as the DAG fractions, were diluted with internal standard-containing chloroform–methanol (1:2) with 5 mM ammonium acetate. TAG and DAG were then quantified by direct infusion (shotgun) on a QTRAP 5500mass spectrometer (Sciex, Concord, Canada) equipped with a robotic nanoflow ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA) performed in positive ion mode by neutral loss detection of 10 common acyl fragments formed during collision-induced dissociation, according to previous work (Murphy et al., 2007). Lipid class-specific internal standards were used of either deuterated or diheptadecanoyl (C17:0) containing FAs.

Ceramides were analysed using ultra-performance liquid chromatography–tandem mass spectrometry according to previous work (Amrutkar et al., 2015). Prior to ceramide analysis the total extract was exposed to alkaline hydrolysis (0.1Mpotassium hydroxide in methanol) to remove phospholipids that could potentially cause ion suppression effects. After hydrolysis the samples were reconstituted in chloroform–methanol–water (3:6:2). Ceramides were then quantified using a QTRAP 5500 mass spectrometer equipped with an Infinity quaternary ultra-performance pump (Agilent Technologies).

5.3.7 Statistical analysis

All data were analysed using statistical analysis software (Statistical Package for the Social Sciences for Windows, version 27.0, Chicago, IL, USA). A mixed-design ANOVA was used to interrogate all time and condition-dependent data. Subsequent Bonferroni adjustment post-hoc analysis was used to examine main effects and interactions. Variables with skewed distributions were log-transformed prior to analysis. Data are presented as means $\pm$ S.D. Significance was accepted at $P<0.05$.

5.4 Results

5.4.1 Physiological data, energy expenditure and substrate oxidation

RER did not change over time during the treadmill walk (*P*=0.29), although mean RER during exercise was higher in the Acipimox trial (0.89 ± 0.02) compared to the control trial (0.86 ± 0.03; *P*=0.046). Accordingly, the mean rate of carbohydrate oxidation during exercise was greater in the Acipimox trial (1.21 ± 0.46 $g.min^{-1}$) compared to the control trial (1.01 ± 0.38 $g.min^{-1}$; *P*=0.002). Carbohydrate oxidation increased throughout exercise in the Acipimox trial but was decreased during the second half of the control trial (figure 5.4.1). No significant changes in fat oxidation occurred throughout exercise, although the mean rate of fat oxidation was significantly greater in the control trial (0.38 ± 0.10 $g.min^{-1}$) compared to the Acipimox trial (0.28 ± 0.07 $g.min^{-1}$; *P*=0.009, Fig. 5.4.1).

Table 5.2. Physiological data and energy expenditure during 45-minute walking at 45% $VO_{2\ max}$ following acipimox ingestion or no acipimox ingestion.

Condition	**Time (min)**					
	15		**30**		**45**	
	Control	**Acipimox**	**Control**	**Acipimox**	**Control**	**Acipimox**
VO2 ($L.min^{-1}$)	1.39 ± 0.28	1.32 ± 0.35	1.46 ± 0.28	1.48 ± 0.45	1.49 ± 0.32	1.51 ± 0.47
VCO_2 ($L.min^{-1}$)	1.24 ± 0.25	1.20 ± 0.33	1.26 ± 0.24	1.30 ± 0.43	1.28 ± 0.27	1.32 ± 0.43
$\%VO_{2\ max}$	44 ± 3	42 ± 5	46 ± 3	46 ± 8	47 ± 4	47 ± 9
HR (bpm)	112 ± 1	102 ± 40	117 ± 21	106 ± 43	122 ± 21	121 ± 20
Energy Expenditure ($KJ\ min^{-1}$)	27.1 ± 14.7	28.3 ± 10.1	29.1 ± 11.6	30.1 ± 10.0	31.6 ± 7.7	30.5 ± 9.6

Values are mean ± SD

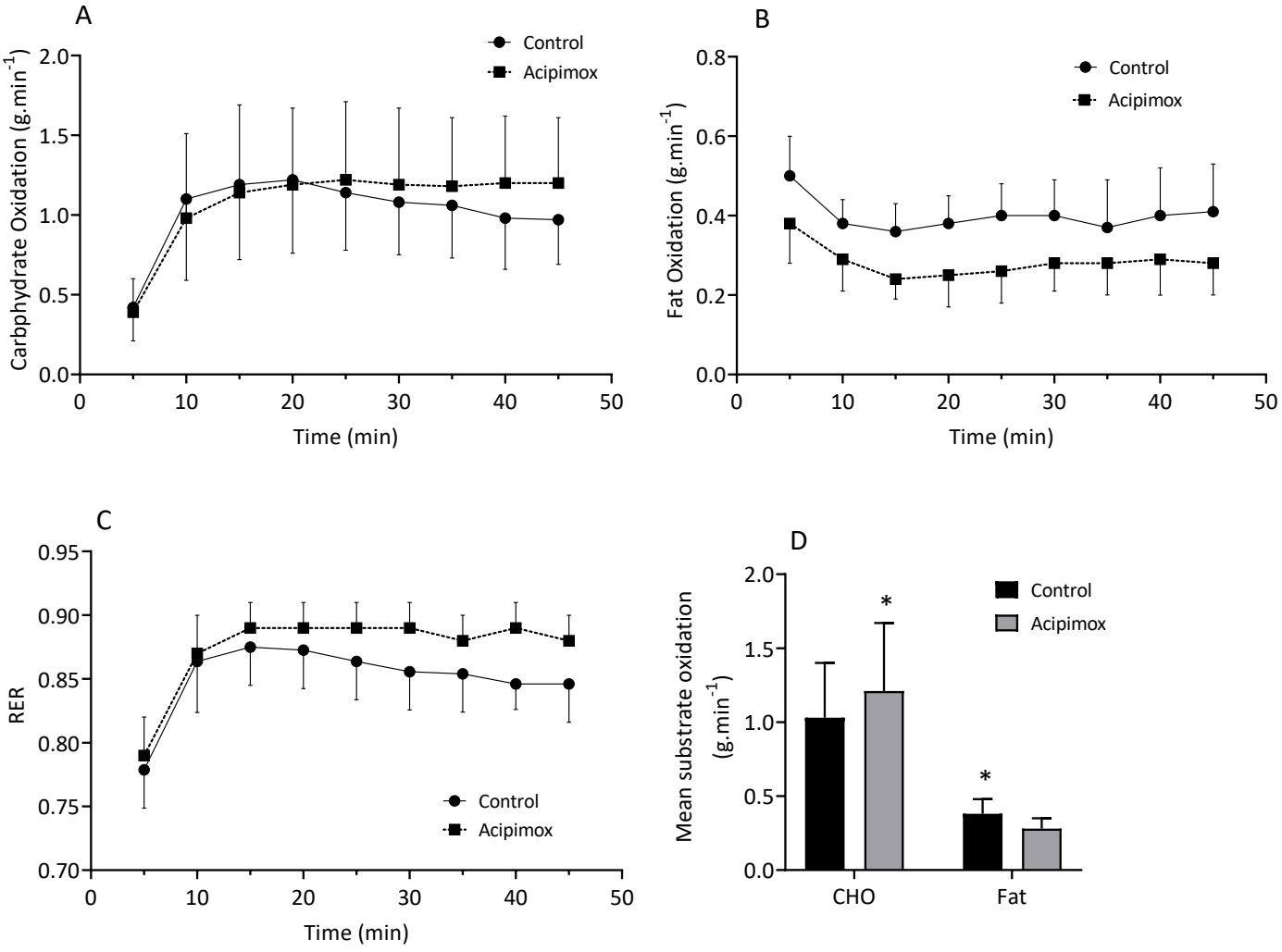


Figure 5.2. Respiratory exchange ratio (RER) (A), carbohydrate oxidation (B), fat oxidation (C) and mean rates of substrate oxidation (D) for 45 minutes walking at 45% $VO_{2\ max}$ during the control trials and acipimox trials. CHO oxidation was significantly greater in the acipimox trial and fat oxidation significantly greater in the control trial (* represents P<0.05). Data are presented as mean ± SD.

5.4.3 Blood metabolites

Plasma FFA concentrations in the control trial increased throughout the treadmill walking exercise period and reached a peak at 15 min post-exercise. After this point, plasma FFA concentrations declined and reached similar levels to those observed at baseline by the end of the 3 h recovery period (*P*>0.05). In contrast, plasma FFA concentrations in the Acipimox

trial decreased during exercise, reaching their lowest point at the end of the exercise bout, and this level was sustained throughout the recovery period. Overall, plasma FFA concentrations were significantly lower in the Acipimox trial compared to control from 60 min onwards (Main time x trial effect; $P<0.05$). Plasma glycerol concentrations increased throughout exercise and decreased during the recovery period in both trials. Importantly though, plasma glycerol concentrations were significantly lower in the Acipimox trial compared to the trial from 75 min (Main time x trial effect; $P<0.001$). Plasma glucose concentrations were unaltered at rest, during exercise, or throughout the recovery period ($P = 0.81$).

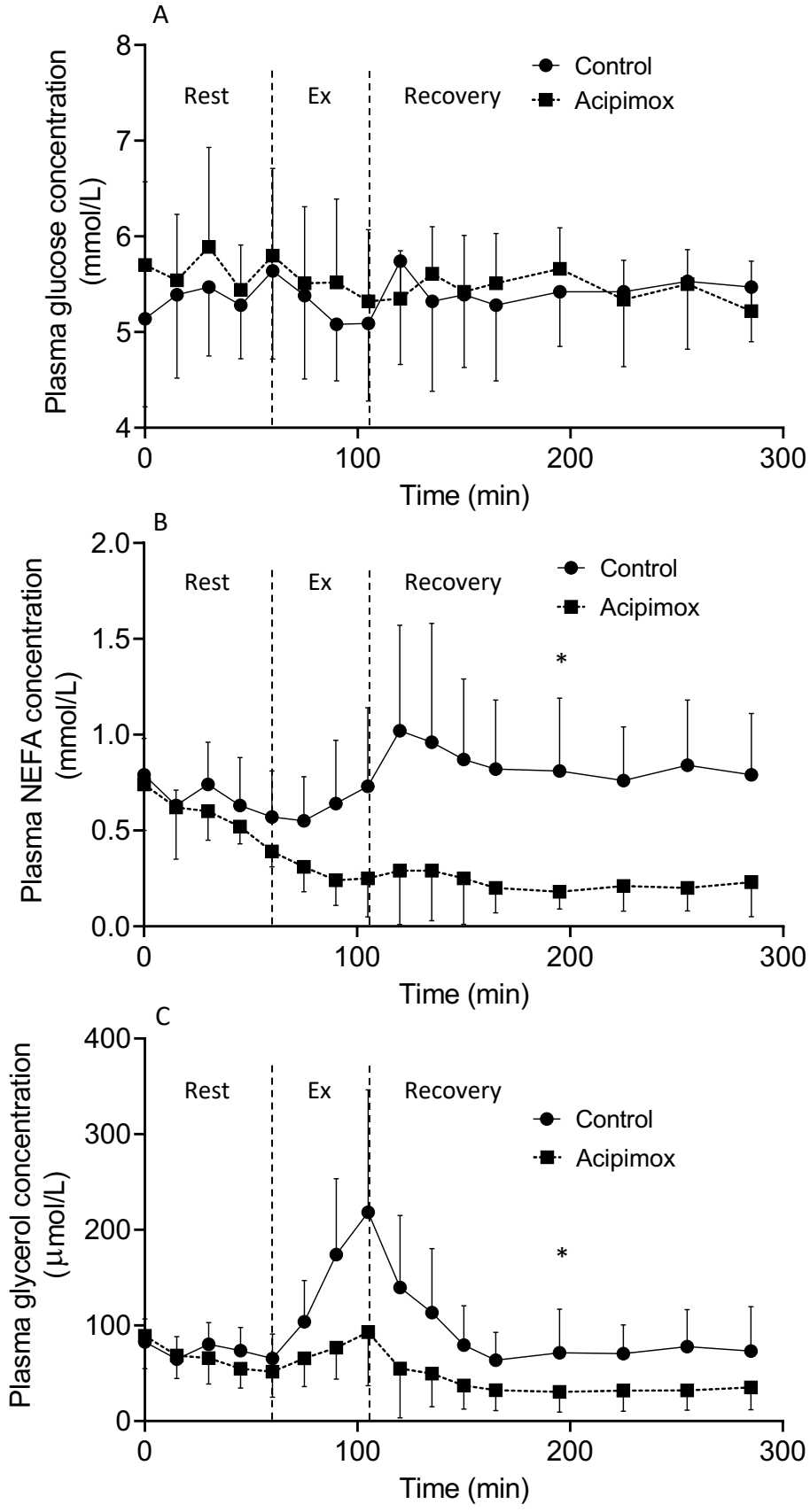

A
Plasma glucose concentration (mmol/L)
Rest
Ex
Recovery
Control
Acipimox
Time (min)
B
Plasma NEFA concentration (mmol/L)
*
C
Plasma glycerol concentration (μmol/L)
*

Figure 5.3. Mean glucose (A), NEFA (B) and glycerol (C) concentrations over the 5-hour study protocol for the control trials (solid line with a circle marker) and the Acipimox trials (dashed line with a square marker). Plasma FFA concentrations were significantly lower in the Acipimox trial compared to control from 60 min onwards and plasma glycerol concentrations were significantly lower in the Acipimox trial compared to the trial from 75 min (* represents P<0.05). Data are mean ± SD.

5.4.4 Lipidomics

Diacylglycerol: Total DAG concentrations were not significantly altered in response to exercise or during recovery (main time effect; *P*=0.20), with no difference between conditions. This was also true for concentrations of saturated DAGs (main time effect; *P*=0.89), DAGs with 1 unsaturated FA (main time effect; *P*=0.51) and DAGs with 2 unsaturated FA (main time effect; *P*=0.10). When examining specific species of DAGs, a number of DAGs with 2 unsaturated FA were significantly increased (DAG 16:1 18:2, DAG 18:1 18:3, DAG 18:2 22:6, main time effect; *P*<0.05) or had a tendency to be increased (DAG 16:1 18:1, DAG 18:1 20:1, DAG 18:1 20:2, *P*=0.05 to 0.09 for time) from pre to post-exercise. Post hoc analysis revealed that following the post-exercise increase in DAG 18:1 18:3, this specific DAG was significantly reduced at 3 hours post-exercise compared to the post-exercise time-point (*P*=0.043).

Ceramides, Glucosylceramides, Lactosylceramide: Total ceramide concentrations were not significantly altered in response to exercise or during recovery (*P*=0.34). This was also the case for the concentrations of specific ceramide species, except for Cer 20:0, which was significantly lower at 3 h post-exercise in the Acipimox trial compared to the control trial (main effect time x trial; *P*=0.018). GluCer 24:0 and GluCer 24:1 concentrations were both increased immediately following exercise (main time effect; *P*<0.05), but no other GluCer species or total GluCer was altered by exercise in either condition. Total LacCer concentrations were significantly increased by exercise (main time effect; *P*=0.02), as were a number of LacCer species, including LacCer 20:0, LacCer 22:0, LacCer 24:0 (all *P*<0.05) and a tendency for LacCer 24:1 to be reduced (*P*=0.06). Again, no differences between conditions were apparent. Notably, exercise-induced increases in LacCer concentrations appeared to be sustained following 3 hours of recovery.

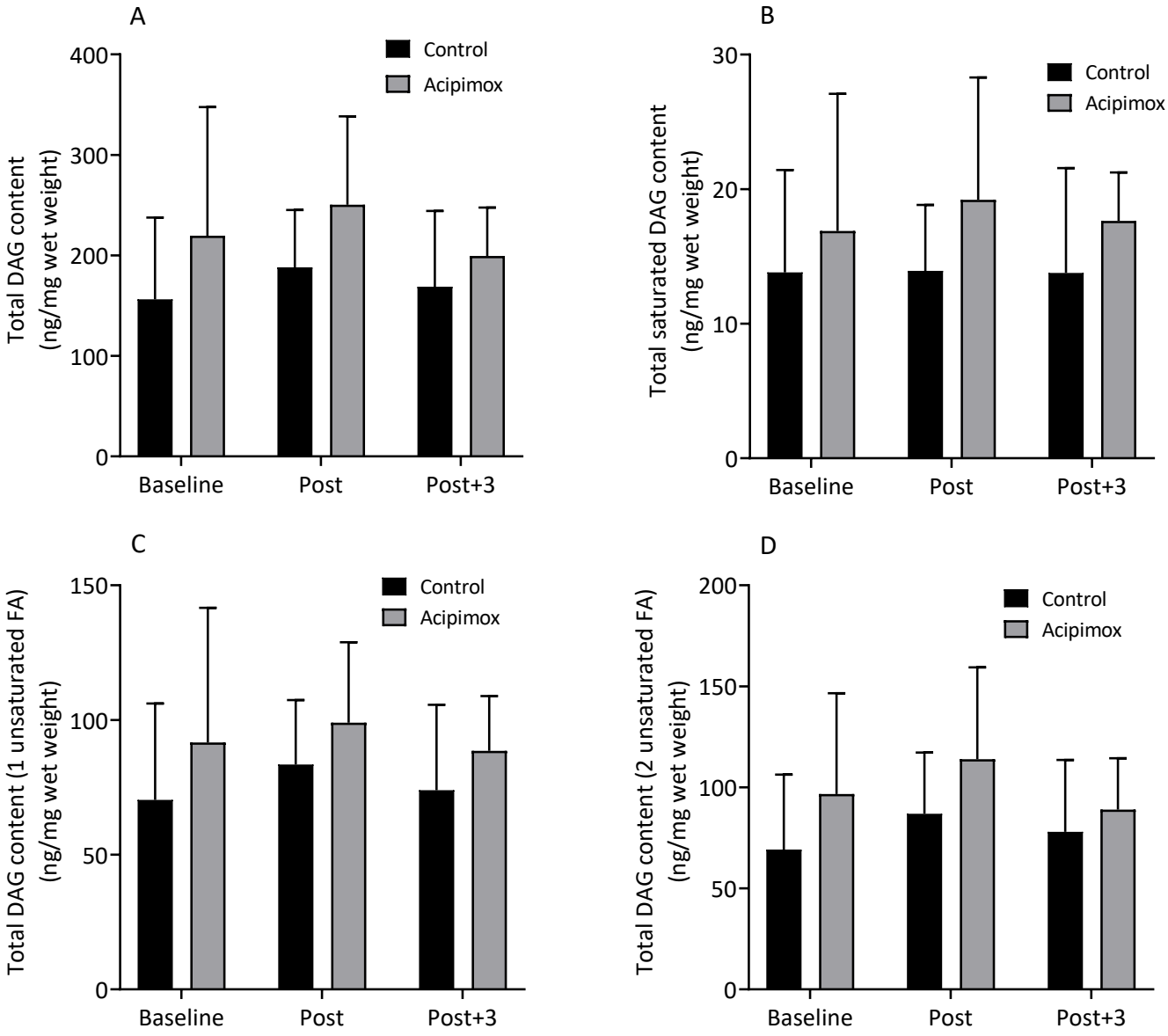


Figure 5.4. DAG content in vastus lateralis muscle. Total DAG content (A), total saturated DAG content (B), and total DAG content with 1 (C) and 2 (D) unsaturated fatty acids. There were no significant differences between the control trial and acipimox trial at all three time points for total DAG content, saturated DAGs, or unsaturated DAGs on 1 or 2 FA ($P>0.05$). Data are presented as mean $\pm$ SD.

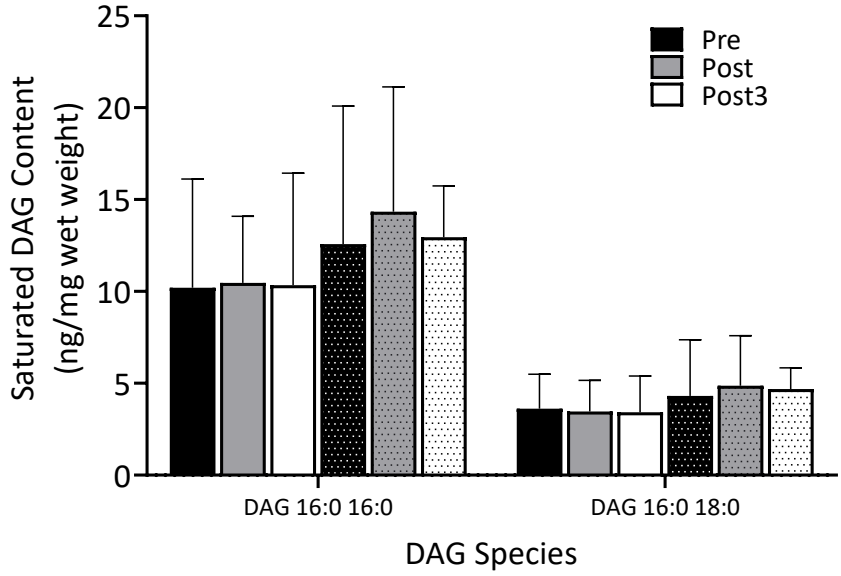
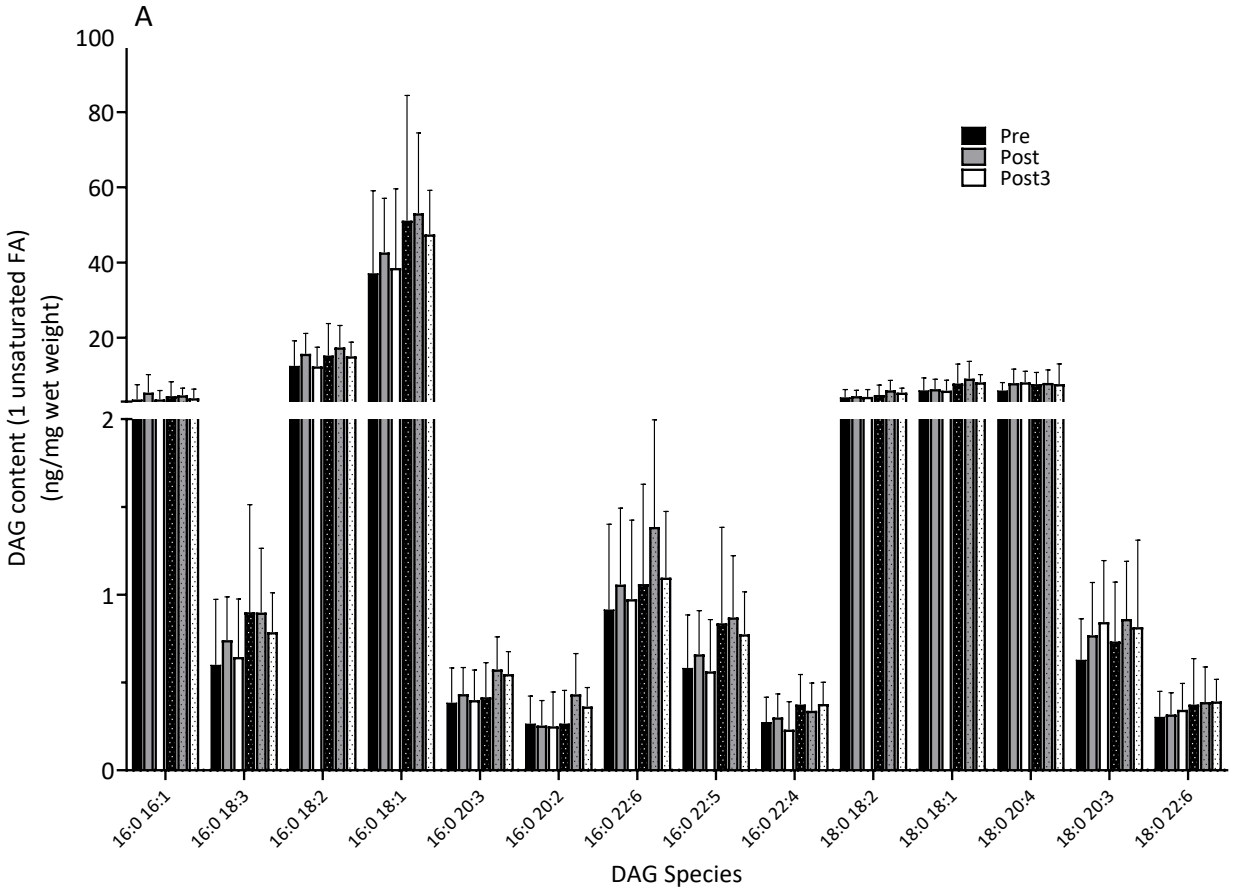


Figure 5.5. Individual species of saturated DAG at baseline (black), immediately post exercise (grey) and 3 hours post exercise (white). Plain bars represent the control trial and spotted bars represent the Acipimox trial. There were no significant changes in saturated DAG content ($P>0.05$). Data are presented as mean $\pm$ SD.



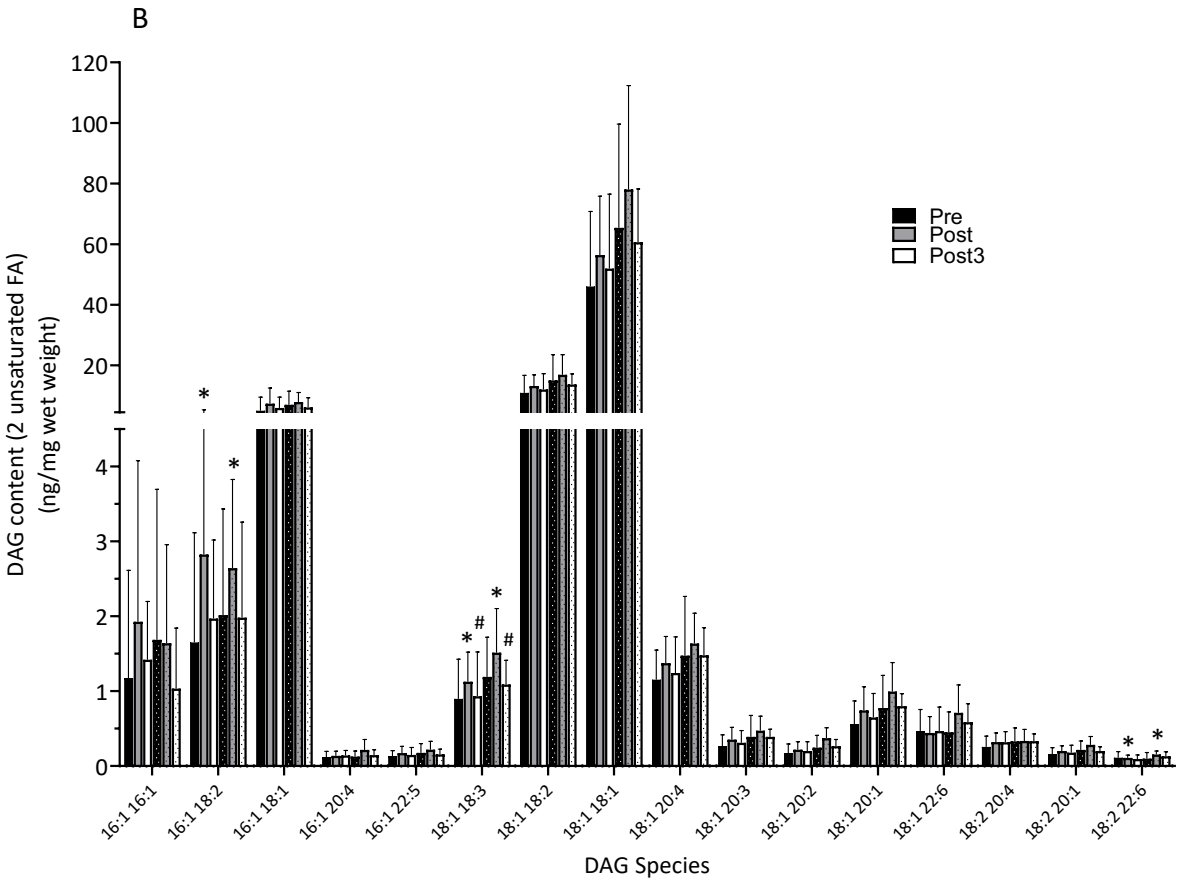
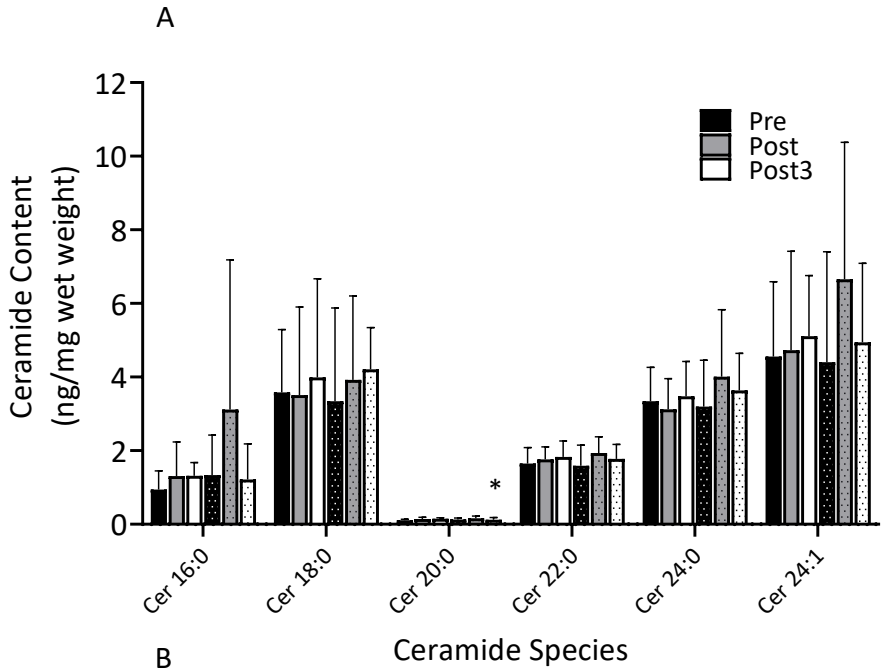
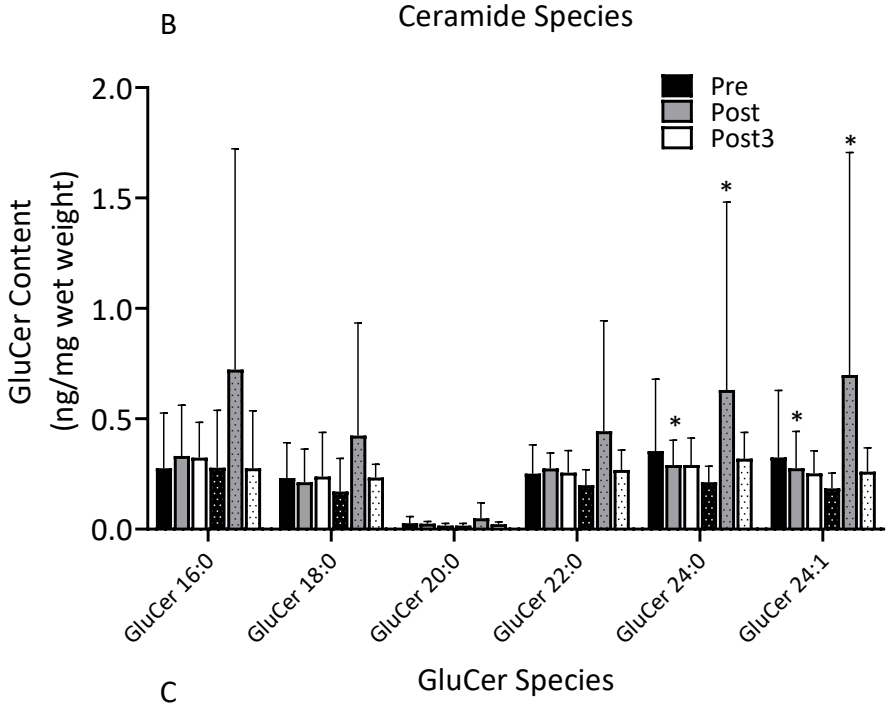


Figure 5.6. Individual species of DAG with 1 unsaturated FA (A) and 2 unsaturated FA (B) at baseline (black), immediately post exercise (grey) and 3 hours post exercise (white). Plain bars represent the control trial and spotted bars represent the Acipimox trial. DAG 16:1 18:2, DAG 18:1 18:3, DAG 18:2 22:6, were significantly increased form pre to post exercise (*) and DAG 18:1 18:3 was significantly reduced at 3 hours post exercise (#). Significance set at $P<0.05$. Data are presented as mean $\pm$ SD.


A
Ceramide Content
(ng/mg wet weight)
12
10
8
6
4
2
0
Pre
Post
Post3
*
Cer 16:0
Cer 18:0
Cer 20:0
Cer 22:0
Cer 24:0
Cer 24:1
Ceramide Species


B
GluCer Content
(ng/mg wet weight)
2.0
1.5
1.0
0.5
0.0
Pre
Post
Post3
*
*
*
*
GluCer 16:0
GluCer 18:0
GluCer 20:0
GluCer 22:0
GluCer 24:0
GluCer 24:1
GluCer Species

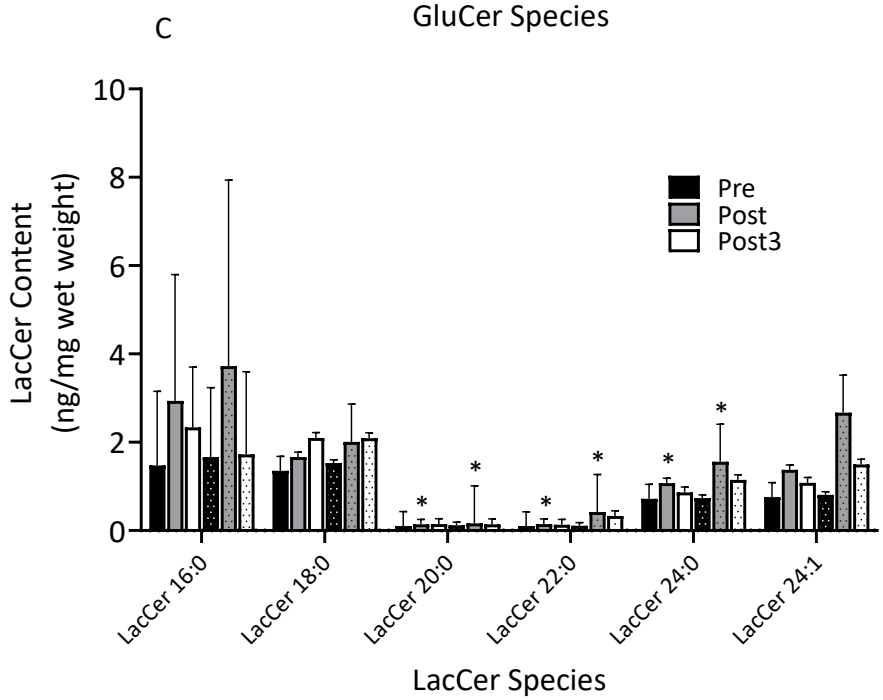

C
LacCer Content
(ng/mg wet weight)
10
8
6
4
2
0
Pre
Post
Post3
*
*
*
*
*
*
LacCer 16:0
LacCer 18:0
LacCer 20:0
LacCer 22:0
LacCer 24:0
LacCer 24:1
LacCer Species

Figure 5.7. Individual species of ceramides (A), glucosylceramides (B) and lactosylceramides (C) at baseline (black), immediately post exercise (grey) and 3 hours post exercise (white). Plain bars represent the control trial and spotted bars represent the Acipimox trial. Cer 20:0 was significantly lower at 3 h post-exercise in the Acipimox trial compared to the control trial, GluCer 24:0 and GluCer 24:1 concentration both increased immediately following exercise, as were LacCer 20:0, LacCer 22:0, LacCer 24:0 (* represents $P<0.05$). Data are presented as mean ± SD.

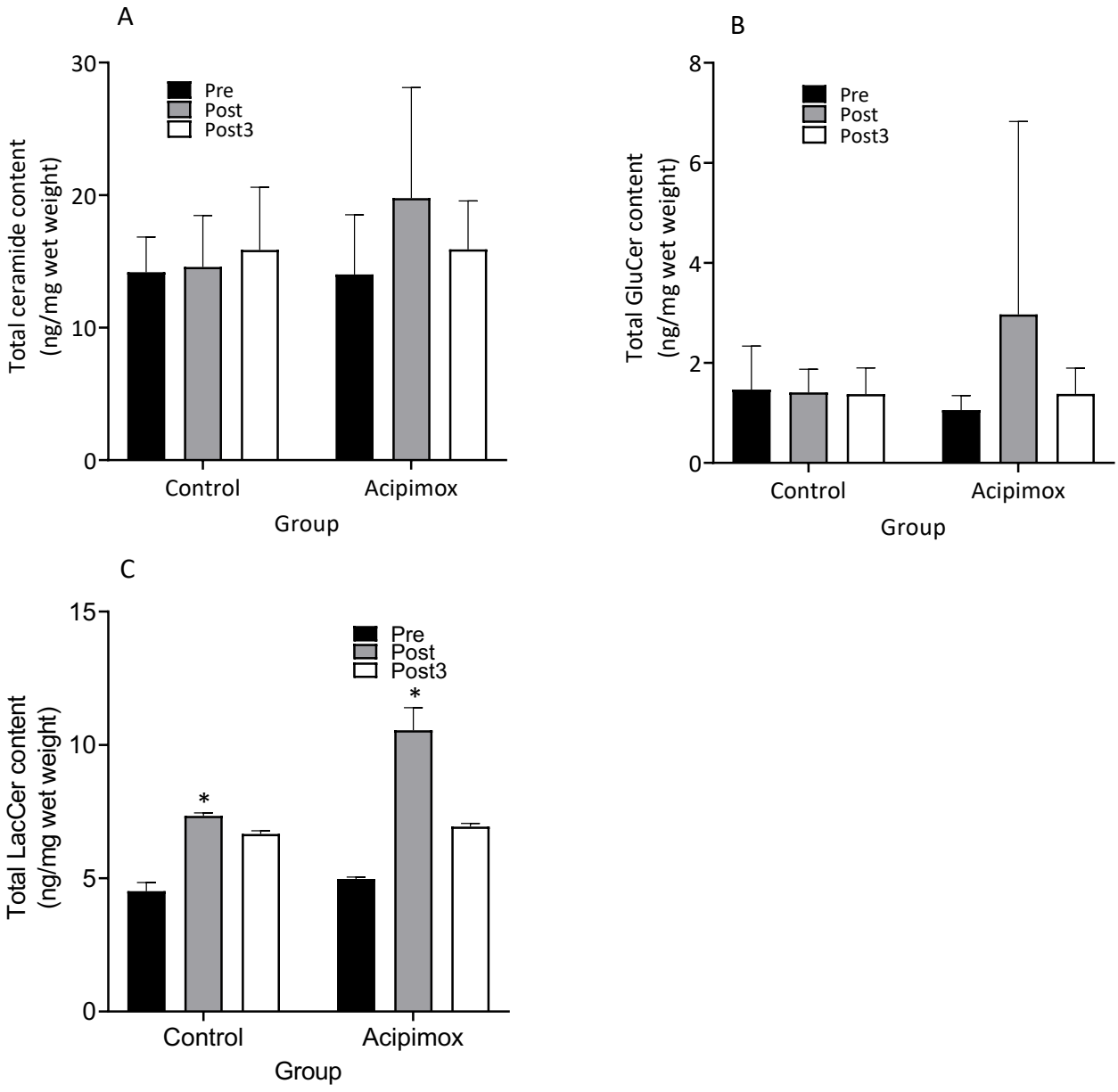


Figure 5.8. Mean ceramides in vastus lateralis muscle. Total lactosylceramide content (a) and individual species of lactosylceramides (b) at baseline (black), immediately post exercise

(grey) and 3 hours post exercise (white). Plain bars represent the control trial and spotted bars represent the Acipimox trial. Total LacCer concentrations were significantly increased by exercise (* represents P<0.05). Data are presented as mean ± SD.

5.5 Discussion

The aim of the present study was to investigate the effects of reducing plasma FFA during a single bout of exercise on the concentration of specific species of DAGs and ceramides. Here, we show that brisk walking for 45 min does not significantly alter the concentration of DAG or ceramide in skeletal muscle. However, exercise did lead to an increase in a number of unsaturated DAG species, as well as lactosylceramides. Importantly though, these changes (or lack of) occurred independent on whether plasma FFA concentrations were reduced or not.

Acipimox is a specific inhibitor of adipose tissue lipolysis, and ingestion of Acipimox before and during moderate intensity cycling exercise in lean, healthy individuals (van Loon et al., 2005b) and T2D patients (van Loon et al., 2005a) significantly reduces plasma FFA and glycerol concentrations. This results in a decrease in whole-body fat oxidation, with a reciprocal increase in rates of whole-body carbohydrate oxidation (van Loon et al., 2005a; van Loon et al., 2005b). In the present study, Acipimox ingestion also led to a significant decrease in plasma FFA and glycerol concentrations during and throughout recovery from a bout of brisk walking exercise, as well as an increase in carbohydrate oxidation and decrease in fat oxidation, in comparison to the control trial. Despite the decrease in fat oxidation, it has previously been reported that Acipimox ingestion actually increases IMTG utilisation during exercise (van Loon et al., 2005a; van Loon et al., 2005b). On this premise, we sought to determine if reducing plasma FFA concentrations during exercise would subsequently reduce the concentrations of DAGs and ceramides in skeletal muscle.

We observed no significant changes in total, saturated, or unsaturated DAG concentrations immediately following the exercise bout, or during the 3 h recovery period. However, several DAG species with two unsaturated FA were increased immediately following exercise, and

this occurred independent of the availability plasma FFA. Even in the obese individuals who participated in the present study we would expect exercise to increase IMTG turnover (i.e., utilisation). This premise is based on the observations of Bergman et al. (Bergman et al., 2018), who reported that in obese individuals and T2D patients the rate of IMTG synthesis is increased during exercise but no exercise-induced changes in IMTG content are observed; thus, IMTG turnover is increased in the absence of a net decrease in IMTG content, as typically observed in healthy, lean individuals. Based on this, the increase in several DAG species immediately post-exercise is to be expected, given that DAG is a breakdown product of triglyceride. Although measures of IMTG content and utilisation are yet to be made, we would hypothesise that IMTG use during exercise would be greater in the Acipimox compared to the control trial. However, assuming that changes in DAG concentrations reflect turnover of the IMTG pool, this hypothesis may not hold true. It is important, though, to consider the potential relevance of the post-exercise increase in specific DAG species with two unsaturated FA. It has previously been reported that DAG saturation is more closely linked to insulin sensitivity than total DAG content (Bergman et al., 2010; Bergman et al., 2012), although this is not without controversy (Amati et al., 2011). Nevertheless, an exercise-induced increase in DAG species with two unsaturated FA, as a result of IMTG breakdown, could be potentially 'safer' than creating a situation where saturated (or partly saturated) DAGs accumulate. Given that the subcellular location of DAGs may be important in mediating the relationship with insulin sensitivity (Bergman 2012), future work should investigate whether exercise effects the accumulation of DAG species (saturated and unsaturated) in membrane and cytosolic fractions in skeletal muscle.

While it has been shown that chronic endurance exercise training reduces ceramides in skeletal muscle (Bruce et al., 2006; Dube et al., 2008; Dube et al., 2011), acute exercise actually appears to lead to an increase in ceramide concentrations muscle (Helge et al., 2004; Bergman et al., 2016). In the present study, 45 min of brisk walking did not significantly alter skeletal muscle ceramide concentrations, and this continued throughout the 3 h recovery period, independent of condition. When considering the specific ceramide species though, we observed a significant decrease in Cer 20:0 at 3 h post-exercise in the Acipimox trial, whereas in the control trial Cer 20:0 remained elevated at 3 h post-exercise. Saturated ceramide species more readily form lipid 'rafts' in membranes, whereas ceramide species

containing double bonds disrupt these rafts (Pinto et al., 2011). Rafts containing saturated ceramide species decrease membrane fluidity which can subsequently influence insulin receptor function in the membrane (Storlien et al., 1991). It is worth noting though that Cer 20:0 is typically observed at a much lower abundance than Cer 18:0 concentrations, which have been shown to be linked to insulin sensitivity (Bergman et al., 2016). Furthermore, Cer 20:0 is not different between athletes, obese individuals and T2D patients (Amati et al., 2011). Therefore, whether the exercise-induced changes and/or turnover of Cer 20:0 is of physiological relevance in mediating insulin sensitivity requires further clarification.

In the present study, total lactosylceramide (LacCer) concentrations, as well as a number of LacCer species, were elevated immediately post-exercise, and were reduced but not restored to pre-exercise levels after 3 hours of recovery. LacCer is fmormed from glucosylceramide (GluCer), which in turn is generated from ceramide. Given the exercise-induced increase in LacCer observed in the present study, it could be expected that GluCer concentrations would be reduced (and similarly a reduction in ceramide concentrations). However, exercise did not lead to a decrease in GluCer concentrations (or ceramide, as discussed above). Thus, it could be speculated that both the rate of synthesis and degradation of ceramides and GluCer is being increased by exercise, resulting in greater formation of LacCer. Of course, direct measures of ceramide and GluCer synthesis are required to establish if this is true. The physiological relevance of the increase in LacCer is also not clear, since LacCer are precursors for the formation of gangliosides, which have been shown to promote insulin resistance in mice (Yamashita et al., 2003) via their localisation to the plasma membrane (Lipina et al., 2015). Moreover, LacCer has been shown to accumulate in the sarcolemmal fraction of muscle from T2D patients and may therefore play a role in the development of insulin resistance in humans (Perreault et al., 2018). However, in lean individuals LacCer accumulates in the nuclear fraction (Perreault et al., 2018), although the role of LacCer at the nucleus in skeletal muscle is not yet known. Investigating exercise-induced changes in the subcellular location of ceramides (and derivatives of ceramides) would provide additional insight, especially if combined with measures of ceramide synthesis.

A strength of the current study was that the timing and dose of Acipimox was successful in reducing plasma FFA concentrations during exercise and throughout the 3 h recovery period,

and this subsequently led to a reduction in whole-body fat oxidation and an increase in whole-body carbohydrate oxidation. Whether this strategy leads to a net reduction in IMTG content will be investigated using transmission electron microscopy. This approach was chosen because it permits subcellular analysis of lipid droplet morphology (lipid droplet number and size), which is important because the size of subsarcolemmal lipid droplets is closely related insulin resistance (Nielsen et al., 2017). Whether the increase in whole-body carbohydrate oxidation is accompanied by greater muscle glycogen utilisation, as has been previously reported (van Loon et al., 2005a; van Loon et al., 2005b) will also be investigated. This is important because an exercise-induced decrease in muscle glycogen concentrations augment insulin sensitivity (Perseghin et al., 1996; Wojtaszewski et al., 1997; Wojtaszewski et al., 2003a). Whilst recognising that specific species of lipids are linked to reduced insulin sensitivity (Bergman et al., 2016), this still oversimplifies the multifaceted reality of lipids within skeletal muscle. For example, lipids reside in many subcellular compartments and are continuously being trafficked between these, so changes to compartmentation and trafficking of lipids may reveal differences between trained individuals and T2D patients (Bergman and Goodpaster, 2020). Future work using exercise to understand the turnover of different lipids in skeletal muscle should also consider subcellular distribution.

In summary, oral administration of an anti-lipolytic drug suppresses plasma FFA concentrations and alters whole-body substrate utilisation during 45 min of brisk walking in obese individuals. However, this combined exercise and pharmacological strategy did not differentiate from exercise alone with respect to changes in the concentration of specific species of DAGs and ceramides in skeletal muscle. Future work will determine whether exercise combined with oral administration of an anti-lipolytic drug enhances subcellular IMTG and glycogen utilisation.

Chapter 6. A randomised free-living walking-based exercise trial improves HbA1c in individuals with obesity, with additional benefits of nutritional status on metabolic health.

6.1 Abstract

Regular exercise improves insulin resistance and glycaemic control, but 'real world' programmes are not universally effective. Laboratory studies show that post-breakfast exercise improves postprandial glucose excursions with subsequent meals while pre-breakfast exercise augments whole-body fat oxidation. Whether these acute effects translate to greater changes in metabolic health with exercise training is not clear. To investigate if exercise before/after breakfast leads to differential improvements in metabolic health and/or postprandial glycaemic response. Thirty-four people with obesity (43±12 y, BMI 35.1±5.1 kg/m^2) undertook a 12-week walking-based programme, with two continuous and two interval sessions each week (for 30-60 min, at 50% HR_{max} *vs.* alternating 3 min at 85% HR_{max} and 3 min 50% HR_{max} respectively). Participants exercised before (FAST) or after (FED) breakfast (*n*=17 per group). Body composition, HbA1c, blood lipids and liver transaminases (ALT/AST) were assessed pre- and post-intervention. Postprandial glucose responses were assessed in week 1 and 12 using flash glucose monitoring. Adherence (FAST: 93%, FED: 95%) and compliance (FAST: 85%, FED: 88%) was high in both groups. Body mass, BMI, waist-to-hip ratio and HbA1c decreased similarly between groups (all *P* <0.001*,* HbA1c *P*=0.01). However, ALT decreased after FAST (-16%; *P*=0.001), but not FED training (-2%; *P*=0.720). In week 1, continuous exercise lowered postprandial responses to lunch (FAST; *P*=0.064) and dinner (FED; *P*=0.047) versus interval exercise. In week 12, the postprandial glucose responses to lunch and dinner after interval exercise were lower compared to week 1 (*P*=0.008). Free living exercise, pre- or post-breakfast improved body composition, glycaemic control and enhanced the postprandial-lowering effect of interval exercise. Pre-breakfast exercise had a greater impact on liver biochemistry. Exercise, pre-breakfast, may be advantageous for people with obesity and fatty liver.

6.2 Introduction

The prevalence of overweightness and obesity continues to grow, with an estimated 68% men and 60% of women in England currently classified as being overweight or obese (England, health and Health Survey for England, 2022). Importantly, obesity predisposes individuals to dyslipidaemia, hypertension, and chronic low-grade inflammation, all of which are linked to the accumulation of lipid in non-adipose tissues such as skeletal muscle, liver and the pancreas. This cluster of metabolic issues all contribute to the lowering of insulin sensitivity and glucose intolerance (Lorenzo et al., 2003) which can manifest as an elevation in postprandial glucose responses following ingestion of mixed meals throughout the day. Consequently, being overweight or obese places individuals at a six-times greater risk of developing type 2 diabetes mellitus (T2D) compared to lean, healthy individuals (Schnurr et al., 2020). Therefore, developing effective strategies that delay or prevent the transition from obesity to T2D remains a priority for healthcare researchers.

Given the role of hyperglycaemia in the development of pathologies linked to T2D, understanding how exercise and/or nutrition can influence glycaemic control is of significant interest. Common laboratory methods such as oral glucose tolerance testing, however, may not accurately reflect the influence of exercise on glycaemic regulation (Mikus et al., 2011; Mikus et al., 2012a; Mikus et al., 2012b), and so assessing post-prandial glucose control in free-living conditions is particularly important. Continuous glucose monitors provide novel data on the direction and magnitude of glycaemic excursions in response to mixed meals and therefore can provide unique insight into postprandial glycaemic responses over the course of the day under free-living conditions (Monnier and Colette, 2008). Studies examining the impact of exercise on free-living glucose control using CGM in individuals without T2D are sparse, but van Dijk et al. (2012) reported that a single session of either continuous moderate intensity exercise or resistance exercise improved glycaemic regulation in participants with impaired glucose tolerance (i.e., prediabetes). Thus, CGM appears to be a valuable tool to assess the impact of exercise on glucose control in individuals with, or at risk of, T2D.

Regular exercise is the cornerstone for the prevention of insulin resistance (Bjorntorp et al., 1970; Seals et al., 1984; Mikines et al., 1988; Rogers et al., 1988; Burstein et al., 1990; Cononie

et al., 1994; Dela et al., 1996). The most effective exercise training interventions that improve insulin sensitivity and glycaemic control in overweight/obese individuals appear to employ high frequency (≥4 sessions per week) and/or high intensity (≥70% VO_{2max}) exercise sessions (DiPietro *et al.,* 2006; Karstoft *et al.,* 2014; Kaarstoft *et al.,* 2014; Liubaoerjijin *et al.,* 2016). Therefore, a combination of interval exercise and continuous type exercise should be incorporated when designing an exercise programme aimed specifically at improving insulin sensitivity and glycaemic control. Brisk walking at a lower intensity (~50% VO_{2max}) is well tolerated by overweight/obese individuals (Morikawa *et al.,* 2011; Memoto *et al.,* 2007), likely due to their low starting exercise capacity and physical capabilities coupled with the low risk of injury that accompanies walking (Tse *et al.,* 2012). As well as being well tolerated, walking interventions are effective in improving insulin sensitivity (Yamanouchi et al., 1995). Acutely, one interval walking exercise session improves glycaemic control in T2D individuals when compared with an oxygen consumption- and duration-matched continuous walking exercise session (Karstoft et al., 2014), and may have greater and longer lasting postprandial glucose-lowering effects than continuous exercise (Little et al., 2014). After 12-weeks of interval versus continuous walking, interval walking led to significant improvements in very low-density lipoprotein cholesterol in an obese population (Campbell et al., 2010). However, it is important to note that intervention-induced changes in insulin sensitivity and glucose tolerance may simply be related to the effects of the last exercise session. In this case, it is important to understand whether the post-prandial lowering effect of an acute exercise session is altered following an exercise intervention.

When designing exercise programmes for clinical populations, there is often little consideration for the timing of exercise relative to meal ingestion. However, manipulating the timing of meal ingestion relative to exercise is an important consideration, and could influence the long-term effectiveness of an exercise programme. For example, exercising after breakfast (i.e., in the postprandial state) leads to greater improvements in glycaemic responses to subsequent meals (lunch and/or dinner) compared to exercise undertaken before breakfast (Nygaard et al., 2017). However, longer-term exercise training (12 weeks) in fasted state has been associated with greater improvements in insulin sensitivity in healthy subjects compared to training after breakfast (Hansen et al., 2017). Whether regularly exercising before or after breakfast in individuals with obesity culminates in a greater effect

on postprandial glucose responses and glycaemic control is not yet clear, since only two long-term studies have been conducted with contrasting results(Edinburgh et al., 2020; Verboven et al., 2020). One study in obese individuals reported increased plasma glucose disposal during subsequent meals following 6 weeks of fasted exercise training (Edinburgh et al., 2020), whereas another study in T2D patients observed greater improvements in HbA1c following 12-weeks of fed exercise compared to fasted exercise (Verboven et al., 2020). Another issue is that studies investigating the nutrient-exercise interactions during chronic interventions have been lab-based. Under these conditions, tight control of diet and exercise allows for accuracy in the measurement of the study outcomes. However, these studies are not directly translatable to everyday living. Therefore, whether the timing of exercise relative to the first meal of the day determines the response to exercise training in the real world is not known. With this in mind, the primary aims of the study were to 1) determine whether changes to glycaemic control following an exercise intervention differ when exercise is completed in the fasted or fed state, and 2) assess changes to body composition, HbA1c, blood lipids, and markers of liver function following a 12-week free-living exercise intervention. A secondary aim of the study was to investigate the acute glucose responses to continuous or interval exercise sessions dependent on nutritional state in a free-living situation.

6.3 Methods

6.3.1 Participant information

This study took place during the COVID-19 global pandemic, between May 2020 and April 2021. All data collection and the intervention itself was conducted remotely, using equipment and services that were available, or made available to the participant themselves, thereby minimising the risk of virus transmission. Thirty-four sedentary overweight individuals were recruited from all areas of the UK via social media (see Table 6.1 for characteristics). Participants were free of metabolic or cardiovascular disease. They were deemed sedentary if they performed less than two 30-minute structured exercise sessions per week for the last year and were unable to take part if they were taking any diabetes medication. Eligible participants provided written informed consent, and the study was approved by the Liverpool

Central NHS Research Ethics Committee (17/NW/0042) and conformed to the Declaration of Helsinki.

6.3.2 Experimental protocol

Using a parallel groups design, participants took part in a 12-week walking-based exercise programme where each prescribed exercise session was undertaken either before or after breakfast (detailed below). Participants were randomised upon recruitment in an alternating group allocation approach whereby participants 1, 3 ,5 etc would be in the fed group, and participants 2, 4, 6 etc would be in the fasted group for ease of distance monitoring. The lead researcher was not blinded to the group allocations. Before and after the intervention assessments of body composition, fitness, blood lipids, markers of liver function, and free-living glycaemic control were made. Following receipt of written consent, participants were initially sent a study pack by post which contained all equipment and information required for the study. This included a Bluetooth polar heart rate (HR) monitor, two Freestyle Libre continuous glucose monitors (Abbott, UK), a fabric tape measure, and an instruction booklet containing a timetable for the study, details of each assessment and the outline for the training programme. A commercially available capillary blood sampling kit (Thriva, UK) was also delivered directly to the participant. Before undertaking any baseline assessments, all participants had a video call with the lead researcher (JB) to talk through the assessments (alongside the explanation provided in the instruction booklet) and answer any questions. Full details of each assessment follows below.

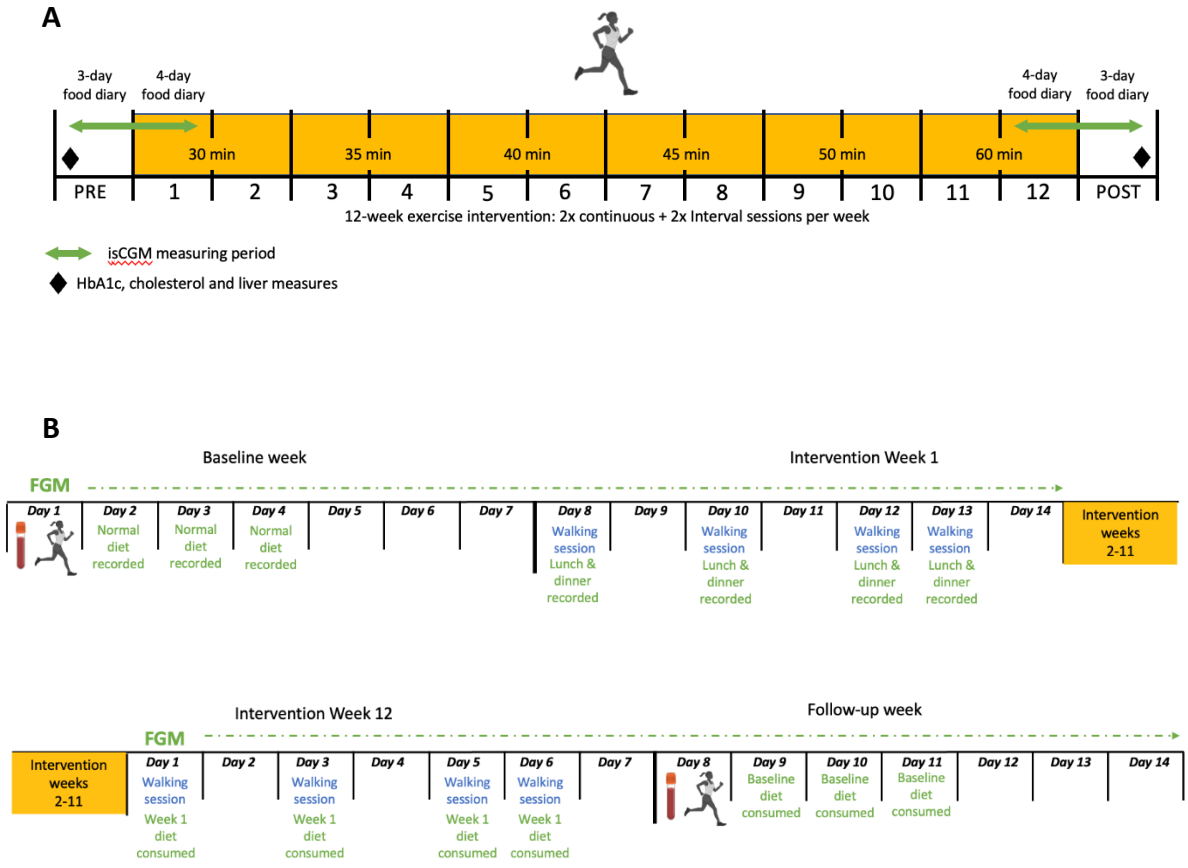


Figure 6.1. (A) Overview of the study protocol, including data collection weeks at the start of the 12-week intervention (baseline and week 1) and then following the intervention (week 12 and follow-up). In further detail below (B) Individuals inserted the intermittently scanned continuous glucose monitoring (isCGM) on day 1 of the baseline week which then remained in place for two weeks. During the baseline week, participants carried out a finger prick blood sample, a fitness test and kept a food diary for 3 days. During week 1 of the intervention, the isCGM was still in place and participants recorded their lunch and dinner on the days they completed their first 4 exercise sessions. An isCGM was then re-inserted at the start of week 12 of the intervention and on the 4 days of exercise in this week participants were asked to consume the same diet they did in week 1 of the exercise intervention. In the follow-up week, participants repeated the finger prick blood sample, fitness test and were asked to consume the exact same diet as they did in the 3 days recorded in the baseline week.

6.3.3 Body Composition

Participants were provided with a tape measure and scales (if required), to measure their height (cm), weight (kg), waist and hip circumference (cm). The instruction booklet sent in the study packs contained details on the correct area to measure waist and hip circumference. This was then used to calculate waist-to-hip ratio.

6.3.4 Fitness test

Participants were asked to carry out the Harvard step test as a measure of fitness (Brough et al., 1943). Briefly, participants were required to step onto, and back down from a step ~30cm high at a rate of 30 completed steps per minute (one second up, one second down) for 5 minutes. The individuals immediately sat-down following completion of the test, and the total number of heart beats were counted from 1 to 1½ minutes after finishing, from 2 to 2½ minutes after finishing, and finally from 3 to 3½ minutes after finishing. The participants wore the HR monitor during the test to record beats per minute, and from this, a 'fitness index' measure was calculated using the following equation:

$$\text{Fitness index} = 30000 \div (\text{pulse1} + \text{pulse2} + \text{pulse3})$$

The fitness index was then compared to normative data available for 16-year-old athletes (Beashel and Taylor, 1997). No normative data for our population is currently available.

6.3.5 Blood sampling

Participants were sent a commercially available home blood testing kit (Thriva, UK) in order to measure triglyceride and cholesterol concentrations, several markers of liver function (gamma-GT, alanine transferase, alkaline phosphatase, globulin, albumin, bilirubin) and HbA1c. Participants were asked to collect the blood sample in the morning following an overnight fast. Clear and detailed instructions on sample acquisition were provided to the participants as part of the kit. Briefly, participants used an alcohol wipe to clean one finger, pierced the skin using a lancet, and then filled two 1.5 ml Eppendorf tubes with blood. These tubes were then packaged as instructed and posted the same day to an accredited Thriva laboratory. Following analysis, results of the test were uploaded to an online platform which was available to view by the participant and the lead researcher.

6.3.6 Intermittently scanned Continuous Glucose Monitor (isCGM)

Participants were required to wear an intermittently scanned continuous glucose monitor on the back of their upper arm on Day 0 which then remained in place for 2 weeks. Participants were also required to download the Freestyle Libre app and an individual log-in was provided by the researchers so that both the participant and research team had access to their isCGM data. The sensor was inserted on the upper arm using the instructions provided on the Freestyle Libre app and supervised by a researcher on a video call. The sensor requires scanning (using the Freestyle Libre app) at least every 8 hours which notified participants when they had 1-hour of the 8 remaining to scan. Following a 24 h 'bedding in' period, data was collected from Day 1 to Day 15, and therefore spanned the pre-intervention assessment week and week 1 of the intervention (Figure 1). Diet was recorded in a self-reported food diary for 3 days during the pre-intervention assessment week, and for the 4 exercise days in week 1 of the intervention. The sensor was removed by the participant from the upper arm on Day 15. A second FGM sensor was inserted at the beginning of week 12 and remained in place for 2 weeks, such that isCGM glucose data was collected during the final week of the intervention and 7 days post-intervention. Participants were sent their food diary from the beginning of the intervention and were asked to consume an identical diet for the 4 exercising days in week 12 and for the 3 days in the post-intervention assessment week.

6.3.7 Exercise and diet Intervention

The exercise intervention was identical in all respects for all participants, only differing by the nutritional status under which the exercise sessions were completed (detailed below). The intervention included four exercise sessions per week for 12-weeks, incorporating two continuous walking sessions and two interval walking sessions. The continuous session required participants to walk at a pace that maintained their HR between 50 and 60% (identified as the pale blue zone in the Polar Beat app) of estimated HR maximum (HR_{max}; calculated as 220 minus age). The interval sessions required participants to alternate between 3 minutes of walking at 50-60% HR_{max} and 3 minutes at 80-90% HR_{max} (identified as the orange zone in the Polar Beat app). All exercise sessions in week 1 were 30 minutes in duration and increased every two weeks by 5 minutes for the continuous walking sessions and 6 minutes

for the interval walking sessions. Participants were allocated to either the fasted (FAST) or fed (FED) exercise group matched for gender, age and weight. Those in FAST were instructed to complete their prescribed exercise session after waking and before consuming anything other than water. They were then required to consume their habitual breakfast within 30-90 minutes of completing the exercise session. In FED, participants were required to first consume their habitual breakfast, and then complete their prescribed exercise session 30-90 minutes after. Participants had a weekly video call with the lead researcher to check they had completed all their sessions and discuss any issues they were having with the programme.

6.3.8 Training monitoring and exercise data analysis

All participants were provided either a Polar H10 chest strap heart rate monitor or a Polar Verity Sense optical heart rate sensor as part of their study pack. Participants were thus provided with real-time HR readings during the exercise sessions when paired with the Polar Beat app, in order to help them meet the desired exercise intensity for the session. The HR data was also used to assess adherence and compliance to the exercise programme. All HR data from each exercise session was stored on a cloud-based system, available at www.flow.polar.com to the research team. For each MICT session, data pertaining to exercise duration and mean HR (as a % of HRmax) was extracted. For each interval session, the number of intervals completed, peak HR on each interval, % of intervals achieving the criterion HR (≥80% HRmax), and time spent above the criterion HR were recorded. Adherence was calculated as the % of prescribed sessions completed during the programme. Compliance to the prescribed exercise is defined differently for continuous exercise sessions and interval sessions, and has previously been detailed by Hesketh et al., (2021), but generally refers to the achievement of both a prescribed duration and intensity. For the continuous exercise sessions, duration was adjusted for the exercise intensity to produce a HR physical activity score (HRPAS = min × %HRmax) for each session (Miller et al., 2014). If the session HRPAS was equal to or greater than the prescribed HRPAS, the session was compliant. Interval session compliance was defined as achieving a HR ≥80% HRmax during the session and performing the prescribed number of intervals.

6.3.9 Dietary analysis

At baseline, participants recorded their habitual diet in an electronic diary for 3 consecutive days. During week 1 of the intervention, participants also recorded their food intake on the days on which they performed their prescribed exercise sessions. These diaries were collected by the research team and subsequently returned to the participants towards the end of the intervention, with the request to replicate their meals on the exercise days in week 12 and for 3 consecutive days during follow-up (Figure 1). Diaries were analysed using Nutritics software (Nutritics Ltd., Co. Dublin, Ireland) for total energy (kcal) and macronutrient intake. All analyses were carried out by a single trained researcher in order to minimise any potential variation in data interpretation.

6.3.10 Statistical analysis

All data were analysed using statistical analysis software (Statistical Package for the Social Sciences for Windows, version 27.0, Chicago, IL, USA). A mixed-design ANOVA was used to interrogate all time and group-dependent data, whereas an independent t-test was used to investigate differences in adherence and compliance to the exercise intervention between FAST and FED. Data are presented as means $\pm$ S.D. Significance was set at $P<0.05$.

6.4 Results

6.4.1 Anthropometrics

Throughout the COVID-19 pandemic, 34 participants were recruited for the study (n=17 per group). Two participants in FED withdrew their participation, meaning the data presented includes 17 individuals in FAST and 15 individuals in FED. Baseline characteristics are reported in Table 1. At baseline, there were no significant differences between the two groups for age or any anthropometric variables. The 12-week training intervention significantly reduced weight (-4% in FAST, -3% FED; P <0.001), BMI (P <0.001) and waist and hip circumferences (both P <0.001), with no difference between the two groups (P>0.05).

Table 6.1. Data concerning changes in body composition are reported. Training reduced weight, BMI, as well as waist and hip circumference (*P*<0.001), with no difference between the two groups.

Variable	FAST		FED		Time effect	Time x group
	Baseline	Follow-up	Baseline	Follow-up	(p value)	(p value)
n (M/F)	7/10		5/10		-	-
Age (years)	47±11		43±12		-	-
Height (m)	1.71±6.8		1.69±9.8		-	-
Weight (kg)	101.3±17.1	97.5±15.9	98.5±20.5	95.4±21.0	<0.001	0.620
BMI ($kg.m^{-2}$)	34.8±5.1	33.6±5.0	34.1±4.8	33.1±4.9	<0.001	0.369
Waist circumference (cm)	110 ± 16	103 ± 16	108 ± 17	103 ± 16	<0.001	0.812
Hip circumference (cm)	117 ± 8	111 ± 8	116 ± 11	110 ± 11	<0.001	0.868
Waist:hip ratio	0.94±0.13	0.93±0.12	0.94±0.11	0.93±0.10	0.290	0.871
Fitness Index	98±21	110±17	98±20	108±15	<0.001	0.662

Data are presented as means ± S.D

6.4.2 Adherence and compliance to exercise sessions

The mean duration and HR responses to the exercise sessions over the 12-week programme are reported in Tables 2 and 3. Overall adherence to the exercise programme was similar between conditions (*P* = 0.456), with 94% and 95% sessions completed in the FAST and FED groups, respectively. Considering each type of exercise session, similar rates of adherence were observed between groups to both the continuous sessions (FAST 95%; FED 96%; P = 0.656) and interval sessions (FAST 93%; FED 95%; P = 0.639).

Compliance to the prescribed exercise is defined differently for continuous exercise sessions and interval sessions, and has previously been detailed by Hesketh et al., (2021), but generally refers to the achievement of both a prescribed duration and intensity. For the continuous exercise sessions, duration was adjusted for the exercise intensity to produce a HR physical activity score (HRPAS = min × %HRmax) for each session (Miller et al., 2014). If the session HRPAS was equal to or greater than the prescribed HRPAS, the session was compliant. Using this approach, compliance to the continuous walking sessions was not different between groups (FAST 84%, FED 85%; *P* = 0.955). Interval session compliance was defined as achieving

a HR ≥80% HRmax during the session and performing the prescribed number of intervals, and again, compliance to the interval walking sessions was similar between groups (FAST 86%, FED%; *P* = 0.671). Thus, total compliance to all the exercise sessions was 85% for FAST and 87% for FED.

Table 6.2. Reporting the total duration and mean HR for every 2-week period of continuous session.

Variable	**Continuous**					
Weeks	1+2	3+4	5+6	7+8	9+10	11+12
Duration (min:sec)						
Fasted	31:27 ± 2:26	35:54±2:48	42:05 ± 2:51	45:45 ± 2:44	56:34 ± 11:00	01:01:39 ± 4:02
Fed	34:08 ± 8:56	39:04 ± 10:06	43:00 ± 5:40	30:08 ± 4:57	55:33 ± 9:39	01:02:11 ± 5:15
HR_{mean} (%HR_{max})						
Fasted	69 ± 7	69 ± 8	69 ± 7	69 ± 7	69 ± 7	68 ± 6
Fed	70 ± 5	69 ± 6	69 ± 7	68 ± 5	67 ± 6	67 ± 6

Table 6.3. Reporting the total duration, peak HR and time above HR max for every 2-week period of interval session.

Variable	**Interval**					
Weeks	1+2	3+4	5+6	7+8	9+10	11+12
Duration (min:sec)						
Fasted	32:59 ± 4:34	36:35 ± 01:54	44:06 ± 3:28	49:44 ± 6:21	55:11 ± 4:08	1:05:36 ± 13:05
Fed	33:40 ± 8:47	39:37 ± 12:04	44:48 ± 7:23	50:33 ± 5:41	55:20 ± 7:35	1:02:11 ± 5:15
HR_{peak} (%HR_{max})						
Fasted	81 ± 8	82 ± 10	82 ± 9	83 ± 8	84 ± 9	83 ± 8
Fed	82 ± 4	88 ± 24	85 ± 9	82 ± 6	81 ± 9	81 ± 8
Time above 80% HR_{max}						
Fasted	1:11 ± 2:24	1:29 ± 3:40	1:31 ± 3:11	2:04 ± 4:13	1:45 ± 3:12	1:55 ± 3:36
Fed	0:43 ± 1:09	1:29 ± 1:40	2:24 ± 1:40	1:27 ± 2:16	1:14 ± 1:59	1:36 ± 2:14

Data are mean ± SD of time spent for each variable.

6.4.3 Energy intake and macronutrient composition

Dietary data was first collected on three consecutive days during baseline and follow-up. Importantly, total energy intake and the macronutrient composition (in both absolute and

relative terms) of each meal was similar across the three days during baseline and follow-up (Table 6.4). Dietary data was also collected on the prescribed exercise days during week 1 and week 12 of the intervention. Total energy and macronutrient intake for breakfast, lunch and dinner for the 3-day food diary at baseline and follow-up, as well as the 4-days recorded lunch and dinner on the exercise days in weeks 1 and 12 of the intervention, were analysed to determine whether participants adhered to the requirements of eating the same macronutrients over the intervention. Carbohydrate, protein and fat intake at breakfast, lunch and dinner was not different at baseline compared to follow-up ($P > 0.05$). Nor was there a difference in carbohydrate, protein or fat intake between groups at breakfast ($P > 0.05$).

As with the baseline and follow-up weeks, the macronutrient and total energy intake on the exercise days did not differ between week 1 and week 12 of the exercise intervention for lunch (CHO, P = 0.492; PRO, P = 0.942; FAT, P = 0.930) or dinner (CHO, P = 0.783; PRO = 0.624; FAT, P = 0.732). Macronutrient intake did not differ between groups at lunch (CHO, P = 0.636; PRO, P = 0.367; FAT, P = 0.423) or dinner (CHO, P = 0.126; PRO, P = 0.552; FAT, P = 0.473) during intervention weeks 1 and 12 also.

Table 6.4. Reporting the mean macronutrients for breakfast, lunch and dinner consumed over the 3 recorded days at baseline and follow-up.

Baseline – Follow-up	FAST		FED	
(g, unless stated)	Baseline	Follow-up	Baseline	Follow-up
Breakfast				
Carbohydrate	45 ± 22	43 ± 20	39 ± 20	45 ± 39
Fat	13 ± 11	13 ± 10	11 ± 9	11 ± 8
Protein	16 ± 11	17± 11	14 ± 10	17 ± 11
Total Energy (kcal)	355 ± 193	347 ± 176	309 ± 135	324 ± 141
Lunch				
Carbohydrate	78 ± 42	74 ± 36	74 ± 38	76 ± 45
Fat	22 ± 12	19 ± 9	20 ± 12	21 ± 14
Protein	26 ± 11	23 ± 9	24 ± 11	24 ± 12
Total Energy (kcal)	607 ± 284	562 ± 251	567 ± 265	586 ± 316
Dinner				
Carbohydrates	98 ± 31	102 ± 28	106 ± 43	107 ± 42
Fat	35 ± 12	38 ± 12	37 ± 13	38 ± 14
Protein	41 ± 15	38 ± 12	44 ± 16	45 ± 16
Total Energy (kcal)	866 ± 183	861 ± 165	926 ± 237	995 ± 374

Data are mean ± SD for mean macronutrient intake in grams.

Table 6.5. Reporting the mean macronutrients for breakfast, lunch and dinner consumed over the 4 recorded days in intervention weeks 1 & 12.

Intervention week 1 – week 12	FAST		FED	
(g, unless stated)	Week 1	Week 12	Week 1	Week 12
Breakfast				
Carbohydrate	45 ± 22	43 ± 20	39 ± 20	45 ± 39
Fat	13 ± 11	13 ± 10	11 ± 9	11 ± 8
Protein	16 ± 11	17 ± 11	14 ± 10	17 ± 11
Total Energy (kcal)	355 ± 193	347 ± 176	308.9 ± 135	324 ± 141
Lunch				
Carbohydrate	77 ± 36	78 ± 37	74 ± 38	79 ± 43
Fat	21 ± 11	16 ± 10	20 ± 12	22 ± 12
Protein	24 ± 10	28 ± 10	24 ± 11	26 ± 11
Total Energy (kcal)	587 ± 251	521 ± 255	567 ± 265	617 ± 286
Dinner				
Carbohydrates	100 ± 39	99 ± 40	114 ± 45	111 ± 44
Fat	35 ± 11	35 ± 11	37 ± 15	39 ± 16
Protein	42 ± 16	42 ± 16	44 ± 15	44 ± 16
Total Energy (kcal)	884 ± 220	850 ± 195	965 ± 296	972 ± 299

Data are mean ± SD for mean macronutrient intake in grams.

6.4.4 Blood lipids, HbA1c, and markers of liver function

Blood lipids, HbA1c, and liver function measures are reported in Table 6. At baseline, there was no group differences observed for any of these measures. Twelve weeks of walking exercise training did not alter total cholesterol, triglyceride, LDL cholesterol or HDL cholesterol concentrations in either group (P>0.05). Importantly though, the 12-week training intervention significantly reduced HbA1c (P = 0.01), although the magnitude of the reduction was relatively small and similar between conditions (P>0.05); the change in HbA1c for FAST ranged from -4 to +1, and -from 7 to +2 in the FED group. The training intervention also had a significant effect on the circulating concentrations of the key liver enzyme ALT, with a decrease being observed following training (P = 0.006). Post-hoc analysis revealed the decrease was specific to the fasted group (-16 %, P = 0.001) but was not apparent in the fed group (-2%, P = 0.720). For all other liver protein measures, there was no significant changes following training (see Table 6.6).

Table 6.6. Changes in blood lipids, HbA1c and markers of liver function in response to FAST or FED training.

Variable	FAST		FED		Time effect	Time x group
	Pre	Post	Pre	Post	(p value)	(p value)
HbA1c (%)	38±4	35±4	37±5	36±4	0.010	0.763
Triglycerides ($mmol.L^{-1}$)	1.73±0.6	1.70±0.6	2.03±1.0	1.82±0.7	0.343	0.498
Cholesterol ($mmol.L^{-1}$)	5.7±0.8	5.6±0.8	5.6±1.1	5.5±1.0	0.440	0.909
LDL-cholesterol ($mmol.L^{-1}$)	3.5±0.6	3.5±0.7	3.3±0.9	3.3±0.8	0.783	0.672
HDL-cholesterol ($mmol.L^{-1}$)	1.4±0.3	1.4±0.3	1.3±0.2	1.3±0.2	0.243	0.611
Gamma-GT (IU/lL)	36.4±36.8	34.3±39.0	57.5±131.7	58.3±148.2	0.779	0.566
Alanine Transferase (IU/L)	33.9±17.5	28.4±14.9*	28.3±19.6	27.8±20.4	0.006	0.021
Albumin (g/L)	41.5±2.4	40.9±2.2	42.6±2.4	42.4±2.0	0.294	0.576
Alkaline Phophatase (IU/L)	83.0±25.9	87.2±30.0	81.7±21.7	80.8±22.0	0.374	0.180
Globulin (g/L)	30.2±3.3	30.4±3.1	30.7±4.2	30.9±3.4	0.623	0.919
Bilirubin (umol/L)	10.5±4.0	9.5±3.3	9.7±2.3	10.1±2.5	0.504	0.178
Total Protein – (albumin & bilirubin combined) (g/L)	71.7±4.0	66.9±16.3	73.2±5.7	73.3±4.9	0.234	0.124

Data are mean ± SD, significance <0.05. Post hoc analysis indicates significant change post intervention in the FAST group (*).

6.4.5 Intermittent scanning continuous glucose monitoring outcomes

Glycaemic control at baseline and follow-up on non-exercise days

Mean glucose over the 3-days of recorded diet at the start of the intervention was 6.0 ± 1.0 $mmol{\cdot}L^{-1}$ for FAST and 6.1 ± 1.5 $mmol{\cdot}L^{-1}$ for FED. The coefficient of variation for the mean glucose over the 3-days at baseline was 10 ± 4% for FAST and 14 ± 8 % for FED with no difference between groups (P>0.05). Following the intervention mean glucose tended to be reduced in FAST (5.8 ± 1.2 $mmol{\cdot}L^{-1}$; P = 0.09) but was unchanged in FED (6.2 ± 1.4 $mmol{\cdot}L^{-1}$; P = 0.90). The intervention did not alter the glucose coefficient of variation in either FAST (12% ± 9) or FED (11% ± 4; P>0.05).

The postprandial responses to each meal, defined as AUC for the 3 hours following the time participants recorded as the time of each meal, were also examined (see figure 2). At baseline, there was a main effect of meal detected (*P*=0.040), whereby glucose AUC was significantly greater following dinner compared to breakfast (*P*=0.047), which corresponded to mean glucose also being greater following dinner compared to breakfast (*P*=0.020).

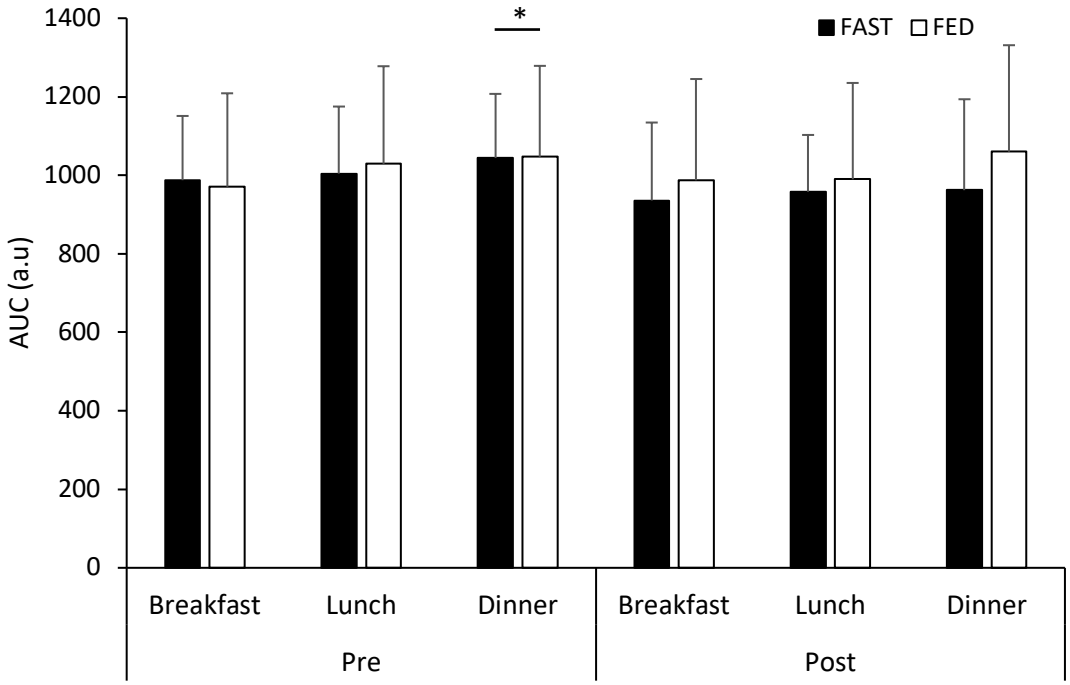


Figure 6.2. The area under the curve responses to breakfast, lunch and dinner during the baseline week and follow-up week. AUC was significantly greater following dinner (*) compared to following breakfast (*P* >0.05). Data are mean ± SD.

When examining post-prandial glucose responses at baseline compared to follow-up, there was a significant time × meal interaction (main effect; P = 0.047). However, the post hoc analysis revealed no significant changes in postprandial glucose AUC to any of the three meals from baseline to follow-up. Rather, there was only a difference in glucose AUC in response to breakfast versus dinner prior to the training intervention (post-hoc analysis of time X meal effect; *P* = 0.046), whereas following the intervention this meal-specific difference disappeared (post-hoc analysis of time X meal effect; *P* = 0.135). Peak glucose during the post-prandial period was greater following dinner compared to breakfast (Main meal effect; P =

0.043), with no difference between groups or from baseline to follow-up (*P* >0.05). Overall, then, it appears that the intervention did not affect free-living postprandial glucose responses on non-exercise days.

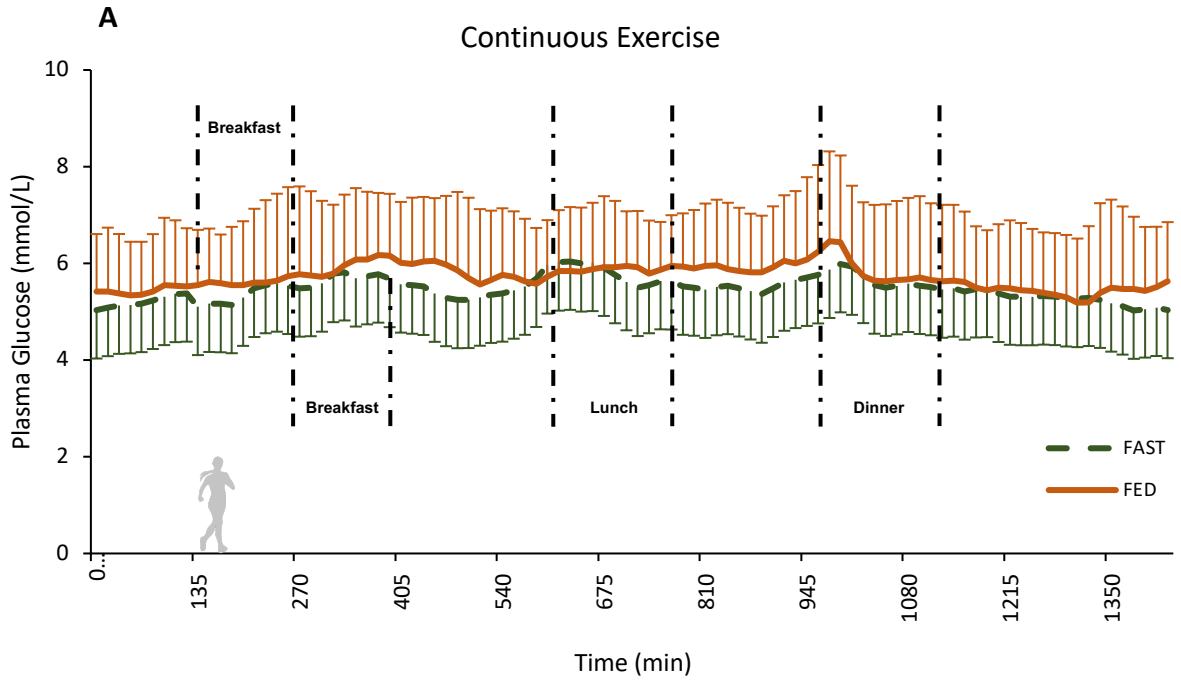
Glycaemic control following exercise

Free-living glucose data was also collected during the first (week 1) and final week (week 12) of the intervention, specifically focusing on glucose control on each of the exercise days during these weeks.

Analysis of the 24 h isCGM response to the exercise sessions in week 1 of the intervention showed no difference in the mean glucose values after continuous or interval walking sessions (main time effect; *P* = 0.812), nor was there a difference in this 24 h mean glucose between FAST or FED groups (main group effect; *P* = 0.601). Furthermore, glucose CV was not significantly different between groups or exercise types at baseline (P <0.05). In week 12 of the intervention, there was no significant difference in mean 24 h glucose compared to week 1 in either FAST or FED, or when comparing the interval and continuous exercise sessions (main exercise type effect; P <0.05). There was, however, a significant group × exercise type interaction where the FED group had a significantly greater glucose CV following the interval exercise sessions compared to the FAST group (4.0% vs 2.5%). Within the FED group, there was a significantly greater glucose CV following the interval exercise compared to the FED response to continuous exercise sessions (4.0% vs 2.6%).

When examining the acute post-prandial glucose response to continuous or interval sessions in week 1 of the intervention, there was a significant 3-way interaction (main exercise type X meal X group effect; *P* = 0.012) which revealed a trend for a lower glucose AUC for lunch in the fasted group following continuous exercise compared to interval exercise (main exercise type X meal X group effect; *P* = 0.064), and a significantly lower glucose AUC for dinner in the fed group following continuous exercise compared to interval exercise (main exercise type X meal X group effect; *P* = 0.047). Consequently, the fasted group tended to experience a lower mean glucose following lunch on days when they did continuous exercise compared to interval exercise (main exercise type X meal X group effect; *P* = 0.052), whereas the fed group experienced a lower mean glucose following dinner when they did continuous exercise (main

exercise type X meal X group effect; $P = 0.041$). Thus, in the first week of the intervention continuous exercise led to a lower postprandial glucose response compared to interval exercise, with the meal at which the postprandial-lowering effect manifested being dependent on whether exercise was undertaken before or after breakfast.



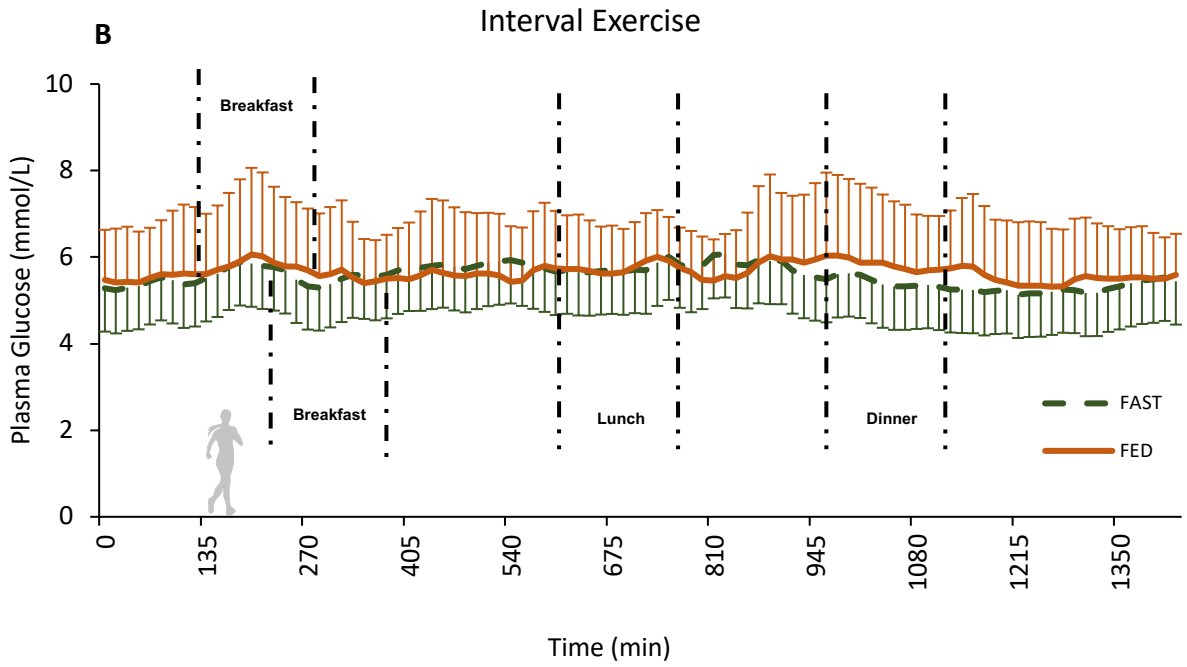


Figure 6.3. Mean isFGM trace on a continuous (A) and on an interval exercise day (B). Solid orange line represents the fed group (FED), dashed green line is the fasted group (FAST). Meals are represented by dotted lines and the timing of the exercise session is depicted along the x axis. Data are mean ± SD.

When examining post-prandial glucose responses following exercise in week 1 compared to week 12, there were several key observations. First, there was a significant time × exercise type interaction, where the post-prandial glucose response (AUC) to meals following the interval exercise sessions in week 12 were lower compared to the response to the interval exercise sessions in week 1 (P = 0.008). Thus, training improves the postprandial glucose-lowering effect of interval exercise. Further interrogation of the data revealed that when the FAST group performed interval exercise, they experienced a lower post-prandial glucose response to lunch in week 12 compared to week 1 (P = 0.002), whereas when the FAST group performed continuous exercise they experienced a lower post-prandial glucose response to dinner in week 12 compared to week 1 (P = 0.048). No such responses were observed for the FED group. Therefore, it appears that regularly training before breakfast improves the postprandial glucose lowering effect of exercise compared to training after breakfast.

A

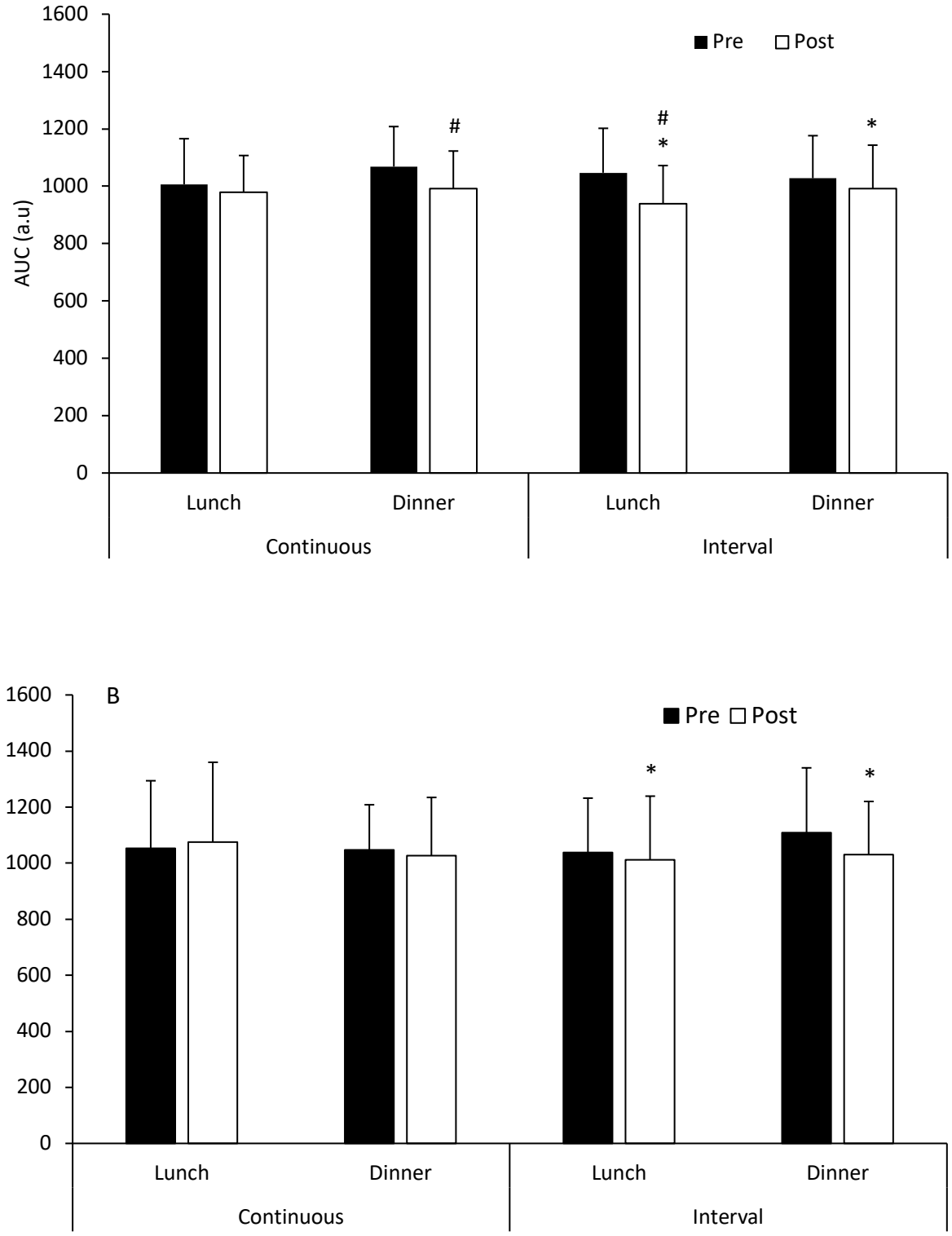


Figure 6.4. AUC following continuous and interval exercise sessions before and after the intervention in the FAST group (A) and the FED group (B). There was a significant time × exercise type interaction, where AUC to meals following interval exercise sessions in week 12 were lower than the response to the interval exercise sessions in week 1 (*). Following interval exercise, the FAST group experienced a lower AUC to lunch in week 12 compared to

week 1, yet when the FAST group do continuous exercise they experienced a lower AUC to dinner in week 12 compared to week 1 (#). Data presented are mean ± SD.

The mean glucose response following meals from pre- to post-intervention, there was a significant time × exercise type interaction where the post-prandial mean glucose response to meals was lower after an interval session in week 12 compared to after an interval session in week 1 (P = 0.009). A significant 4-way interaction (group × time × meal × exercise type) revealed that when exercising in the fasted state, the mean glucose response to lunch after an interval session is lower after 12-weeks of training compared to the response to an interval session in the fasted group after lunch in week 1 (P = 0.002). The fasted group also showed a lower response to dinner on the continuous exercise days after the 12-week intervention compared to dinner in week 1 (P = 0.030).

Peak glucose data showed a similar response, with a significant time × exercise type interaction (P = 0.022), with post hoc analysis showing a significant decrease in the peak post prandial glucose values following the interval, but not the continuous, exercise sessions in week 12 compared to week one (P = 0.004). There was also a significant 4-way interaction, showing that there was a significant reduction in the post-prandial peak glucose value to lunch after an interval walking session following 12-weeks of FAST training (P = 0.005), as well as a significant reduction in the post-prandial peak glucose value to dinner after a continuous walking session following 12-weeks of FAST training (P = 0.030).

6.5 Discussion

The overlying aim of the current study was to investigate whether a free-living, 'real world' walking-based intervention in overweight individuals has greater benefits to overall health when exercise is undertaken either before or after breakfast. We demonstrate that this walking-based intervention involving both continuous and interval walking exercise sessions is suitable for sedentary overweight and obese individuals and is effective at reducing HbA1c concentrations and inducing weight loss. Furthermore, we demonstrate that regularly training before breakfast, rather than after, appears to improve the post-prandial lowering effect of exercise. This observation in particular may be an important influence in the design

of exercise programmes aimed at improving metabolic health and reducing the risk of developing type 2 diabetes.

Suitability of the programme for overweight and obese individuals

Whilst the free-living nature of the programme was chosen due to the ongoing COVID-19 pandemic, this provided the opportunity to develop and test whether our exercise intervention could be successfully implemented into the daily lives of overweight and obese individuals. Here, we show that this 12-week combined continuous and interval walking intervention had high adherence in which participants completed ~96% of the prescribed exercise sessions with a high level of compliance (~86%). Previous studies which aimed to implement free-living exercise interventions in sedentary overweight and/or obese individuals generally report low rates of adherence, with only 39-48% (Hesketh et al., 2021), 36-67% (Roy et al., 2018) and 25-30% (Jung et al., 2020) of prescribed exercise sessions actually completed. When compared to these studies, we report remarkably high rates of adherence to our exercise programme, importantly, the adherence to the programme was similar whether individuals were randomised to exercise before or after breakfast, and there were several features of the programme that likely contributed to this effect.

Previous research studies in individuals with T2D show that where interventions include supervised exercise sessions, they also increase total PA (Mosalman Haghighi et al., 2021). However, the resources (such as the cost of exercise physiology staff and space for exercise sessions) for the implementation of such interventions are not feasible in many countries (Balducci et al., 2010). Supervision of exercise sessions also helps to overcome the issue of motivation, which is one of the major barriers to physical activity in overweight/obese individuals (Burke et al., 2008; Leone and Ward, 2013; Piana et al., 2013; Venditti et al., 2014; Martinez-Ramos et al., 2015). In the current study, all participants received a video call once per week from the lead researcher (JB). Feedback from participants at the end of the study showed that the regular contact with the lead researcher provided them with the motivation to continue with the programme, as well as acting as a form of accountability. Knowing the researcher could check their exercise sessions each week, since they were recorded on the HR monitor and uploaded to a cloud-based system, also likely contributed to the sustained motivation and adherence to the programme. Participants also felt that having a heart rate

monitor alongside target HR zones for each walking session gave them a structure to follow. These observations are in line with a recent study from our laboratory showing that the same HR monitoring system was successful in improving adherence to structured home-based exercise programmes in individuals with obesity and an elevated cardiovascular disease risk (Scott et al., 2019). Together, this study demonstrates that weekly contact with an exercise physiologist paired with a simple form of wearable technology can result in high rates of adherence and compliance to a free-living exercise intervention.

Effect of the programme on body composition and blood parameters

Likely due to high levels of adherence to the programme, the 12-week exercise intervention was successful in significantly reducing weight (~3 kg reduction). Although weight loss was significant pre- to post-intervention with a 4% reduction in the FAST group and a 3% reduction in the FED group, a >5% decrease in weight is considered clinically relevant (Stevens et al., 2006). An ~3 kg weight reduction appears to be typical in the literature for a 12-week exercise intervention in obese individuals (King et al., 2008; Christiansen et al., 2010). We also report a significant reduction in BMI, waist circumference (~7 cm reduction), hip circumference (~ 6cm reduction) and waist-to-hip ratio. Waist circumference shows a strong association with all-cause (Pischon et al., 2008; Cerhan et al., 2014) and cardiovascular mortality (Zhang et al., 2008; Song et al., 2015) and is therefore necessary to be included in clinical practice when stratifying obesity-related health risks (Ross et al., 2020). Here, we see a 7 cm (6.0 %) decrease in waist circumference in the FAST group and a 5 cm (4.6%) decrease in the FED group. A reduction of 3 to 6.8 cm in waist circumference is considered clinically relevant meaning that 12-weeks of both fasted and fed exercise training resulted in a clinically relevant reduction in waist circumference (Verweij et al., 2012).

Importantly, this 12-week intervention was effective in reducing HbA1c values by a small, but significant, value of ~3$\pm$4 mmol/mol in FAST and ~1$\pm$4 mmol/mol in FED (~7% reduction in the FAST group and 2% reduction in the FED groups). Therefore, it appears that the walking exercise intervention alone, independent of the nutritional state in which exercise was undertaken, was of sufficient power to improve glycaemic control. A clinically relevant reduction in HbA1c is defined as a decrease of $\geq$5 mmol/mol (NICE guidelines, 2015), whereas

our groups saw an average reduction of 3±4 mmol/mol in FAST and 1±4 mmol/mol in FED, with only 1 individual in each group exhibiting a reduction greater than 5 mmol/mol. These data differ from data in T2D from Verboven et al (2020) who reported minor and non-significant changes in HbA1c following 12-weeks of exercise undertaken in the fasted or fed state. In those with T2D, exercise of moderate intensity in the fed state has been identified as more consistent in improving the post-prandial rise in glucose concentrations compared to fasted exercise (van Dijk et al., 2013; Oberlin et al., 2014; Chacko, 2017), and specifically 30 – 90 minutes post-meal was the optimal time to undertake exercise (Chacko, 2017; Teo et al., 2018). Although HbA1c serves as a marker of glucose variability, the use of continuous glucose monitoring provides the opportunity to assess short-term glycaemic control and examine changes in glucose control over time. Using isCGM in the present study, we didn't observe any change in free-living postprandial glucose responses to daily meals, or any markers of glycaemic variability, in response to the exercise programme in either group. It is possible that the free-living nature of our study design may have contributed to this observation, although it is important to note that the dietary intake at baseline and follow-up was almost identical, which should have limited some of the variation between monitoring periods. Furthermore, in a more controlled study investigating the effect of six weeks moderate intensity cycling in overweight/obese males undertaken in the fasted or fed state, there was also no improvement in glucose control during an oral glucose tolerance test (Edinburgh et al., 2020). Overall, then, it remains to be determined whether exercise-nutrient interactions are an important modulator of short-term glycaemic variability and an improvement in HbA1c in the longer-term.

Exercise is known to reduce inflammation and oxidative stress in obesity-related liver disease, and Burgert et al (2016) reported that deterioration in glucose and insulin metabolism may emerge as a function of an increase in circulating ALT concentrations and hepatic fat accumulation (Burgert et al., 2006). Therefore, we included markers of liver function in our blood analysis. For all indirect markers of liver function, ALT, a marker of hepatic lipid accumulation, was the only one that was significantly reduced following the 12-week exercise intervention. Importantly, this reduction was significant following 12-weeks of fasted exercise, but not 12-weeks of fed exercise. A recent systematic review and meta-analysis clarified that exercise interventions can reduce liver fat despite minimal or no weight loss,

however this does not correspond with reduced ALT (Keating et al., 2012). The lack of a reduction in ALT concentrations observed following our intervention despite a significant weight loss in the fed group may suggest that in the real world when exercise isn't tightly controlled, an additional nutritional component is required to induce a reduction in ALT concentrations. We observed a 16% reduction in ALT concentrations in the fasted group versus 2% in the fed group, and therefore only the fasted exercise group experienced a clinically significant reduction in ALT concentrations (a reduction >5%). Exercising in the fasted state leads to greater mobilisation of fat stored in subcutaneous and visceral adipose tissue (Horowitz et al., 1997; De Bock et al., 2005) and contribution of fat to energy expenditure. Therefore, it is likely that during an intervention where exercise is repeatedly undertaken in the fasted state that there will be increased whole-body lipid turnover, including in tissues such as the liver. Consequently, this will lead to a decrease in markers of hepatic lipid accumulation, such as ALT. Whether the reduction in ALT manifests as a reduction in liver fat requires further investigation. It is also important to note that while none of our participants had received a diagnosis of NAFLD prior to entering the study, the baseline blood tests showed that 8 individuals had elevated ALT concentrations (>50 IU/L); 5 in the FAST group and 3 in the FED group. Importantly, all 8 of these individuals experienced a reduction in ALT concentrations following the intervention, and 4 of those 8 (2 in each group) were now within the normal range (15 – 50 IU/L) at the follow-up blood test. Thus, the exercise programme was effective in reducing ALT concentrations in those most at risk of, or who have pre-existing NAFLD.

Glycaemic Control - isCGM - Baseline

The use of isCGM first enabled a comparison of the postprandial glucose responses to an acute bout of continuous versus interval walking exercise at the beginning of the intervention. Initially, we observed that the postprandial glucose response to the continuous exercise sessions in the first week of the intervention was lower compared to the interval exercise sessions. Interestingly, our acute isCGM findings contradict the general consensus that high intensity interval exercise reduces the postprandial glucose responses more so than continuous exercise (Little et al., 2014). Moreover, it was previously reported that a single aerobic interval exercise session improved glycaemic control in T2D patients to a greater extent than an oxygen consumption- and time duration-matched continuous exercise session

(Karstoft et al., 2014). We also report that nutritional status influences the meal at which the lower postprandial glucose response is observed. Specifically, doing the continuous exercise session fasted resulted in a lower postprandial glucose response to lunch, whereas doing the exercise session in a fed state led to a lower postprandial glucose response to dinner. Taken together, it appears that at the start of the intervention continuous exercise, compared to interval exercise, induced a lower postprandial glucose response to meals later that day, but the specific meal (lunch or dinner) at which the lower postprandial glucose occurred was dependent on whether the continuous exercise session was completed in the fasted or fed state. The latter observation is partly in line with the findings of Nygaard et al (2017) who also reported a lower postprandial glucose response at dinner in hyperglycaemic individuals following 60 min of morning treadmill walking in the fed, but not fasted state. Thus, it appears that it is the second meal following exercise in which a reduction in postprandial glucose occurs. This effect has been termed the "second-meal phenomenon" (Gonzalez, 2014), whereby exercise-induced increases in glucose rate of disappearance are matched or exceeded by the rate of appearance of exogenous (meal-derived) glucose. This effect explains why exercise may deteriorate (King et al., 1995; Folch et al., 2001; Rose et al., 2001; O'Connor et al., 2006)) or not alter glucose tolerance (Venables et al., 2007; Long et al., 2008), at least in healthy individuals. Exercise-induced improvements in insulin sensitivity are maintained for 24-48 hours though (Wojtaszewski et al., 2000a), and this likely explains the decrease in postprandial glucose tolerance to the second meal following exercise. It is important to note though that the exercise-induced improvement in postprandial glucose disappears by the third meal of the day following exercise (apparent in the fasted group). Whether this is a reflection of the low-intensity nature of the continuous exercise requires a future mechanistic investigation.

isCGM - Pre to Post Intervention

Using isCGM, we were, for the first time, able to examine differences in the postprandial glucose response to exercise sessions completed in the final week of the intervention compared to the first week. On diet-replicated days, exercise reduced the postprandial glucose response to meals specifically on days when participants did interval exercise. This was observed independent of nutritional state under which exercise was performed. This can

likely be explained by the fact that in week 12 of the intervention, the participants were performing double the number of intervals compared to week 1. This will have induced a greater reduction in muscle glycogen in week 12 compared to week 1, improving the capacity for postprandial glucose uptake as the need for post-exercise glycogen replenishment will have been enhanced. Further inspection of the data revealed that performing interval exercise before breakfast improved the postprandial glucose response to lunch in week 12 compared to week 1 of the intervention. In contrast, performing continuous exercise before breakfast improved the postprandial glucose response to dinner in week 12 compared to week 1 of the intervention. Exercising before breakfast potentiates the use of muscle glycogen (De Bock et al., 2005), presumably because liver glycogen will be low, enhancing post-exercise glucose uptake and improving insulin sensitivity for up to 48 hours. Why this manifests at specific meals under free-living conditions is less clear and requires further investigation. Nevertheless, it appears that regularly training before breakfast improves the postprandial glucose-lowering effect of exercise, compared to training after breakfast.

Strengths and Limitations

A strength of the study is that it was completed during the COVID-19 global pandemic, and as such, the exercise programme and all outcome measures was done so with no researcher-participant contact. Despite this, we were able to observe significant improvements in HbA1c, body composition, and in individuals who exercised fasted a decrease in ALT concentrations. Therefore, we have successfully developed an exercise intervention that can be rolled out remotely to individuals at risk of T2D and therefore could be considered as a tool in community settings for diabetes prevention. It is important to also acknowledge limitations of the study. An important drawback of this study is the lack of a non-exercising control group limiting our ability to state that changes we see at the end of the 12-weeks was due to the exercise programme. However, we can be confident that our participants maintained their normal diet throughout the 12-week programme and did not begin any medication that could affect cardio-metabolic health markers. The incapacity for in-person testing to a remote study meant that measures such as the fitness index had to be utilised rather than a gold-standard VO_{2max} test. Furthermore, a measure of liver fat would enhance the differential changes we see in ALT, and an oral glucose tolerance test or hyperinsulinemia euglycemic clamp to

measure glucose tolerance and insulin sensitivity before and after the intervention would give support to the improvements, we observed in HbA1c.

Conclusion

This current study provides a rationale for the continuous use of free-living and home-based interventions. We show significant benefits to key health markers from a combined continuous and interval walking exercise programme, and more importantly suggest that liver health represented by a surrogate measure of liver fat accumulation (ALT) is improved more so after 12-weeks of exercising in the fasted state.

Chapter 7. General Discussion

Overview

During the last ~100 years, the lifestyle lead by the general population has changed significantly, with an increase in the consumption of energy-dense food that has poor nutritional value and a reduction in physical activity. This has ultimately caused an increase in the prevalence obesity and the occurrence of non-communicable diseases such as insulin resistance and type 2 diabetes, cardiovascular disease, and metabolic syndrome. It has become a priority to target prevention of such diseases through the development of interventions that are both effective but also feasible for individuals to incorporate into their daily lives. To do so, researchers need to unveil the underlying mechanisms that contribute to diseases such as insulin resistance and metabolic syndrome. Skeletal muscle is the major tissue responsible for glucose clearance from the circulation following the ingestion of a mixed meal (Ferrannini et al., 1985; Capaldo et al., 1999) , and is therefore an important focus of research in the maintenance of glucose homeostasis. Dysregulation of skeletal muscle lipid metabolism is associated with a reduction in insulin sensitivity and glucose clearance leading to very high and/or very low episodes of plasma glucose concentrations. Acute exercise bouts are associated with improved glucose clearance to subsequent meals, and chronic exercise training is associated with improved insulin sensitivity. The link between the two is due, in part, to an increase in the capacity for IMTG breakdown and therefore increased regulation of synthesis and breakdown of the IMTG pool.

Elevated IMTG content is observed in both insulin resistant overweight and obese individuals, yet also in insulin sensitive trained individuals; a phenomenon now well known as the "athlete's paradox" (Goodpaster et al., 2001). The main discrepancy between the two groups is the capacity for IMTG utilisation, which is high in trained individuals, but low in overweight or obese individuals and T2D patients. In individuals who are insulin sensitive, a greater capacity for IMTG utilisation during exercise also enhances of the IMTG pool in the post-exercise period, and therefore overall turnover rates of the IMTG pool are high (Perreault et al., 2010). As a result, there is little opportunity for lipid metabolites, such as DAGs and ceramides, to accumulate and directly disrupt the insulin signalling cascade. Several studies have demonstrated that exercise training increases IMTG content and IMTG utilisation during an acute bout of moderate-intensity exercise alongside improvements in insulin sensitivity

(highlighted in Chapter 2). The success of these interventions in individuals at risk of developing T2D is mixed. Fundamental though, is that interventions which enhance the effect of exercise training on lipid metabolism could lead to improvements in insulin sensitivity and glycaemic control in people at risk of developing T2D.

On this basis, the overall aim of this thesis was to investigate IMTG turnover in skeletal muscle; specifically focussed on the mechanisms of impaired insulin sensitivity with elevated plasma FFA, as well as the effectiveness of two exercise-based strategies which influence lipid metabolism on acute and chronic outcomes related to insulin resistance and metabolic health. Specifically, Chapter 4 determined whether GLUT4 translocation is impaired when plasma FFA availability is increased (using an intralipid infusion) in trained and sedentary individuals. Chapter 5 investigated whether suppression of plasma FFA could alter substrate metabolism during a single bout of brisk walking exercise and subsequently reduce the concentration of lipid metabolites. Finally, Chapter 6 examined the effectiveness of a free-living walking-based exercise programme with exercise undertaken before or after breakfast to improve glycaemic control and several cardio-metabolic outcomes.

Visualisation and identification of GLUT4 clusters in skeletal muscle

Over 100 years ago, contraction-induced skeletal muscle uptake was observed through arteriovenous glucose differences and venous outflow during chewing in equine muscle. In the 1920's and 30's, the importance of glucose as a fuel for endurance exercise in humans was identified, and the links between fatigue and hypoglycaemia (Christensen, 1939). Then, the identification of the insulin- and contraction-regulated glucose transporter isoform GLUT4 (Birnbaum, 1989; James, Strube and Mueckler, 1989; Charron and Katz, 1998) paved the way for enhanced understanding of glucose transport and muscle glucose uptake during exercise. The ability to investigate GLUT4 translocation within skeletal muscle has improved in recent years, with the first confocal immunofluorescence microscopy technique utilised by Bradley et al (2014; 2015). Within these two studies, Bradley et al demonstrated comprehensively how to visualize GLUT4 translocation to the plasma membrane in skeletal muscle, and separate GLUT4 vesicles by large and small storage vesicles to understand their origin and the

difference in translocation between the two. Missing from this analysis was the separation of fibre types. Within Chapter 4, the fibre-specific difference in GLUT4 translocation is reported for both trained and sedentary individuals. No fibre type differences in GLUT4 translocation were observed, corresponding with early reports that there is no significant relationship between fibre type and GLUT4 protein content (Anderson and Mark, 1993). Recent reports further confirm this notion with no fibre type difference in glucose disposal during hyperinsulinaemic euglycaemic clamp in lean and obese individuals (Koh et al., 2021). We did report, however, that training status appears to determine the number of small and large GLUT4 spots present. Specifically, we show that at baseline, trained individuals exhibit a great number of small GLUT4 spots at the plasma membrane. This adaptation may represent a larger, more mobile, pool of GLUT4 in trained individuals compared to sedentary individuals which would support the greater rates of glucose clearance previously observed in this population.

Use of an intralipid infusion to investigate the lipid-mediated dysregulation of insulin signalling in skeletal muscle

As detailed in depth in Chapter 2, obese individuals and T2D patients exhibit elevated IMTG content due to the over consumption of energy-dense food and limited time spent participating in physical activity. It has become clear over recent years of research that elevated IMTG content is not directly responsible for impaired insulin signalling. Rather it is lipid metabolites, such as DAGs and ceramides, that accumulate as a result of a reduced rate of IMTG turnover and elevated blood lipids that cause direct disruption to insulin signalling in skeletal muscle. As a result, GLUT4 translocation and glucose uptake into skeletal muscle is reduced in obese, T2D individuals. The mechanistic details of how elevated lipid availability results in reduced GLUT4 translocation is not yet clear, and so in Chapter 4 we examined GLUT4 translocation using confocal immunofluorescence microscopy in muscle samples of trained and sedentary individuals who previously underwent either a glycerol or intralipid infusion alongside a hyperinsulinaemic euglycaemic clamp (Chow et al., 2012; Chow et al., 2014). Within this chapter, the key observation is that GLUT4 translocation is affected by a lipid infusion compared to a glycerol control infusion. To our knowledge, this study was the first to use confocal immunofluorescence microscopy to investigate whether there are fibre-

type specific differences in GLUT4 translocation, and despite baseline differences, there were also no fibre-type specific differences in GLUT4 translocation during either infusion. Importantly though, the results of Chapter 4 suggest that suppression of glucose uptake in response to a lipid infusion (reported previously; REF) is not due to GLUT4 availability at the plasma membrane. It could be questioned, therefore, whether using an intralipid infusion as a model for understanding the mechanisms of lipid-induced insulin resistance and the impact on GLUT4 translocation may not be appropriate.

Other methods for investigating lipid-induced insulin resistance have been utilised within the literature, with one approach being short-term overfeeding studies, sometimes also paired with reduced physical activity as a method to induce whole-body insulin resistance and mimic the metabolic effects of observed in obesity and individuals with T2D. Short-term high-fat high-calorie feeding studies of only 5-14 days are sufficient in reducing hepatic insulin sensitivity (Brons et al., 2009), peripheral insulin sensitivity (Bakker et al., 2014), and whole-body insulin sensitivity (Hulston, Churnside and Venables, 2015; Parry et al., 2017). Therefore, they may provide an alternative, and more physiologically relevant, model of lipid-induced insulin resistance. Indeed, like a lipid infusion, high-fat high-calorie interventions also lead to IMTG accumulation in skeletal muscle (Gemmink et al., 2017; Whytock et al., 2020). However, DAG and ceramide concentrations are not elevated (Whytock et al., 2020), and despite a decrease in whole-body insulin sensitivity, there is no impairment of insulin signalling intermediates (Parry et al., 2020). Therefore, in the context of investigating whether GLUT4 translocation is impaired when lipid availability is high, such overfeeding studies may also not be the answer.

Strategies to enhance intramuscular lipid turnover: Acute outcomes related to insulin resistance

Chapter 2 explained that IMTG utilisation during acute exercise in lean, healthy individuals typically leads to a post-exercise net reduction in IMTG content. However, in obese individuals or T2D patients, a net decrease in IMTG content in response to acute exercise does not occur. This is likely related to an inability to simultaneously reduce rates of IMTG synthesis during exercise (which occurs in lean, healthy individuals), due to the elevated

plasma FFA availability and uptake in obese individuals and T2D patients. This imbalance between the rate of FA uptake, IMTG synthesis, storage, breakdown and FA oxidation in skeletal muscle has been linked to the accumulation of lipid metabolites, such as DAGs and ceramides, which subsequently impair insulin signalling. In Chapter 5, we sought to determine whether reducing plasma FFA concentrations during exercise would acutely reduce species of DAGs and ceramides in skeletal muscle of obese individuals. Oral administration of Acipimox successfully reduced plasma FFA concentrations during exercise and throughout the 3 h recovery period. However, this did not translate into differential exercise-induced changes in DAGs and ceramides, compared to the control condition (i.e. no Acipimox). Overall, exercise led to an increase in several species of DAG with two unsaturated FA. Whether this is simply a reflection of triglyceride hydrolysis generating these specific DAG species is not known, since the mass spectrometry method used in Chapter 5 did not permit determination of the position of the FA on the DAG species. Indeed, it is known that ATGL-mediated hydrolysis of triglyceride generates sn-1,3 DAG and sn-2,3 DAG (Eichmann et al., 2012), whereas sn-1,2 DAG is the subtype recognised for possessing insulin desensitizing properties (Boni and Rando, 1985; Hannun et al., 1986). Given the link between saturated DAG and insulin resistance (Bergman et al., 2009; Bergman et al., 2010), and that exercise improves insulin sensitivity (Dube et al., 2011), it would make sense that the DAG species generated from the hydrolysis of triglyceride would be unsaturated and of the sn-1,3 DAG and sn-2,3 DAG subtypes, although further work would be required to confirm this.

An interesting observation of Chapter 5 was the exercise-induced increase in total, and several species of, lactosylceramide (LacCer). LacCer is synthesised from glucosylceramide, the concentration of which was not changed by exercise. Glucosylceramide is formed from ceramide, which was also not changed by exercise. Based on these data alone, it is tempting to speculate that the flux through the ceramide pathway is increased by exercise, although this would require ceramide synthesis (and glucosylceramide synthesis) to match the rate of ceramide degradation. Ceramide can be synthesised through the sphingomyelin and salvage pathways, as well as through de novo synthesis with the long chain fatty acid palmitate as the substrate (see figure 8.1 (Chavez and Summers, 2003; Holland et al., 2011). Dihydroceramide is the immediate precursor to ceramide via de novo synthesis, and it has previously been shown that exercise increases the concentration of dihydroceramide (Bergman et al., 2016),

suggesting that flux through the de novo synthesis pathway is increased during exercise. Concentrations of other intermediates of the de novo synthesis pathway, as well as sphingomyelin and sphingosine (of the salvage pathway), would provide additional insight into changes in ceramide metabolism induced by exercise. Importantly though, the results of Chapter 5 suggest that exercise could be used as a model to understand ceramide metabolism in skeletal muscle.

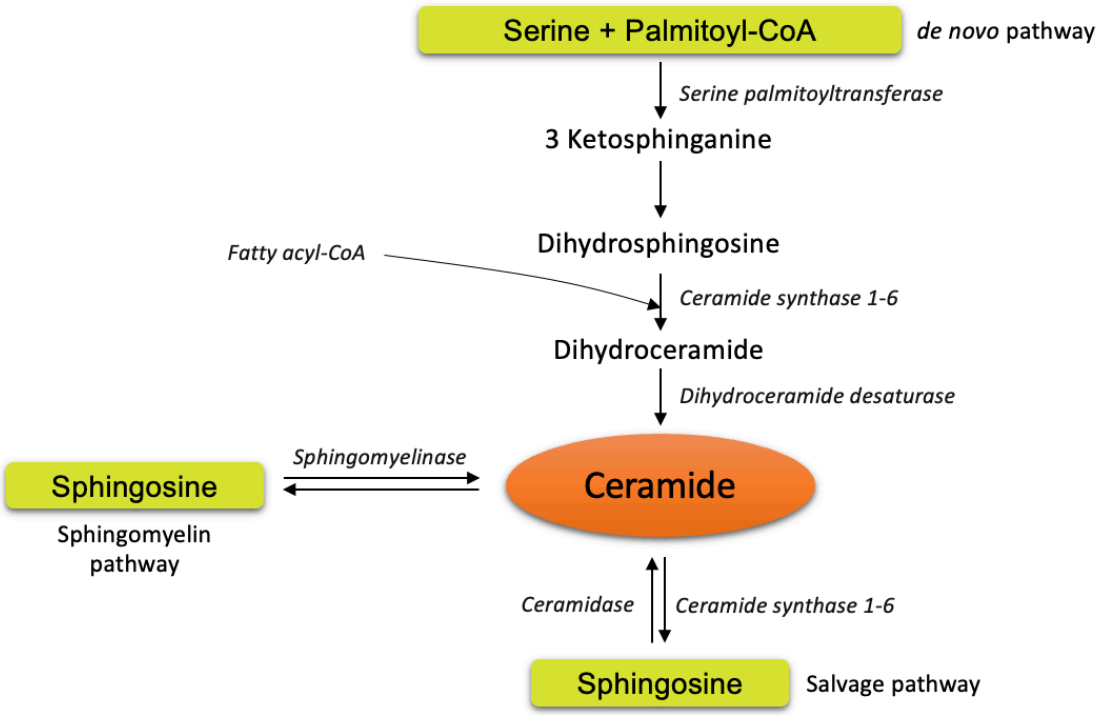


Figure 7.1. Ceramide synthesis and degradation pathway. Ceramide de novo synthesis from serine and palmitate is catalysed by serine palmitoyltransferase. The salvage pathway forms ceramides from sphingosine catalysed by ceramide synthases (CerS1-CerS6) with tissue and ceramide species synthesis specificity.

Strategies to enhance intramuscular lipid turnover: Chronic outcomes related to insulin resistance

As detailed in Chapter 2, another approach to increase the utilisation of IMTG during exercise is to undertake exercise before breakfast (i.e. following an overnight fast). Indeed, in lean, healthy individuals, IMTG utilisation is greater when exercise is undertaken before breakfast (De Bock et al., 2005; Lane et al., 2015; Hansen, De Strijcker and Calders, 2017). Importantly though, in overweight/obese individuals exercising before breakfast also leads to a net decrease in IMTG content, compared to exercising in the postprandial state (Edinburgh et al., 2020). Thus, exercising before breakfast stimulates greater turnover of the intramuscular lipid pool. Whether regularly undertaking exercise before breakfast therefore leads to greater benefits to insulin sensitivity and glycaemic control in obese individuals or T2D patients is less clear, with one study reporting a benefit of fasted exercise training (Edinburgh et al., 2020), and another reporting no difference compared to regularly training after breakfast (Verboven et al., 2020). This provided the impetus (together with the COVID-19 pandemic) to investigate whether an exercise intervention based on brisk walking was most beneficial to glycaemic control and markers of health when each training session was undertaken before or after breakfast (Chapter 6). The intervention was entirely field-based, which meant that participants could undertake the walking sessions in an environment of their choosing. Importantly, high rates of adherence and compliance to the intervention were observed, which demonstrates that it is possible to implement nutrition and exercise interventions with no face-to-face contact with a research team member. This alone demonstrates that within the community, a similar intervention that requires little staff involvement could be utilised in both weight loss and diabetes prevention services.

The programme was similarly effective whether exercise was undertaken before or after breakfast for improving HbA1c and inducing weight loss. Interestingly, when examining blood markers related to liver health, only the group who exercised before breakfast exhibited a decrease in blood ALT concentrations. ALT is a marker of liver function and is related to hepatic lipid accumulation (Schindhelm et al., 2006). Regularly exercising before breakfast, which permits a greater contribution of fat to be oxidised to meet the energy demands of exercise, means that whole-body lipid turnover would presumably be higher in these individuals. One would hypothesise that this elevated rate of lipid turnover would lead to a decrease in hepatic lipid concentrations. Measuring hepatic lipid content using MRI in future

studies would help to confirm whether exercising before breakfast does in fact lead to a greater reduction in hepatic lipid accumulation in individuals at risk of developing T2D.

Future research directions

Investigating intramuscular lipid turnover at the fibre type and subcellular level

Measuring turnover of the IMTG pool - that is, simultaneous measures of synthesis and degradation – has been attempted using a number of techniques. Research demonstrating minimal, if any, utilisation of IMTG during moderate-intensity exercise (Kelley and Simoneau, 1994; Blaak and Wagenmakers, 2002) only measured the arterial-venous exchange of plasma FFA across the human forearm, and importantly did not measure IMTG content, nor did these research groups use ^{13}C-enrichment (using U-^{13}C palmitate as intravenous tracer) of IMTG in the muscle biopsies. Bergman et al. (2018) is the only group to have measured the IMTG content and [U-^{13}C]palmitate enrichment in muscle biopsies taken before, immediately after 90 min of exercise at 50% VO_{2max}, and 2 h after recovery from the exercise. As a total lipid extraction method of the muscle biopsies was used in Bergman et al (2018), no discrimination can be made between the fractional synthesis rate (FSR) of IMTG in the type I fibres and of the FSR of IMTG in type IIa and type IIb fibres. While type IIb fibres are only recruited during exercise at higher intensities (75-90% VO2max range), type IIa fibres still make an important contribution during moderate-intensity exercise. This implies that previous conclusions that obese individuals and T2D patients do in fact utilise and synthesize IMTG simultaneously during exercise at 50% VO_{2max} may not apply to all the muscle fibre types. For example, it is not possible to exclude that type I fibres break IMTG down, while net synthesis of IMTG occurs in type IIa and type IIb fibres. Thus, in order to explicitly determine the turnover of the IMTG pool in skeletal muscle, future studies should aim to determine the FSR of the IMTG stores in the various fibre types at 50% VO_{2max} alongside a measurement of the [U-^{13}C] palmitate enrichment in the three fibre types.

As described in the methods section of Chapter 5, a portion of the muscle samples obtained from participants were processed for transmission electron microscopy (TEM) analysis (see figure 7.2). Image capture of these samples is in progress and analysis will be soon started.

These samples will be used primarily to measure changes in the content and morphology and IMTG-containing lipid droplets. The reason to use TEM, over immunofluorescence microscopy for example, is the greater magnification and resolution of the images and information which can be obtained. Thus, using TEM it is possible to investigate changes in lipid droplet content and morphology at a fibre type and subcellular specific level. Specifically, two different LD pools will be measured; the subsarcolemmal LD pool and the intermyofibrillar LD pool. This is important, because it has been previously shown that intermyofibrillar LD in type I fibres are preferentially used during exercise in trained individuals (Koh et al., 2017; Jevons et al., 2020), whereas accumulation of large LD in the subsarcolemmal region of type II fibres is associated with insulin resistance (Nielsen et al., 2017). Only one previous study has investigated the effect of acute exercise on lipid droplet content in individuals with metabolic impairments. Chee et al. (2016) demonstrated that 1 h of exercise at 50% VO_{2max} reduced intermyofibrillar LD in both young and old lean individuals. While intermyofibrillar LD were also reduced in old overweight individuals, exercise actually led to an increase in subsarcolemmal LD in this group (Chee et al., 2016). This highlights the importance of investigating changes in LD at the subcellular level to fully understand the effect of exercise on intramuscular lipid turnover. Furthermore, based on the data of Chee et al. (2016), it could be hypothesised that subsarcolemmal LD may also be increased post-exercise in the control trial in Chapter 5, whereas oral administration of Acipimox will increase the utilisation of IMTG-containing lipid droplets (as previously observed by van Loon et al., 2005a, 2005b) and therefore prevent an increase in subsarcolemmal LD following exercise. If true, this would have important implications in terms of developing strategies to improve insulin sensitivity, given the previously mentioned link between subsarcolemmal lipid droplets and insulin resistance (Nielsen et al., 2017).

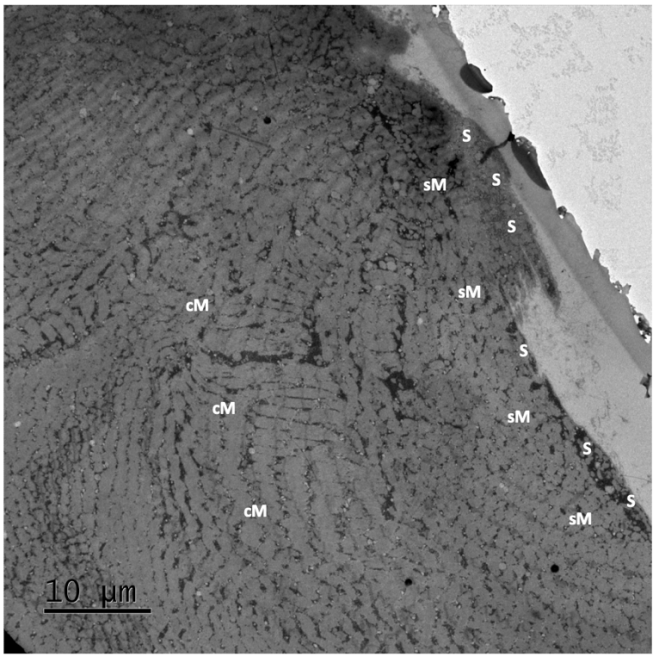


Figure 7.2. Location of transmission electron microscopy (TEM) images. The location of TEM images was systematically randomized representing the subsarcolemmal region (S), the superficial myofibrillar region (sM), and the central myofibrillar region (cM) of each fibre.

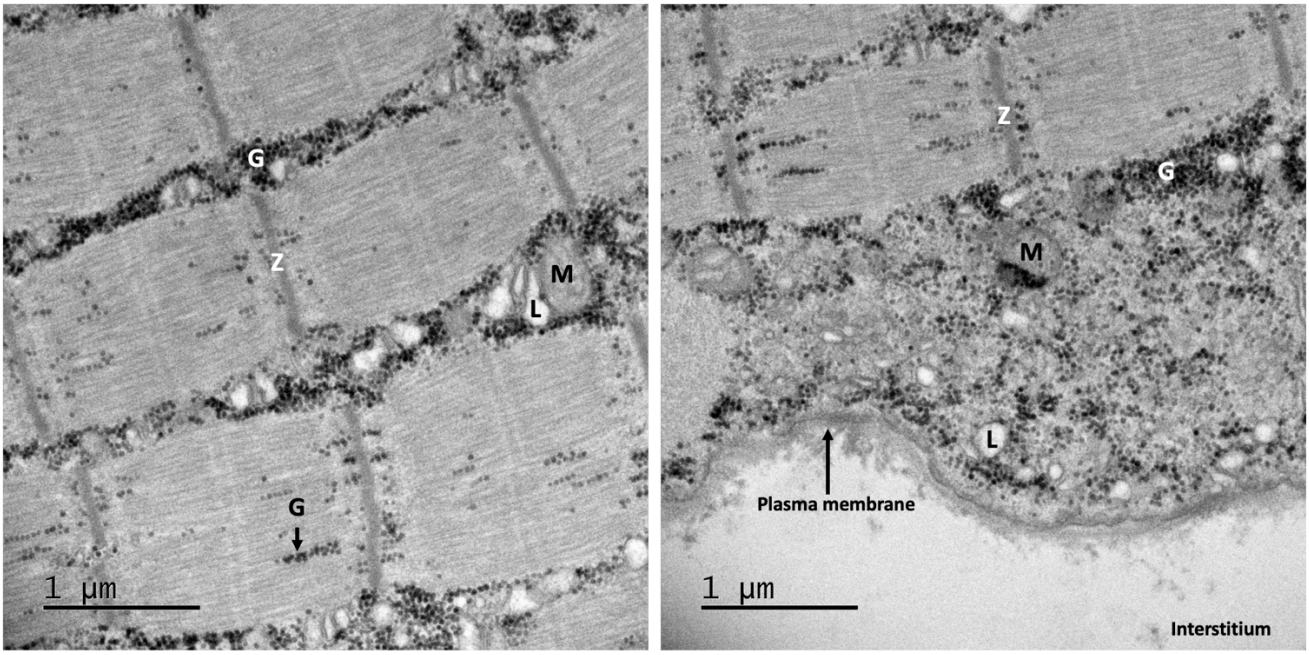


Figure 7.3. Skeletal muscle intramyocellular lipid, glycogen, and mitochondria. TEM images of the myofibrillar region (A) and subsarcolemmal region (B) of skeletal muscle fibre. L, lipid droplet; M, mitochondria; G, glycogen particles (black dots); Z, Z-line. In B, glycogen is located

both in the intermyofibrillar (IMF) space (white G) and intramyofibrillar space (black G). Magnification set at 9300x.

On the discussion of considering subcellular location when investigating lipid metabolism in skeletal muscle, it is pertinent to consider the importance of also measuring lipid metabolites, such as DAGs and ceramides, at this level too. This may be particularly relevant when investigating DAGs, given that in recent years the long-held belief that DAG content is greatest in insulin resistant individuals has been challenged by the "DAG paradox" (Amati et al., 2012). This paradox was based on the observation that DAG content in skeletal muscle was actually higher in trained athletes (with high insulin sensitivity) compared to obese individuals (Amati et al., 2011). Rather, it may be the subcellular location of DAG that links this lipid intermediate to insulin resistance. Indeed, Bergman et al. (2012) reported that DAG concentrations were highest in the membrane fraction of skeletal muscle from obese individuals and T2D patients, compared to trained athletes, and that membrane DAG correlated with insulin sensitivity. The same is also true for ceramides, with Perreault et al. (2018) reporting an inverse relationship between sarcolemmal ceramide concentrations and insulin sensitivity. Taken together, these data suggest that the subcellular localisation of muscle lipids should be considered when understanding their potential role in mediating insulin resistance. Of course, this should also include the measurement of specific DAG and ceramide species in each subcellular location, given that it is starting to be understood that some species of these metabolites are more closely linked to insulin resistance (Bergman and Goodpaster, 2020).

Developing interventions which target intramuscular lipid turnover to improve insulin sensitivity

As outlined in Chapter 2, although short-term Acipimox treatment alone (i.e. 250 mg, two-to-three times per day) induces positive changes to blood lipids and insulin sensitivity (Bajaj *et al.,* 2005; Daniele *et al.,* 2014; Phielix *et al.,* 2014; van de Weijer *et al.*, 2015), longer term Acipimox treatment results in a rebound rise in fasting plasma FFA (Fulcher *et al.*, 1992; Vaag and Beck-Nielsen, 1992; Saloranta *et al.,* 1993) and both hepatic and skeletal muscle insulin sensitivity is unchanged (Makimura *et al.*, 2016). Given that oral administration of Acipimox increases IMTG utilisation during exercise (van Loon 2005a, 2005b) – and further evidence for

this may come once the TEM sample analysis has been completed as part of the wider study described in Chapter 5 – the question then arises as to whether this strategy could be employed in a longer-term intervention to maximise the exercise training-induced improvements in insulin sensitivity in individuals either with, or at risk of developing T2D. In addition to an effect on IMTG utilisation, acute Acipimox treatment can also increase the utilisation of muscle glycogen during exercise (van Loon 2005a), where depletion of muscle glycogen is linked to the insulin-sensitising effect of exercise (Jensen et al., 2011). Not only this, a reduction in muscle glycogen concentration is a stimulus for augmenting the activation of signalling proteins which lead to elevated gene expression of mitochondrial proteins (Hawley and Morton, 2014). Augmenting IMTG utilisation may also support the adaptive response to exercise, since ATGL-mediated hydrolysis of triacylglycerol activates PGC-1α/PPARα signalling via SIRT-1 (Khan et al., 2015; Najt et al., 2020). Thus, the combination of increased muscle glycogen and IMTG utilisation during exercise when preceded by oral administration of Acipimox could provide a powerful stimulus to not only increase the turnover of intramuscular substrates, but also activate the intracellular signalling responses leading to mitochondrial biogenesis and upregulation of the fatty acid oxidation programme. The high rates of adherence and compliance reported in Chapter 6 provide confidence that an intervention combining both interval and continuous walking exercise sessions can be successfully implemented into the daily lives of individuals at risk of developing T2D. Importantly, the high rates of adherence and compliance were achieved with no direct face-to-face contact with the lead researcher, although it should be acknowledged that the weekly check-ins with a member of the research team likely played a key role in the success of the intervention. This suggests that weekly contact with an exercise physiologist (or other health professional) should be integrated into future free-living exercise interventions, which otherwise do not always yield high rates if adherence and compliance (Hesketh et al., 2021).

The results of the intervention described in Chapter 6 were promising and revealed the potential of the programme to improve glycaemic control and induce weight loss. In addition, when each exercise session was commenced before breakfast (compared to after breakfast) there was also a decrease blood ALT concentration, which is a marker of hepatic liver accumulation. A decrease in intrahepatic lipid content is a consistent finding from well-controlled, laboratory-based studies (reviewed in (Brouwers et al., 2016)), but it could be

speculated from the results of Chapter 6 that when exercise training is undertaken in a more free-living environment then additional intervention combined with exercise is required facilitate a reduction in intrahepatic lipid content. The obvious follow up to Chapter 6 is to determine whether the same exercise programme where each training session is commenced before breakfast, compared to exercising after breakfast or in the postprandial state, is more effective for reducing hepatic lipid concentrations. Combining this with measures of hepatic and peripheral insulin sensitivity using a hyperinsulinaemic euglycaemic would then provide clear evidence as to whether a combined exercise and nutrition programme can be effectively implemented in the daily lives of individuals at risk of developing T2D. Of course, it would then be intriguing to investigate whether the same intervention could be applied with similar effectiveness to NAFLD patients or individuals who are already living with T2D.

Final conclusions

The work contained within this thesis has expanded on novel immunofluorescence microscopy techniques to determine the impact of elevated plasma free fatty acids on insulin signalling, specifically fibre-type GLUT4 translocation. In this regard, evidence that GLUT4 translocation is not reduced following an intralipid infusion, nor does this differ between fibre types, is reported. Additionally, the effect of inhibiting adipose tissue lipolysis during a low-intensity exercise bout reveals novel information for the effect of acute exercise on lipid metabolite concentrations in skeletal muscle and opens up several new questions to answer to understand how exercise may impact intramuscular lipid turnover.

Finally, the work in this thesis provides important evidence that improvements in body composition and HbA1c occur in response to a free-living low-intensity walking-based exercise training programme. Therefore, this adds to the growing body of literature in home-based settings and free-living situations demonstrating that a simple, low-contact training strategy is effective to induce positive health outcomes in individuals with obesity. It is expected that the evidence obtained in this thesis will inform future research into interventions that aim to tackle the worldwide health threat associated with physical inactivity.

Chapter 8 References

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