Exploring intramuscular energy storage. Implications for health and exercise performance.

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Abstract.

Chapter 1 of this thesis focuses on the role of skeletal muscle on carbohydrate storage and glucose control. Responsible for ~80% of the body's carbohydrate stores and a modest, yet important quantity of fat, skeletal muscle is a crucial site for energy storage in the human body. As skeletal muscle has large plasticity and has a finite storage capacity, it is important for energy to be stored efficiently, in order to supply metabolic processes. Lifestyle factors such as physical activity and energy intake can cause skeletal muscle to structurally adapt which can influence its metabolism. This is noticeable when comparing polarizing populations, such as trained individuals who undergo large volumes of exercise activity, compared to individuals with metabolic disorders, including type 2 diabetes (T2D). **Chapter 2** of this thesis focuses on how nutritional stimuli can affect the ability of skeletal muscle to store energy and discuss the subsequent effects that this can have on exercise performance. An emerging area of exercise and health nutrition is polyphenols, a naturally occurring compound, found in many fruits and vegetables. New Zealand blackcurrant (NZBC) extract is highly concentrated in polyphenols and has been purported to aid exercise performance through improve blood flow and glucose uptake into muscle. We provide evidence that short-term supplementation of NZBC can improve carbohydrate storage in skeletal muscle. **Chapter 3** then considered the differences between energy storage between individuals who are healthy, compared to those with T2D. To do this, we employed immunofluorescence microscopy to examine proteins involved in carbohydrate and lipid metabolism in skeletal muscle of individuals with T2D and healthy participants. SNAP23 is a membrane associated protein, which appears particularly important in attaching the glucose transporter (GLUT4) to the plasma membrane, therefore allowing for glucose uptake. SNAP23 is also localised on lipid droplet (LD) membranes

and accumulation of intramuscular triglycerides, appear to cause lipid droplets (LD) to fuse together and grow. SNAP23 does not appear to be synthesised, therefore in order for LD to fuse it must 'hijack' SNAP23 away from the plasma membrane, which could attenuate glucose uptake. This chapter provides evidence for SNAP23 to be less colocalised at the plasma membrane in individuals with T2D, compared to lean individuals. However, this was not in conjunction with greater colocalisation of SNAP23 with LD. Therefore, this chapter also includes our speculation behind these unexpected findings.

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Chapter 1: General Introduction

1.1 Skeletal muscle, insulin sensitivity and glucose uptake

1.1.1 Glucose homeostasis

The process of glucose homeostasis is fundamental to human life, as there is ever changing fluctuations in the demand for glucose as a substrate for energy production. Circulating plasma glucose is critical in providing substrate to energy demanding tissues, such as the brain and skeletal muscle with glucose, in times of increased energy demand (i.e., exercise). As a result, plasma glucose concentrations fall and require further glucose provision. The fundamentals of glucose homeostasis include two variables in its equation, glucose input (appearance) and glucose output (disappearance); For glucose homeostasis to be maintained, both sides of the equation must be working in Unisom. In humans, plasma glucose can be taken up at a number of sites e.g. skeletal muscle, liver, brain and adipose. Skeletal muscle is the largest tissue in the human body and consumes most of the circulating glucose (figure 1.1). In response to insulin infusion (hyperinsulinaemic euglycemic clamp), skeletal muscle is estimated to remove 80% of circulating glucose in lean healthy individuals (DeFronzo et al., 1981) and is responsible for ~50% of glucose disposal in response to a mixed meal in older individuals (Capaldo et al. 1999). Skeletal muscle is therefore fundamental in maintaining plasma glucose homeostasis. The liver is also an important storage site for carbohydrate (glycogen, figure 1.1), for maintenance of blood glucose concentrations. Unlike skeletal muscle, the liver regulates glucose homeostasis by adding glucose to the blood, compared to removing it. The liver also regulates blood glucose homeostasis through glycogenolysis and gluconeogenesis which maintain blood glucose concentrations especially during periods of fasting. Therefore, there is a sophisticated relationship between skeletal muscle and the liver in the maintenance of glucose homeostasis, as one organ is responsible for adding

glucose, while the other removes it. Typically, in healthy conditions, fasting blood glucose concentrations are between 4-5.5 mmol.L^{-1} (American Diabetes, 2015). In response to dietary carbohydrate consumption, carbohydrates are digested in the gut, before being absorbed and released into blood circulation as glucose and resulting in increased blood glucose concentrations. Many physiological processes function as a result of homeostatic concentrations of blood glucose, therefore is of upmost importance that this system is well regulated. Glucose is the largest contributing source of energy in the human body, feeding vital tissues, such as skeletal muscle and the brain. Fortunately, skeletal muscle has a modest reservoir of glycogen, whereby in times of need, can be hydrolysed into its constituent parts, glucose. However, skeletal muscle glycogen stores are depleted relatively quickly in response to vigorous exercise, therefore, once glycogen concentrations are significantly reduced, skeletal muscle will soon be rid of any glucose and energy production will reduce drastically, resulting in exercise induced fatigue. To circumvent this, homeostatic blood glucose concentrations will supply skeletal muscle with adequate glucose to maintain a sustained flow of energy production. To achieve this, blood glucose must be maintained via exogenous glucose consumption. In addition, not all glucose utilising tissues have the capacity to synthesis glucose, for example, the human brain, meaning that should blood glucose should become too low and hypoglycaemic (~ <3.3 mmol. L^{-1}, Rozance and Hay. 2010), the brain will simply not receive enough energy, and this can result in problematic health symptoms, ranging from less serious (Sweating, headache, fatigue, anxiety dizziness etc), to more serious (Confusion, loss of coordination, slurred speech) and even result in individuals to have seizures and become unresponsive/lose consciousness) (Kalra et al. 2013). Conversely, blood glucose concentrations can become too high (hyperglycaemia), a

response typically seen in type 2 diabetes (T2D), discussed in further detail in **section *1.6.3***. Often a result of a chronic state of a high glucose influx overwhelming the bodies capacity to remove blood glucose into glucose consuming tissues, blood glucose reaches excessive concentrations and become problematic for the regulation of glucose uptake into tissues, such as skeletal muscle, discussed in **section *1.6.3.*** In healthy conditions and in conjunction to the consumption of carbohydrate is an increase in circulating insulin; carbohydrate ingestion initiates pancreatic insulin secretion into blood (Wilcox. 2005). Postprandial rises in circulating insulin concentrations augments skeletal muscle blood supply via an increased recruitment of the microvasculature (Keske et al. 2016), which in turn, increases the delivery of insulin and dietary metabolites, such as glucose, fatty acids, lipids and amino acids (Wagenmakers et al. 2016). Tissues that are sensitive to insulin contain insulin receptors that receive insulin to initiate blood glucose uptake. Elevated blood glucose concentrations promote glucose uptake to insulin sensitive/glucose consuming tissues, while hepatic glucose production is simultaneously attenuated, to reinstate blood glucose homeostasis (Edgerton et al 2017). It is well established that in the postprandial period, following carbohydrate ingestion, blood glucose and insulin concentrations are elevated and then followed by insulin mediated glucose uptake into insulin sensitive tissues, such as skeletal muscle (accounting for ~80% of this disposal). The intracellular mechanisms underpinning glucose uptake in skeletal muscle is a major component to this thesis.

A) ***B)***

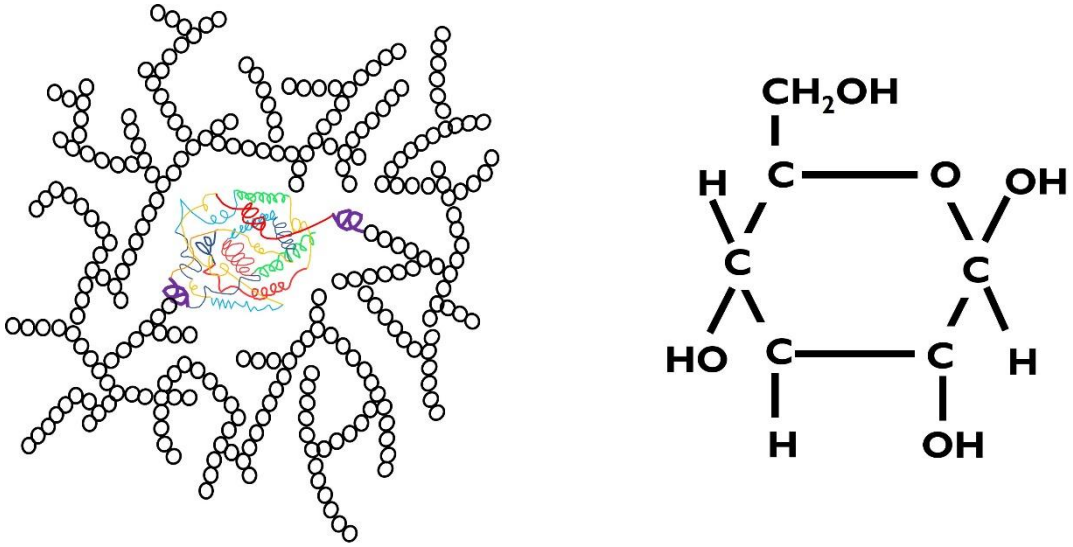

Figure 1.1. *The structure of the glycogen (A), a multibranched glucose polysaccharide, a key site of energy storage in humans and (B) the chemical structure of glucose.*

1.1.2 Insulin mediated glucose uptake into skeletal muscle via the signalling cascade.

Glucose uptake into skeletal muscle predominantly occurs through diffusion via glucose transporter proteins. Currently, 14 isoforms exist for glucose transporter proteins (Uldry and Thorens. 2004), of which glucose transporter 4 (GLUT4) is the most active for insulin mediated glucose uptake to skeletal muscle (Watson and Pessin, 2001). Under fasting conditions, GLUT4 resides in intracellular cytosolic microvesicles, however in response to feeding (that increases plasma insulin concentrations) GLUT4 microvesicle structures translocate to the plasma membrane of the skeletal muscle fibre (Watson et al. 2004; Bradley et al. 2015). The translocation process of GLUT4 has been the subject of many studies (Goodyear et al. 1996; Watson and Pessin. 2001; Richter. 2021), with the findings showing the process to be heavily regulated and to involve various intracellular signalling events.

Insulin binds to an extracellular insulin receptor (IR) on the cell membrane, which in turn, stimulates the insulin-signalling cascade. The insulin receptor is composed of two extracellular α-subunits and two transmembrane β-subunits, both stimulated by the binding of insulin. The binding of insulin to the extracellular α-subunit of the receptor evokes conformational alterations to the e tyrosine kinase domain in the transmembrane β-subunit. This insulin receptor tyrosine phosphorylation activates and recruits a number of proteins to the IR, such as the insulin receptor substrate proteins (IRS1-4) and the Shc adapter protein isoforms, signal regulatory protein family members, Gab-1, Cbl, and adaptor protein with pleckstrin homology and Src homology (APS) (Pessin & Saltiel. 2000). Activation of the insulin receptor leads to the activation of two known insulin signalling pathways, both important in mediating glucose uptake from blood; The IRS-PI3-K signalling cascade and the APS/CAP/Cbl pathway. More is understood about the IRS-PI3-K signalling cascade and its importance in glucose uptake. When phosphoinositide 3-kinase (PI3-K) is inhibited, or a dominant interfering mutant regulating PI3-K is overexpressed, GLUT4 translocation and glucose uptake is attenuated (Cheatham et al. 1994; Okada et al. 1994). However, Pharmacological activation of the IRS-PI3-K pathway does not augment glucose uptake (Egawa et al., 2002; Konrad et al., 2002). Moreover, when IRS-PI3-K is pharmacologically inhibited, there is not a complete inhibition of glucose uptake, the therefore, alternative pathway(s) must work in conjunction with the IRS-PI3-K pathway (figure 1.2).

1.1.3 IRS-PI3-K-AKT pathway

A cascade of events is initiated through the binding of insulin to the IR, ultimately results in GLUT4 translocation and glucose uptake into skeletal muscle (figure 1.2).

Activation of the IR phosphorylates several tyrosine sites, situated at the C-terminus of IRS-1. Specifically, activation of the pair of YXXM motifis at positions 612 and 632 of IRS-1 (Tyr^{612} and Tyr^{632}) appears important for the binding and activation of PI3-K and subsequent GLUT4 translocation in humans (Esposito et al., 2001). IR tyrosine kinase activity phosphorylates IRS-1 on a number of tyrosine residues which subsequently bind to molecules containing src-homology 2 (SH2) domain binding sites. PI3-K is composed of a p85 subunit, containing two SH2 domains which binds with the phosphopeptides of IRS-1, and a p110 subunit (Świderska et al. 2020) Once phosphorylated, SH2-domain binding sites and a p110 subunit are exposed (Myers et al. 1992). Following activation, PI3-K translocates to the inositol ring whereby it phosphorylates PI to produce phosphatidylinositol-3-phosphates, including the formation of phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5)P_3 or PIP_3). PIP3 formation excites its downstream targets phosphoinositide-dependent kinase 1 (PDK1) Akt 10 (protein kinase B) and atypical PKCs (Manna and Jain. 2013). Once PDK1 and Akt are colocalised, PDK1 phosphorylates Akt at Thr308 and Ser473 thereby completing kinase activity (Alessi et al., 1996). Activation of Akt leads to downstream phosphorylation of glycogen synthase kinase-3 (GSK-3) and Akt substrate of 160 kDa (AS160) (Świderska et al. 2020. Akt phosphorylates and in turn, inactivates GSK-3 at Ser9 which attenuates the inhibition of glycogen synthase, increasing its activity and promoting glycogen synthesis. AS160 withholds a Rab-GAP (GTPase-activating protein) domain as well as Akt phospho-motifs (Miinea et al., 2005). Rab proteins are important in vesicle trafficking and exocytosis of glucose transporters. Under basal conditions Rab-GAP contain Rab proteins loaded with GDP. Akt activation phosphorylates AS160 on Akt phospho-motifs, which inhibits Rab-GAP, thus allowing for GTP loading, thus promoting GLUT4 excytosis and reducing its

endocytosis, resulting in GLUT4 accumulation at the plasma membrane (Sano et al. 2007). The PI3-K-dependent insulin signalling pathway is an important sequence of events that regulate GLUT4 translocation to the plasma membrane (figure 1.2), however, alternative insulin signalling pathways for glucose uptake do exist (PI3-K-independent pathway).

1.1.4 APS-CAP-Cbl pathway

Lipid rafts are plasma membrane microdomains, enriched in cholesterol and are proposed to be the initiation site of the APS/CAP/Cbl pathway, an insulin-dependent, PI3-K-independent pathway for GLUT4 translocation (Patel and Insel. 2009). Once bound with cholesterol, caveolin forms caveolae, which are small invaginations of the plasma membrane. Caveolae are saturated with signalling proteins, such as the IR (Gustavsson et al. 1999). Adaptor protein with a PH and SH2 domain (APS) is an IR substrate responsible for binding various subunits to the IR via the SH2 domain (Ahmed et a. 1999). Association and phosphorylation of APS with the insulin receptor creates a binding site for the SH2 domain of Cbl. Cbl has multiple tyrosine residues that can be phosphorylated by the IR but requires the association of Cbl adaptor proteins, most notably CAP (Cbl-associated protein) (Baumann et al. 2000). Cbl and flotillin bind to the C-terminus and N terminus of CAP, respectively; Thereby the CAP/Cbl complex is assimilated into the lipid raft. Further Cbl recruitment through tyrosine phosphorylation incorporates the adaptor protein CrkII and guanine nucleotide exchange factor CG3 to the lipid rafts. As a result, TC10 is activated, a reaction important in the translocation and plasma membrane tethering of GLUT4 (Bogan. 2012).

1.1.5 GLUT4 translocation

GLUT4 translocation to the plasma membrane can occur by either polymerized microtubules or actin cytoskeleton pathways (Fletcher et al. 2000; Lopez et al. 2009). PI3-K and protein kinase C (PKC) are the likely candidates to mediate actin polymerization following insulin signalling (Liu et al. 2006b). The abundance of GLUT4 translocation and localisation at the plasma membrane is determined by GLUT4 vesicle endocytosis and exocytosis into the plasma membrane. Invagination of the plasma membrane results in t-tubules extending into the cell. *In vivo* and *in vitro* models show ample GLUT4 to reside at the plasma membrane in basal conditions (Kanzaki. 2006). Then, following insulin stimulation or glucose feeding, there is greater localisation and presence of GLUT4 at plasma membrane sites, allowing for greater glucose uptake (Cushman and Wardzala. 1980; Lauritzen et al. 2006; Fazakerley et al 2010; Bradley et al. 2015). Time-lapse immunofluorescence microscopy has shown that in male C57BL/6 mice insulin stimulation of skeletal muscle lead to translocation of GLUT4 containing microvesicles along the plasma membrane (Lauritzen et al. 2008). The kinetics between insulin stimulated GLUT4 translocation differs between the plasma membrane and t-tubules. Initially insulin reaches the sarcolemma, before its delayed diffusion into t-tubules, local interaction with insulin receptors, triggering local insulin signalling and local GLUT4 translocation. GLUT4 depot vesicles are predominantly depleted locally in the sarcolemma or t-tubule regions and do not require translocation over long distances. (Lauritzen. 2009). More recent immunofluorescence microscopy analysis partly visualises the effects of GLUT4 translocation following glucose feeding (75g) in human skeletal muscle, whereby GLUT4 increased its colocalization with the plasma membrane 30 mins post glucose

ingestion (Bradley et al. 2015). This finding, paralleled with the aforementioned studies demonstrates the kinetic response from GLUT4 in response to insulin stimulation. The initiation of GLUT4 trafficking is explained by insulin signalling pathway, however the terminal processes mediating the incorporation of GLUT4 to the plasma membrane is yet to be discussed. The movement of GLUT4 relies on tethering, docking and fusion to the plasma membrane in order to function as a transporter for extracellular glucose.

1.1.6 GLUT4 docking and fusion

The presence of GLUT4 at the plasma membrane is fundamental for glucose entry into the muscle. Following a finely regulated translocation trafficking process via the insulin signalling cascade, GLUT4 must then undergo fusion and docking to the plasma membrane (St-Denis and Cushman. 1998; Foster et al. 1999; Kawanishi et al. 2000; Jaldin-Fincati et al. 2017). GLUT4 requires the aid of SNARE (Soluble N Ethylmaleimide Sensitive Factor Attachment Protein Receptor) proteins to fuse to the plasma membrane. SNAREs are membrane-associated proteins fundamental to many fusion and docking processes (Foster et al. 1999; Kawanishi et al. 2000). There are 36 SNAREs which all contain a distinct coiled coil structure and a SNARE “motif” formed of 60-70 amino acids (Chen and Scheller, 2001; Jahn and Grubmüller. 2002; Söllner, 2004; Duman and Forte. 2003). Dependent on their role, SNAREs are classified accordingly; target-SNAREs (t-SNAREs) are located at the plasma membrane, whereas vesicle-SNAREs (v-SNAREs) are found on transport vesicles. The formation of complete v-t SNARE complexes is fundamental for target proteins to fuse to the target membrane to perform their role. The SNARE proteins implicated primarily in GLUT4 docking are the v-SNARE vesicle associated membrane protein-2

(VAMP2) and t-SNAREs; synaptosomal associated23 (SNAP23) and syntaxin 4. NSF and alpha SNAP (soluble NSF associated protein) are responsible for conformational changes to SNARE proteins in order to form an active SNARE complex (Sollner et al. 1993).

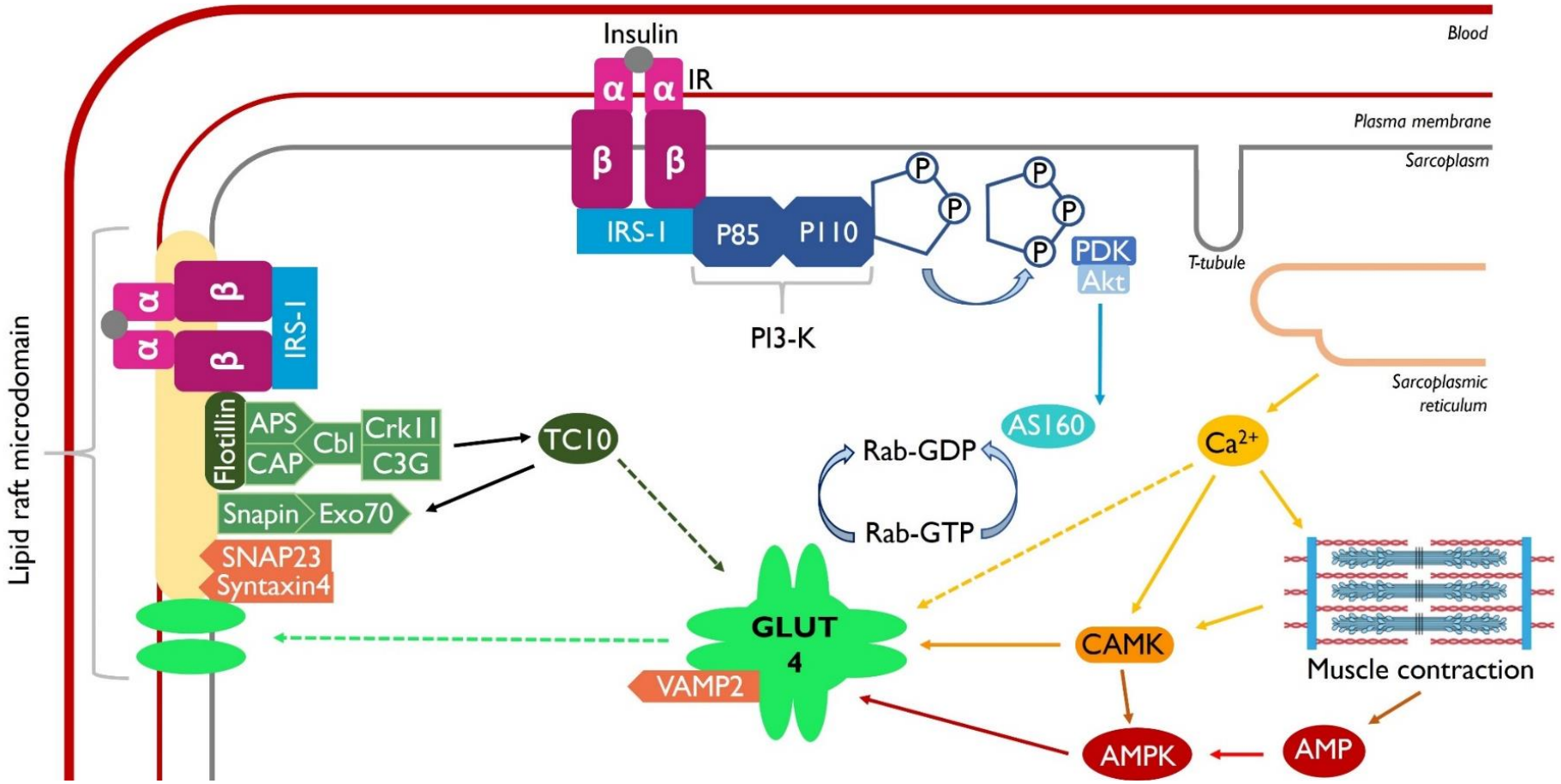

Figure 1.2. *Skeletal muscle glucose transport, insulin-dependent and independent pathways. AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; APS, adaptor protein with a PH and SH2 domain; AS160, Akt substrate of 160 kD; Ca2+, calcium; CAMK, calmodulin-dependent protein kinase; CAP, Cbl-associated proteain; 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.*

TC10 activation (through the action of APS/CAP/Cbl) causes activation and translocation of Exo70 to the lipid raft, whereby it binds with snapin. Exo70 binding with snapin activates SNAP23, a t-SNARE that couples with a complementary membrane SNARE, Syntaxin (1, 2, 3 and 4) to bind with v-SNARE (VAMP1 and 2), allowing for exocytosis of the targeted protein. SNAP23 (and the accompanying v-SNAREs) appears to be important in insulin mediated glucose uptake, with direct association with GLUT4 docking and fusion to the plasma membrane (Foster et al. 1999; Kawanishi et al. 2000). These *In vitro* studies transfected adipocytes with an inactive mutant of SNAP23 or microinjected an antibody to SNAP23 and showed reduced translocation and docking of GLUT4 to the plasma membrane, resulting in reduced glucose uptake (Foster et al., 1999). In addition to the plasma membrane, SNAP23 is located at other intracellular organelle membranes, including lipid droplets (LDs) and mitochondrial networks where it may mediate fusion processes (Strauss et al 2015). However, SNAP23s functional properties remain understudied and require clarity.

1.1.7 Mechanisms of Insulin-independent glucose uptake

The entry of glucose into skeletal muscle can occur in the absence of insulin. For example, during exercise, glucose uptake is increased, irrespective of insulin concentrations (Ivy et al. 1987). Similar to insulin induced glucose uptake, muscle contraction promotes GLUT4 vesicle trafficking to the plasma membrane and t-tubules, resulting in increased glucose uptake into skeletal muscle fibres (Rose and Richter, 2005; Richter and Hargraeves. 2013). Interestingly, inhibition of PI3-K with wortmannin, had no effect on contraction-mediated GLUT4 translocation in rat soleus

muscle (Lund et al. 1995). Moreover, the activation of IRS-1, PI3-K and Akt/PKB are unchanged in response to electrically stimulated contraction of isolated skeletal muscle (Goodyear et al. 1995; Wojtaszewski et al. 1996). Therefore, there must be alternative mechanisms responsible for contraction induced GLUT4 translocation. Muscle contraction increases the AMP to ATP ratio which activates AMP-activated protein kinase (AMPK) (Wojtaszewski et al., 2000b). AMPK activation enhances GLUT4 translocation to the plasma membrane and glucose uptake (Kurth-Kraczek et al. 1999; Merrill et al. 1997), even when incubated with wortmannin (Hayashi et al. 1998), thus suggesting that AMPK stimulated glucose uptake is not affected by insulin signalling. An important finding to note is that knockout of AMPK reduced contraction-mediated glucose uptake by just 40%, (Mu et al. 2011), meaning that other mechanisms must also play a role in contraction-mediated glucose uptake. Muscle contraction acts upon the sarcoplasmic reticulum to release the metabolite calcium (Ca^{2+}), which forms a complex with calmodulin (CaM). In turn, CaM binds to and then activates Ca2+/CaM-activated kinase (CAMK). Inhibition of CAMK reduces glucose uptake thereby potentially presenting a Ca^{2+} modulated pathway in GLUT4 translocation (Wright et al. 2005; Wright et al. 2004). Ca^{2+} upregulates PKC, which has previously been associated with glucose uptake; PKC inhibition has been shown to attenuate contraction-meditated glucose uptake (Ihlemann et al. 1999), however in this study, the method used to inhibit PKC was unable to distinguish between any of the twelve known isoforms of PKC. Of the subclasses of PKC isoforms, both conventional PKC (cPKC) and novel PKC (nPKC) are activated in response to electrical stimulation of rat skeletal muscle (Richter et al. 2003), but this response was not replicated in human skeletal muscle (Rose et al. 2004). However, the activity of atypical PKC (aPKC) isoforms have been shown to be upregulated following muscular

contractions (Rose et al. 2004). Interestingly, it has also been shown that exercise mediated glucose uptake is not dependant on aPKC activity per se (Sajan et al 2009). Rather, aPKC could act upon insulin and therefore influence glucose uptake into the cell (Maarbjerg et al 2011). The clarity surrounding PKC activity and glucose uptake remains uncertain in both insulin-independent and dependent contexts. Furthermore, activation of aPKC is coupled with an increase in extracellular related kinases (ERK). However, ERK activation can only partly account for contraction induced glucose uptake, since ERK inhibition does not fully block contraction induced glucose uptake. Similarly to ERK, p38, and c-Jun N-terminal kinase (JNK) are also mitogen-activated protein kinase (MAPK) isoforms, shown to be upregulated by muscular contraction. To add to this, muscle contraction has been demonstrated to phosphorylate p38 (Sakamoto and Goodyear, 2002) and these conditions simultaneously result in increased glucose uptake (Chambers et al., 2009), moreover, p38 inhibition negates these responses to glucose uptake (Chambers et al., 2009). In response to this, however, the p38 inhibitor used in the aforementioned study has also been purported to directly inhibit GLUT4 itself, as described by (Jensen and Richter, 2012). Therefore, if GLUT4 action was simultaneously inhibited, this could negate p38's proposed role in GLUT4 activity and glucose uptake. Once in the muscle, the fate of glucose is dependent on current metabolic requirements, such that glucose is either diverted towards oxidative pathways or glycogen synthesis (discussed in section 1.4.3). It is evident that there are many aspects of contraction-mediated glucose uptake which require further investigation.

1.2 Intracellular fate of glucose

1.2.1 Glycogenolysis and glycolysis

The importance for glucose uptake into skeletal muscle is to maintain glycolytic flux and energy production. When energy demand increases (i.e. exercise), skeletal muscle energy demands increase. In turn, energy production is upregulated, which in part, is reliant on carbohydrate metabolism. Intracellular carbohydrate metabolism is regulated by allosteric effectors and covalent modification, which when energy demand increases, begin to initiate signalling cascades in order to produce more sustainable energy in the form of glucose-6-phosphate. An increase in allosteric effectors (ADP, AMP, Pi) and intracellular Ca2+ concentrations (i.e., following muscle contraction) activate glycogen phosphorylase, an important step in glycogen breakdown (glycogenolysis), whereby glycogen is converted into glucose-1-phosphate (figure 1.3). Furthermore, glucose 1 phosphate is then phosphorylated and forms glucose 6 phosphate under the action of phosphoglucomutase. Alternatively, glucose 6 phosphate can be derived from extracellular substrate, plasma glucose. Following transportation into the cell, glucose is initially converted to glucose-6-phosphate via the enzyme hexokinase; the action of hexokinase is important as the reaction is irreversible, thus meaning glucose will remain within the muscle cell. Next, glucose 6 phosphate undergoes a number of steps, which formulate the process known as anaerobic glycolysis (figure 1.3); this series of reactions include the activity of phosphofructokinase (PFK), which is the glycolytic rate limiting enzyme. Overall, anaerobic glycolysis produces a net total of 2 pyruvate molecules and 2 ATP. The reason for this discrepancy is due to 1 ATP being required to convert extracellular derived glucose into glucose 6 phosphate. Not only that, but anaerobic glycolysis also

produces lactate and pyruvate which are important in oxidative phosphorylation, the metabolic pathway in which yields the greatest amount of ATP.

1.2.2 Glycogenesis

Glucose uptake is not only crucial to maintain glycolytic flux and energy production but is also key for glucose storage when the metabolic demands of the body are reduced and glycogen storage is a priority (such as following a glycogen depleting bout of exercise). Skeletal muscle is the body's largest site for glycogen storage, which will be discussed at greater length in section 1.4.3. Glycogen storage is governed by several factors: Firstly, as glycogen stores are finite, glycogen concentrations cannot already be fully saturated, as skeletal muscle will already be 'full' of glycogen. Exercise training can increase skeletal muscles glycogen storage capacity (Bergstrom & Hultman, 1962; Hargreaves, 2006; Hearris et al., 2018; Murray & Rosenbloom, 2018). Secondly, a concentration gradient must exist whereby blood glucose is greater than the concentration of intracellular glucose. To achieve this, enzymes associated with glycogen storage are upregulated, such as hexokinase. Meanwhile glycolytic enzymes, such as phosphofructokinase (PFK) are downregulated (Layzer et al., 1967; Mandarino et al., 1987; Pelley, 2007; Vestergaard et al., 1994). Furthermore, using ATP, glucose is rapidly converted to glucose 6 phosphate upon entry into the cell, mediated by the enzyme hexokinase to maintain a concentration gradient between blood and intracellular glucose (figure 1.4). Next, the enzyme phosphoglucomutase acts upon glucose 6 phosphate by converting the position of the phosphate to glucose's first carbon, producing glucose 1 phosphate. UDP-Glucose is then formed via glycogenin – 1, which is then joined with other glucose molecules to form small

glucose polymers through glycogen synthase. Lastly, the formation of glycogen particles are a result of many glucose polymers being joined together in branch like structures, through the action of glycogen branching enzymes. Existing glycogen particles receive glucose molecules thereby growing by 1 glucose particle through each 'cycle' of glycogenesis (glycogen synthesis, figure 1.4).

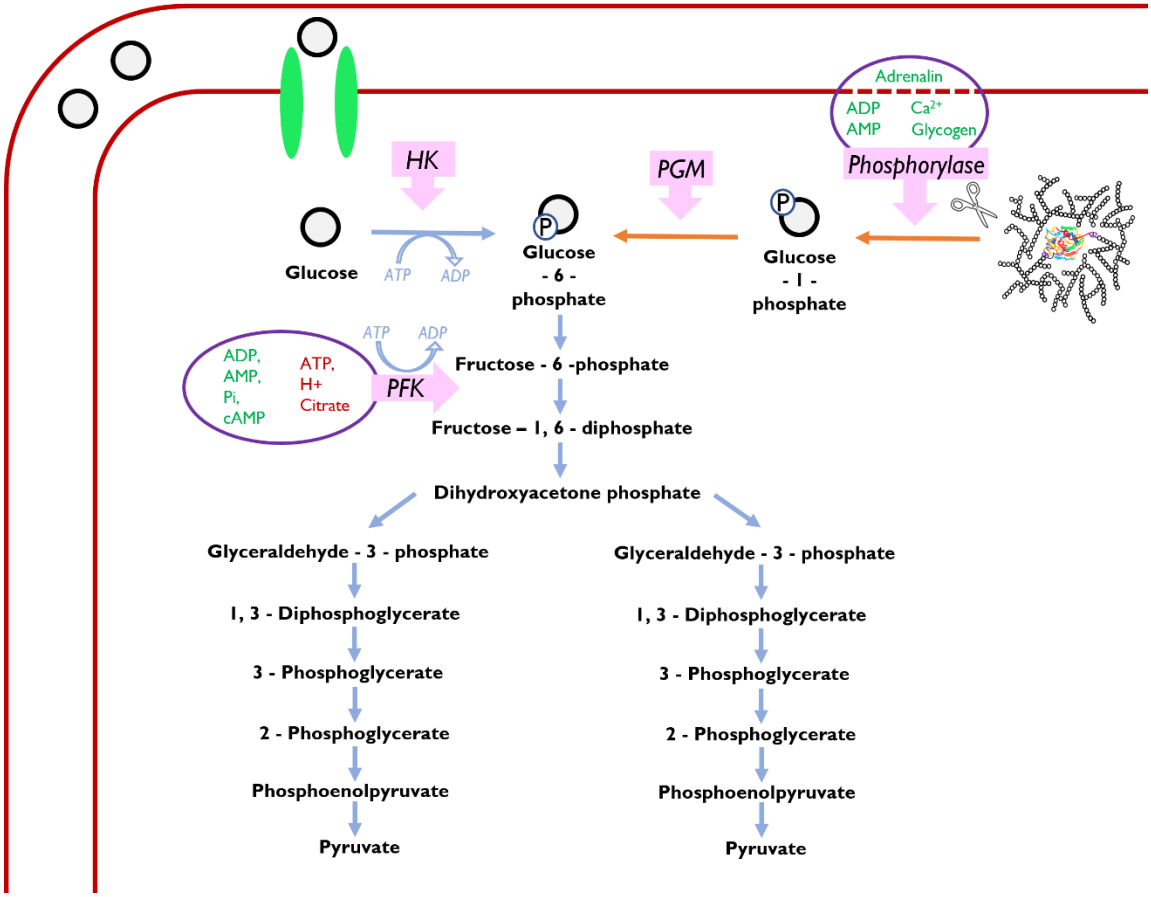

Figure 1.3. *Glycogenolysis and glycolysis. Series of events in the metabolic pathway for glycogenolysis (orange arrows) and glycolysis (blue arrows). Demonstrating the process by which glycogen and extracellular glucose are converted to two pyruvate molecules. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; HK, hexokinase; H+, hydrogen; PFK, phosphofructokinase; Pi, phosphate; PGM, phosphoglucomutase; Phosphorylase, glycogen phosphorylase.*

1.3 Substrate utilisation, exercise & glycogen

Carbohydrate metabolism is an important consideration prior to, during and following exercise. There are a number of factors that influence the metabolic process that provide energy provision in and around exercise, such as: substrate availability, the abundance of transport proteins for the movement of substrates across plasma and mitochondrial membranes and, regulatory enzymes activity (Hearris et al. 2018). During exercise, cellular metabolism regulates skeletal muscle excitation contraction-coupling in order to perform locomotion. These processes require energy and thus, for sustained muscle contraction, a sustained influx of substrate is required.

The two main energy yielding macronutrients are fat and CHO. Their contribution towards energy production rely on a number of factors, including but not limited to: exercise intensity, exercise duration, environmental conditions, energy availability, training status etc (Van Loon et al. 2001; Watt et al. 2002; Hawley and Leckey. 2015; Hargreaves and Spriet. 2020). Classical data by Van Loon et al, (2001) showing that as exercise intensity increases from 40 to 55 to 75% Wmax, the demand for carbohydrate (muscle glycogen and plasma glucose) increases. To further explain the magnitude of carbohydrate demand during high intensity exercise, this study shows that when exercising at higher intensities (75% Wmax), carbohydrate metabolism accounts for 76% of energy expenditure (muscle glycogen, 58%; plasma glucose 18%). Therefore in high intensity exercise, when energy demand is high, we must maintain a high flux through carbohydrate metabolism. As a result of limited storage capacity and the rapid nature of carbohydrate metabolism, glycogen depletion can occur in much less time than many endurance events (Hearris et al. 2020). Muscle glycogen utilisation is most rapid in the earlier stages of exercise and decreases later in exercise as a result of lower glycogen availability (Gollnick et al. 1974). Over the course of 240 minutes of moderate intensity exercise (55% VO2 max), there is an

inverse relationship between carbohydrate and fat metabolism, with carbohydrates provision to energy expenditure linearly decreasing (Watt et al. 2002). As a result, exogenous carbohydrate provision is important to maintain carbohydrate metabolism, and in turn allow for sustained energy production, thus proving important to performance in endurance exercise. Therefore, to sustain prolonged moderate-high intensity exercise, athletes must firstly have a large pool of muscle glycogen to utilise and secondly, when exercise lasts long enough to deplete these stores, CHO must be consumed during exercise (Costill and Hargreaves, 1992). Alternitivley, there is reduced substrate availability to maintain metabolic flux, which could cause the athlete to fatigue prematurely. Vøllestad and Blom (1985) investigations surrounding the effects of varying exercise intensities on exercise induced glycogen depletion found a linear progression in the amount of fibres that experienced glycogen depletion from low, moderate and high intensity exercise (40%, 60% and 90% VO2 max). These results suggest that when a muscle is repeatedly stimulated through contraction, it will reduce its glycogen content and the magnitude of glycogen loss is partly a result of the intensity of the muscle contraction; Thus, prolonged high intensity exercise would lead to skeletal muscle glycogen depletion. Glycogen depleting exercise illustrates skeletal muscle's demand for carbohydrate during exercise, while also demonstrating the limitations surrounding the human bodies glycogen storage capacity.

1.3.1 Pre exercise glycogen influence on exercise performance

Carbohydrate and exercise performance has been an area of study for over a century. Findings from the 1920's unveiled that exercise performance appears to be influenced by diet, particularly carbohydrate consumption; showing that high carbohydrate diets

prolong the onset of fatigue during exercise (Krogh and Lindhard. 1920). In addition, another classical study investigated “sugar level” and “physical condition” upon completion of the 1923 Boston marathon. Interestingly, runners who had low glucose concentrations (<70 mg/dL) were also classified as being in “poor” or “very poor” conditions. Contrastingly, athletes who had normal glucose concentrations, not only appeared in better physical condition, but were also self-reportedly feeling better and ultimately, performed better. Another valuable finding from this study, was that the overall race winner, who performed a world record time, displayed one of the highest pre- and post-race glucose values (Levine et al. 1923). These early investigations suggest that prolonged exercise requires sufficient carbohydrate provision. Work across the 1930’s identified that carbohydrate can improve endurance performance and high intensity exercise (Haggard and Greenberg. 1935; Christensen and Hansen 1939), however, due to methodological limitations, it was not until decades later that the mechanisms of carbohydrate metabolism could be investigated. The development of percutaneous needle biopsy methodologies in the 1960s led to assays which could directly measure substrate utilisation and metabolic processes (Bergstrom. 1962). The percutaneous needle biopsy technique enabled researchers to collect 100-200 mg samples of muscle tissue from participants, allowing researchers to investigate the effects of exercise and diet on muscle metabolism and other biochemical, metabolic, histological, and contractile characteristics of muscle. Bergstrom's experiments leveraging the percutaneous needle technique to investigate the impact of substrate utilisation during exercise established that muscle glycogen is depleted in an intensity-dependent manner during exercise, and that a high carbohydrate diet increased muscle glycogen storage and thus improved exercise capacity (Bergstrom et al. 1967). This study compared the effect of low (0.63 g/ 100 g), moderate (1.75/100 g) and high

(3.31 g/ 100 g) muscle glycogen content before completing a high intensity exercise task to exhaustion (75% VO2 max). Time to exhaustion strongly correlated with pre-exercise glycogen stores ($r = 0.92$, $p < 0.001$), whereby starting exercise with high glycogen allowed participants to exercise for longer (167 min) compared to starting exercise with moderate (114 min) and low muscle glycogen concentrations (57 min). The key conclusions from this study were that muscle glycogen stores have a direct impact on exercise performance, whereby promoting greater glycogen stores prior to exhaustive exercise, allows individuals to exercise for longer. Following the work of Bergstrom and colleagues, the scientific literature has repeatedly demonstrated that muscle glycogen availability is a key determinant of exercise capacity during prolonged (>90 min) and high-intensity intermittent exercise (Perkow & Saltin,1971; Gollnick et al.,1972; Hargreaves et al.,1995; Hargreaves,1999). Furthermore, the opposite is also true, whereby reduced muscle glycogen availability impairs exercise performance, resulting in early-onset fatigue (Hermansen et al., 1967). The key conclusions from these studies were that muscle glycogen stores have consistently been proven to directly impact exercise performance; therefore, pre exercise glycogen concentrations must be a consideration should athletes wish to delay fatigue and improve performance.

In 1981, Sherman and colleagues demonstrated that muscle glycogen stores of trained participants could be maximally increased. The group demonstrated that a moderate carbohydrate diet (5 $g.kg^{-1}$ BW) for three days, followed by a high carbohydrate diet (8 $g.kg^{-1}$ BW) (combined with an exercise taper), led to comparable levels of muscle glycogen storage. Current recommendations (table 2) suggest that skeletal muscle glycogen can be fully saturated within a 24-48 hour period (Bussau et al. 2002).

Carbohydrate metabolism during exercise depends on a number of factors, including exercise intensity and duration. As exercise intensity shifts from moderate (~65% VO_{2max}) to high (>85% VO2max), carbohydrate metabolism (muscle/liver glycogenolysis and glucose uptake) increases, while lipid metabolism (plasma FFA and intramuscular triglyceride oxidation) is reduced (Van loon et al. 2003; Gonzalez et al. 2004). On the other hand, metabolism for prolonged steady state exercise is characterised by increased oxidation of lipid and reduced carbohydrate oxidation as exercise duration progresses (Watt et al. 2002). More specifically, metabolism of substrates derived from intramuscular stores (skeletal muscle glycogen and intramuscular triglycerides) is reduced, compared to increased oxidation rates of plasma free fatty acids (Watt et al. 2002). As exercise duration increases, carbohydrate availability is decreased (muscle glycogen depletion), meaning there is insufficient substrate to feed carbohydrate metabolism and thus, reducing glycolytic flux (Watt et al. 2002; Hearris et al. 2018). Mechanistic studies have attributed this shift in metabolism in part to the downregulation of the rate limiting enzyme, pyruvate dehydrogenase (PDH). In conditions of high rates of carbohydrate metabolism, the action of PDH is responsible for converting pyruvate (product of glycolysis) into acetyl CoA (substrate for downstream oxidation). The downregulation of PDH that occurs as exercise duration progresses is thought to be a result of reduced pyruvate flux and increased PDH kinase activity (Watt et al. 2002; 2004). Similar observations are seen across the literature, in that exercise intensity and duration are powerful mediators for the magnitude of substrate availability, as well as directly influencing substrate utilisation. Furthermore, as carbohydrate metabolism is vulnerable to relatively quick depletion, but is also fundamental for high intensity exercise, pre-exercise glycogen

concentrations must be maximised when performing prolonged high intensity exercise and glycogen depletion is a limiting factor to performance.

Table 1. Daily dietary carbohydrate guidelines for athletes

Training volume	**Carbohydrate**
Moderate exercise programme (1 hour per day)	5-7 $g.kg^{-1}$ BW
Endurance programme (1-3 hours per day moderate-high intensity exercise)	6-10 $g.kg^{-1}$ BW
Extreme commitment (>4-5 hours per day moderate-high intensity exercise)	8-12 $g.kg^{-1}$ BW

*(table adapted from *Burke LM, Hawley JA, Wong SHS, et al. Journal of Sports Sciences. 2011;29(1):S17-S27)*

Table 2. Acute carbohydrate strategies for athletes

	Type of training	**Carbohydrate**
General fuelling	Events < 90 mins exercise duration	7-12 $g.kg^{-1}$ BW per 24 hours
Carbohydrate loading	Events > 90 min exercise duration (sustained/intermittent)	10-12 $g.kg^{-1}$ BW per 24 hours for 36-48 hours
Pre-Event fuelling	Before exercise >60 mins	1-4 $g.kg^{-1}$ BW consumed 1-4 hours pre exercise

*(table adapted from *Burke LM, Hawley JA, Wong SHS, et al. Journal of Sports Sciences. 2011;29(1):S17-S27)*

1.3.2 Post exercise glycogen resynthesis

As discussed in **section 1.3,** muscle glycogen depletion can occur relatively fast and as this is directly associated with exercise fatigue and therefore must be avoided to maintain endurance exercise (Bergstrom et al. 1967; Hultman. 1967). High performing athletes undergo large weekly training volumes, in which multiple sessions contain high intensity work. Therefore, a priority during recovery from exercise sessions is the restoration of muscle glycogen stores, so that athletes are prepared for the next exercise training session. Indeed, when carbohydrate is consumed following exercise, rates of muscle glycogen resynthesis can reach ~40-50 mmol/kg wet wt, per hour (Hearris et al, 2018). The recommended post exercise carbohydrate intake depends on the time available for recovery, as it isn't uncommon for endurance athletes to train multiple times per day. If the athlete has <8 hours to recover, consuming carbohydrate immediately after exercise at a rate of 1.2 $g.kg^{-1}.h^{-1}$, in 15-60 minute intervals, for >4 hours remains the most efficient protocol (Van loon et al. 2000; Jentjens and Jeukendrup. 2003). Whereas, if the athlete has >8 hours to recover, then resuming their everyday energy needs should suffice (Parkin et al. 1997; Burke et al. 2017). The post exercise feeding window has received much attention in sports science, due to the importance of efficient recovery for athletes. Much of this research concerns carbohydrate, due to the report effects of exercise on skeletal muscle glucose sensitivity (Price et al. 1994). Glucose entry into skeletal muscle is typically modulated through insulin-dependent signals to increase glucose transport from blood circulation into muscle. The pattern of glycogen synthesis following exercise occurs in a biphasic manner (Price et al. 1994). Exercise, much like insulin, also increases glucose transport, and therefore upon exercise completion skeletal muscle is primed for glucose uptake. Consequently, glucose disposal into skeletal muscle can be maintained at a much faster rate, irrespective of insulin concentrations (Burke et al.

2017; Richter et al. 2021). Lasting 30-60 minutes, this insulin-independent pathway for glucose uptake is suggested to be reliant on muscle glycogen concentrations being <128-150 mml/kg dw and for carbohydrate to be fed immediately (Price et al. 1994; Jentjens and Jeukendrup. 2003). Following this rapid influx of glucose during the insulin-independent phase, the insulin-dependent phase follows at a more sustained, but slower rate.

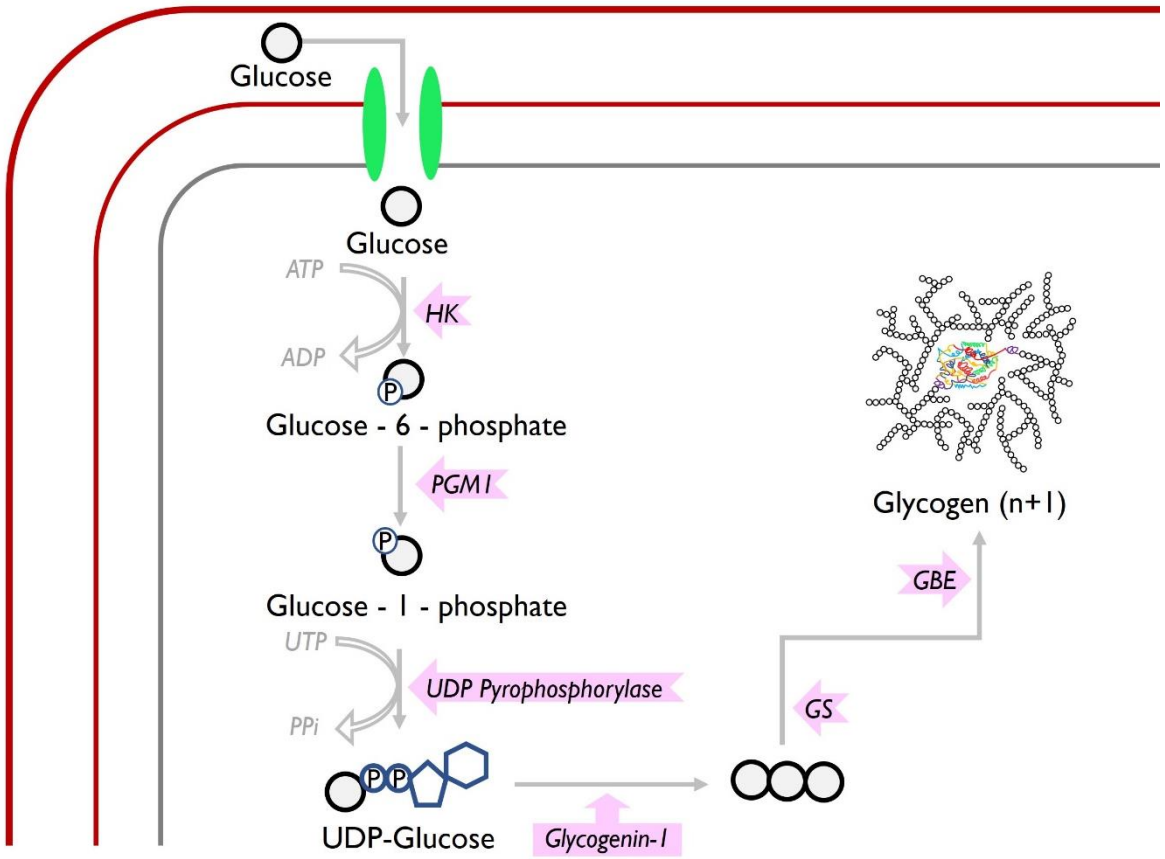

Figure 1.4. *Glycogen synthesis pathway. ADP, adenosine diphosphate; ATP, adenosine triphosphate; GBE, glycogen branching enzyme; GS, glycogen synthase; HK, hexokinase; PGM1, phosphoglucomutase 1; PPi, pyrophosphate; UDP, uridine diphosphate.*

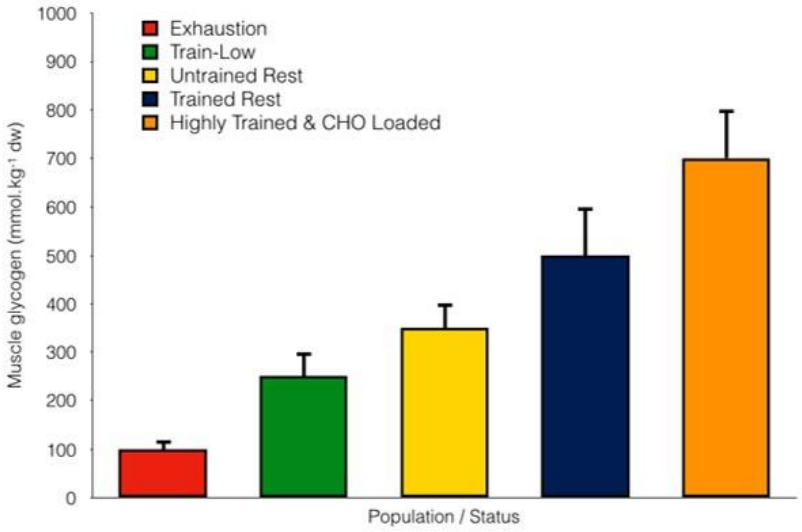

Figure 1.5. *Figure used from Hearris et al. (2018). 'Variations in muscle glycogen storage according to fatigue status, training status and dietary carbohydrate (CHO) intake (data are compiled from males only and from several studies including Taylor et al. ; Bartlett et al. ; Arkinstall et al. ; Gollnick et al. ; Coyle et al.).*

1.3.3 Increasing glycogen resynthesis with co-ingestion of carbohydrate and other nutrients

Although carbohydrates have the most profound effects on glycogen synthesis, it is possible that synthesis rates can be influenced by the presence of other nutrients. Furthermore, athletes will often consume a mixed-meal postexercise, therefore it is important to understand how co-ingestion with other nutrients effects the rate of glycogen synthesis. This could be particularly important in times when glycogen resynthesis is critical (when refuelling period is limited e.g. stage races in cycling) or when the carbohydrate dose is suboptimal for maximal glycogen synthesis, which is often the case for amateur athletes managing moderate-to-high training loads and full time work commitments.

Because athletes eat real food in addition to supplements post exercise, there are some key considerations to be made when aiming to promote glycogen synthesis via post-exercise feeding e.g. the effect of gluconeogenic substrates, digestion

processes, insulin secretion and signalling and satiety. Due to its insulinotropic response, protein ingestion has received the most attention in this area. The provision of amino acids/ protein can augment postprandial insulin secretion, leading to downstream increases to skeletal muscle glucose uptake and glycogen synthase activity (Margolis et al. 2021). Critically though, the benefit of protein co-ingestion only appears to have an additional benefit to glycogen synthesis when the carbohydrate dose is sub optimal (<1 $g.kg^{-1}.h^{-1}$), with the optimal protein dose currently suggested to be 0.3-0.4$g.kg^{-1}$ BM, the same amount recommended to promote optimal muscle protein synthesis (Burke et al. 2017; Margolis et al. 2021). In addition to protein coingestion, other dietary supplements have been proposed to promote glycogen synthesis. Chronic and short term supplementation with creatine monohydrate appears to potentially augment muscle glycogen synthesis. Van Loon and colleagues (2004), implemented a 5 day creatine loading (20g per day) protocol, which was matched with significantly greater glycogen concentrations (18%), compared to placebo. Similarly, 5 days of creatine supplementation (20g per day), has been shown to increase glycogen concentrations by 40 and 15% in soleus and red gastrocnemius in rats (Eijnde et al. 2001). Roberts and colleagues (2016), pre-loaded recreationally active males with 6 days of creatine (20g per day) or placebo supplementation, before completing a prolonged bout of exhaustive cycling and subsequent carbohydrate refeeding, following exercise. Creatine supplementation lead to significantly greater glycogen concentrations both, after 1 and 6 days of creatine loading, with the difference being attributed after the initial 24 hours and the difference thereby being maintained for the subsequent days (Roberts et al. 2016). Although, as of yet, there is no consensus on the mechanisms involved (Van Loon et a. 2004; Burke et al. 2017). Suggestions include upregulation of mRNA of genes associated with glycogen

synthetic proteins and/or altered cellular osmolarity (Safdar et al. 2008). The need for further study is paramount for a true understanding in this area. Future studies must target trained athlete populations and explore the previously proposed mechanisms. Another compound of interest is the effects of caffeine on insulin sensitivity, glucose tolerance and glycogen synthesis. Although, caffeine is purported to negatively affect carbohydrate metabolism and reduce glucose disposal and glycogen synthesis in resting humans (Han and Bonen. 1998; Graham et al. 2001; Greer et al. 2001; Thong et al. 2002), exercise appears to negate these effects. Indeed, the co-ingestion of carbohydrate with caffeine during exercise increases rates of carbohydrate oxidation during prolonged exercise (Yeo et al. 2005) and does not attenuate glycogen resynthesis following glycogen depleting endurance exercise (Battram et al. 2004). This signal to increase glucose uptake following muscle contraction appears to override the negative responses that have been shown in non-exercising participants. A later study tested post exercise co-ingestion of caffeine in two equal doses immediately after exercise and after 2 h of recovery (8 mg.kg BM^{-1}) with carbohydrate (1g.kg $BM^{-1}.h^{-1}$), following glycogen depleting exercise. The co-ingestion of caffeine and carbohydrate resulted in greater skeletal muscle glycogen stores following four hours recovery, compared to consuming the same amount of carbohydrate only (Pedersen et al. 2008). More recently it was shown that following exhaustive exercise, the ingestion of coffee and milk (8 $mg.kg^{-1}$ BM caffeine) ingestion increased post-exercise muscle glycogen resynthesis as well as glycaemic and insulinemic responses over 4 hours, compared to milk only (Loureiro et al. 2021). However, divergent findings have also been reported when caffeine (1.7 $mg.kg^{-1}.h^{-1}$) is provided with carbohydrate over a six hour post-exercise, re-feeding/recovery period (Beelen et al. 2012). A clear explanation behind these contrasting results is not yet known (Burke et al. 2017),

however, one noticeable difference is the doses used, so larger doses of caffeine may attenuate rates of glycogen synthesis (table 2.3). To summarise, when aiming to optimise muscle glycogen stores, adequate provision of carbohydrate at a rate of $1.2g.kg^{-1}.h^{-1}$, in 15-60 minute intervals, for >4 hours, proves the best strategy to do so (Van loon et al. 2000; Jentjens and Jeukundrup. 2003). However, this is often unrealistic and practically difficult to achieve for many athletes, especially those who are not of a professional nature, because they have other priorities, less or no nutritionist/product support from sponsors. Therefore, a priority area for future research is the identification of strategies that support glycogen resynthesis to compensate for sub-optimal carbohydrate doses. One such strategy may be to augment postprandial insulin sensitivity and skeletal muscle glucose uptake to increase the delivery of substrate (glucose) to glycogen synthase.

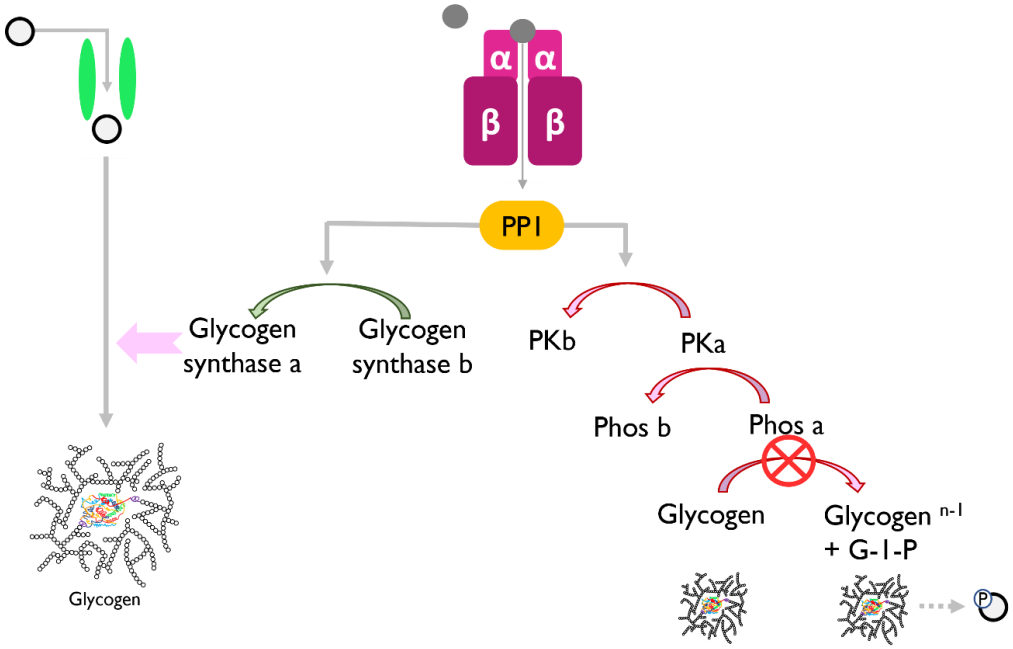

Figure 1.6. *Glycogen synthesis regulation. PP1, protein phosphatase 1; PK, protein kinase; Phos, glycogen phosphorylase; G-1-P, glucose – 1 – phosphate.*

Table 2.3. Rates of glycogen resynthesis in response to carbohydrate and co ingestion of carbohydrate with other nutrients

Study	Treatment	Sample size	Exercise	Recovery	CHO does ($g.kg^{-1}$ BW)	Co – nutrient dose ($g.kg^{-1}$ BW)	Glycogen Synthesis
Alghannam et al.	CHO + Protein	6 (5M, 1F)	Running TTE @ 70 VO2 max (82 min)	4 hours	0.8	0.4	41.7a
	CHO				1.2		37.4a
Beelen et al.	CHO + Protein	14 (M)	Cycling, Intermittent @ 50%-90% VO2max (106 min)	4 hours	1.8	0.45	34a
	CHO				1.8		31a
Betts et al.	CHO + Protein	6 (M)	Running @ 70% VO2 max (90 min)	4 hours	0.8	0.3	12.1a
	CHO				0.8		12.3a
Carrithers et al	CHO + Protein	7 (M)	Cycling @ 70% VO2 max; Sprint to exhaustion (82.5 min)	4 hours	0.86	0.14	28a
	CHO				1		31a
Cogan et al	CHO + Protein	11 (M)	Cycling @ 70% VO2 max (120 min)	4 hours	0.52	0.08	0.52b
	CHO				0.6		0.58b
Detko et al*	CHO + Protein	7 (M)	Intermittent cycling @ 70% and 120% VO2 max (120 min)	4 hours	0.8	0.4	3.7 c
	CHO				1.2		5.8c
Ferguson-Stegall et al.	CHO + Protein	10 (5M, 5F)	Cycling @ 70% VO2 max; Intervals @ 45% and 90% VO2 max (100 min)	4 hours	0.51	0.16	23.9 d
	CHO				0.67		30.6d

Howarth et al.	CHO + Protein	6 (M)	Cycling, intermittent @ 50% and 80% VO2 max (120 min)	4 hours	0.9	0.3	25a
	CHO				1.2		25a
Ivy et al.*	CHO + Protein	9 (M)	Cycling @ 65% - 75% VO2 max; sprint to exhaustion (150 min)	4 hours	0.54	0.19	12b
	CHO				0.54		8.4c
Jentjens et al.	CHO + Protein	8 (M)	Cycling, intermittent @ 50% - 90% VO2 max (135 min)	3 hours	1.2	0.4	25a
	CHO				1.2		40a
Kammer et al	CHO + Protein	8 (M, 4F)	Cycling @ 60% - 65% VO2 max (120 min)	2 hours	1.08	0.27	7.3c
	CHO				1.1		6.2c
Lunn et al.	CHO + Protein	6 (M)	Running @ 65% VO2 max (45 min)	3 hours	0.25	0.07	0e
	CHO				0.32		-0.1e
Roy et al	CHO + Protein	10 (M)	Resistance exercise, whole body @ 3 x 80% 1RM; 9 exercises.	4.5 hours	0.29	0.1	23a
	CHO				0.44		19.3a
Tarnopolsky et al.	CHO + Protein	16 (8M, 8F)	Cycling @ 65% VO2 max (90 min)	4 hours	0.38	0.05	24.6a
	CHO				0.5		37.2a
Van Hall et al.	CHO + Protein	5 (M)	Cycling, intermittent @ 50% - 90% VO2 max	4 hours	0.42	0.13	36.5a
	CHO				0.42		39.8a
Van Hall et a.	CHO + Protein	8 (M)	Cycling, intermittent @ 50% - 90% VO2 max	4 hours	0.8	0.3	34a

	CHO				0.8		28a
Van Loon et al.	CHO + Protein	8 (M)	Cycling, intermittent @ 50% - 90% VO2 max (100 min)	5 hours	0.8	0.4	35.4a
	CHO				0.8		16.4a
Williams et al.	CHO + Protein	8 (M)	Cycling, intermittent @ 65% - 85% VO2 max (120 min)	4 hours	0.39	0.1	39.8a
	CHO				0.16		17.3a
Yaspelkis et al.	CHO + Protein	12 (M)	Cycling, intermittent @ 50% - 80% VO2 max (120)	3 hours	1.0	0.08	8.2c
	CHO				1.0		6c
Zawadzki et al.	CHO + Protein	9 (M)	Cycling, intermittent @ 50% - 85% VO2 max (120)	4 hours	0.77	0.28	35.5d
	CHO				0.77		26.7d
Beelen et al	CHO + Caffeine	14 (M)	Cycling, to exhaustion @ 50%-90% VO2 max.	6 hours	1.2	1.7 mg.kg $^{-1}$ .h^{-1}	31a
	CHO				1.2		31a
Pedersen et al	CHO + Caffeine	9 (M)	Cycling, to exhaustion @ 50%-90% VO2 max.	4 hours	1.0	8 mg.kg^{-1}	57.7a
	CHO				1.0		38a
Loureiro et al	CHO + Caffeine	14 (M)	Cycling, to exhaustion @ 50%-90% PPO	4 hours	1.2	0.3 g.kg^{-1} protein, 8 mg.kg^{-1} caffeine	25.6a
	CHO				1.2	0.3 g.kg^{-1} protein	10.1a
Nelson et al.	CHO + Creatine	12 (M)	Cycling, to fatigue (~150 min)	5 days	6.6 g.kg-1 .day-1	20 g.day-1	241a
	CHO				6.6 g.kg-1 .day-1		164a

Op 't Eijnde et al.	CHO + Creatine	22 (13M, 9F)	Resistance training, 10 weeks	3 weeks	N/A	20 g.day^{-1} (2 weeks), followed by 5 g.day^{-1} (5 days)	660a
	CHO				N/A		520a
Roberts et al.	CHO + Creatine	14 (M)	Cycling, to fatigue @ 70% VO2 peak	6 days	~8 g.kg^{-1} .day^{-1} (37.5 Kcal kg^{-1} day^{-1})	20 g.day^{-1}	410a
	CHO				~8 g.kg^{-1} .day^{-1} (37.5 Kcal kg^{-1} day^{-1})		225a
Robinson et al.	CHO + Creatine	14 (M)	Cycling, to fatigue (1 leg) @	5 days	N/A	5 g.day^{-1}	73% of non-exercising limb (baseline)
	CHO				N/A		69% of non-exercising limb (baseline)

Table 2.3. *Rates of glycogen resynthesis in response to carbohydrate (CHO) and CHO + congestion of other nutrients, such as, protein, caffeine and creatine. All values are presented as means. a, mmol·kg^{-1} muscle dry weight·h^{-1}, b; µg·mg^{-1} muscle wet weight·h^{-1}; c, mmol·L^{-1}·h−1; d, µmol·g^{-1} muscle wet weight·h^{-1}; e, g per 100 g wet muscle weight·h^{-1}. M, males; F, females.*

1.4 Anthocyanins

1.4.1 Polyphenols and Anthocyanins

A growing area in sports nutrition is the potential ergogenic effects of micronutrients such as those naturally occurring in plants, including plant extracts, phytochemicals and vitamins. These supplements are a growing area of research as they are perceived to lead to less side effects than manufactured substances (Kim et al. 2011; Knapik et al. 2016); a particular compound that is emerging in the literature are polyphenols (Somerville et al. 2017). Found in plants and their by-products, polyphenols are widely consumed in everyday foods including (but not limited to): berries, cacao, coffee and many fruits and vegetables that are rich in colour (Williamson. 2017). Polyphenol is a term used to describe flavonoids, stilbenes, lignans phenolic acid and despite not being classed as an essential nutrient, polyphenols are believed to contribute significant health benefits (Cory et al. 2018; Fraga et al. 2019). The process in which polyphenols are metabolised is beyond the scope of this thesis (Scalbert et al. 2002; Manach et al. 2004; Van Duynhoven. 2011, but to summarise, polyphenols are non-nutritive, therefore, they evoke different metabolic handling within the gut microbiota in the colon. Following ingestion, polyphenols are poorly absorbed into the blood, however, the concentration of polyphenolic metabolites that enter the blood are significantly greater than the parent compound (Scalbert et al. 2002). In fact, the bioavailability of polyphenolic metabolites are typically >10-100 fold greater than the original parent polyphenol and have the capacity to remain in the circulation for up to 48 hours, whereas maximal plasma concentrations occur within 2 to 4 hours and typically return to baseline in <12 hours (Scalbert et al. 2000; Kay et al. 2005; Williamson et al. 2018). Historically, scientific investigations have focused on the effects of polyphenol supplementation on health-

related outcomes, with evidence showing positive cell modulation and preventative adaptations on metabolic syndrome risk factors (Manach et al. 2004), aging (Dhib-Jalbut et al. 2006) and immune health (Somerville et al. 2016). The effect of polyphenols on athletic performance is growing, but as a result of the paucity of available data, little is known. Due to their antioxidant properties, they may reduce muscle damage and fatigue, but simultaneously interfere with training adaptation (Braakhuis and Hopkins. 2015).

Another purported effect of polyphenols is increasing blood flow through the action of nitric oxide on endothelial function, by increasing vasodilation (Bassett. 2000; Edirisinghe et al. 2011). With over 8000 individual molecules, with several classification groups and more sub-classes of each group, polyphenols are a complex, yet fascinating micronutrient.

The largest (and most researched) group of polyphenols are flavonoids. Flavonoids are found in foods such as, cherries, blackcurrants, coffee, tea, cacao/chocolate and many more (Manach et al. 2004). There is ever growing evidence to support the use of flavonoids to improve performance and recovery (MacRae and Mefferd. 2006; Davis et al. 2010; Overdevest et al. 2018; Ruiz-Iglesias et al 2021). Anthocyanins, a sub-class of flavonoids, give many fruits and vegetables their rich blue/red/purple colour and are gaining increasing interest in the literature. In a recent systematic review and meta-analysis by Ruiz-Iglesias et al. (2021), involving fifty-one studies (1288 human participants), concluded that the most effective polyphenol supplementation on exercise performance were attributed to anthocyanin intake. More specifically, anthocyanin intake/supplementation has been shown to improve exercise performance in the following performance parameters: cycling and running time trials (Braakhuis et al. 2014; Cook et al. 2015; Levers et al. 2016; Morgan et al. 2019;

Nielman et al; 2020; Costello et al. 2020), maximal isometric contractions (Cook et al. 2017; Fryer et al. 2020), running distance covered (Perkins et al. 2015), work performed in cycling exercise (Bell et al. 2014) and climbing and running time to exhaustion (Tascano et al. 2015; Fryer et al. 2020; Potter et al. 2020). Interestingly, Ruiz-Iglesias et al (2021), not only attribute anthocyanins to be the polyphenol with the greatest performance effects, but also show that of the 13 studies included in this review, 8 used blackcurrants as the anthocyanin source and more specifically 7 of which were New Zealand blackcurrants (discussed further in section 1.5.2). In most cases, the mechanistic understanding underpinning the effect of anthocyanins on exercise performance is lacking (Cook et al. 2015; Strauss et al. 2015; Cook et. 2017). The most suggested mechanism of action appears to surround anthocyanins positively modulating endothelial function (Cook et al. 2015), via an increase in endothelial cells nitric oxide production and inhibit nitric oxide free radicals, which could promote skeletal muscle blood flow and contractability (Bailey et al. 2010; Boushel et al. 2002; Cook et al. 2015).

1.4.2 New Zealand blackcurrant

Blackcurrants (Ribes nigrum) have naturally occurring, high concentrations of the flavonoid subclass, anthocyanins. More specifically, blackcurrants are rich in the anthocyanins: delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside. Studies comparing blackcurrants from different geographical locations observed those grown in New Zealand to naturally produce the highest anthocyanin content (Schrage et al. 2010). Schrage et al, (2010) reported New Zealand Blackcurrants (NZBC) to contain approximately 150% of the anthocyanin

content compared to non-NZBC (336-850 mg/100 mL and 170 to 310 mg/100). The greater anthocyanin content is thought to be due to greater sun and UV exposure (Braakhuis et al. 2020). An important consideration is the dose of NZBC and the corresponding anthocyanin intake, as this will affect the response. In an athletic context and from a research perspective, concentrating NZBC (i.e. into liquid, powder or capsules) makes this a more practical approach and provides athletes with a more manageable method of consumption.

1.4.3 Metabolic and performance effects of NZBC

Epidemiological studies associate higher consumption of anthocyanins with favourable effects on insulin sensitivity (Knekt et al. 2002; Virtanen et al. 2014). Furthermore, a study of >10,000 men showed the prevalence of T2D to be related to berry consumption, with the prevalence of T2D being lower in people who consume diets high in anthocyanin containing berries (Knekt et al. 2002). Postprandial glucose response was recently investigated in our group following acute (single dose) and chronic (8 days) of NZBC extract supplementation in overweight and obese individuals (Nolan et al. 2020). Contrary to previous findings in blackcurrant consumption and postprandial glucose concentrations, there was no decrease in postprandial glycaemia following acute NZBC extract supplementation (Castro-Acosta et al. 2016; 2017; Schel et al. 2019). This finding was attributed to the inclusion of protein and fat into the meal to more closely replicate real world scenarios, as fat consumption slows gastric emptying (Little et al. 2007) and prolongs absorption process of carbohydrate in the gut (Collier et al. 1984; Gentilcore et al. 2006), whereas previous investigations have predominantly used simple glucose drinks/foods and excluded other

macronutrients (Castro-Acosta et al. 2016; 2017; Schel et al. 2019). Furthermore, 8 days of NZBC supplementation reduced postprandial glucose concentrations which could be attributed to increased postprandial insulin sensitivity and glucose handling (Nolan et al. 2020). Nolan et al (2020), found comparable postprandial peak glucose concentrations, thus suggests that the mechanism responsible for improved glucose handling may be downstream of the digestion and absorption process. Therefore, we must examine the potential influence of skeletal muscle in postprandial glucose uptake following chronic NZBC supplementation.

In response to a fixed amount of CHO ingestion, chronic supplementation of NZBC attenuates postprandial glucose area under the curve, despite no difference in peak glucose concentrations when compared to placebo controls (Nolan et al. 2020). This finding indicates that glucose absorption into the blood is unchanged in response to NZBC, but suggests that the rate of clearance is enhanced, therefore skeletal muscle glucose uptake could be a candidate to explain this finding (figure 1.7), as the predominant tissue responsible for glucose clearance (Koistinen and Zierath. 2002). This ability of skeletal muscle to modulate glucose uptake following chronic anthocyanin intake is supported by studies using cell culture models (figure 1.7). Blackcurrant anthocyanins have been shown to improve insulin sensitivity (Stull et al. 2010; Różańska and Regulska-Ilow. 2018; Naseri et al. 2018), upregulate GLUT4 translocation (Prasad et al. 2010) and expression (Inaguma et al. 2006) in 3T3-L1 cells, as well as accentuating glucose uptake in C2C12 cells (Martineau et al. 2006) and 3T3-L1 adipocytes (Prasad et al. 2010). As a result, blackcurrant derived phenolic metabolites appear to upregulate glucose uptake *in vitro* (see figure 1.7), however whether this happens *in vivo* is, as yet, unknown. While *in vitro* models have a plentiful list of advantages, such as a precise control over the environment, less invasive and

a greater throughput, the method also has its limitations. Cell culture studies do not mimic living organisms, as cells are isolated and unaffected by extracellular tissues and stimuli (Vavrova et al. 2011; Frohlich et al. 2014). Currently, our understanding of anthocyanins, specifically NZBC effect on glucose handling is limited to *in vitro* work.

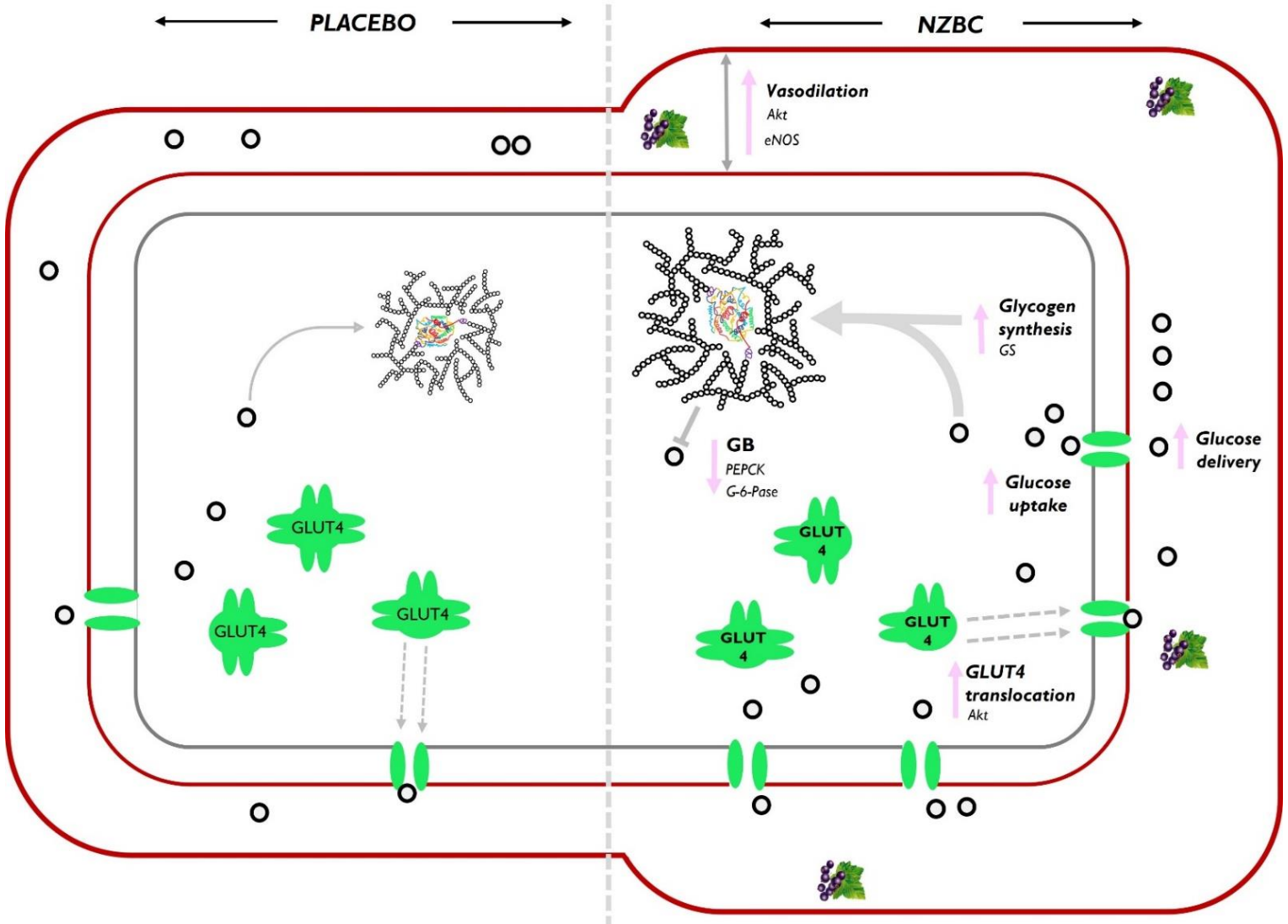

Figure 1.7. *Proposed mechanisms of action: anthocyanins potential effects on vascular and intramuscular responses. Implications and hypothesis for glycogen synthesis. Akt, protein kinase B; eNOS, endothelial NOS (nitric oxide synthase); GS, glycogen synthase; G-6-Pase, glucose 6 phosphatase; PEPCK, Phosphoenolpyruvate carboxykinase*

1.5 Physical inactivity and overfeeding

As of the latest Active Lives Adult Survey report, nearly 40% of the UK population were failing to meet the recommended physical activity guidelines (Sport England. 2022), thus reducing exercise induced energy expenditure. Physically inactive individuals require less energy provision and consequently, if energy intake is not reduced, a positive energy balance ensues which induces weight gain. The Westernised lifestyle enables convenient access to energy dense foods that are lacking nutritional quality, thus prompting increased energy intake. Coupled with reduced energy expenditure, this results in an excessive positive energy balance, in turn causing weight gain. Westernised lifestyles, perhaps unsurprisingly, have led to increasing prevalence of obesity and type 2 diabetes (T2D). As of 2016, 1.9 billion adults (39% of the global adult population) were classified as overweight, which is an increasing trend from statistics from 1975 (World Health Organisation. 2016). At present, there is a mismatch between our 'physically active genotype' and the inactive nature of modern lifestyles, which drastically increases risk for the development of metabolic diseases (Mokdad et al. 2004).

1.5.1 Skeletal muscle plasticity

As skeletal muscle is the largest tissue in the body in which houses thousands of muscle fibres each containing an array of organelles, it is highly responsive and adaptability. For example, physical activity and nutritional stimuli directly drive skeletal muscle transcriptional activity which over time can cause the tissue to adapt or maladapt (Brook et al. 2016; Hoppeler. 2016). Metabolic disorders such as insulin resistance, T2D and obesity are closely associated with reduced insulin sensitivity. As

discussed previously, maintaining insulin sensitivity in skeletal muscle for efficient handling glucose is of utmost importance for blood glucose homeostasis and good health, in order to prevent the development of disease, such as T2D (Petersen et al. 2007; DeFronzo & Tripathy. 2009). Therefore, to understand disease development and potential prevention, we must understand the intracellular maladaptions that lead to the cells inability to uptake glucose and result in metabolic dysfunction. While the microvascular systems influence on insulin mediated skeletal muscle glucose uptake must not be overlooked, it is beyond the narrative of this thesis, for further information, see a recent symposium review from our laboratory (Wagenmakers et al. 2016).

1.5.2 Lipid induced insulin resistance and lipid spill over

Obesity is closely correlated with impaired insulin sensitivity and insulin resistance. Chronic exposure to excessive energy intake and overfeeding results in gains in fat mass, predominantly stored as TAGs in adipose tissue and over time leads to obesity. Following ingestion, fat is emulsified in the lumen of the intestine, allowing fatty acids to be absorbed by the epithelial cell of the intestine, before linking with other fatty acids to form TAGs. Formed in the golgi apparatus, chylomicrons are a specialised lipoprotein formed of a hydrophobic centre containing TAG and cholesterol, surrounded by a monolayer of phospholipids and free cholesterol (Feingold and Grunfeld, 2018). Chylomicrons transport TAGs from the small intestine into peripheral tissues and the liver (Tso and Balint, 1986). The rise in plasma insulin following fat ingestion suppresses adipose tissue lipolysis through inhibition of hormone sensitive lipase (HSL) and upregulates TAG uptake in into adipocytes and this is a protective mechanism to avoid ectopic lipid accumulation (Frayn, 2002). As a result, when this

buffering process is impaired, an overspill of circulating TAG/FAs occurs, leading to TAG to ‘spillover’ into non adipose tissues, such as skeletal muscle and liver.

The obese, insulin resistant phenotype attenuates insulin-stimulated skeletal muscle glucose uptake and hepatic glucose secretion. Thus, in obesity, lipid accumulation in non-adipose tissues has been associated with insulin resistance. However, intramuscular triglycerides (IMTG) accumulation per se does not appear to lead to insulin resistance. The work of Goodpaster and colleagues (2001) demonstrated a paradox in the content of IMTGs between elite athletes and insulin resistance. This phenomenon discusses that IMTG accumulation in type 2 diabetic patients and obese is associated with insulin resistance, however, IMTG content is even greater in endurance-trained athletes (who are extremely insulin sensitive). This therefore suggests that IMTG content alone is unable to explain insulin resistance and there must be a mechanistic difference in the way lipids are stored in those who are insulin resistant vs those with high insulin sensitivity. Long term endurance training adaptations equip endurance athletes with the capacity to upregulate fat metabolism during exercise, which is demonstrated by athletes possessing greater rates of IMTG utilisation during exercise, than untrained individuals, which in turn, acutely reduces IMTG content (Klein, Coyle and Wolfe. 1994; Coggan et al. 2000; Purdom et al. 2018). As a result, following exercise, the demand for IMTG storage is increased, thus upregulating IMTG synthesis. It is the combination to regularly utilise and replenish IMTG that results in high lipid turnover (lipolysis and synthesis) which is thought to avoid accumulation of fatty acid (FA) metabolites (Lund et al. 2018; Barrett et al 2022). Accumulation of metabolites are thought to lead to insulin resistance and reduced skeletal muscle glucose uptake (Ellis et al. 2000; Itani et al. 2002; Kim et al. 2004; Amati et al. 2011; Dube et al. 2011). Low IMTG turnover in physically inactive

individuals results in a stagnant IMTG pool, which appear to be bigger in size than that observed in healthy (He et al. 2004; Nielsen et al. 2017; Barrett et al. 2022). Therefore, despite similar IMTG content, the lipid profiles between athletes and T2D appear to differ drastically. High exogenous fat provision, coupled with low lipid flux may encourage growth of IMTGs and has also been proposed to stimulate the synthesis of harmful metabolites (Covington et al., 2017; Chadt et al, 2018).

1.6.3 The effect of insulin resistance on skeletal muscle glucose uptake

Insulin responsive tissues vary in their functionality and sensitivity to respond to insulin depending on an individual's phenotype. Modern day lifestyles in the western world have strayed from that of our ancestors. Rapidly evolving technological advances have drastically increased individuals day to day reliance on technology. Modern lifestyles have shifted the norm for individuals behavioural traits and habits, to many people are increasingly turning to convenient energy dense foods, using motorised transport, working sedentary based jobs and steering away from physical activity and exercise (Farhud. 2015; Gremaud et al. 2018; Guthold et al. 2018). The ramifications of these lifestyle shifts are likely harmful for human health, in fact, in 2010 the World Health Organisation (WHO, 2010) attributed physical inactivity as a leading cause of mortality. Physical inactivity is also attributed with metabolic diseases and components of metabolic syndrome, including obesity, insulin resistance and T2D. Insulin resistance is characterised by a reduced capacity for tissues to respond to increases in plasma insulin concentrations following carbohydrate (or meal) ingestion and thus prolonging periods of hyperglycaemia (Obrien et al. 1998; Lautt. 2007; Manios et al. 2017). In insulin resistant individuals' pancreatic secretions of insulin fail to account for

elevations in circulating glucose, therefore more insulin must be secreted to adequately promote glucose uptake into skeletal muscle at rates to restore euglycemia. Insulin resistance can develop further and ultimately progress into T2D; whereby postprandial rises in insulin can no longer traffic adequate glucose into skeletal muscle, leading to prolonged hyperglycaemia and hyperinsulinemia.

Pathogenesis in the development of diabetes is characterised by impairment to β-islet cells of the pancreas, causing a lack of control of blood glucose. Pancreatic β-cells are small clusters of endocrine cells that are localised within the islets of Langerhans. These cells typically account for ~1–2% of the total pancreatic mass, however, Individuals with type 2 diabetes have decreased β-cell mass compared with nondiabetic individuals of similar BMI. In fact, there appears to be a threshold, whereby blood glucose elevates if β-cell mass is below ~1.1% (Ritzel et al 2006). Interestingly, β-cell mass is increased in nondiabetic obesity, and second, there is decreased β-cell mass in both lean and obese patients diagnosed with type 2 diabetes (Linnermann et al. 2014). β-cells are important role in ensuring that blood glucose homeostasis in healthy individuals, whereas this regulation is disrupted in T2D. In a healthy condition, there is a continuous feedback loop between β-cells and insulin-sensitive tissues; If the adipose tissue, liver, and muscles demand glucose, this will instruct the β-cells to initiate increased insulin secretion. As a result, when β-cell function is disrupted, insulin signalling to insulin sensitive tissues is downregulated, thus attenuating postprandial glucose tolerance. As explained in section 1.1.2, the postprandial clearance of glucose into skeletal muscle is a important regulatory mechanism of plasma glucose concentrations. Similarly, the postprandial clearance of chylomicrons/TAG into adipose tissue is an important regulatory mechanism for plasma TAG concentrations (Explained in section ***1.5.2***). Impairments to this lipid

buffering can result in elevated plasma TAG, which increase the clearance of TAG into non-adipose tissues, including the liver, pancreatic β cells and skeletal muscle (Frayn et al. 2002). It has been postulated that the longer TAG remain plasma circulation, the increased likelihood of it being deposited into non adipose tissue, such as skeletal muscle and liver (Bickerton et al. 2008). Accumulation of TAG in insulin-responsive tissues such as skeletal muscle (Goodpaster et al. 1997; Forouhi et al. 1999) and liver (Goto et al. 1995; Ryysy et al. 2000) is associated with insulin resistance. In addition, when human pancreatic islets are exposed to FA for extended periods, insulin secretion is attenuated (Paolisso et al., 1995; Zhou & Grill, 1995). The impairment of postprandial TAG clearance into adipose encourages TAG disposal into insulin responsive tissues, in which overtime, leads to ectopic accumulation of TAG which appears to disrupt postprandial glucose tolerance and insulin sensitivity.

Associations have been shown between T2D and BMI, which indicates a link between obesity and T2D (Edelstein et al., 1997); obesity predisposes individuals to develop diseases, such as T2D (Kivmaki et al. 2022). A meta-analysis reported individuals with obesity to have a 7 times greater risk of developing T2D (Abdullah et al., 2010) Most obese individuals, despite being insulin resistant, do not experience fasting hyperglycaemia (Algoblan et al. 2014), meaning the pancreas can release adequate amounts of insulin that are able to maintain regular glucose tolerance (Roder et al. 1998). Evidence shows larger adipocytes to be less responsive to insulin stimulation (Olefsky, 1977). Moreover, larger adipocytes appear less efficient at buffering FA flux in the postprandial period, resulting in elevated plasma TAG concentrations. While T2D does not appear indicative of obesity per say, the obese phenotype does appear more susceptible to develop insulin resistance and subsequent T2D.

1.5.4 IMTG and the athletes paradox

Previously, the accumulation of TAG in non- adipose tissue, such as skeletal muscle, was associated with reduced insulin sensitivity and this was pronounced in obese, sedentary and T2D individuals (Pan et al., 1997; Kelley et al., 2001; van Loon, 2004). However, a study by Goodpaster and colleagues (2001), demonstrated that highly insulin sensitive endurance trained athletes had high content of intramuscular TAGs (IMTGs). Named 'the athletes paradox', this phenomenon describes how athletes and insulin resistant individuals can share similar IMTG content, yet have vastly different insulin sensitivity (See figure 1.9). This finding has since been shared by other investigators (Van Loon et al. 2004) and demonstrates that IMTG content alone, cannot mechanistically explain insulin resistance. Instead, lipid metabolite concentrations are typically much higher in obese or T2D individuals, when compared to healthy lean or endurance trained individuals (Hulver et al., 2003; Adams et al., 2004; Straczkowski et al., 2007; Coen et al., 2010; Bergman et al., 2012). Moreover, expression of metabolites have been shown to lead to insulin resistance and reduce skeletal muscle glucose uptake (Ellis et al. 2000; Itani et al. 2002; Kim et al. 2004; Amati et al. 2011; Dube et al. 2011). A potential reasoning for the differences in metabolite concentrations between populations could be down to the contrasting metabolic demands of everyday life. For example, the high oxidative capacity of endurance trained athletes coupled with high lipolytic flux means that lipid stores (i.e. IMTG) are regularly being used and replenished. In fact, greater IMTG turnover both at rest (Kanaley et al., 2009) and during exercise (Guo et al., 2000) has been shown in healthy populations. On the other hand, obese individuals have been shown to have impaired IMTG turnover which in turn leads to IMTG accumulation (Perreault et al. 2010). Not only does overfeeding and physical inactivity promote IMTG accumulation, but, as mentioned in section *1.6.2,* the low turnover of IMTG, coupled with excessive

exogenous fat consumption appears to affect the morphology of lipid droplets, when compared to healthy and trained populations. More specifically, provision of high caloric, high fat diet, for 8 weeks, induced insulin resistance in sedentary individuals (Covington et al. 2017). An important observation in this study, was that the lipid induced insulin resistance was not thought to be by virtue of changes to IMTG number, but rather, resulted in growth of LDs. Contrastingly, when an exercise programme is coupled with a weight loss intervention in overweight or obese individuals, LD size appears to be reduced, irrespective of any changes to LD number (He, Goodpaster and Kelley. 2004). Furthermore, the reduced LD size is associated with improved insulin sensitivity, suggesting that LD growth could be a problematic adaptation in regard to insulin sensitivity (He, Goodpaster and Kelley, 2004). To support this, the lipid droplet profiles of healthy lean and trained individuals are small in size, but scattered across large regions of the muscle cell, thereby creating a large surface area in which is thought to encourage the binding of proteins and lipolytic enzymes associated with fat metabolism, thereby creating an efficient environment for the liberation and release of FA from IMTG.

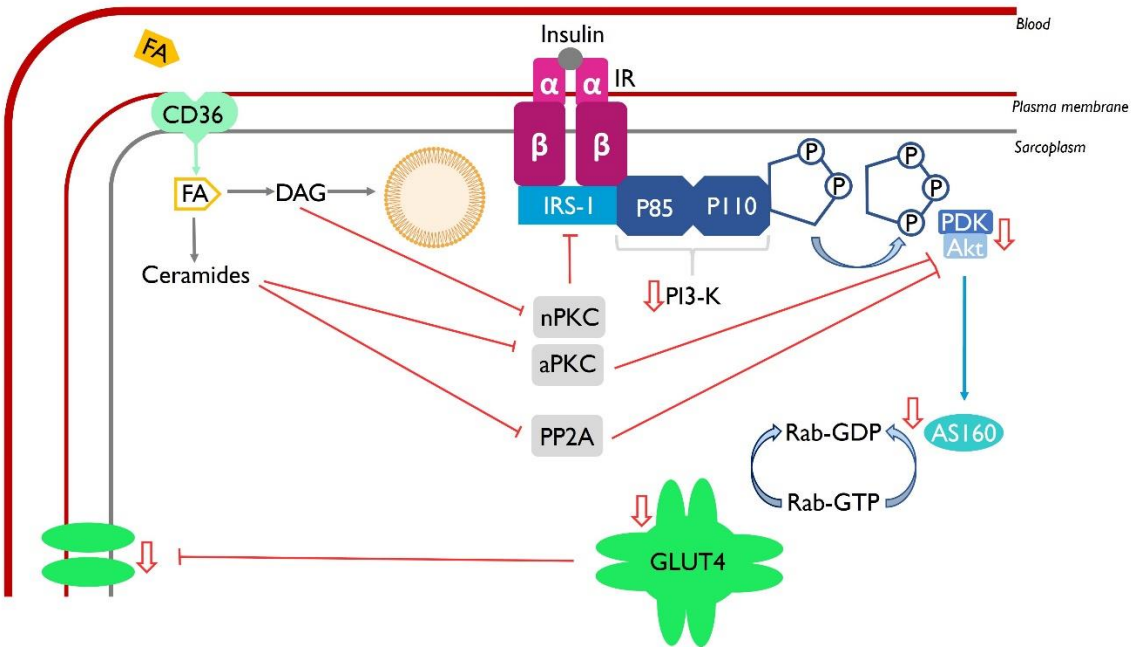

***Figure 1.8**. Lipid induced skeletal muscle insulin resistance. aPKC, atypical protein kinase C; AS160, Akt substrate of 160 kD; DAG, diacylglycerol; FA, fatty acid; fatty acyl-CoA; GLUT4, glucose transporter-4; IMTG, intramuscular triglyceride; IR, insulin receptor; IRS-1, insulin receptor substrate-1; nPKC, novel protein kinase C, PDK, phosphoinositide-dependent kinase 1; PI3-K phosphoinositide 3-kinase; Rab-GDP, Rab guanosine disphosphate; Rab-GTP, Rab guanosine triphosphate*

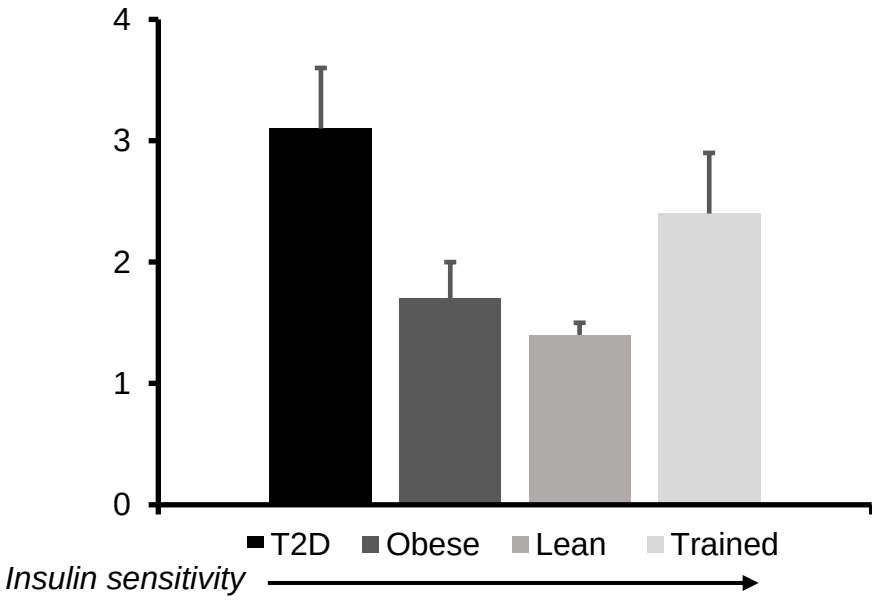

***Figure 1.9.** The athletes paradox, intramuscular triglyceride content of T2D, lean, obese and trained individuals. Figure adapted from Goodpaster et al. (2001). * = P < 0.05 vs lean and obese groups. ** P < 0.05 vs Lean group only.*

1.5.5 Lipid metabolites and PI3K-Akt pathway

Despite being closely correlated with insulin resistance, it does not appear by virtue of IMTG accumulation, per se. Whilst adipose tissue has the capacity to store a vast amount of lipid, other tissues such as skeletal muscle do not. Lipid overspill is thought to stimulate ectopic lipid accumulation, including in skeletal muscle. Lipid accumulation appears to be accompanied with various lipid species, in which contain signalling effects that could downregulate insulin signalling. Lipid species, such as diacylglycerol (DAG), fatty acyl CoA and ceramides are attributed to being a link between lipid accumulation and insulin resistance (Amati et al., 2011).

The role of DAGs within insulin resistance is complex. Previously, DAG concentrations have been associated with insulin resistance in animal and human models (Turinsky et al .1990; Itani et al. 2002). However, a study by Amati et al (2011) unexpectedly found greater DAG concentrations in (insulin sensitive) endurance-trained individuals when compared to normal weight and overweight sedentary individuals (insulin resistant), however, contrasting data exists (van Hees et al., 2011; Bergman et al. 2012). The authors of this study hypothesised that specific DAG species and their subcellular localisation may have effect their metabolic function (Amati et al. 2011). Subsequently, Bergman and colleagues (2012) showed only DAGs localised in the plasma membrane fraction (not cytosolic) were inversely related with insulin sensitivity. Furthermore, of 16 DAG species, only one was associated with insulin sensitivity (di-C18:0). Mechanistically, DAGs have been proposed to stimulate protein kinase C (PKC) isoforms, which has downstream implications on serine phosphorylation of IRS-1 (Itani et al., 2000; 2002). Serine phosphorylation of IRS-1 downregulates tyrosine phosphorylation, which inhibits the insulin-signalling cascade (Yu et al., 2002; Kim et al., 2003). It appears that total DAG content is an unreliable

predictor of insulin resistance, however, specific species of plasma membrane DAG are likely to have negative implications on PKC phosphorylation and subsequent downregulation on insulin signalling cascade. As a result, DAG species (i.e. di-C18:0) must be considered as a potential contributing factor in the development of insulin resistance and T2D.

Ceramides belong to the sphingolipid family and are strongly associated with impaired insulin signalling, insulin resistance and T2D (Adams et al., 2004; Amati et al., 2011). Enhanced concentrations of ceramides have been shown to directly reduce GLUT4 translocation and glucose uptake through the inhibition of AKT/PKB (Hajduch et al., 2001; Powell et al., 2003; Sokolowska and Blachnio-Zabielska., 2019). Ceramides effect insulin action through down regulating Akt/PKB in a number of ways. Firstly, ceramides increase the activity of aPKC, which in turn, inhibits insulin stimulated glucose uptake, through reduced GLUT4 translocation. Secondly, ceramides mediate protein phosphatase 2a (PP2a), which has the ability of dephosphorylating Akt, which again, reduces insulin mediated GLUT4 translocation and glucose uptake (Stratfor et al. 2004). Both of these mechanistic pathways regarding ceramides association with insulin resistance are outlined in the following reviews (Lipina & Hundal. 2011; Chavez & Summers. 2012). Ceramides appear to have a role in the development of insulin resistance, predominantly through inhibition of Akt/PKB, which downregulates AS160 activity, ultimately reducing GLUT4 translocation to the plasma membrane.

1.5.6 SNAP23 high jacking hypothesis

SNAP23 binds with syntaxins and VAMPs, forming SNARE complexes to facilitate membrane fusion. SNAP23 is predominantly localised at the plasma membrane

whereby it mediates GLUT4 docking and fusion to the plasma membrane in response to insulin mediated GLUT4 translocation (Foster et al. 1999; Kawanishi et al 2000). SNAP23 is also colocalised at LD-mitochondrial networks reportedly fusing the LD to mitochondria to allow for FA channelling (Jagerstrom et al. 2009) . Furthermore, SNAP23 is localised at the LD, whereby it is suggested to augment LD-LD fusion (Bostrom et al. 2009). Insulin resistance is suggested to increase the size of LD, due to low lipolytic flux and intramuscular lipid accumulation. One theory suggest that increased LD size is a result of LD-LD fusion, mediated by SNAP23 (See figure 1.10). Bostrom et al (2007) presents a hypothetical model of the role of SNAREs in LD fusion, whereby SNAP23 intertwines with syntaxin5 and VAMP4 in a helix bundle, resulting in each LD monolayer to be fused together, exposing a connection between the 2 hydrophobic centres into one droplet. Ectopic LD accumulation in skeletal muscle could cause SNAP23 to translocate from the plasma membrane to cytosolic regions to promote LD fusion. SNAP23 gene knockdown in NIH-3T3 fibroblast cells decreased the rate of fusion and size of LD (Bostrom et al., 2007). Oleic acid treatment to cardiomyocytes increased intracellular LD volume, which increased LD associated SNAP23, while simultaneously attenuating the pool of SNAP23 in the plasma membrane (Bostrom et al., 2007). In addition, this reduced SNAP23 mediated GLUT4 vesicle exocytosis and in turn attenuated insulin-stimulated glucose uptake. Furthermore, insulin stimulated glucose uptake was restored following transfection of SNAP23 in oleic acid treated cardiomyocytes (Bostrom et al., 2007), this has been attributed to restoration of SNAP23 localised at the plasma membrane. The findings from this paper lead to the SNAP23 high jacking hypothesis (Sollner, 2007), which postulates that in response to ectopic lipid accumulation, SNAP23 is sequestered away from the plasma membrane to cytosolic regions to facilitate LD-LD fusion. This

theory would result in a reduction of SNAP23 at the plasma membrane, which in turn may reduce the fusion and docking of GLUT4 storage vesicles which could therefore lead to decreased insulin stimulated glucose uptake and therefore hyperglycaemia (see figure 1.11). This hypothesis lends itself to a potential theory that explains the association between obesity and insulin resistance, in that LD highjack SNAP23 from the plasma membrane for LD-LD fusion (See figure 1.10 and 1.11), thus reducing GLUT4s capacity to dock at the plasma membrane as the end point of the insulin signalling cascade.

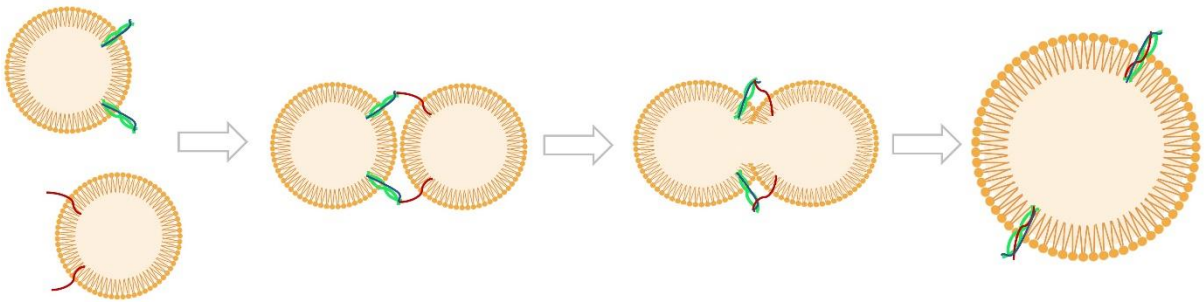

Figure 1.10. *The growth of lipid droplets via SNARE mediated fusion: The formation of the four-helix bundle of SNAP23 (green), syntaxin5 (blue) and VAMP4 (red) fuses the monolayers of two lipid droplets to one another. As a result, the hydrophobic cores meet and conjoin with one another, which seemingly produces one larger lipid droplet.*

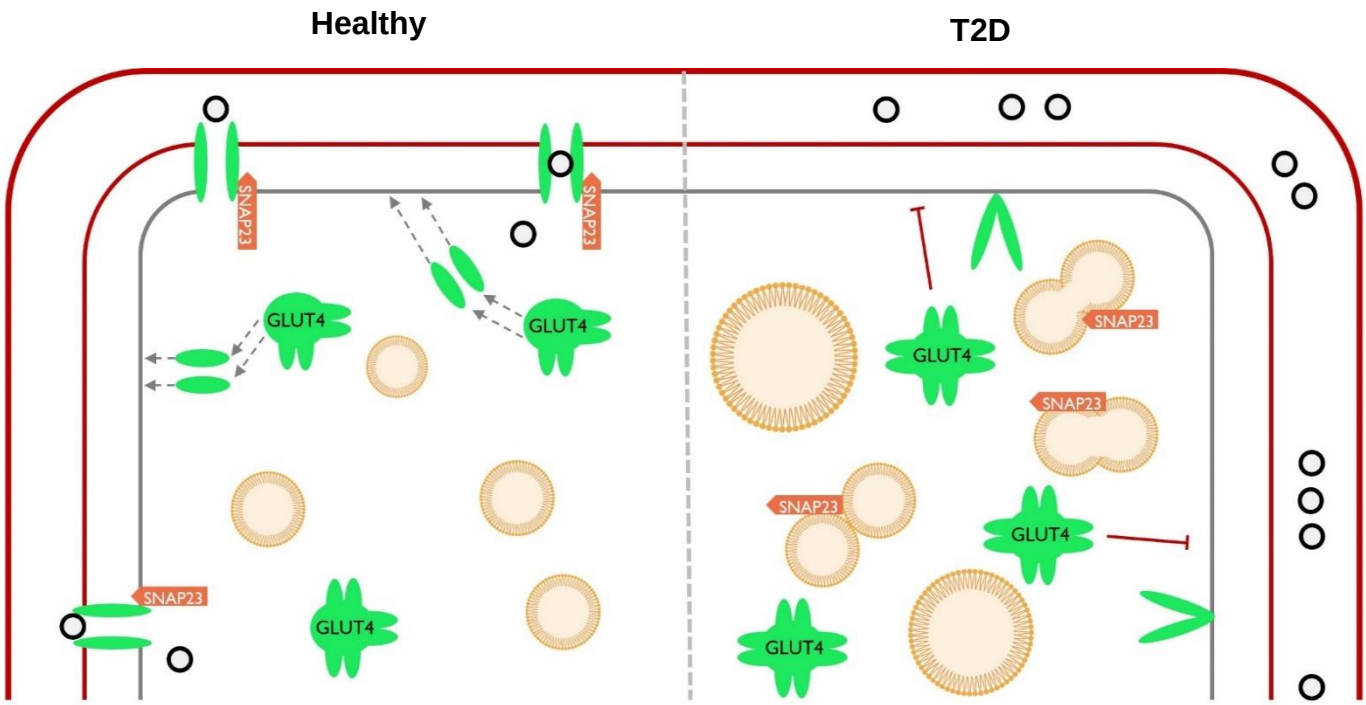

Figure 11. *SNAP23 high jacking hypothesis: Skeletal muscle cell metabolism in response to circulating glucose. In healthy conditions (left), increased glucose concentrations stimulate the insulin signal cascade that upregulates GLUT4 translocation to the plasma membrane. SNAP23 facilitates the fusion and docking of GLUT4 t the plasma membrane to allow for glucose entry to the cell. In T2D (right), lipid spill over results in ectopic intramuscular lipid accumulation, in turn promoting lipid droplet growth via SNAP23 mediated fusion. Consequently, the demand for SNAP23 at the lipid droplet attenuates SNAP23 at the plasma membrane, therefore reducing the ability to fuse and dock GLUT4 to the plasma membrane, thus reducing glucose uptake.*

Chapter 2

Short-term supplementation with New Zealand blackcurrant extract enhances post-exercise muscle glycogen resynthesis

2.1 Abstract

Athletes often train multiple times per day, thus requiring quick recovery, including replenishing muscle glycogen stores. Current guidelines suggest a carbohydrate intake of 1.2 $g.kg^{-1}$ per hour to maximise glycogen synthesis, yet this proves an impractical dose for many athletes. Anthocyanin-rich New Zealand blackcurrant (NZBC) extract improves glucose clearance following carbohydrate intake, while cell culture models show anthocyanins to augment glucose transport and cellular uptake. It is not yet known whether this happens in humans or if it leads to greater glycogen storage. As a result, we investigated the effects of 7 days of NZBC supplementation on post-glycogen depleting exercise plasma glucose concentrations and skeletal muscle glycogen synthesis in response to 4 hours of post exercise carbohydrate feeding. The aim of the study was to determine whether NZBC supplementation can augment post exercise glycogen synthesis, when the carbohydrate dose is suboptimal and provide athletes with a potential strategy to enhance their post exercise glycogen storage. Ten amateur cyclists (age, 23 ± 4 years; height, 178.9 ± 6.8 cm; weight, 75.9 ± 6.4 kg; BMI, 23.7 ± 1.6 kg/m^2; $VO_{2max,}$ 55.6 ± 7.0 $ml.min^{-1}. Kg^{-1}$; PPO, 324 ± 29 W; habitual anthocyanin intake, 28 ± 10 mg day^{-1}) completed a randomised, cross-over, double-blind, placebo-controlled study. On day 6 of NZBC supplementation, participants performed a cycling protocol lasting ~90–120 min in an attempt to deplete muscle glycogen followed by a low-carbohydrate evening meal. The following morning, participants performed a fasted steady state cycling bout for 45 min at 50% Wmax, prior to an exercise capacity test. Following exercise, participants consumed carbohydrate (0.8 $g.kg^{-1}. h^{-1}$), in the form of maltodextrin drink, for 240 mins, given in 15 min boluses, while muscle biopsies were taken at 0-, 60- and 240-min post exercise. Our results report no difference in blood glucose area under the curve

between trials (Placebo, 1363 ± 72; NZBC, 1334 ± 56, P = 0.179). The rate of glycogen resynthesis was similar between groups after 60 min (~54 mmol.kg^{-1}, P=0.538), however, after 240 min, NZBC supplementation lead to significantly greater glycogen concentrations (NZBC, 258±34 mmol.kg^{-1} ; PLA, 236±29 mmol.kg^{-1} dw; P=0.005). Our study shows 7 days of NZBC supplementation to augment post exercise glycogen resynthesis after 4 hours of carbohydrate ingestion. These findings demonstrate NZBC to positively modulate post exercise carbohydrate storage in skeletal muscle. As a result, NZBC supplementation could improve an athlete's capacity to replenish energy stores, at least, when carbohydrate intake is suboptimal, which could improve short term recovery.

2.2 Introduction

It is well established that muscle glycogen availability is a determining factor for high intensity and prolonged endurance exercise. Endurance athletes, even at the sub-elite level, habitually undertake a high volume of training, typically consisting of multiple exercise sessions per day. As a result, regular energy provision is required to fuel and recover from one session to the next. Typically, carbohydrate is the largest contributing macronutrient to energy intake and is a determining factor for exercise performance. Consequently, it is well established that the provision of exogenous carbohydrate is the primary determinant of glycogen accumulation following exercise (Burke et al., 2017). To replenish muscle glycogen stores following high intensity and/or prolonged exercise, a carbohydrate intake of 1.2 g.kg^{-1}.h^{-1} is recommended for up to four hours post-exercise (Jentjens and Jeukendrup, 2003; Kerksick et al., 2008; Jentjens et al., 2001; van Loon et al., 2000). However, a high training volume often necessitates a short turn-around period between training bouts, and under these circumstances the recommended post-exercise carbohydrate intake can become difficult to achieve. This

is especially true in sub-elite or top-performing amateur athletes, who balance their training with other commitments, including part-time or full-time work, meaning their nutrition can often be compromised (Doering et al., 2016b; McLeman et al., 2019). On this basis, a number of studies have investigated whether the co-ingestion of other macronutrients or dietary agents alongside a sub-optimal dose of carbohydrate (<1 $g.kg^{-1}.h^{-1}$) can enhance post-exercise muscle glycogen accumulation. While protein co-ingestion with carbohydrate appears effective in augmenting muscle glycogen resynthesis (Ivy, 2004; Margolis et al., 2021), evidence for supplements such as caffeine to enhance post-exercise muscle glycogen accumulation are less clear (Pedersen et al., 2008; Beelen et al., 2012). Mechanistically, the augmentation of muscle glycogen accumulation could potentially occur at multiple sites, including absorption of glucose from the gut, regulation of glucose delivery to skeletal muscle via the microvascular system, skeletal muscle glucose uptake, or through direct effects on the rate limiting steps and enzymes regulating muscle glycogen synthesis. For example, protein-carbohydrate co-ingestion evokes insulin secretion and upregulates insulin-mediated glucose entry into skeletal muscle.

New Zealand blackcurrant (NZBC) contains the highest concentration of anthocyanins, polyphenolic compounds with anti-inflammatory, antioxidant and insulin sensitising properties (Gavrilova et al., 2011; Strauss et al., 2018; Willems et al., 2019). Emerging evidence showcases the potential use for NZBC supplementation with the purposes to improve exercise performance. More specifically, supplementation of NZBC extract has been shown to improve cycling time trial performance (Cook et al., 2015) maximal isometric contraction force (Cook et al., 2017; Fryer et al., 2020), running distance covered (Perkins et al. 2015) and climbing and running time to exhaustion (Fryer et al. 2020; Potter et al., 2020). Although the

mechanisms to explain these results are less clear, NZBC has been shown to elicit metabolic adaptations including augmenting rates of fat oxidation during moderate intensity exercise (Cook et al., 2015; Strauss et al., 2018) and improving blood flow (Edirisinghe et al., 2011a; Nakamura et al., 2002). From a health perspective, blackcurrant anthocyanins have been shown to improve insulin sensitivity (Stull et al., 2010; Rózańska and Regulska-Ilow, 2018; Naseri et al., 2018) and enhance muscle glucose uptake in cultured cells (Martineau et al., 2006). Moreover, short-term (7 days) supplementation of NZBC extract improves insulin sensitivity and attenuates the postprandial glucose response in lean, healthy (Willems et al., 2019) and overweight/obese individuals (Nolan et al., 2021). In the latter study, an improved postprandial glucose response following supplementation with NZBC extract occurred despite peak postprandial glucose concentrations being similar to the placebo condition, suggesting that NZBC extract increased the rate of glucose clearance (Nolan et al. 2021). As such, NZBC extract could have potential benefits for the rate of glycogen storage, through improving blood glucose clearance upon carbohydrate feeding into skeletal muscle, which could subsequently lead to greater rates of glycogen synthesis. On this basis, the aim of this study was to test the hypothesis that 7 days of NZBC extract supplementation augments post-exercise skeletal muscle glycogen resynthesis at greater rates when compared to supplementation of placebo.

2.3 Methods

Ten moderately trained male cyclists (participants characteristics, table 2.1) volunteered to participate in this study. Written, informed consent was obtained from volunteers following a verbal and written explanation of the nature and risks involved in the experimental procedures. The study was approved by the Liverpool John

Moores University Research Ethics Committee (approval no. 21/SPS/037) and abided by the declaration of Helsinki.

Experimental procedures

Preliminary testing

Having abstained from vigorous exercise, alcohol and caffeine for 24 h prior, participants attended the laboratory to undergo a two-part incremental cycle test (Lode Excalibur Sport, Groningen, Netherlands). Following measurements of height and weight, participants first completed a submaximal graded exercise test, in which participant's initially cycled at 80 W, after which the workload was increased by 30 W every 4 min until a blood lactate ≥4 mmol $^{L-1}$ (Biosen C-Line, EKF Diagnostic, Cardiff, UK) was reached. After ~10 min seated recovery, participants completed a progressive test to exhaustion on the same cycle ergometer to determine maximal oxygen uptake (VO_{2max}) using an online gas collection system (Metalyser 3B, Cortex, Germany). Briefly, participants cycled at 80 W for 4 min, after which the workload was increased by 30 W every 1 min until a cadence of ≥60 rpm could not be maintained. VO_{2max} was deemed to have been reached if one or more of the following end-point criteria were met: (1) heart rate within 10 b.min^{-1} of age-predicted maximum, (2) respiratory exchange ratio >1.15, and (3) plateau of oxygen consumption despite increased workload (Gilman 1996). The test end time and power output of the final stage from the second part of the incremental cycling test was used to calculate peak power output (PPO) using the following equation (Kuipers et al., 1985):

$$PPO = W_{max} + ([t/60] \cdot PI)$$

Where W_{max} is the power output of the final completed stage in watts, *t* is time spent in the final uncompleted stage (seconds), 60 is the duration of each stage (seconds)

and *PI* is the increase in power output between each stage (W). The preliminary testing was completed >7 days before commencing the study protocol, as outlined below.

Table 2.1. Participant characteristics

	Means ± SD
n	10
Age (years)	23 ± 4
Height (cm)	178.9 ± 6.8
Weight (kg)	75.9 ± 6.4
BMI (kg/m^2)	23.7 ± 1.6
VO2 max (ml/min/kg)	55.6 ± 7.0
VO2 max (L/min)	4.20 ± 0.38
PPO (W)	324 ± 29
Habitual anthocyanin intake (mg day^{-1})	28 ± 10

Study protocol

In a repeated-measures, randomised, cross-over design, participants consumed 2 capsules (600 mg) of concentrated NZBC extract or a visually identical placebo for 7 days (figure 2.1). Each 300 mg NZBC capsule contained 105 mg of anthocyanins, consisting of 35–50% delphinidin-3-rutinoside, 5–20% delphinidin-3-glucoside, 30–45% cyanidin-3-rutinoside, and 3–10% cyanidin-3-glucoside (CurraNZ™, Health Currancy Ltd, Surrey, UK). Each placebo capsule contained 300 mg microcrystalline cellulose. On day 6 of supplementation, participants reported to the laboratory at approximately 5pm to perform a ~90 min bout of intermittent glycogen-depleting cycling exercise. Following a 5 min warm up at 100W, participants cycled repeatedly for 2 min at 90% PPO, followed by 2 min recovery at 50% PPO (figure 2.1). This work

to rest ratio was continued until the participant could no longer achieve 2 min at 90% PPO (cadence <60 rpm), after which the exercise period was reduced to 1 min. Once the participant could no longer maintain this power output for 1 min, the exercise intensity was lowered to 80% PPO for 2 min, and then down to 1 min when the power output could not be maintained. When the participant failed to achieve this, this pattern was continued at 70% PPO and then 60% PPO. The activity pattern for the glycogen-depleting exercise was recorded for each participant during their first trial and replicated for repeated trials. Heart rate (HR) was measured continuously throughout exercise (Polar H10, Kimpele, Finland). Water intake was consumed ad libitum throughout the trial. This intermittent pattern of exercise was employed in an attempt to induce glycogen depletion in both type I and II fibres, and has previously been successfully used by our laboratory (Taylor et al., 2013). Following the exercise session, participants consumed a standardised, low carbohydrate meal consisting of a protein bar, casein protein drink, and mixed nuts (Energy, 533 kcal; CHO, 30.3 g; Fat, 19.8 g; Pro, 55.4 g). On day 7 of supplementation, participants arrived at the laboratory in the morning (~7.30 am) in a fasted state. Following a resting capillary blood sample, participants initially cycled for 45 min at 50% PPO (167 ± 15 W), to further deplete glycogen stores, measure substrate utilisation and include a exercise capacity measure. Heart rate and expired air (Metalyser 3B, Cortex, Germany) were collected at 15 min intervals during exercise. Rates of carbohydrate and fat oxidation during steady state exercise were calculated according to the equations of Jeukendrup and Wallis (2005). Following the steady state component of the protocol, participants remained on the bike and performed 5 min of active recovery (50W) followed immediately by an exercise capacity test. This test required participants to perform 1 min intervals (1:1 ratio) at 80% (259 ± 23 W) and 40% (130 ± 12 W) of their PPO,

respectively, until volitional fatigue. All exercise was conducted in a temperature-controlled laboratory (21°C). Immediately following completion of the exercise test, participants underwent a muscle biopsy of the vastus lateralis, and then

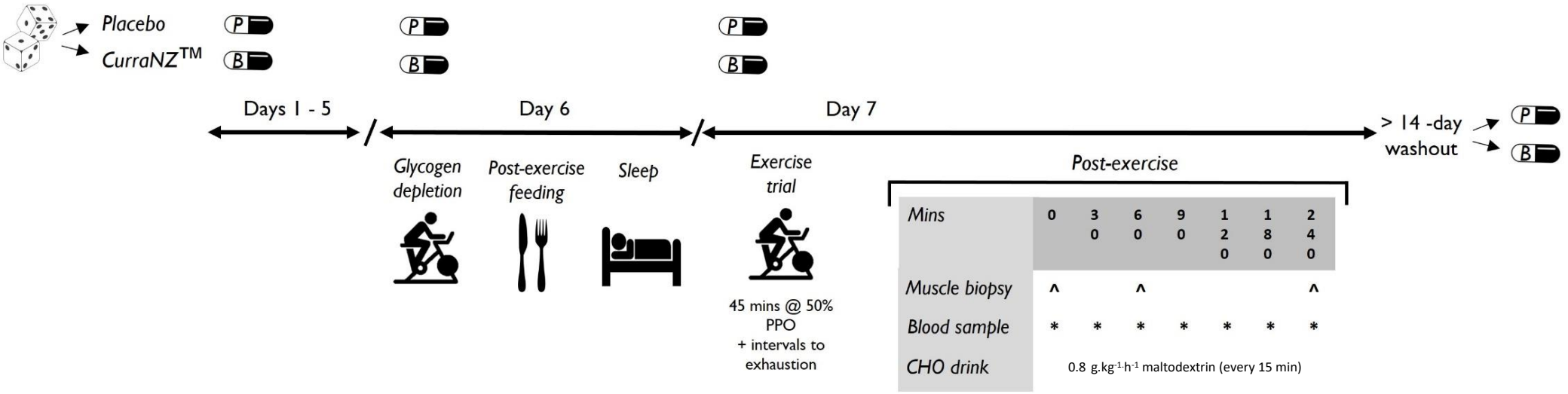

Figure 2.1 . Study protocol and design. *Following a repeated-measures, randomised, cross-over design, participants were randomly assigned to either undergo 7 days of NZBC (CurraNZ, B) or placebo (P) ingestion, with participants and researchers blinded to the conditions. On day 6 of supplementation of either B or P, participants attended the laboratory to complete a glycogen depletion protocol and were administered there evening meal. On day 7, following an overnight fast, participants completed the exercise trial (45 min @ 50% PPO and intervals to exhaustion). Following exercise, participants consumed a sub-optimal dose of carbohydrate (CHO) for 4 hours (0.8* g.kg^{-1}.h^{-1}*, consumed in 15-minute intervals). Skeletal muscle biopsies were obtained 0-, 60- and 240-min post exercise, with capillary blood sampled at 0, 30, 60, 90, 120, 180, 240 mins. Participants then completed a washout period (>14 days), whereby no supplement was consumed, before repeating the protocol under the alternate condition.*

consumed a carbohydrate drink. Carbohydrate intake continued every 15 min throughout the 240 min recovery period. The drink was in the form of isotonic maltodextrin (20% maltodextrin, 80% distilled water), and provided 0.8 g CHO.kg^{-1} BW.h^{-1}). Subsequent muscle biopsies were obtained at 60 min and 240 min post-exercise (figure 2.1). Capillary blood samples were also obtained at 0, 30, 60, 90-, 120-, 180- and 240-min post exercise to be immediately analysed for plasma glucose concentrations (Biosen C-Line, EKF Diagnostic, Cardiff, UK). A >14-day washout period was adopted before supplementation started for the second exercise trial. In a previous study, an anthocyanin intake for 1 month at a dose greater than that used in the present study required a 15-day washout period for biomarkers of antioxidant status to return to baseline (Alvarez-Suarez et al. 2014).

Muscle biopsies

Skeletal muscle biopsies (100–150 mg) were obtained from the lateral portion of the m. vastus lateralis at 0-, 60- and 240-min following the exercise capacity test. Muscle biopsies were obtained using the Weil-Blakesley conchotome technique under local anaesthesia (0.5% Marcaine) and were taken from the same region and depth on alternating legs (2 from one leg, 1 from the contralateral leg), with incisions separated by ∼5 cm with care to avoid damage from multiple biopsies. Once collected, samples were quickly dissected from fat and connective tissue and divided into multiple pieces on an irradiated sterile Petri dish using surgically sterile tweezers and a scalpel. A large sample portion of the muscle tissue was then prepared for glycogen analysis, with the muscle biopsy being immediately (snap) frozen in liquid nitrogen and stored at −80˚C for later analysis.

Muscle glycogen concentration

Whole muscle glycogen concentrations were determined according to the acid hydrolysis. Approximately 2–5 mg of freeze-dried muscle tissue was powdered, dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation in 500 µl of 1 M HCl for 3 h at 100˚C. After cooling to room temperature, samples were neutralised by the addition of 250 µl 0.12 mol l−1 Tris-2.1 mol l−1 KOH saturated with KCl. Following centrifugation, 200 µl of the supernatant was analysed in duplicate for glucose concentration according to hexokinase method using a commercially available kit (GLUC-HK; Randox Laboratories, Antrim, UK).

Habitual dietary anthocyanin consumption

A food frequency questionnaire was used as a low burden, high compliance method to assess habitual dietary anthocyanin consumption was recorded through which lists anthocyanin-containing foods and drinks compiled from the Phenol Explorer database (Neveu et al., 2010). Daily anthocyanin intake was calculated, by multiplying the anthocyanin content of the portion size by the total consumption frequency of each food.

Statistical analysis

All data are expressed as means ± SD. Significance was set at the 0.05 level of confidence. Interpretation of $0.05 > P \leq 0.1$ signified a trend effect, as according to guidelines by Curran-Everett and Benos, (2004). Time-dependent changes of

glycogen synthesis and plasma glucose concentrations following exercise, between trials using a within-subjects repeated measures ANOVA. Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis. All other data was compared using a paired t test.

2.4 Results

RER decreased across the 45 min steady-state cycling bout, in both conditions (main time effect; *P* = 0.010, Fig. 1A), and there was no difference in RER between NZBC and placebo conditions (*P* = 0.666). There was a trend for carbohydrate oxidation to be reduced during steady state exercise (*P*=0.085), where carbohydrate oxidation was lower at 45 min compared to 15 min. However, there was no difference between conditions (*P* = 0.589; Fig. 1B), and there was no difference (*P* = 0.595) in mean carbohydrate oxidation across the 45 min steady state exercise bout (Fig. 1D). Fat oxidation was increased during steady state exercise (*P*=0.011; Fig. 1C), where fat oxidation was higher at 45 min compared to 15 min. Again though, there was no difference between NZBC and placebo (*P* = 0.399). There was no difference (*P* = 0.406) in mean fat oxidation across the 45 min steady state exercise bout (Fig. 1D).

Exercise capacity was no different (P = 0.174) between trials (placebo = 409 ± 166 s; NZBC = 445 ± 220 s). However, 7 of the 10 participants exercised for longer following NZBC supplementation.

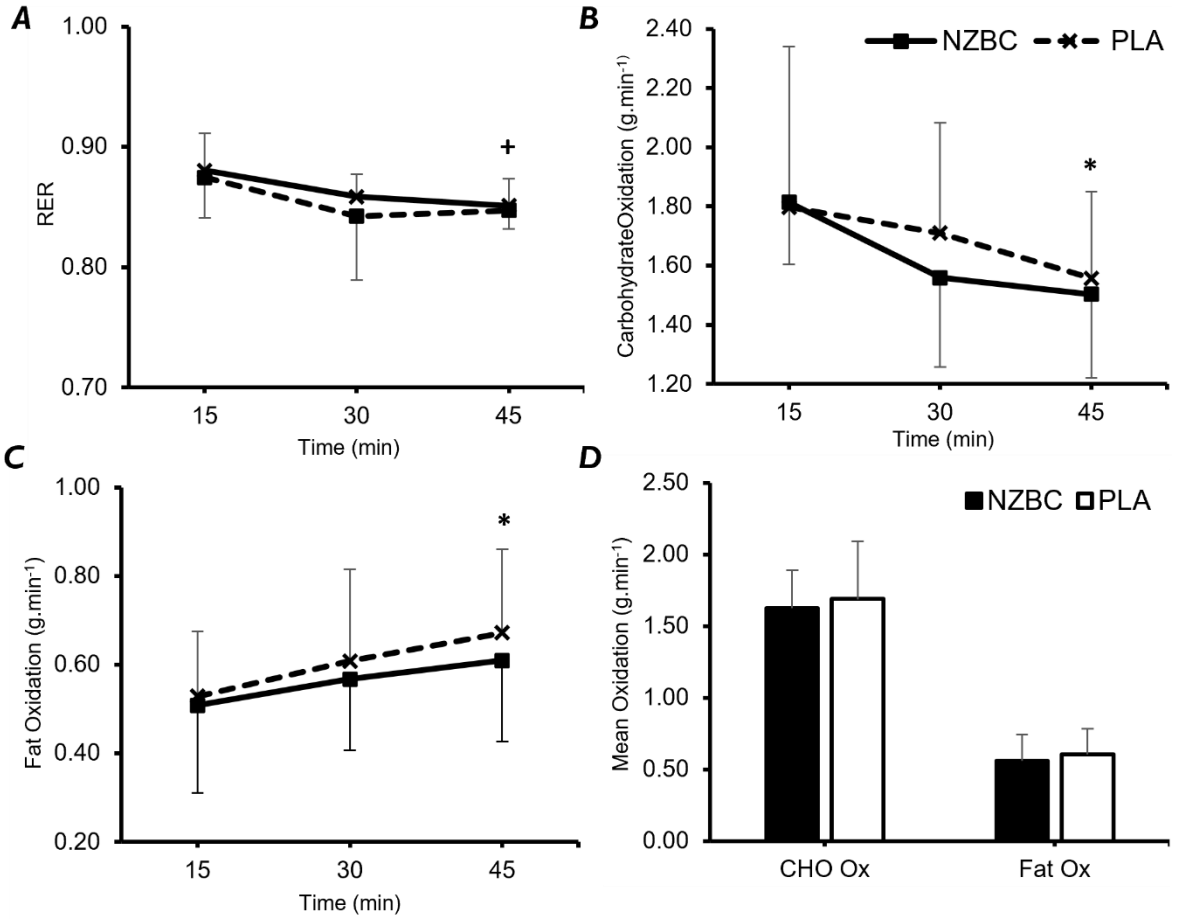

Figure 2.2. *Respiratory exchange ratio (RER) (A), carbohydrate oxidation (B), fat oxidation (C) and mean substrate oxidation (D) across 45 min steady state exercise at 50% PPO, following 7 days of supplementation with NZBC or placebo (PLA). * Indicates significant effect of time in both conditions (P <0.05). + indicates a trend effect of time in both conditions (P < 0.1).*

Muscle glycogen

Immediately following the exercise capacity test, muscle glycogen was depleted to ~70 mmol.kg^{-1} dw with no difference between conditions (NZBC: 69±22 mmol.kg^{-1} dw, PLA: 69±27 mmol.kg^{-1} dw; P=0.975, figure 2.2). Following 60 min of recovery, muscle glycogen concentrations were increased by ~90% in both trials (NZBC: 122±19 mmol.kg^{-1} dw, PLA: 116±30 mmol.kg^{-1} dw; P=0.538, figure 2.3A). Thus, the net glycogen synthesis rate during the first 60 min recovery was 52±12 mmol.kg^{-1} dw.h^{-1} and 47±24 mmol.kg^{-1} dw.h^{-1} for NZBC and PLA, respectively (figure 3A). The increase in muscle glycogen concentration from 0 min to 240 min post-exercise was not different between conditions (time × condition; *P*=0.137). However, after 240 min of recovery muscle glycogen concentrations were significantly greater following NZBC

supplementation (258±34 mmol.kg^{-1} dw) compared to PLA (236±29 mmol.kg^{-1} dw; P=0.005, figure 2.3). This can be attributed to significantly greater glycogen synthesis across 240 minutes of carbohydrate feeding following NZBC supplementation (188.7 ± 38.2 mmol.kg-1 dw), compared to PLA (167.6 ± 39.4 mmol.kg-1 dw; P = 0.044). Accordingly, when considering the net glycogen synthesis rate during the entire 240 min recovery period, this was greater (*P*=0.047) following NZBC (47±10 mmol.kg^{-1} dw.h^{-1}) compared to PLA (42±10 mmol.kg^{-1} dw.h^{-1}; figure 2.3B).

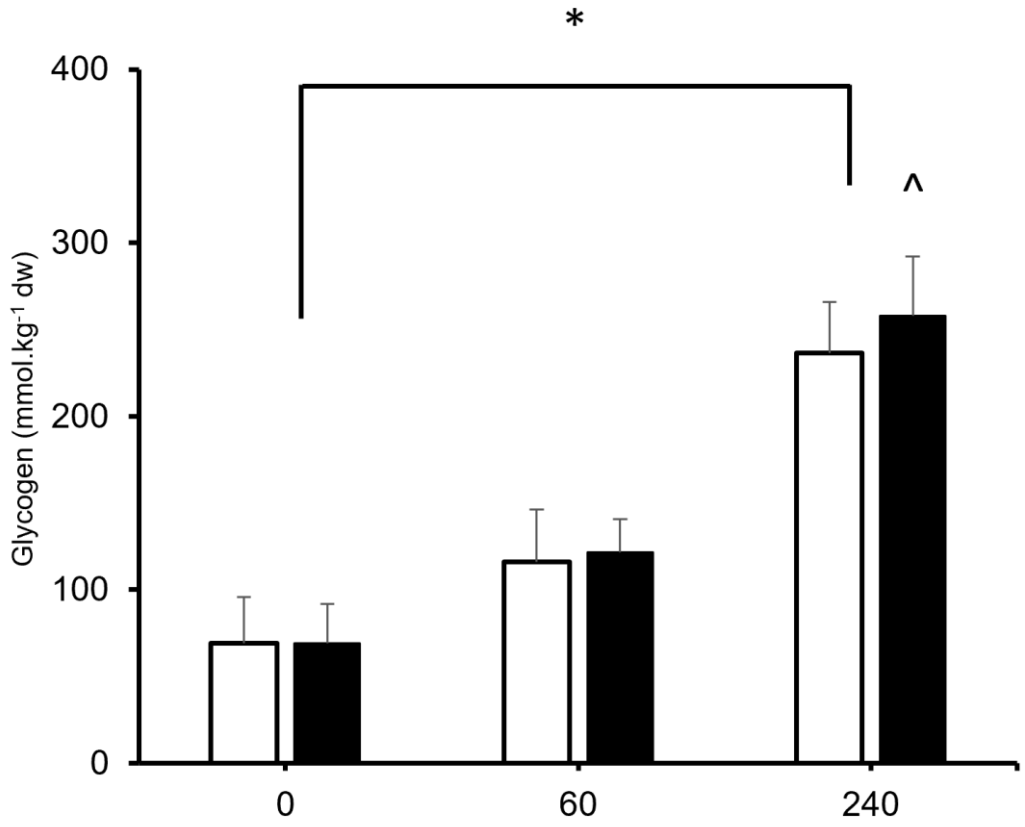

Figure 2.3. *Glycogen concentrations at 0, 60 and 240 of post exercise carbohydrate feeding in placebo (PLA, white) and NZBC (black) conditions. * Signifies significant effect of time in both conditions (P<0.05). ^ Indicates significance in glycogen between conditions (P<0.05).*

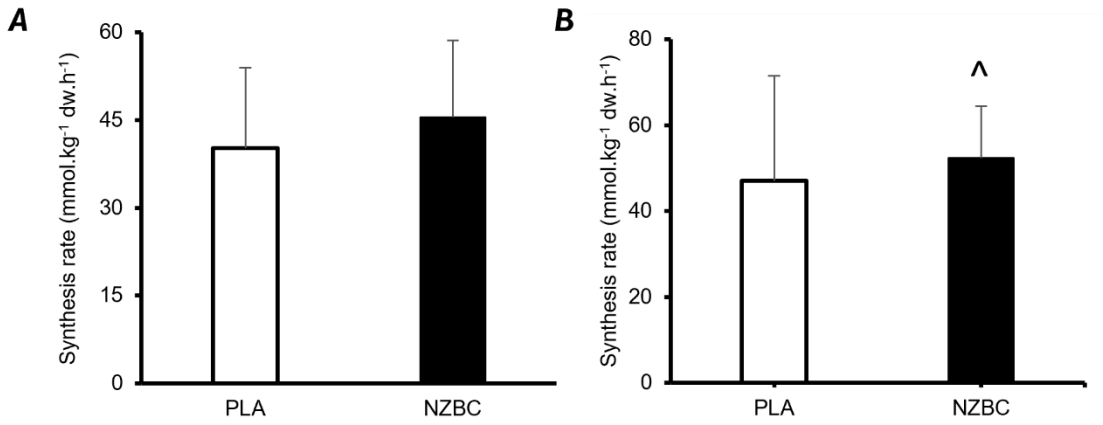

Figure 2.4. *Glycogen synthesis rates from 0-60 min (A) and from 0-240 min (B), in response to post exercise carbohydrate feeding, in placebo (PLA, white) and NCBC (black) conditions. ^ indicates a significance (P < 0.05) between conditions.*

Plasma glucose responses

There was a significant main effect for time (*P*<0.001) for plasma glucose concentrations in response to post-exercise carbohydrate feeding. Plasma glucose concentrations significantly increased from 0 to 30 min post-exercise (P=0.039, Fig. 4A) and were then sustained at ~5.5mmol/L for the remainder of the recovery period. Plasma glucose concentrations were not different between the NZBC and placebo trials (P=0.30, Fig. 4A). Furthermore, plasma glucose area under the curve (AUC) during the recovery was not different between conditions (P=0.179, Fig. 4B).

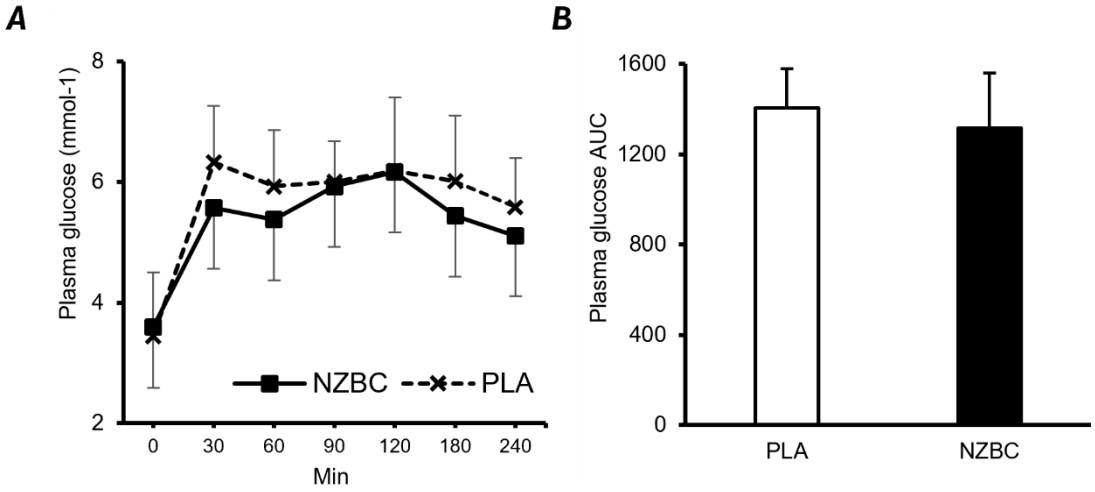

Figure 2.5. *Plasma glucose concentrations (A) and plasma glucose area under the curve (AUC) across 240 min of post exercise carbohydrate feeding.*

There was a modest degree of variability in habitual anthocyanin intake, although this did not influence the results, since there was no correlation between habitual anthocyanin intake and glycogen synthesis in response to NZBC (r = 0.056, P = *0.879)* or placebo (r = 0.075, P = 0.837) supplementation.

2.5 Discussion

The aim of this study was to investigate the effect of 7-days of NZBC extract supplementation on post-exercise muscle glycogen resynthesis in the face of sub-optimal carbohydrate feeding. The novel findings from the present study are: (1) 7-day supplementation of NZBC extract resulted in greater skeletal muscle glycogen concentrations in response to carbohydrate feeding during 4 h recovery from glycogen-depleting exercise, compared to placebo. Because plasma glucose concentrations were similar between conditions throughout the recovery period, this implies that mechanism for the greater rates of synthesis may be beyond the glucose delivery and uptake processes and perhaps directly on the muscles storage capacity.

Glycogen synthesis responses to carbohydrate ingestion (alone, or in combination with other nutrients) have been thoroughly studied, adopting various exercise protocols and feeding strategies in attempt to explore ways of optimising glycogen resynthesis following exhaustive exercise (for extensive reviews, see the following references: (Jentjens and Jeukendrup, 2003; Betts and Williams, 2010; Jensen and Richter, 2012; Burke et al., 2017). Although in the current study, a sub-optimal dose of carbohydrate was provided (0.8 $g.kg^{-1}.h^{-1}$), we observed comparable rates of glycogen synthesis (40-50 $mmol.kg\ dw^{-1}.h^{-1}$) to those previously reported in response

to more optimal post-exercise carbohydrate provision (~1.2 $g.kg^{-1}.h^{-1}$, Ivy. 2004). We chose to provide a sub-optimal dose of carbohydrate because this is a more representative quantity seen amongst athletes, particularly those who compete at a sub-elite level. This is supported by a recent study in amateur runners, in which <50% of their participants (*n*=100) achieved the recommended carbohydrate intake (Mcleman et al., 2019). The low prevalence of amateur athletes achieving optimal carbohydrate is a finding repeatedly reported by many studies (Foo et al., 2021; Hargreaves et al., 2004; Praz et al., 2015; Sparks et al., 2018; Thomas, 2016; Wardenaar et al., 2015). However, Doering et al (2016), showed that young amateur triathletes (*n*=182) do meet the current carbohydrate guidelines, although the standard deviation reported (~0.7 $g.kg^{-1}.h^{-1}$) also demonstrates a large variation, and therefore many are likely to be below optimal practices. The contrasting finding reported by Doering et al (2016) could be indicative of a generational shift, with younger athletes potentially adhering to more optimal practices, although this is speculative and further evidence must be explored to support this theory.

The accumulation of skeletal muscle glycogen following glycogen-depleting exercise and carbohydrate feeding occurs in a biphasic pattern. First, the rapid or insulin-independent phase occurs for around 30-60 min post-exercise, before the slow or insulin-dependent phase is initiated and sustained for several hours. Due to the biphasic manner of glycogen synthesis, we obtained skeletal muscle biopsies after 60 min, representing the insulin-independent phase, and after 240 min, for the insulin-dependent phase. Our findings appear to support this biphasic response of glycogen synthesis, since the rate of synthesis across the first 60 minutes (~54 $mmol^{-1}.h^{-1}$) tended to be greater than the rate of synthesis from 60 min to 240 min (~43 $mmol^{-1}.h^{-1}$). The low muscle glycogen concentrations observed immediately post-exercise (~69

mmol.kg^{-1} dw) indicate that exercise successfully depleted glycogen concentrations. Importantly, our study found muscle glycogen synthesis rates to be similar between conditions after 60 minutes of carbohydrate feeding, which could suggest that NZBC extract has little impact during the first phase of post-exercise muscle glycogen resynthesis.

There is a paucity of mechanistic studies on the effects of NZBC extract or supplements with comparable anthocyanin profiles on glycogen resynthesis or post-exercise metabolism. Some evidence suggests that NZBC supplementation could augment glucose delivery and skeletal muscle glucose uptake. Our data shows post-exercise glucose concentrations to be comparable between conditions, thus suggesting that blood glucose clearance occurs at similar rates. Therefore, if NZBC were to effect skeletal muscles glucose uptake, the similar plasma glucose profile observed in the placebo condition must be explained by greater glucose uptake by the liver. On the other hand, the differences in glycogen synthesis between the groups could be explained by mechanisms with the muscle itself.

Greater post exercise glycogen accumulation was observed following 7 days of NZBC supplementation. The reasoning for this could be virtue of intramuscular alterations in metabolism. Previously, anthocyanin containing mulberry extract supplementation in vivo has been shown to promote glycogen synthesis, via upregulation of Akt phosphorylation and glycogen synthase kinase-3β (GSK3β) (Yan et al., 2016). In turn, enzymes associated with glycolysis and glucose oxidation, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were downregulated, which together, would drive glycogen synthesis and inhibit glycogen breakdown. Limitations of this study are that it was performed in human HepG2 cells, and using a different supplement, therefore cannot be directly compared

to our work (Yan et al. 2016). Nevertheless, intramuscular signalling responses related to glycogen synthesis, enzyme activity and phosphorylation are worthy candidates for future investigations to explain our data. More specifically, studies should investigate the responses of human skeletal muscle glucose transporters and proteins responsible for glycogen synthesis following post-exercise carbohydrate feeding.

In accordance with previous studies, we employed a 7-day supplementation period. A recent study from our laboratory showed that short-term (7 days) supplementation of NZBC extract was required to improve postprandial glucose response and insulin sensitivity, compared to a single dose. Previous studies using NZBC extract have also found that chronic supplementation is needed in order to induce metabolic responses during exercise (Cook et al. 2015; Strauss et al. 2018). While these interventions were concerning different metabolic processes than the current study, they suggest that a loading period is required in order to induce measurable metabolic adaptations. Following consumption, anthocyanin appearance in the blood has been reported to be ~12%, which implies a meagre level of bioavailability (Czank et al., 2013). However, anthocyanin intermediates have a longer lasting effect, with a presence within blood circulation for up to 48 hours post ingestion (Kay et al., 2005). This further supports the use of a more chronic supplementation period in order to induce metabolic changes. Habitual anthocyanin intake of participants in the present study (~28 $mg.day^{-1}$) was seven-to-eight times lower than when taking the NZBC extract supplement (210 $mg.day^{-1}$). There was a modest degree of variability in habitual anthocyanin intake, although this did not influence the results, since there was no correlation between habitual anthocyanin intake and glycogen synthesis in response to NZBC or placebo. Therefore, using the supplement appears to have the potential

to augment the increased glycogen synthesis rates irrespective of habitual anthocyanin intake.

Our data shows 7 days of NZBC to have no effect on carbohydrate or fat oxidation during moderate intensity exercise (see figure 2.1). The pattern of substrate utilisation across the 45 minutes of moderate intensity exercise were similar between conditions and replicate classical exercise metabolism principles, in that with increasing exercise duration, carbohydrate oxidation is reduced, whereas fat oxidation increases (Watt et al., 2002). Previously, 7 days of NZBC supplementation has been shown to promote fat oxidation during cycling at 65% VO2max in trained males (Cook et al., 2015, 2017) and females (Strauss et al., 2018). Despite participants being of a comparable training status and exercising at a similar intensity, it is unsurprising that the current study does not replicate previous findings in substrate utilisation. Reasons for this could be that exercise is being conducted fasted and, in a glycogen depleted state, thus reducing carbohydrate availability and potentially increasing the reliance for fat for energy provision, regardless of the supplemental condition. Furthermore, as fat utilisation increases over time and the exercise bout in the current study was much shorter than the studies cited above, any potentially detectable differences in fat utilisation may have appeared after several hours of exercise.

In conclusion, our study is the first to investigate the effect of 7-day NZBC on muscle glycogen synthesis in response to 4 hours of a sub optimal carbohydrate feeding, following glycogen depleting exercise. The key findings from this study are that in response to 240 minutes of sub optimal carbohydrate consumption, 7 days of NZBC intake leads to glycogen synthesis concentrations that are comparable to that previously seen in response to the current guidelines for optimal glycogen synthesis. The significance to magnitude of the difference between conditions is unexplored and

undetermined to if there is any performance or recovery effect. At this stage, with limited comparative research, this supplementation protocol could be implemented to augment short term glycogen synthesis, which could be important for athletes who require fast carbohydrate replenishment, such as when performing multiple training sessions per day.

Chapter 3

Increased colocalization of SNAP23 with the plasma membrane in lean skeletal muscle, compared to individuals with type 2 diabetes: Implications for SNAP23 high jacking hypothesis.

3.1 Abstract

As the largest storage depot for glycogen, the regulation of skeletal muscle glucose uptake is vital for the maintenance of glucose homeostasis. Type 2 diabetes (T2D) is characterised by an inability for glucose to be sufficiently cleared from the blood and leads to dysfunctional skeletal muscle glucose uptake. SNAP23 is a SNARE protein responsible for membrane fusion, such as joining the skeletal muscle glucose transporter (GLUT4) to the plasma membrane, allowing glucose to enter the muscle. It appears that the growth of lipid droplets (LD), as seen in the development of T2D, requires SNAP23 to fuse LDs together. A hypothesis suggests that in order to grow, LDs must high jack SNAP23 from the plasma membrane in order to facilitate LD-LD growth, thus reducing plasma membrane SNAP23. This could reduce GLUT4 at plasma membrane and therefore, potentially reduce glucose uptake. Using immunofluorescence microscopy, we examine the skeletal muscle localisation of SNAP23 in lean individuals and individuals with T2D, to test the proposed SNAP23 high jacking hypothesis. Our results revealed part colocalization of SNAP23 with the plasma membrane, with significantly greater ($P < 0.05$) colocalization of SNAP23 in lean participants ($r = 0.19 \pm 0.04$) compared to T2D individuals ($r = 0.15 \pm 0.03$). Staining of SNAP23 and the LD dye BODIPY revealed part colocalization, with a trend ($P = 0.095$) for greater colocalization of SNAP23 with LD in individuals with T2D ($r = 0.25 \pm 0.02$), compared to lean individuals ($r = 0.22 \pm 0.03$). Staining of SNAP23 was also partially colocalised to the mitochondria, but no significant difference ($P = 0.322$) between the colocalization of mitochondria in lean individuals ($r = 0.34 \pm 0.04$) and individuals with T2D ($r = 0.31 \pm 0.06$). Our results suggest that there is greater SNAP23 at the plasma membrane of lean individuals, compared to individuals with T2D. The presence of SNAP23 to be at plasma membrane regions is important for

glucose uptake into the muscle. As a result, reduced plasma membrane SNAP23 in individuals with T2D, could potentially reduce glucose uptake and be partly responsible for hyperglycaemia often seen when they consume carbohydrate. However, GLUT 4 transport, nor glucose uptake was measured in this study, so the effects of our results remain speculative. Our study indicates that elements of the hypothesis that LDs high jack SNAP23 away from the plasma membrane, could prove to be true, but it is unclear what is responsible for reduced plasma membrane SNAP23 in individuals with T2D. The LD size between lean participants and individuals with T2D is not known in this study and as it is the process of LD growth that is proposed to increase LD associated SNAP23, future studies should also measure LD size.

3.2 Introduction

Skeletal muscle is the largest tissue in the human body and the target for the majority of glucose disposal. In response to insulin infusion (hyperinsulinaemic euglycaemic clamp), skeletal muscle is responsible for clearance of up to 85% of circulating glucose (DeFronzo et al., 1981). Increased plasma insulin leads to translocation of the skeletal muscle glucose transporter (GLUT4) to the plasma membrane, resulting in facilitated glucose diffusion into the muscle cell. As the predominant site of glucose disposal, skeletal muscle plays a critical role in the maintenance of plasma glucose by increasing its uptake when plasma glucose concentrations rise, therefore maintaining insulin sensitivity of the tissue is paramount to regulate euglycemic conditions (DeFronzo and Tripathy, 2009; Bird and Hawley, 2017). As glucose predominantly relies on transporters to be present at the plasma membrane, tethering of the transporter to the membrane is a necessity to regulate plasma glucose concentrations. It appears that insulin sensitivity and plasma glucose regulation are attenuated by periods of physical inactivity, sedentary behaviour and overfeeding (Knudsen et al., 2012; Costill et al., 1976; Hawley et al., 2011; Hoppeler, 2016). It is considered that physical activity guidelines and appropriate nutritional intake allows human physiology to function (Bird and Hawley, 2017).

The presence of GLUT4 at the plasma membrane is fundamental for glucose entry into the muscle. Following a finely regulated translocation trafficking process via the insulin signalling cascade, GLUT4 must then undergo fusion and docking to the plasma membrane (St-Denis and Cushman. 1998; Foster et al. 1999; Kawanishi et al. 2000; Jaldin-Fincati et al. 2017). GLUT4 requires the aid of SNARE (Soluble N Ethylmaleimide Sensitive Factor Attachment Protein Receptor) proteins to fuse to the plasma membrane. SNAREs are membrane-associated proteins fundamental to many

fusion and docking processes. SNAREs are defined by their distinct characteristics; all 36 SNAREs contain a coiled coil structure (figure 3.1) and contain a SNARE “motif” formed of 60-70 amino acids (Chen and Scheller, 2001; Jahn and Grubmüller. 2002; Söllner, 2004; Duman and Forte. 2003). Dependent on their role, SNAREs are classified accordingly; target-SNAREs (t-SNAREs) are located at the plasma membrane, whereas vesicle-SNAREs (v-SNAREs) are found on transport vesicles. The formation of complete v-t SNARE complexes is fundamental for target proteins to fuse to the target membrane to perform their specific role.

Synaptosomal-associated protein 23 (SNAP23) is a t-SNARE that couples with fellow membrane SNAREs, Syntaxin (1, 2, 3 or 4) to bind with v-SNARE (VAMP1 or 2), allowing for exocytosis of the targeted protein. SNAP23 (and the accompanying v-SNAREs) appears to be important in skeletal muscle glucose uptake, with direct association with GLUT4 docking and fusion to the plasma membrane (Foster et al. 1999; Kawanishi et al. 2000), which is likely required for glucose uptake into the muscle and subsequent utilisation or storage. In healthy, lean individuals, SNAP23 resides predominantly at the plasma membrane and is present in smaller pools at LDs and mitochondria (Strauss et al., 2016). While not directly measured in humans, the available evidence suggests that in insulin sensitive, healthy conditions, SNAP23’s presence at the plasma membrane could be an important component for GLUT4 mediated glucose uptake (Foster et al. 1999; Strauss et al. 2016). As occurs in obesity, lipid oversupply in cardiomyocytes show SNAP23 to be sequestered away from the plasma membrane and channelled to LD regions, where it promotes LD-LD fusion (Bostrom et al. 2007). Furthermore, following SNAP23 ablation in NIH 3T3 cells, there is a reduced rate and magnitude of LD-LD fusion (Bostrom et al. 2007). These studies suggest an important role for SNAP23 in GLUT4 docking to and fusion with the plasma

membrane for subsequent glucose uptake and suggest that the localisation of SNAP23 is dynamic and can directly respond to influx of lipid. Immunofluorescence microscopy has also visualised SNAP23 to be located at intracellular organelle membranes, including LDs (Strauss et al. 2016) whereby it is thought to be involved in LD-LD fusion and mitochondrial networks (Jagerstrom et al. 2009; Strauss et al. 2016). In healthy conditions, LD are often positioned in close proximity to mitochondria which is thought to allow for efficient direct channelling of LD derived FA into the mitochondria to be oxidised (Pu et al. 2011; Gemmink et al. 2020). The formation of LD-mitochondria complexes is attenuated following the ablation of SNAP23 in NIH 3T3 fibroblasts, resulting in reduced β oxidation (Jagerstrom et al. 2009). As a result, this suggests that SNAP23 is important not only for the physical interaction of LD-mitochondria, but also the functionality of LD metabolism.

Endurance training increases the number, but not the size, of LD (Tarnopolsky et al, 2007). Training adaptations in athletic populations cause LDs to undergo adaptations to LD morphology, to increase the efficiency and capacity to oxidise lipid during exercise (Bergman et al. 1999; Martin et al. 1993). However, insulin resistance is associated with increased LD size, likely due to low lipolytic flux and intramuscular lipid accumulation. Oleic acid induced lipid accumulation in cardiomyocytes results in SNAP23 translocation away from the plasma membrane to LD regions, thereby aiding LD growth via LD-LD fusion (Bostrom et al. 2007). Not only does this suggest that LD accumulation promotes LD growth, but also that LD accumulation attenuates GLUT4 fusion to the plasma membrane (as SNAP23 is sequestered away to the fusing LD's), potentially hindering the cells capacity for glucose uptake. As a result, LD accumulation is hypothesised to lead to LD growth through SNAP23 facilitated LD-LD fusion. Proposed models for LD-LD fusion illustrate SNAP23 to be intertwined with

syntaxin5 and VAMP4 in a helix bundle, via which the monolayers from each LD fuse together, exposing a connection between the 2 hydrophobic centres into one droplet (figure 1.10). This provides a potential explanation to why skeletal muscle from obese, insulin resistant and T2D is often associated with larger lipid droplets and that accumulation of LD may attract SNAP23 (Bostrom et al 2007; Covington et al. 2017; Nielsen et al. 2017; Barrett et al. 2022). These cellular changes in response to LD accumulation suggest that LD fusion directly interferes with the cell's ability to uptake glucose, thereby providing potential mechanisms that underpin the development of insulin resistance in humans. This potential response has been termed the 'SNAP23 high jacking hypothesis' (Bostrom et al 2007), whereby LD accumulation upregulates LD growth via SNAP23 mediated LD-LD fusion, which consequently, reduces abundance of SNAP23 at the plasma membrane (see figure 1.11).

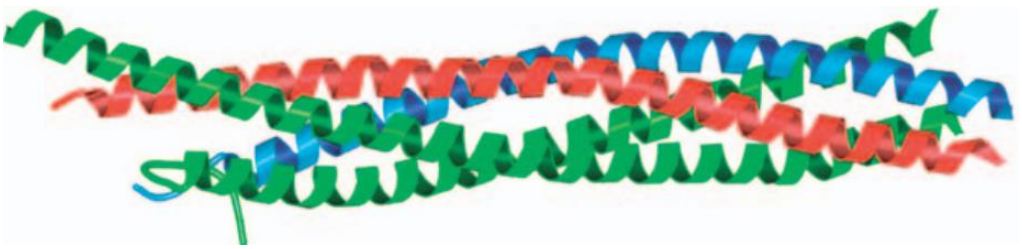

Figure (3.1) SNARE complex structure. Each individual SNARE motif has a coil structure which is intertwined with other SNARES, as seen above. SNAREs shown are syntaxin4 (red), SNAP23 (green), and VAMP2 (blue). The C-terminal ends of the helices are positioned on the right, which points towards the direction of the membrane.

Investigations have unveiled a functional relationship for LD-mitochondrial interactions, which allows efficient FA trafficking into the mitochondria (Sturmey et al., 2006; Moro et al., 2008; Scharwy, Tatsuta and Langer., 2013). Electron and confocal microscopy work have visualised LD to be in close proximity to mitochondria (Hoppeler 1999; Shaw et al., 2008). Confocal microscopy visualised localisation of SNAP23

between LD and mitochondria demonstrating at least a structural role for the SNARE protein in NIH 3T3 fibroblasts (Jagerstrom et al. 2009), which has since been visualised in human skeletal muscle (Strauss et al., 2016). SNAP23 ablation results in reduced LD-mitochondrial interactions paralleled with significantly less β-oxidation, thus attenuating mitochondrial function and oxidative phosphorylation (Jagerstrom et al. 2009). Mitochondrial dysfunction has previously been suggested to contribute to the pathogenesis of insulin resistance and T2D (Kelley et al., 1999; Simoneau., 1999; Sergi et al., 2019), yet the mechanistic understanding behind this is still debated (Di Meo, Lossa and Vendetti., 2017). Mitochondrial dysfunction reduces the cells capacity to oxidise substrate and appears to lead to the accumulation of lipotoxic metabolites, such as DAG and ceramides (Samuel et al., 2010; Sergi et al., 2019). LD and mitochondria interactions, facilitated by SNAP23 appears to promote efficient delivery of FA to mitochondria and it appears that absence of SNAP23 may attenuate FA oxidation. As insulin resistance is associated with reduced oxidative capacity, it could be speculated that the localisation of SNAP23 at both the LD and mitochondria is important. To understand this further, cross sectional investigations must compare the role of SNAP23 in LD-mitochondrial interactions in healthy conditions compared to obese and T2D patient populations.

The present study sought to investigate and visualise the proposed high jacking response in skeletal muscle biopsies from lean participants and patients with type 2 diabetes (T2D). Using an extensively validated SNAP23 immunofluorescent assay, confocal immunofluorescence microscopy will be used for identification of SNAP23 colocalisation with the plasma membrane, LDs and mitochondria in lean individuals and those with T2D. It is hypothesised that the skeletal muscle of individuals with T2D will result in greater colocalisation of SNAP23 with LD when compared to the plasma

membrane and that this will be greater than lean individuals. It is also expected that healthy individuals will have a greater colocalisation of SNAP23 with the plasma membrane compared to the LD's and greater SNAP23 colocalisation with the plasma membrane compared to biopsies obtained from T2D individuals. SNAP23 colocalisation with the mitochondria is expected to be greater in lean individuals compared to T2D individuals. This study will develop what is known about the understudied relationship between SNAP23 and insulin resistance.

3.3 Methods:

Participants and ethical approval

8 lean males (age, 22 ± 1 years; height, 1.74 ± 0.02 m; body mass, 75.1 ± 3.0 kg; BMI, 24.80 ± 0.08 kg.m^2) and 8 males with type 2 diabetes (age, 61 ± 3 years; height, 1.76 ± 0.02 m; body mass, 109.7 ± 5.4 kg; BMI, 35.40 ± 1.70 kg.m^2) were included in this study. All subjects were fully informed of the procedures and any potential risks before the respective experiments took place and verbal and written consent was obtained.

Muscle samples

Percutaneous muscle biopsies were obtained from the vastus lateralis of lean, previously sedentary males, following a 6-week high intensity interval training intervention (see Shepherd et al. 2013). Following an incision to the sterile area of sample collection, ~100mg of muscle was obtained, before removing any visible excess blood, fat or collagen from the sample. ~30 mg of the sample was embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cork board which was immediately frozen in liquid nitrogen-cooled

isopentane (Sigma-Aldrich, Dorset, UK) and stored in pre-cooled cryotubes at - 80 ºC for histological analyses.

Percutaneous muscle biopsies were obtained from the *m. gluteus maximus* of T2D males during elective orthopaedic surgery. All participants were undergoing elective total hip arthroplasty at Russells Hall Hospital, Dudley, UK and provided informed consent for their participation. The sample collection was approved by the local NHS Research Ethics Committee. Following an incision to the sterile area of sample collection, ~300mg of muscle was obtained, before removing any visible excess blood, fat or collagen from the sample. ~30 mg of the sample was embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) on a cork board which was immediately frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich, Dorset, UK) and stored in pre-cooled cryotubes at - 80 ºC for histological analyses.

Immunohistochemistry

Staining protocol

Serial cryosections (5 µm) were cut at -30°C using a microtome (Bright Instrument Company Limited, Huntingdon, UK). Samples were transferred onto pre cleaned glass slides (VWR International Ltd, Leicestershire, UK). Samples were fixed for 1 hour in 3.7% formaldehyde, before being rinsed in double distilled water (ddH_2O) for 3 x 30s. The sections were then treated with 0.5% triton-X-100 for 5 min, before being washed for 3 x 5 min in Phosphate Buffered Saline (PBS, 137mM sodium chloride, 3 mM potassium chloride, 8 mM sodium phosphate dibasic and 3mM potassium phosphate monobasic, pH of 7.4). Slides were then incubated for 1 h with the appropriate primary

(1ry) antibodies. Following this, slides were then washed 3 x 5 min in PBS, incubated with the appropriate secondary (2ry) antibodies for 45 min, followed by further 3 x 5 min PBS washes. For the identification of LDs, a further step was included. Completed in darkness due to the light sensitive nature of the dye, BODIPY (Difuoro{2-[1-(3,5-dimethyl-2H-pyrrol-2-ylidene-N)ethyl]-3,5-dimethyl-1H-pyrrolato-N}boron) was applied for 20 min, followed by 3 x 5 min wash in PBS. Lastly, coverslips were mounted to the dried slides using 20µl mowiol (for analysis of *SNAP23, plasma membrane and mitochondria*) or vectashield (for analysis of *SNAP23, BOPIDY,* WGA) and sealed with nail varnish.

Antibodies

Snap 23, plasma membrane and mitochondria:

All antibodies underwent pre experimental investigations to determine their optimal working concentration in PBS. SNAP23 was stained using anti-SNAP23 (1:50 dilution, Synaptic Systems, Goettingen, Germany) followed by Alexa Fluor 488 conjugated goat anti mouse IgG (1:200 dilution). The plasma membrane was identified using mouse anti- dystrophin (1:50 dilution, D8168; Sigma-Aldrich) followed by an Alexa Fluor 633 conjugated goat anti-rabbit IgG2b (1:200 dilution). Dystrophin is a membrane marker found in the sarcolemma which binds to the protein actin forming a bond between the membrane and cytoskeleton (Haenggi and Fritschy 2006). Mitochondria were labelled using mouse anti-cytochrome c oxidase (COX) (1:50 dilution, 459600; Invitrogen, Paisley, UK) followed by the application of an Alexa Fluor 546 conjugated goat anti-mouse IgG2a (1:200 dilution).

Snap 23, BOPIDY, WGA.

SNAP23 was stained using anti-SNAP23 (1:50 dilution, Synaptic Systems, Goettingen, Germany) followed by alexa fluor 546 conjugated goat anti mouse IGg2b (1:200 dilution). WGA was included in the secondary antibody at a dilution of 1:50. A working solution of BODIPY was diluted from the stock solution as supplied (Sigma Aldrich, UK). The stock solution was diluted in PBS at a ratio of 1:50 to create a working solution. BODIPY was applied to the muscle samples for 20 min before a 5 min wash in PBS. Due to the light sensitive nature of BODIPY dye, all work with BODIPY including the final washes and mounting of coverslips was completed in a dark room.

Image capture

Cross sectional images of skeletal muscle fibres were used to investigate the localisation of SNAP23. Images were captured using an inverted confocal microscope (Zeiss LSM710; Carl Zeiss AG, Oberkochen, Germany), with a 63x (1.4 NA) oil immersion objective. Images were captured at a 1.1 zoom allowing for one complete cell to fill the field of view. An argon laser was used to excite the Alexa Fluor 488 fluorophore whilst a helium–neon laser excited the Alexa Fluor 546 and 633 fluorophores. Images showing cross sectional skeletal muscle fibres were initially acquired with a 40 °ø 0.7 NA oil immersion objective to examine the cellular distribution of SNAP23. All image capture settings were kept consistent across participants to ensure quality control and accurate comparison between participants.

Image processing and data analysis

All image processing and analysis was undertaken using Image-Pro Plus, version 5.1 (Media Cybernetics, Bethesda, MD, USA) and was kept consistent throughout.

Colocalization between SNAP23 and the stain used to depict the plasma membrane, mitochondria and lipid droplets, were determined using a Pearson's correlation coefficient (PCC) to denote the overlap between the red and green signals. On average, 10-15 fibres were analysed per participant, per stain. Each image contained 1 fibre, therefore an average of 10-15 fibres per participant (per stain) underwent analyses for colocalization. Non-matched sections were also analysed for colocalization using the same image capture settings and Pearson's correlation analysis ($P < 0.05$). This was done by overlaying randomly assigned non matched images of different cells to detect the correlation value that is achieved by chance. This is a method used to act as a control to validate the colocalization results, previously described by Lachmanovich et al. (2003) and if the non-matched pairs show significantly less correlation, the colocalization of matched pairs was deemed significant.

Co-localisation

Immunofluorescence staining of SNAP23, the plasma membrane, lipid droplets and mitochondria was performed to visualise the localisation of each protein within skeletal muscle cells. The image capture process produced individual images for each labelled protein combination, which allowed images to be overlayed to identify protein to protein colocalization. With the images labelled in red and green, a greater correlation represents a greater overlap (colocalization) of green and red pixels and therefore that two proteins are closely positioned to one another (within 200nm as this is the resolving power of the microscope). As a result, a greater number of yellow pixels will be present in the images denoting green and red pixel overlap (see figures 3.3 and 3.4, H-J).

Statistical Analysis

The PCC data from immunofluorescence images were analysed for statistical significance using paired samples t-tests using SPSS. Statistical significance was classified as $P < 0.05$ and all data is presented as means ± SD.

3.4 Results

Immunofluorescence staining of SNAP23 in human skeletal muscle cross sections, reveal a more intense stain in plasma membrane regions compared to intracellular regions. This was quantified by combining labelling of SNAP23 and the plasma membrane marker, dystrophin, which revealed part colocalization of SNAP23 with the plasma membrane. There was significantly greater ($P < 0.05$) colocalization of SNAP23 in lean participants ($r = 0.19 \pm 0.04$) compared to T2D ($r = 0.15 \pm 0.03$). SNAP23 is heterogeneously localised at the plasma membrane with some regions showing greater colocalization (greater density of yellow pixels, see figure 3.3, I-J).

In addition to the plasma membrane, staining of SNAP23 revealed punctate fluorescence within the intracellular regions of the cell. Co staining of SNAP23 and the LD dye BODIPY revealed part colocalization between SNAP23 and LD. Moreover, there was a trend ($P = 0.095$) for greater colocalization of SNAP23 with LD in individuals with T2D ($r = 0.25 \pm 0.02$), compared to lean individuals ($r = 0.22 \pm 0.03$).

The punctate intracellular staining of SNAP23 was also partially colocalised to the mitochondria. However, there was no significant difference ($P = 0.322$) in the colocalization of mitochondria between lean individuals ($r = 0.34 \pm 0.04$) and individuals with T2D ($r = 0.31 \pm 0.06$).

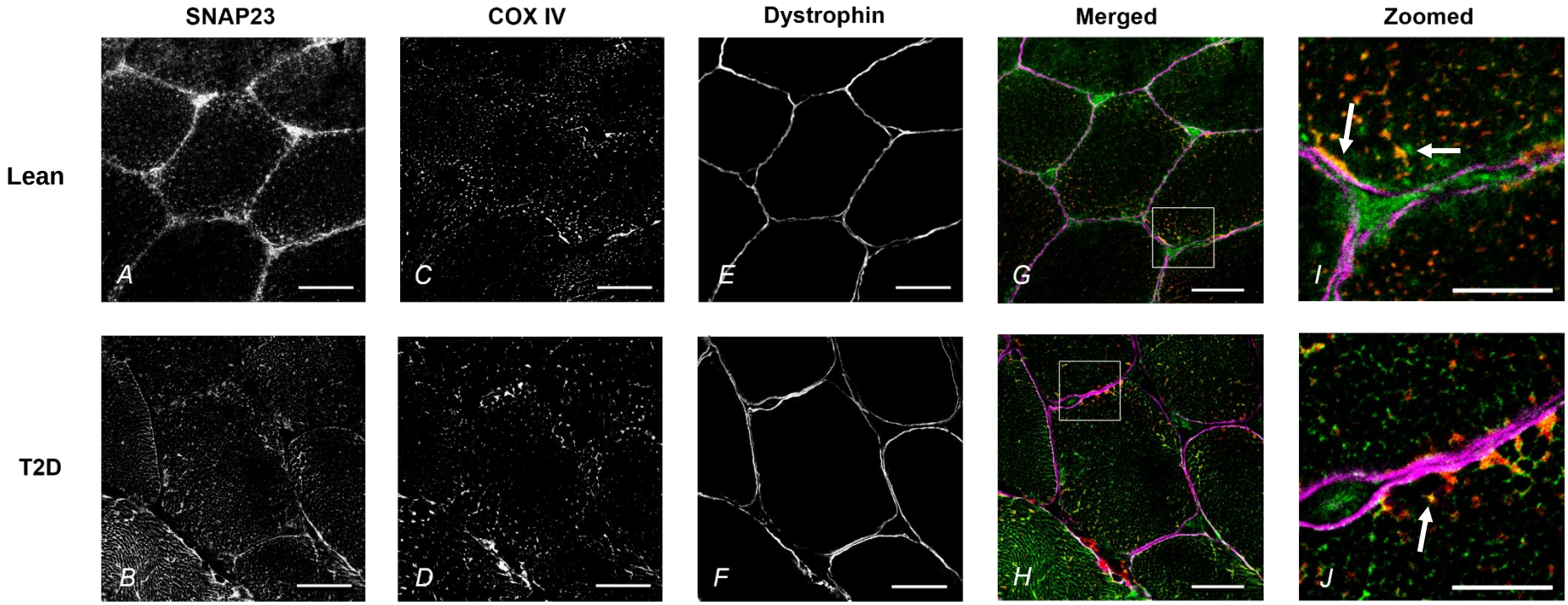

Figure 3.3.
Individual channels for this image can be seen for SNAP23 (A,B) labelled using anti-SNAP23, mitochondria (C, D) labelled using anti-COX IV and the plasma membrane labelled using anti-dystrophin (E, F). Merged images of SNAP23 (green), mitochondria (red) and the plasma membrane (pink) can be seen (G, H). White scale bars represent 25 µm in images A-H. Images I and J represent areas of interest shown by white box in G and H and visualise colocalization of stained proteins, depicted by greater portion of yellow pixels (signifying overlap of red and green channels) and highlighted by arrows. Scale bars on I and J represent 100 µm. All images were obtained from confocal microscope with a 63x oil immersion objective and 1.1 digital zoom..
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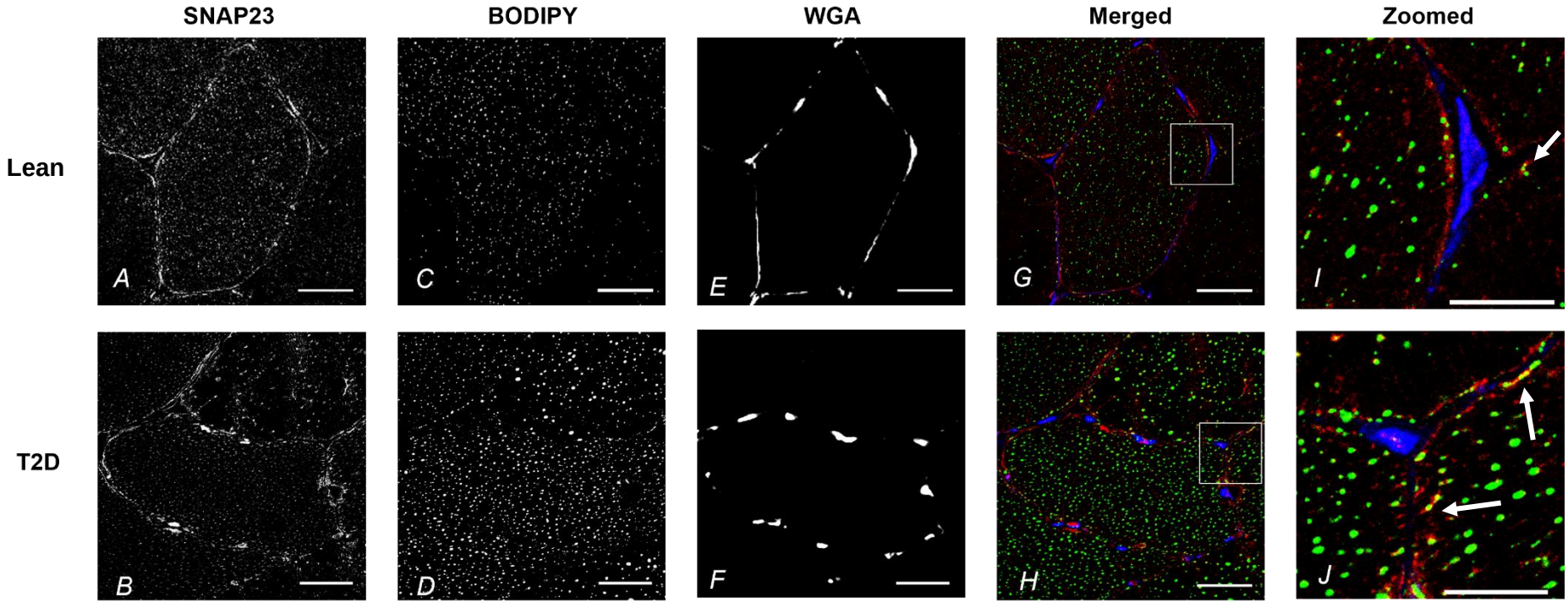

Figure 3.4. *Individual channels for this image can be seen for SNAP23 (A,B) labelled using anti-SNAP23, lipid droplets (C, D) labelled using BODIPY and the plasma membrane labelled using WGA (E, F). Merged images of SNAP23 (red), lipid droplets (green) and the plasma membrane (blue) can be seen (G, H). White scale bars represent 25 μm in images A-H.*
Images I and J represent areas of interest shown by white box in G and H and visualise colocalization of stained proteins, depicted by greater portion of yellow pixels (signifying overlap of red and green channels) and highlighted by arrows. Scale bars on I and J represent 100 μm. All images were obtained from confocal microscope with a 63x oil immersion objective and 1.1 digital zoom..

3.5 Discussion

The aim of this study was to apply immunohistochemical analyses to visualise the sub cellular co-localisation between SNAP23 and the plasma membrane, lipid droplets and mitochondria in both lean participants and in individuals with T2D. Previous research proposed a potential redistribution in SNAP23 localisation in response to intracellular lipid accumulation whereby it is redistributed from the plasma membrane to the lipid droplets (Bostrom et al. 2007). We aimed to visualise this proposed redistribution in human skeletal muscle obtained from lean individuals and T2D patients. Our investigations show a greater co-localisation between SNAP23 and the plasma membrane in skeletal muscle of lean men, compared to men with T2D. There was also greater co-localisation between SNAP23 and lipid droplets in the T2D individuals compared to lean individuals. Lastly, we observed no difference in the colocalisation of SNAP23 and the mitochondria between skeletal muscle of males with T2D and lean trained individuals.

We show co-localisation between SNAP23 and the plasma membrane marker, dystrophin, in both populations. Previous findings that propose a functional role for SNAP23 in the docking of GLUT4 vesicles at the plasma membrane in 3T3-L1 adipocytes (Foster et al. 1999; Kawanishi et al. 2000) support our findings, in that SNAP23 is situated in plasma membrane regions. Similarly, using immunofluorescence microscopy, a study from our group (Strauss et al, 2016), illustrated a correlation (PCC) between SNAP23 and the plasma membrane in lean, healthy males. An important observation between datasets is that the PCC values in the present study ($r = 0.19 \pm 0.04$ and $r = 0.15 \pm 0.03$) are notably lower than previously seen ($r = 0.50 \pm 0.01$). The reasonings for the contrasting values are unclear but could be in large part due to differences in the microscope systems that were used to obtain

images. Microscopy is a (semi)quantitative method that uses system specific algorithms to perform deconvolution and downstream analysis of images captured from confocal microscopy, to determine, in this case, colocalization between proteins of interest. Furthermore, the staining process requires the application of protein specific antibodies to human tissue, and as a result, the images captured, and subsequent analysis is performed on the protein specific antibody and not the protein itself, which may result in small amounts of staining that is non-specific to the protein of interest (Walker. 2006). Earlier work by Cheatham and colleagues (1996), also showcased the involvement of SNARE proteins in GLUT4 trafficking and docking in 3T3-L1 adipocytes suggesting a potential interaction between SNAP23 and GLUT4 containing vesicles in plasma membrane fractions. This was later affirmed by St-Denis et al, (1999), where SNAP23 was highly present in plasma membrane fractions of rat adipocytes. The combining of tSNAREs (syntaxin 4 and SNAP23) and vSNAREs (VAMP2) forms a complete and functioning SNARE complex. The subsequent fusion of SNAREs to the plasma membrane is reviewed by Bryant et al (2002), which splits the fusion process into three distinct stages, vesicle tethering, vesicle docking and membrane fusion. Following vesicle tethering, whereby the small GTPase Rab family of proteins tether transport vesicles to the appropriate target membranes, a stable SNARE complex begins to form, allowing for vesicle docking. Finally, the docked vesicle can then undergo fusion to the target membrane, requiring NEM (N-ethylmaleimide) sensitive factor and its binding partner αSNAP. The exact roles of individual SNAREs remain ambiguous, and despite being speculative, it appears that the proposed fusion properties of SNAP23 are present in both populations in our study, depicted by the correlation between SNAP23 and the plasma membrane. The interaction between insulin and SNARE complexes could be that they share

localisation with insulin signalling cascade molecules responsible for GLUT4 translocation; Cbl and TC10 (Baumann et al. 2000; Chiang et al. 2001; Watson 2001). This would mean that the lipid raft and its specific embedded proteins (i.e. SNAREs) are directly engaging with the cells insulin receptors, when activated. Using subcellular fractionation techniques in 3T3-L1 adipocytes, Chamberlain and Gould (2002) confirmed the localisation of SNAP23 within lipid rafts. Our current data shows SNAP23 to be heterogeneously present at the plasma membrane. Moreover, immunofluorescence images of our current study and previous work in our group (Strauss et al. 2016), suggest that SNAP23 content is not uniform around the plasma membrane, but rather, appears more dense in certain plasma membrane regions. Whilst this can only be speculated at this stage, it seems plausible that the clusters of SNAP23 around the plasma membrane represent areas of lipid rafts.

As previously described, and in line with our hypothesis, we demonstrate co-localisation between SNAP23 and the plasma membrane stain, dystrophin. In addition, the correlation between SNAP23 and dystrophin was significantly greater in lean individuals. Disruption of insulin mediated glucose transport is associated with insulin resistance and development of T2D. The inability to adequately clear elevations in plasma glucose following carbohydrate ingestion is a main characteristic of peripheral insulin resistance. Given that skeletal muscle is the primary tissue responsible for glucose disposal in the body and coupled with the fact that insulin is the main stimuli for postprandial skeletal muscle glucose uptake, it is evident that dysfunction to glucose uptake will disrupt plasma glucose homeostasis. For comprehensive reviews of insulin resistance and type 2 diabetes, please see: Davidson and Eddleman (1950); DeFronzo et al (2015); Petersen et al (2018); Yaribeygi (2019).

As discussed, GLUT4 relies on SNARE complexes to dock within the plasma membrane. As our data set shows, there is significantly less colocalisation of SNAP23 with the plasma membrane in skeletal muscle of type 2 diabetes patients compared to lean individuals. As a result, this reduced presence of the SNARE protein, SNAP23, could directly inhibit GLUT4 plasma membrane docking and subsequent glucose uptake. A study by Bostrom and colleagues (2007), provides supporting data for our findings, showing SNAP23 to reside in plasma membrane fractions in cardiomyocytes. Interestingly, with oleic acid treatment SNAP23 content in the plasma membrane fraction was reduced. The oleic acid infusion increased intracellular LD content, thus mimicking the lipid overspill response associated with T2D. Therefore, the work by Bostrom and colleagues, support our colocalization data in human skeletal muscle of lean and type 2 diabetic males, whereby we show a reduced SNAP23 colocalization at the membrane of T2D patients, but also, we found a greater trend in SNAP23 and LD colocalisation which could be indicative of the SNAP23 redistribution response observed by Bostrom et al. (2007). Other evidence shows immunoblotting following incubation of cells in oleic acid resulting in an increase in the coupling of SNAP23 and adipocyte differentiation related protein (ADRP, also known as adipophilin or perilipin 2 (PLIN2)), which is found on the surface of lipid droplets in most mammalian cell types (Listenberger et al. 2008). Further assays in other laboratories, such as electron microscopy suggest that oleic acid treatment could potentially lead to SNAP23 translocation from the plasma membrane to intracellular regions and, more specifically, lipid droplets. At the time of muscle sample collection, the individuals with T2D were all patients undergoing orthopaedic surgery, therefore were extremely sedentary. Furthermore, these individuals were likely to have been immobile prior to surgery, which is alluded to by the subjects physiological and metabolic profiles,

outlined in the subjects' characteristics. The inactive nature of these individuals is an important consideration when interpreting our data and understanding the mechanisms behind the differences we observed between healthy individuals and type 2 diabetic patients. Tail-suspended rat models can be used to simulate and mimic inactivity. Xu and colleagues (2014) applied this model and observed reduced GLUT4 translocation in soleus muscle of tail-suspended rats, following a hyperinsulinemia-euglycemic clamp treatment. This suggests that GLUT4 translocation and/or docking is downregulated in periods of inactivity, even when high concentrations of insulin are present. Consequently, this would reduce the muscle cells ability to consume glucose. Our data, albeit in humans, provides a potential explanation for the reduced GLUT4 translocation and or docking to the plasma membrane in insulin resistance, whereby SNAP23 content and the capacity to consume glucose is likely inhibited.

Although LD content was not measured, intramuscular TAG (IMTG) content is known to increase from lean, to obese, to T2D populations (Pan et al. 1997; Krssak et al. 1999; Goodpaster et al. 2001; Van Loon et al. 2004; Nielsen et al. 2010). Therefore, it is likely that the skeletal muscle obtained from the type 2 diabetic individuals in this study contained a large amount of IMTG.

Our results show that males with T2D have less SNAP23 available at plasma membrane sites, which could be due to increased intracellular lipid. Reduced plasma membrane associated SNAP23 would likely affect GLUT4 docking to the plasma membrane, but as we did not directly measure glucose uptake, we cannot determine the functional effect of this response. Previously, the cell culture work from Bostrom et al (2007), explored the downstream effects of the oleic acid induced changes to metabolism by measuring insulin sensitivity. Oleic acid infusion reduced insulin sensitivity and reduced insulin mediated GLUT4 content in the plasma membrane.

Expressing SNAP23 in these cells restored insulin sensitivity thus reversing the effects on glucose uptake. As a result, it has been proposed that SNAP23 content at the plasma membrane is a rate limiting factor to glucose uptake into skeletal muscle, particularly when LD content is high as the SNAP23 will be sequestered from the plasma membrane to the fusing LD's (Sollner, 2007). Our results show a trend for colocalization of SNAP23 and LD in the skeletal muscle of individuals with T2D compared to those who are classified as lean. We have predominantly discussed SNAP23 docking at the plasma membrane, however, as a SNARE protein, SNAP23 can also be involved in intracellular fusion processes (Jahn and Scheller. 2006; Han et al. 2017). As dynamic organelles (Martin and Parton. 2006), the morphology of LDs is malleable. Marchesan et al (2003) found newly formed LD to be smaller in size, thereby suggesting that LD must have the machinery to grow in size. Combining transmission electron microscopy with hyperinsulinemic-euglycemic clamp methods, Nielsen et al (2017) showed an association between LD size and insulin sensitivity. Greater LD size (diameter) is associated with reduced insulin sensitivity, rather than LD number, at least in healthy untrained males. Similar conclusions were drawn from an earlier study from He et al (2004). This study found a relationship between LD size and insulin sensitivity, with larger LDs being associated with insulin resistance. Following these studies, Daemen, van Polanen and Hesselink, (2018) progressed this research by comparing different populations' lipid profiles. This study reported similar amounts of intramuscular lipid content between T2D and trained individuals, however, insulin sensitivity and lipid storage were vastly different. Reduced insulin sensitivity and larger LD were observed in T2D patients, which suggests that the size of the lipid droplets could influence the insulin sensitivity of the cell. A more recent study analysed the effects of an 8 week, high-calorie, high-fat diet (140% caloric intake, 44% from fat)

in sedentary individuals. This intervention led to the development of insulin resistance and resulted in increased LD size, but no change in LD number (Covington et al., 2017). More recently, our group 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 of the muscle fibre (Whytock et al., 2020). These findings demonstrate the dynamic morphology of LDs and suggest that intramyocellular lipid accumulation can lead to increased LD size (Barrett et al, 2022). We did not measure LD size, nor number in this study, but increased LD size in insulin resistance appears a consistent finding.

There are multiple proposed processes by which LD are thought to grow in size, including through the fusion of two pre-existing LD. The fusion between LDs is catalysed through the action of the SNARE proteins SNAP23, syntaxin-5 and VAMP4. At this time, studies have predominantly looked at the role of SNARE proteins in fusion processes between transport vesicles and target membranes. Fusion at these sites appears to involve the zippering from a four-helix bundle between α-helical SNAREs, which forces membranes together (Sudof and Rothman. 2009; Giraudo et al. 2009). An important consideration is the differences between target membranes outer bilayer and LD amphipathic monolayer. Therefore, the fusion of two LD cores, relies on their monolayers fusing together through the action of α-helical SNARE domains (Olofsson et al. 2009). This could present a mechanism which supports the trend seen in our colocalization findings. Similar to the current investigation, our lab has previously found colocalization between LD and SNAP23 in healthy, lean males, using immunofluorescence microscopy (Strauss et al. 2016). The current investigation furthers the interpretation of these findings by comparing to skeletal muscle of males with T2D. Although modest. the greater colocalisation of SNAP23 and LD in T2D

patients met our hypothesis, as we suggest this process to be a potentially important intracellular response to the accumulation of intramuscular lipid, coupled with poor insulin sensitivity. In *vivo* studies are sparse, in fact, a previous investigation exploring SNAP23 involvement in LD hijacking in human skeletal muscle, was later retracted (Bostrom et al. 2010; 2017), leaving the literature void of evidence. This highlights the importance of our findings. With the hijacking response yet to be demonstrated in human skeletal muscle we are glad to add our findings to the paucity of data available.

In accordance with our previous study (Strauss et al. 2016), we demonstrated colocalization between SNAP23 and mitochondria in the skeletal muscle of lean men, however we also found comparable colocalization data in skeletal muscle of those with T2D. Using similar methodologies to the present study, Strauss et al (2016) previously found colocalization of SNAP23 with the plasma membrane, LD and mitochondria. The relationship between LD and mitochondria has been investigated previously, showing endurance training to elicit greater LD-mitochondria interactions, which theoretically improves FA transport due to the tight coupling and increased efficiency of FA uptake into the mitochondria. When exploring the interaction between LD and mitochondria, Jagerstrom et al (2009) identified SNAP23 to be localised within the LD-mitochondria network in NIH-3T3 cells, using fluorescence microscopy. In addition, following SNAP23 ablation, there was less expression of LD within mitochondrial fractions. Jagerstrom and colleagues (2009), then hypothesised that SNAP23 could be involved in the fusion of LD and mitochondria. This hypothesis was based partly on a study by Olofsson et al. (2008b) that suggested that LD use SNAREs for homotypic fusion (LD-LD). The fusion of LD to mitochondria may therefore be assisted by SNAP23 and this could explain why SNAP23 is colocalised with mitochondria in our

study, as well as providing rationale to previous studies that show SNAP23 to be situated between LD and mitochondria.

An interaction between LD and mitochondria has been reported several times previously (Sturmey et al. 2006; Shaw et al. 2008; Jagerstrom et al. 2009). Tight tethering of LD-mitochondrial networks allows for efficient channelling of FA's from LD directly to mitochondria, thus supplying a rapid exchange of energy. There is a paucity of literature that directly approaches the interactions between LD and mitochondria in different populations. Thus far, the application of TEM analysis has shown LD-mitochondria interaction to be comparable between healthy lean and healthy obese males and females (Devries et al. 2013; Samjoo et al. 2013). Typically, LD and mitochondria have a similar subcellular distribution, whereby the highest density is in the subsarcolemmal regions and gradually reduces towards the central regions of the cell (Stellingwef et al. 2007; Shaw et al. 2008). Confocal and TEM work by Shaw et al (2008) visualised mitochondria and LD in skeletal muscle of trained individuals to be situated in close proximity to one another, almost as if in they are stored in pairs. Endurance training promotes an array of mitochondrial adaptations, such as increasing mitochondrial content, size and density (Nielsen et al. 2017; Granata et al. 2018). In turn, endurance training adaptations augment skeletal muscles oxidative capacity, with not only more sites for oxidative phosphorylation, but also equips the mitochondria with more efficient machinery to oxidise substrate (Nielsen et al. 2017; Granata et al. 2018), which is likely to be in part due to greater LD-mitochondria interaction (Bleck et al. 2018). Alternatively, obesity and T2D appears to lead to maladaptation's to the mitochondrial network and function (Parry and Glancy. 2021; Gemmink et al 2020; Willngham et al 2021), characterized by reduced fat oxidation rates/ oxidative capacity (Kelley et al. 1999; Kim et al. 2000; Abdul-Ghani and

DeFronzo. 2008). As a result of ectopic lipid accumulation (Machann et al. 2004), we hypothesised that there would be a disassociation between LD and mitochondria in the skeletal muscle of individuals with T2D and therefore, we would witness greater SNAP23 colocalization with the mitochondria in lean individuals. The greater association of SNAP23 to mitochondria could be indicative of greater LD-mitochondria interaction, due to its aforementioned role in LD-mitochondria fusion. Contrary to our hypothesis, there was no difference in SNAP23 and mitochondrial correlations in skeletal muscle in individuals with T2D and lean. A potential reasoning for this finding is that the mitochondrial adaptations, such as increased mitochondrial content in response to endurance training could dilute its affinity with SNAP23. Therefore, potential differences in mitochondria number between our groups could affect and distort correlation values, as the trained individuals would likely have greater total number of mitochondria than those with T2D. Because not all LD in the trained state interact with mitochondria regions, future studies should look to detect total SNAP23-mitochondria content for a more precise understanding. Furthermore, we did not co-stain LD with mitochondria, which would not only provide insight into LD-mitochondrial interactions between the groups studied, but also, the co-localisation of SNAP23 with LD-mitochondrial networks, which could provide a more valuable means of analysis in terms of SNAP23s role at the mitochondria. It must be noted, that methodologically, this could prove difficult as immunofluorescence microscopy is limited to the number of proteins you can stain for at a given time, which Is why our study opted for two separate staining protocols. Additionally, although active, it must be noted that these individuals' endurance training history of 6 weeks is relatively short. Although the training intervention of these participants augmented IMTG concentrations, it could be that the training intervention was simply too short of a time frame to promote IMTG

content that replicates an elite athlete nor is it sufficient to induce significant LD and mitochondrial fusion, which could be important when investigating both extremes of the athlete's paradox, as chronic training adaptations associated with elite athletes elicit an array of mitochondrial changes. Investigations should directly measure SNAP23 content within LD-mitochondria regions to add to our findings and perhaps, more polarised populations within the athletes paradox should be compared, such as elite endurance trained athletes and individuals with severe T2D. Although, there is a lack of cross-sectional evidence in LD-mitochondrial interactions between diseased and healthy populations, studies have focussed on the effect of exercise training on the interaction. Samjoo and colleagues experimented what effect a 12-week endurance training programme would have on the amount of intramyofibrillar lipid juxtaposed to mitochondria in healthy, lean and obese participants, which is notably longer than the training intervention of the lean participants in the present study. TEM analysis revealed that endurance training increased the interaction between the two organelles in both populations. While the evidence is not clear, the comparable LD-mitochondria colocalisation data between our groups may be explained by the training status of the lean participants. Future work should both compare individuals with obesity and T2D with well-trained participants and explore the training effect of this interaction in obesity and T2D.

Limitations:

It is important to appreciate and understand the limitations of the present study. Firstly, the resolving power of our confocal microscope (200nm) provides markedly more detailed images than that of a widefield microscope, but does not retrieve as detailed image capture to that of transmission electron microscopy. As a result, it is possible that in protein-protein analysis (i.e SNAP23 and LD), proteins could appear to be in

contact, when they are just in close proximity (<200nm), when using confocal microscopy and analysis, however in reality, they may not be physically interacting. Secondly, confocal microscopy produces z plane images that are approximately 0.53 µm thick. This means that only labelled proteins the z plane are imaged and any labelled proteins that are beyond the resolution of the microscope (> ~0.53 µm) will not be analysed. Importantly, it is not possible to predetermine the depth at which each organelle is cut through, so a uniform central (widest) sectioning of the organelle cannot be guaranteed. While these limitations are important considerations when interpreting our data and comparing with other literature, the confocal method is still an appropriate analytical method for our analysis, as it provides much more resolute image capture than widefield microscope systems and allows visualisation of the native proteins and with no visual shift that would be present in widefield imaging. Furthermore, whilst the greater resolving power of transmission electron microscopy would provide more precise findings for organelle to organelle interactions, it cannot visualise proteins without complex immunogold labelling.

Conclusions

The aim of this study was to use immunofluorescence microscopy to demonstrate the colocalization of SNAP23 with the plasma membrane, lipid droplets and mitochondria we wanted to test the previously proposed ‘hijacking’ hypothesis; whereby intracellular LD accumulation is suggested to result in LDs having greater affinity with SNAP23 and to redistribute SNAP23 away from the plasma membrane. Our findings agree with previous literature that SNAP23 has a greater presence at the plasma membrane in lean individuals than those with T2D. In T2D individuals, the reduced abundance of SNAP23 at the plasma membrane coincides with increased presence of SNAP23 at the lipid droplet, therefore providing some support to the hijacking hypothesis. We

found no difference in SNAP23 colocalization with the mitochondria between groups which was attributed to the lack of endurance training adaptations in our participants and that reduced LD-mitochondria interactions is not due to insulin resistance, per se. Our findings indicate an important role of SNAP23 in the docking of GLUT4 to the plasma membrane, thus allowing glucose entry into skeletal muscle. Accumulation of intramuscular LD leads to LD-LD fusion which requires SNAP23 and therefore leading to LD growth. As a result, LDs hijack SNAP23 from the plasma membrane in order to catalyse LD fusion. This reduction in plasma membrane SNAP23, therefore reduces the muscles capacity to uptake glucose, leading to increased plasma glucose concentrations. As a result, our findings provide support for SNAP23s involvement at the plasma membrane, which could facilitate GLUT4 mediated glucose uptake. Alternatively, those with T2D have less colocalization of SNAP23 and the plasma membrane, which in turn, could reduce the capacity to utilise GLUT4 to facilitate glucose transport into skeletal muscle. This study suggests a need for SNAP23s localisation at the plasma membrane in healthy conditions and that the development of T2D may lead to SNAP23 redistribution to intracellular regions. Our results in part lend itself to the SNAP23 high jacking hypothesis, but also indicate that the development of insulin resistance via this mechanism is more regulated and complex than originally proposed. What does appear clear, is that research must focus on LD adaptations to insulin resistance, as a potential location for mechanisms that lead to the development of T2D.

Chapter 4: General Discussion

4.1 Thesis overview

Chapter 1 outlined the metabolic regulation of glucose homeostasis in healthy human conditions, with particular focus on skeletal muscles role within glucose regulation. The physiological and metabolic responses induced by exercise were then discussed, before focussing on humans' adaptability to training and nutritional stimuli and exploring avenues in which exercise performance parameters can be improved. Moreover, this chapter expands how various lifestyle factors, such as excessive energy intake and physical inactivity can disrupt the metabolic function of humans, with particular focus around the dysregulation of glucose uptake and subsequent development of metabolic disease.

The regulatory role of nutritional status on exercise performance was expanded upon, particularly the use of ergogenic aids in sport to enhance performance. An important consideration for individuals who are extremely active, such as athletes, is the ability to store nutrients quickly and efficiently. This is due to the large training volumes of many athletes, which results in depletion of the glycogen stores to fuel exercise and requires replenishment after training, however many individuals struggle to store adequate energy sources, which would negatively affect subsequent exercise performance. Therefore, ***Chapter 2*** investigates the post exercise skeletal muscle glycogen content in response to feeding with and without the addition of a NZBC supplement.

On the contrary to athletes, who tend to be lean and healthy in order to meet the demands of their sport, obesity is a result of a positive energy balance. This is a result of excessive energy intake and/or inadequate energy expenditure. Prevalence of these lifestyles are growing, which coincides with increasing rates of obesity and T2D.

T2D is characterised by metabolic dysfunction and poor glucose control, as a result of insulin resistance. ***Chapter 3*** was designed to explore potential mechanisms that can further explain how and why insulin resistance develops in response to weight gain. More specifically, this study explored difference of healthy and T2D skeletal muscle and the metabolic ramifications of T2D.

4.2 Key findings

Chapter 2: Short-term supplementation with New Zealand black currant extract enhances post-exercise muscle glycogen resynthesis

The aim of chapter 2 was to explore whether NZBC extract supplementation could augment glycogen re-synthesis in response to sub-optimal post-exercise carbohydrate feeding. Due to the proposed benefits of NZBC on upregulating blood flow and glucose transport, we employed 7 days of NZBC supplementation (or a visually identical placebo) before participants performed a bout of exhaustive exercise and subsequent carbohydrate intake. In an attempt to replicate 'real world' nutritional behaviours of sub-elite athletes, we provided a sub optimal dose of carbohydrate (0.8 $g.kg^{-1}.h^{-1)}$ following the exercise bout and collected post exercise muscle biopsy samples from the vastus lateralis muscle to measure glycogen concentrations at three time points across a four-hour feeding window (0, 60 and 240 min).

A key finding in chapter 2 is that 7 days NZBC extract supplementation resulted in greater rates of glycogen resynthesis following carbohydrate feeding over the entire 240 min recovery period. Notably, the muscle glycogen concentrations were comparable in the NZBC and placebo groups after 60 min of recovery; we can attribute this difference in synthesis rates to the NZBC extract supplement impacting the insulin-

dependent phase of glycogen resynthesis. Subsequently, muscle glycogen concentrations were greater at 240 min of recovery following NZBC extract supplementation compared to placebo. It could therefore be hypothesised that the mechanism of action by which NZBC appears to augment glycogen synthesis rates could be due to upregulation of insulin-dependent glucose transport or via the regulation of intracellular signalling of proteins associated with glycogen synthesis. For example, and as discussed in chapter 2, anthocyanins have been shown to upregulate the PI3K-Akt pathway which leads to GLUT4 translocation (Prasad et al. 2010; Inaguma et al. 2006; Martineau et al. 2006), as well as potentially upregulating enzymes associated with glycogen synthesis (i.e. glycogen synthase) (Yan et al., 2016).

Previously, co-ingestion of carbohydrates with protein and caffeine are two promising nutritional strategies which have been shown to augment glycogen synthesis in response to sub-optimal carbohydrate (Pedersen et al., 2008; Beelen et al. 2012) the same dose of carbohydrate as used in the present study (0.8 $g.kg^{-1}$ $.h^{-1}$) with protein and caffeine has resulted in glycogen synthesis rates of 35 and 57 mmol.kg $dw^{-1}.h^{-1}$, respectively, when employing a similar exhaustive exercise protocol to our experiment (van Loon et al. 2008; Pedersen et al. 2008). Our findings show NZBC to elicit glycogen synthesis rates of 47 mmol.kg $dw^{-1}.h^{-1}$, which is comparable to the previously mentioned studies. An important finding from our study is that in response to a sub-optimal dose of carbohydrate, we demonstrated 7 days of NZBC intake to lead to glycogen synthesis rates comparable to those previously reported in response to larger, optimal carbohydrate doses (i.e. carbohydrate dose of 1 to 1.8 $g.kg^{-1}.h^{-1}$ resulting in glycogen synthesis rates of 40-45 mmol.kg $dw^{-1}.h^{-1}$; see table 3 in chapter 1). Notably, the placebo condition led to glycogen synthesis rates of 42$mmol.kg^{-1}$ $dw.h^{-}$

[1], which is comparable to rates seen in response to optimal carbohydrate provision and higher than the rates seen in earlier studies when carbohydrate was co-ingested with protein (van Loon et al. 2008). However, the study from van Loon and colleagues (2008) used a 5-hour feeding period, which may have attenuated mean glycogen synthesis rates, as glycogen synthesis is known to slow over time following exercise. Nevertheless, it appears that glycogen synthesis rates can vary quite drastically and there are several possible reasons for this, such as the training status and metabolic health of the participants, as well as considering the specific sources of carbohydrate (e.g., single or dual sources, glucose, fructose, maltodextrin etc.) which could influence its metabolic response. In addition, the starting concentration of glycogen could influence glycogen storage. In thus study, the glycogen values in the first biopsy were relatively low, when compared to other studies (table 2.3). The lower values would mean a greater capacity for post-exercise glucose uptake and storage, which could lead to greater glycogen storage concentrations. The present study progresses an array of earlier cell line and rodent studies (Edirisinghe et al 2011; Inaguma et al. 2006) into humans, as well as considering many practical variables that are transferable to real life. As outlined in chapter 2, this study preliminary findings and further work is warranted to further explore the effect of NZBC supplementation on glycogen synthesis, but also the mechanisms that regulate the process. For example, if studies continue to show promising data regarding NZBC augmenting glycogen synthesis, what is the meaningful effect of this? For example, does the additional glycogen that NZBC supplementation elicits, translate to a recovery or performance effect? Furthermore, as a supplement receiving growing attention in athletic use for adaptations to substrate utilisation and health benefits, proper guidelines should be

established to determine protocols of use for athletes to provoke the desired effect (i.e. what time of season, dosing strategy, and considerations for different outcomes).

Chapter 3: **Increased colocalization of SNAP23 with the plasma membrane in lean skeletal muscle, compared to individuals with type 2 diabetes: Implications for SNAP23 high jacking hypothesis.**

Chapter 2 explored the athletic phenotype and how to optimise post exercise recovery through nutritional supplementation. To the contrary, chapter 3 of this thesis included the exploration of different populations, such as lean and individuals with type 2 diabetes. Type 2 diabetes is a metabolic disorder, characterised by dysfunctional endocrine and metabolic responses to increases in blood glucose in response to a meal and most notably characterised by impaired insulin sensitivity (Lebovits., 2001). As a result, following carbohydrate consumption, individuals with T2D have attenuated insulin secretion from the pancreas, which in healthy conditions, is a key process that regulates blood glucose homeostasis by initiating signalling cascades that upregulate blood glucose clearance (DeFronzo and Tripathy., 2009; Warram et al., 1990). The development of T2D is only partially understood, and the current consensus appears to agree that the accumulation of IMTG is partly responsible (Pan et al. 1997). Importantly however, the athletic phenotype is also associated with high IMTG concentrations (Goodpaster et al. 2001, but it appears that the excessive energy intake coupled with physical inactivity leads to harmful lipid metabolites to accompany IMTG in insulin resistant individuals (Park and Seo, 2020). Furthermore, the morphology of the LDs themselves appear to be different between insulin resistant and athletic individuals, in that, insulin resistant individuals have much larger LD (He

et al., 2004b). A combination of IMTG accumulation and low lipolytic flux, causes LD to grow and they can do this via fusion with other LDs. One proposed mechanism for this, is that SNARE proteins, which are found in abundance on cell and organelle membranes, mediate this fusion process, causing them to grow (Bostrom et al. 2007). A particular SNARE protein of interest is SNAP23, which in healthy populations, has previously been found to be localised predominantly at the plasma membrane and it is thought to facilitate GLUT4 fusion with the plasma membrane to allow for glucose uptake into the intracellular space (Foster et al. 1999; Kawanishi et al. 2000). However, it has been suggested that the LD growth requires SNAP23 to mediate the LD fusion processes. But because the cell only has a finite amount of SNAP23, the LD must acquire SNAP23 from the plasma membrane, which would reduce the abundance of the protein at the membrane regions and therefore potentially reduce the ability for GLUT4 to fuse to the plasma membrane and inhibit its ability to uptake glucose (Bostrom et al. 2007). The purpose of chapter 3 was to explore this process and visualise this hypothesis.

Using fluorescence microscopy, we compared skeletal muscle cells of active males with males who have T2D. More specifically, we labelled the muscle samples with antibodies and dyes to detect SNAP23, lipid droplets, plasma membrane and mitochondria. This technique allowed for colocalization analyses between the labelled proteins. Our data showed that in lean individuals, SNAP23 had (expectedly) greater colocalization with the plasma membrane compared to individuals with T2D. Lastly, there was no difference in colocalization of SNAP23 with LDs and mitochondria between populations.

These findings are that using a (semi) quantitative method, we have provided data in humans in an area which previously, has been heavily performed in rodent or cell

models (Foster et al. 1999; Kawanishi et al. 2000; Bostrom et al. 2007). In conjunction with previous experiments, we believe that our data contributes to the body of literature proposing the SNAP23 hijacking hypothesis. . The questions that surround the development of T2D are sophisticated and multifaceted. However, if interpreted with caution, our data can contribute to this knowledge. The significance of our findings show that individuals have reduced SNAP23 at the plasma membrane and could support components of the previously proposed hypothesis that LD can 'highjack' SNAP23 from the plasma membrane to facilitate LD fusion and in turn, reduce the cells ability to uptake glucose via GLUT4 channelling (Bostrom et al. 2007). However, as outlined in this chapter, more work is required and this work should focus on firstly, clarifying this response and secondly, exploring the mechanisms the regulate this potential process.

Future Directions

The contents of this MPhil include two studies that progress the field in the prospective areas. Firstly, Chapter 2 presents novel data with the application of NZBC extract in an unexplored area. The data presents promising results for the application of this supplement and post-exercise glycogen synthesis. Currently, our blood glucose and skeletal muscle glycogen data indicate that upon carbohydrate feeding, plasma glucose concentrations are similar for NZBC and placebo conditions. However, it appears that once in the muscle, across four hours of carbohydrate feeding, NZBC supplementation appears to elicit an intracellular response that upregulates glycogen synthesis. No study, to our knowledge, has explored the intracellular metabolic responses to NZBC supplementation when subsequently fed with carbohydrate. More

specifically, in regards to carbohydrate metabolism, the protein and enzymatic responses to carbohydrate feeding when supplementing with NZBC extract are not known. As our study suggests the effect of NZBC supplementation could be virtue of an upregulation of skeletal muscle glycogen synthesis, we wish to explore proteins involved in this process. Previously, mulberry extract supplementation in vivo in rodents has been shown to also augment glycogen synthesis and the mechanisms that have been attributed to evoke this response are via upregulation of Akt phosphorylation and glycogen synthase kinase-3β (GSK3β) activation (Yan et al., 2016). Meanwhile, enzymes associated with glycolysis and glucose oxidation, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were downregulated, which together, would drive glycogen synthesis and inhibit glycogen breakdown. Although mulberries contain different blend of anthocyanin compounds, it is currently the only study to compare to and lends potential reasoning for our results. As a result, our aim is to explore similar protein responses to carbohydrate feeding, but instead, test this for NZBC supplementation and in response to carbohydrate feeding following exhaustive exercise. Furthermore, a logical next step would be to also examine the response of GLUT4 to NZBC supplementation, when fed with carbohydrate. To do this, researchers could employ immunofluorescence to assess GLUT4 translocation following a meal, using the methodology previously employed by Bradley et al (2015). Upon publication, this study could initiate further interest for the application of this supplement in an exercise recovery context.

The second study, presented in chapter 3, also provides some novel data and begins to answer questions that are long overdue. Previous work in the area began to develop an understanding of SNAP23s potential involvement in glucose uptake, as well as it's

response to lipid oversupply and insulin resistance (Foster et al. 1999; Kawanishi et al. 2000). However, due to a retracted publication (Bostrom et al. 2010), as well as little research in the area, the topic has struggled to evolve. It appears that SNAP23 localisation can change in response to the metabolic conditions of its environment. Should lipid accumulation be combined with the accumulation of lipid metabolites, as well as physical inactivity and low lipolytic flux, LD appear to grow in size and SNAP23 could have a role to play in this process. A natural comparison to this study is when exploring the paradoxical relationship between IMTGs and insulin sensitivity. Goodpaster et al (2001), showed that despite well trained individuals having high insulin sensitivity and individuals with T2D having low insulin sensitivity, they have comparable IMTG concentrations. This was an important study in establishing that unlike previously thought, IMTG accumulation cannot directly cause insulin resistance. Rather, it has since been considered that large LDs could be more indicative of insulin resistance (He et al. 2004; Barrett et al 2022), compared to small LD seen in well trained individuals (Goodpaster et al. 2001). Naturally, as our study explores how IMTG effects SNAP23 colocalization with IMTG (LDs), plasma membrane and mitochondria, a logical future study would be to explore well trained participants, as seen in the athletes paradox. For example, do the smaller LD in athletes correlate with less demand for SNAP23 for LD fusion and if so, what specifically determines changes in LD size vs. number. Furthermore, the application of additional techniques would advance our findings. For example, using immunofluorescence microscopy, measuring IMTG and mitochondrial content and size could provide explanation for the colocalization data. Such as, the comparable colocalization values for SNAP23 and mitochondrial between the groups, could be explained by the training adaptation that augments mitochondrial content, which could dilute the affinity of SNAP23 with

mitochondria, because there is potentially greater mitochondria content. Another valuable assessment would be to detect the size of IMTGs, using immunofluorescence microscopy image analysis, as this is key for the proposed SNAP23 high jacking hypothesis that proposes that LD growth requires SNAP23 to do so. Lastly, the implications for the proposed SNAP23 high-jacking hypothesis, is that reduced SNAP23 at the plasma membrane could reduce GLUT4's ability to dock at the plasma membrane, thus reducing glucose uptake. Therefore, glucose uptake must also be measured to supplement the confocal analysis previously listed, as this would provide the significance behind the potential SNAP23 high jacking hypothesis in T2D.

Final conclusions

To conclude, this thesis has explored the overarching theme of carbohydrate metabolism, with particular interest surrounding the fate of glucose once in skeletal muscle cells. The thesis has considered how skeletal muscle changes in response to health and metabolic disease, such as trained and physically active individuals, as well as individuals with metabolic disorders, such as T2D.

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