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DOCTORAL THESIS

Whole-body insulin sensitivity and changes in skeletal muscle insulin signalling in response to protein ingestion and exercise

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Whole-body insulin sensitivity and changes in skeletal muscle insulin signalling in response to protein ingestion and exercise

by

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A thesis submitted in partial fulfilment with the requirements for the degree of PhD

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Abstract

This thesis investigated the whole-body and local changes in insulin sensitivity in response to protein ingestion with and without resistance exercise training, in individuals both with obesity (Chapter 3) and those with healthy weight (Chapter 4).

Chapter 1 serves as an introduction to the main theme of the thesis, while Chapter 2, delves into the potential mechanisms through which dietary amino acids can modulate insulin signalling. In Chapter 3, we investigated the immediate effects of various protein interventions: moderate protein (50g), high protein (100g), and moderate protein with added fat, serving as a control group. This research demonstrates a decrease in IP6K1 protein content (p=0.048) following the consumption of a high-protein meal. This reduction hints at a possible association with a reduction in insulin resistance. Notably, this IP6K1 decline coincides with an increase in the ratio of phosphorylated Akt^{Thr308} to total Akt2 activity (p=0.046). Nevertheless, despite these protein-related changes, no discernible alterations in insulin sensitivity or glucose effectiveness during the IVGTT were observed.

In Chapter 4, our focus shifts towards the extended effects of a 10-week protein supplementation regimen, both with and without concurrent exercise interventions. The combined approach of exercise and protein supplementation resulted in a reduction in muscle IP6K1 content. Conversely, exercise without protein supplementation led to an increase in muscle IP6K1 compared to the exercised muscle, accompanied by elevated fasting insulin (p=0.039) and HOMA-2IR (p=0.004) post-exercise training in this group. Additionally, protein supplementation had some additional adverse effects in the non-

exercised leg, including a reduction in the ratio of phosphorylated Akt^{Ser473} to total Akt2 activity (p=0.046).

In summary, our findings indicate that high dietary protein intake leads to notable improvements in IP6K1 content acutely. However, for long-term benefits, our research suggests that protein supplementation is most effective when integrated with exercise.

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List of Abbreviations

Abbreviation	Definition
3-HIB	3-hydroxy-isobutyrate
AA	Amino acids
AIR_g	Acute insulin response to glucose
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AS160	Akt substrate of 160 kDa
ATP	Adenosine triphosphate
AU	Arbitrary units
BAIBA	β-Aminoisobutyric acid
BCAA	Branched-chain amino acid
BCKDC	Branched-chained alpha-keto acid dehydrogenase complex
BCKDK	Branched-chain α-ketoacid dehydrogenase kinase
BMI	Body mass index
BSA	Bovine serum albumin
DASH	Dietary Approaches to Stop Hypertension
DI	Disposition index
DKA	Diabetic ketoacidosis
EDTA	Ethylenediaminetetraacetic acid anticoagulant
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FBR	Fractional breakdown rate

FFA	Free fatty acids
FOXO1	Forkhead Box O1
FSR	Fractional synthesis rate
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLP1-RA	Glucagon-like peptide-1 receptor agonists
GLUT	Glucose transporter
GTP	Guanosine-5'-triphosphate
HbA1c	Glycated haemoglobin
HOMA β%	Percent β-cell function
HOMA2-IR	Homeostasis model assessment of insulin resistance index 2
HP	High protein
HPD	High protein dose
iAUC	Incremental area under the curve
IGF	Insulin-like growth factor
IL	Interleukin
IP6K1	Inositol hexakisphosphate kinase 1
IR	Insulin resistance
IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate-1
IV	Intravenous
IVGTT	Intravenous glucose tolerance test
LDL	Low-density lipoprotein
MBCI	Modified β cell index
ml	Mililitres
mmol/l	Milimoles per litre
MPD	Moderate protein dose
MPDAF	Moderate protein dose and added fat
MRM	Multiple reaction monitoring

mTORC1	Mechanistic target of rapamycin complex 1
NGT	Normal glucose tolerance
NO	Nitric oxide
PAD	Peripheral arterial disease
PDK-1	Pyruvate dehydrogenase kinase
PI3K	Phosphatidyl inositol 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein kinase C
PLA	Placebo
PLC	Enzyme phospholipase C
PP	Pancreatic polypeptide
PP2A	Protein phosphatase-2A
PPB	Protein-polyphenol beverage
R_a	Basal rate of appearance
Rac1	Ras-related C3 botulinum toxin substrate1
RCT	Randomised controlled trials
R_d	Rate of disappearance
RDA	Recommended dietary allowance
RET	Resistance exercise training
RMR	Resting metabolic rate
ROS	Reactive oxygen species
RPM	Revolutions per minute
RYGB	Roux-en-Y gastric bypass
S6K1	Ribosomal protein S6 kinase beta-1
SEM	Standard error of mean
Ser	Serine
S_G	Glucose effectiveness
SGLT1	Sodium-glucose linked transports 1
SH2	Src homology 2
S_I	Insulin sensitivity

T2DM	Type 2 diabetes *mellitus*
TBS-T	Tris-buffered saline-Tween
Thr	Threonine
TNFα	Tumour necrosis factor α
UHPLC-MS/MS	Ultra-high-performance liquid chromatography-tandem mass spectrometry
v-ATPase	Vacuolar H^+ adenosine triphosphatase
WHO	World Health Organisation

Chapter 1. General introduction

1.1 Regulation of glucose homeostasis in a healthy state

Glucose homeostasis is a complex process that ensures stable levels of glucose in the blood, despite variations in nutrient intake and energy demands throughout the day. The maintenance of glucose homeostasis is critical for proper physiological function and is regulated by multiple mechanisms that involve hormonal and neural signalling pathways. The pancreas, located within the left upper abdominal cavity, behind the stomach, has important regulatory functions in glucose hemostasias with implications in macronutrient digestion and energy metabolism and homeostasis through its exocrine and endocrine functions (Chandra & Liddle, 2009). The exocrine cells of the pancreas (acinar cells) secrete digestive enzymes such as amylase, trypsinogen and pancreatic lipase and make up the majority of the pancreatic cells. The endocrine cells form the islets of Langerhans, which are cluster-like formations within the exocrine pancreatic tissue, accounting for only 1-2 % of the entire pancreas. Within the islets of Langerhans there are five different known types of cells that release a variety of hormones: α-cells secreting glucagon; β-cells secreting insulin, C-peptide and amylin (Brissova et al., 2005); γ-cells secreting pancreatic polypeptide (PP) (Śliwińska-Mossoń et al., 2017); δ-cells secreting somatostatin and ε-cells that secrete ghrelin (Wierup et al., 2002). Briefly, glucagon's role is to preserve a decline in blood glucose concentrations levels during fasting periods, while insulin's is to decrease them postprandially (Göke, 2008), somatostatin is a glucagon and insulin inhibitor (Hauge-Evans et al., 2009) and PP has functions in the general regulation of endocrine and exocrine functions of the pancreas (Katsuura et al., 2002), all of them regulating glucose homeostasis. For the purpose of this thesis, the focus will be on the functions of the β-cells and insulin secretion.

Maintaining blood glucose concentrations between the narrow range of 4-6 mmol, also referred to as glucose homeostasis, is accomplished through a complex interplay of various physiological mechanisms and organs in the body. The pancreas plays a crucial role in glucose homeostasis through its opposing, yet balanced secretions of insulin and glucagon. During prolonged fasting time, such as sleep or between meals, blood glucose concentrations drop and glucagon is released into the blood stream to promote hepatic glycogenolysis leading to increased blood glucose concentrations (Freychet et al., 1988; Sanvictores et al., 2021). Moreover, during extended periods of fasting when glycogen stores become exhausted, the body increases gluconeogenesis, a metabolic process that involves the synthesis of glucose from diverse substrates such as amino acids, lactate, glycerol, and pyruvate. This intricate mechanism ensures the continuous production of glucose to sustain vital physiological functions in the absence of dietary glucose intake (Rui, 2014) . On the contrast, following a meal, during increased exogenous blood glucose concentrations, insulin is secreted enabling uptake of glucose into muscle or adipose tissue resulting in removing some of the exogenous glucose from the blood stream, hence lowering its level (Khan & Pessin, 2002), as illustrated in **Figure 1.1.**

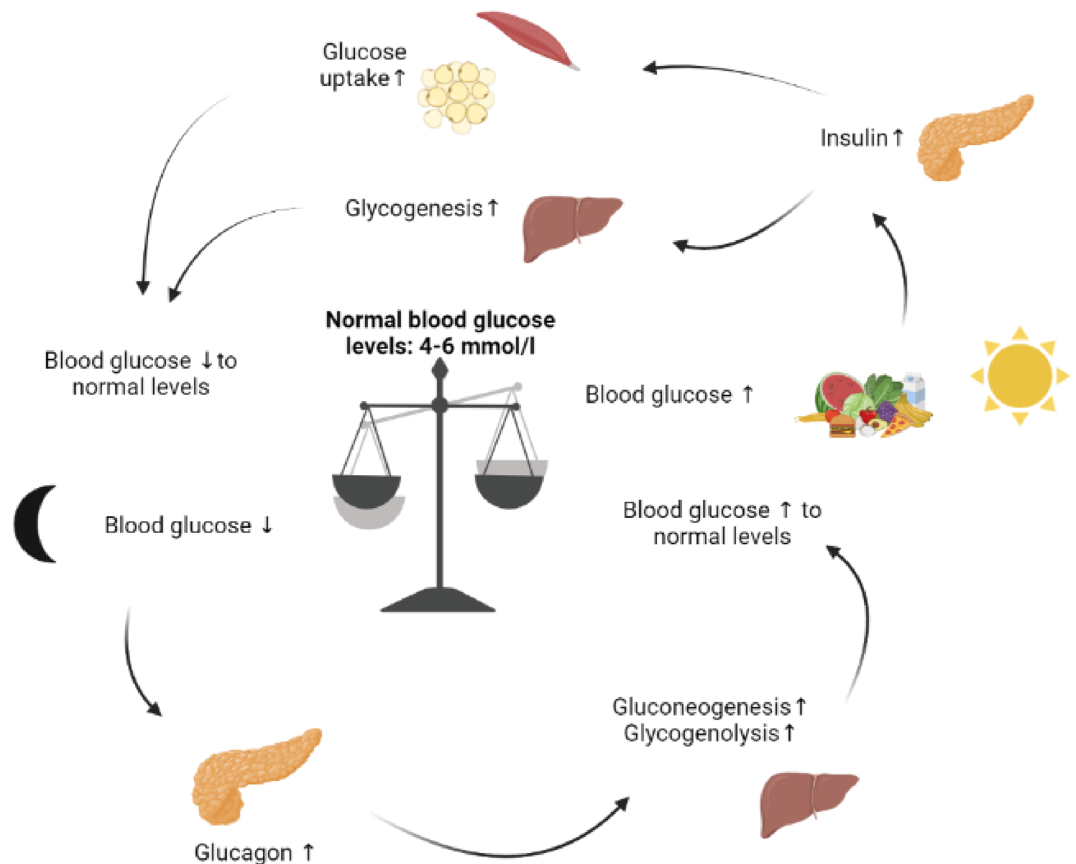

Figure 1.1 Homeostasis of blood glucose levels by the actions on insulin and glucagon. During the night, or fasting times, blood glucose levels are low, the pancreas secretes glucagon, leading to increased blood glucose concentrations, whereas after a meal, the pancreas secretes insulin, which lowers blood glucose concentrations. Figure adapted from (Röder et al., 2016)

The passage of glucose across the plasma membrane is a tightly regulated process mediated by specific carrier proteins. These transporters can be classified into two main groups, namely, the facilitative glucose transporters (GLUTs) and the sodium-glucose linked transports 1 (SGLT1). The SGLT1 transporters are found in the small intestine and exhibit a greater affinity for fructose in the absence of sodium. However, in the presence

of sodium, the transporters switch to a sodium-dependent mechanism, increasing their affinity for glucose and facilitating its transport against its concentration gradient. In contrast, the GLUTs accelerate glucose transport down its concentration gradient (Rea & James, 1997). The presence of these glucose carriers is crucial for maintaining glucose homeostasis, and any disruption in their function can lead to metabolic disorders such as diabetes mellitus (Sinacore & Gulve, 1993).

Skeletal muscle contributes to maintaining normal glycemia levels by removing some of the glucose in the blood in postprandial conditions (DeFronzo et al., 1981) and it has numerous glucose transporters, including GLUT1 and GLUT4. Research conducted in rat skeletal muscle indicates that the majority of glucose carriers are represented by GLUT4, constituting 90-95% of the total number of glucose carriers expressed, while GLUT1 accounts for only 5-10% (Marette et al., 1992). In mouse models, it has been demonstrated that inhibiting GLUT4 with indinavir leads to a significant decrease in basal glucose transport by approximately 60-70% in skeletal muscle, highlighting the predominant role played by GLUT4 in glucose uptake in this tissue (Rudich et al., 2003; Zorzano et al., 2005). Therefore, translocation of GLUT4 to the plasma membrane is the key regulatory mechanism governing glucose uptake. Although this process can be regulated by other factors such as: glucocorticoids, catecholamines or hypoxia, insulin and muscle contractions are the primary stimuli responsible for the mobilisation of GLUT4 from the intracellular storage compartment to the plasma membrane through three different mechanisms (Nesher et al., 1985): 1) increased transport activity due to conformational changes in the cell surface of the transporter, 2) the number of GLUT4 in the plasma membrane is increased and 3) new transporters are synthesised (Hayashi et al., 1997).

Insulin and muscle contraction initiate two distinctive pathways with significant overlap that mediate the glucose transport pathway in the skeletal muscle (Holloszy, 2003), as described into more details in the following sections.

1.1.1 Insulin-induced signalling pathway mediating glucose uptake

In the pancreatic β-cell, insulin is released as a response to elevated blood glucose concentrations following a meal ingestion (Komatsu et al., 2013) and this seems to be the first step in a chain of reactions that promote GLUT4 translocation to the cell membrane leading to glucose uptake in the skeletal muscle. Upon insulin binding to the extracellular α subunit of the insulin receptor, a cascade of events is initiated that results in the autophosphorylation of tyrosine residues in the other subunit of the insulin receptor, the β subunit, that in turn activates a tyrosine kinase intrinsic to this subunit. The activated receptor kinase subsequently phosphorylates non-receptor proteins, such as insulin receptor substrate-1 (IRS-1) (Sun et al., 1991). Tyrosine phosphorylation creates a new binding site on the substrate that has a high affinity for Src homology 2 (SH2) domains, thus recruiting various cellular proteins to the newly created phosphotyrosine site. This recruitment triggers the activation of signalling elements, either directly due to binding or through additional phosphorylation events. The phosphotyrosine-induced recruitment of these signal generators induces independent, parallel signalling pathways that flow into the cell interior (Avruch, 1998). It has been demonstrated that phosphatidyl inositol 3-kinase (PI3K) plays a critical role in intracellular signalling events that lead to GLUT4 mobilisation (Goodyear et al., 1995). The activated PI3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3), which in turn activates proteins including 30-phosphoinositide-dependent kinase-

1 (PDK-1) and Akt (protein kinase B) (Khan & Pessin, 2002). Further on, Akt phosphorylates the AS160 substrate protein, that leads to recruitment of GLUT4 to the cell membrane and therefore finally facilitating glucose uptake by the cell of metabolically active tissues (Saltiel, 2021).

1.1.2 Muscle contraction mediated glucose uptake pathway

The second pathway of glucose uptake is mediated by muscle contraction, which has been deemed to be a more efficient stimulus of glucose uptake in the skeletal muscle than even maximum levels of insulin (James et al., 1985). At present, the molecular signalling mechanisms underlying GLUT4 translocation during muscle contraction remain unclear but there is a general consensus that muscle contractions stimulate GLUT4 translocation via molecular mechanisms distinct from insulin-mediated translocation (Goodyear et al., 1990, 1995; A. D. Lee et al., 1995; Lund et al., 1995; Richter et al., 1985). However, it is worth noting that these two pathways partly converge in their distal parts. Recent studies have identified a number of signalling molecules involved in GLUT4 translocation that are activated by both insulin and muscle contractions, such as TBC1D1 and TBC1D4 (Bruss et al., 2005; Frøsig et al., 2010; Funai & Cartee, 2009; Kramer, Witczak, Fujii, et al., 2006; Kramer, Witczak, Taylor, et al., 2006; Richter et al., 1985) as well as Rac1 (Sylow et al., 2013). Three different TBC1D1 phosphorylation sites have been noticed to be increased following muscle contraction (Ser^{237}, Ser^{660} and Thr^{596}) in an 5’ adenosine monophosphate-activated protein kinase (AMPK) dependent manner (Frøsig et al., 2010; Pehmøller et al., 2009; Vichaiwong et al., 2010). This may suggest that increased AMPK activity may be linked to GLUT4 translocation during muscle contraction. However, in contrast to the association between AMPK activity and GLUT4 translocation during

muscle contraction proposed by some studies, other research suggests a different perspective (McConell, 2020). Specifically, in normal physiological conditions of muscle contractions, it is indicated that while AMPK activation is essential for muscle adaptation following exercise intervention, it may not be a requisite factor for the increased uptake of glucose (Wojtaszewski, Mourtzakis, et al., 2002). These conflicting findings underscore the complexity of the regulatory mechanisms governing GLUT4 translocation and glucose uptake during muscle contraction, prompting a deeper exploration of the intricate interplay between various signaling molecules and physiological conditions.

Apart from the increase in GLUT4 translocation to the sarcolemma and T-tubules during exercise, research studies have also observed coordinated increases in blood flow to the skeletal muscle, as well as capillary recruitment, all of these are important for glucose uptake and oxidation (Richter & Hargreaves, 2013). However, disruptions in the intricate interplay between hormones and peptides in the muscle can result in metabolic disorders, including the widespread and costly type 2 diabetes and its comorbidities, that is described into greater details in the following sections. As such, it is crucial to elucidate and comprehend the mechanisms underlying these interactions to enhance current anti-diabetic treatments and medications, and to develop novel therapeutic strategies.

1.2 Type 2 Diabetes Mellitus - definition and overview

Type 2 diabetes *mellitus* (T2DM) is one of the most common chronic metabolic disorders characterised by high blood glucose levels resulting from impaired insulin secretion by the pancreatic β-cells and inadequate response from insulin-sensitive tissues to insulin production, commonly referred to as insulin resistance (IR) (Roden & Shulman, 2019),

resulting in disturbances in metabolic homeostasis and eventual β-cell failure (Galicia-Garcia et al., 2020). As a consequence, there is a reduction in glucose transport across the cell membrane of the tissues responsible for glucose uptake, namely the liver, muscle cells and adipocyte (Kahn, 1994). Given the increased IR and inadequate levels of insulin production, high blood glucose concentrations, termed hyperglycaemia results. T2DM is the most prevalent type of diabetes, accounting for up to 95% of all diabetes patients (Tripathi & Srivastava, 2006). While individuals suffering from T2DM generally do not require exogenous insulin, unless the blood glucose concentrations are poorly controlled, progression of the condition may result in other complications that detrimentally affect patient quality of life and pose a significant social-economic burden (Zhao et al., 2011).

1.2.1 Complications of diabetes

This chronic condition is associated with a number of short-term and long-term complications (**Figure 1.2**) that have a significant impact on the quality of life of individuals living with T2DM and can even be life-threatening. Acute short-term complications include hypoglycaemia, or dangerously low blood glucose levels, diabetic ketoacidosis (DKA) and hyperglycaemia. DKA is most commonly found in people with type 1 diabetes, but it can also occur in people with type 2 diabetes under certain circumstances. DKA results from a shortage of insulin that causes the body to break down fat for energy instead of glucose, leading to an accumulation of blood acids referred to as ketones in the blood and urine. Symptoms of DKA include excessive thirst, frequent urination, nausea, vomiting, abdominal pain, confusion, and difficulty breathing (Shakoor et al., 2021).

The long-term systemic (chronic) complications of T2DM are generally divided into microvascular and macrovascular, with the former having much prevalence amongst diabetic patients (Deshpande et al., 2008).

Microvascular complications refer to different forms of diabetic neuropathies, including retinopathy, nephropathy and neuropathy, resulting from peripheral and autonomic nervous system damage that affect up to 50% of individuals with diabetes (Patel et al., 2021). These syndromes lead to a range of symptoms, such as numbness and tingling in the feet or hands, pain, and erectile dysfunction (Asmat et al., 2016) as well as increasing the risk of falls and decreasing quality of life (Pop-Busui et al., 2017). The underlying mechanisms of how T2DM targets sensory neurons is still a topic of debate, but it seems to involve retraction of terminal sensory axons in the periphery, with relative preservation in the cell bodies (Patel et al., 2021). There is a characteristic pattern of progression, the damage starts in the longest sensory axons first, leading to the loss of distal leg epidermal axons before the loss in more proximal limbs. Hence, diabetic neuropathy is considered a length-dependent neuropathy. Although this type of nerve damage seen in T2DM is not associated with a demyelinating neuropathy, there is evidence of demyelination in Schwann cells that are targeted by chronic hyperglycaemia, which may lead to alterations in the axons (Dunnigan et al., 2013; Gumy et al., 2008; Mizisin et al., 1998). Various clinical guidelines recommend the use of monofilament and vibration perception tests as screening tools for detecting diabetic peripheral neuropathy (García-Ocaña et al., 2020).

Macrovascular complications include coronary heart disease, heart failure, peripheral arterial disease (PAD) and stroke (Viigimaa et al., 2019). Cardiovascular disease is the leading cause of death in people with type 2 diabetes, and stroke is the most common

cause of disability in this population. Hyperglycaemia and IR are thought to be the leading causes of atherosclerotic changes leading to macrovascular complication in T2DM. Changes in the signalling pathway (described in more details in other sections) lead to release of free fatty acids (FFAs) and inflammatory mediators and increase reactive oxygen species (ROS) production which reduces the production of nitric oxide (NO), an important molecule that maintains the normal function of endothelial cells, therefore leading to atherosclerotic changes (Shulman, 2000). In addition, FFA bind to toll-like receptors which activates nuclear factor NF- κB which further contributes to build up of fatty acids on the blood vessels (Kim et al., 2006).

Furthermore, elevated levels of ROS initiate the activation of protein kinase C (PKC), that can affect the function of other cellular proteins. PKC has been shown to influence permeability, vascular cell growth and apoptosis, cytokine production and extracellular matrix synthesis (Geraldes et al., 2009). Consequently, the activation of PKC alters vascular homeostasis and predisposes individuals to vascular complications. Even more, its activation induces generation of ROS in vascular cells, thereby exacerbating the ongoing pathological cycle (Inoguchi et al., 2000).

Along with atherosclerotic changes, thrombosis is also a key factor in the pathogenesis of macrovascular complications in diabetes. In a normal physiological setting, insulin serves to inhibit thrombosis and increase fibrinolysis. However, insulin resistance leads to a prothrombotic state (Chaudhuri et al., 2004). In addition, insulin deficiency causes calcium accumulation in platelets, thereby promoting platelet aggregation (Vinik et al., 2001), which can further exacerbate the development of cardiovascular disease.

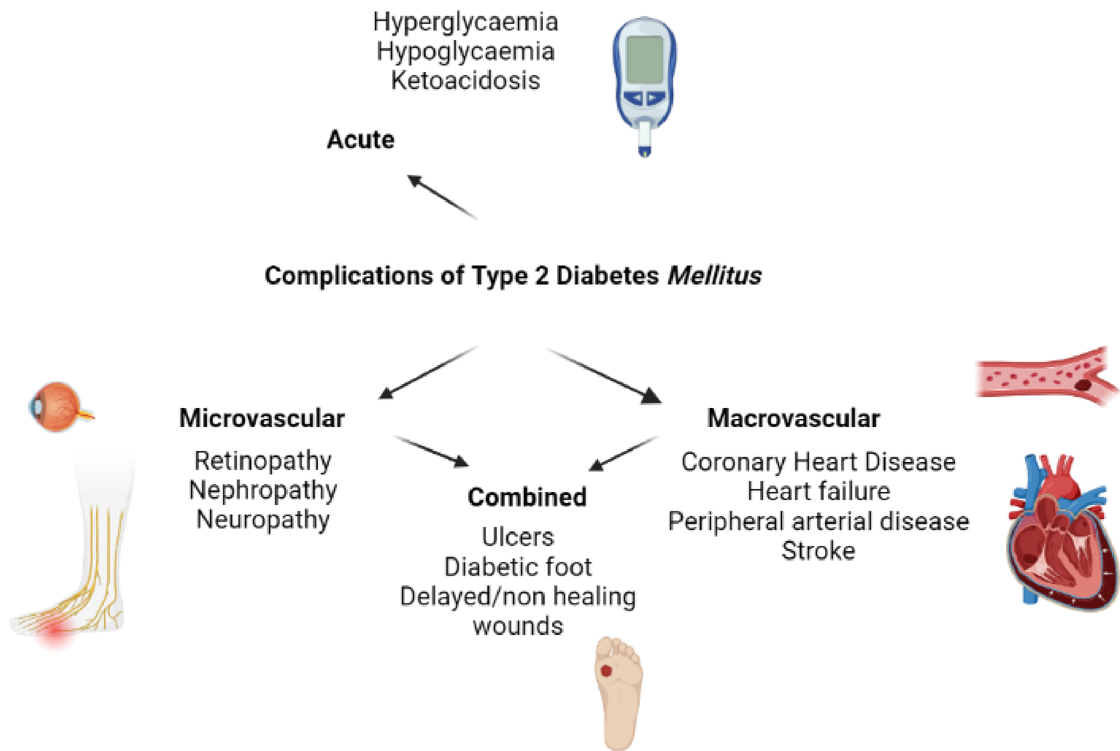

Figure 1.2 Complications of type 2 Diabetes Mellitus

1.2.2 Risk factors for type 2 diabetes

Analysis of statistical epidemiological data has demonstrated that T2DM is as a growing global health problem, impacting both developed countries such as the United States and Italy, as well as developing countries, such as Pakistan and Kuwait (Khan et al., 2020; Wild et al., 2004). However, globally there is a variation in the incidence and prevalence of the condition depending on geographical regions and ethnicity, with South Asians, Hispanics, Native Americans and Japanese populations being at higher risk (Chan et al., 1993; Dabelea et al., 2009; Lin et al., 2020; Liu et al., 2009). No clear factors have been

found for these associations, but an interaction between lifestyle factors, including socioeconomic status, genetic propensity have been proposed to influence its variation.

In addition, researchers have identified a gender difference in diabetes risk. At a global level, men exhibit a higher prevalence of diabetes during younger ages, which has been attributed to factors such as increased skeletal muscle mass, lower adipose tissue, and decreased levels of circulating FFA compared to women. These factors potentially contribute to the development of insulin resistance in women (Mauvais-Jarvis, 2018). However, a shift occurs in diabetes risk after menopause, placing women at a higher susceptibility, likely due to alterations in sexual hormones (Kautzky-Willer et al., 2023). It is widely believed that the underlying causes of gender differences in diabetes risk arise from a combination of genetic predispositions, lifestyle factors, and hormonal changes experienced during pregnancy and menopause, varying across different stages of reproductive life (Mauvais-Jarvis, 2018).

According to the International Diabetes Federation (2021), the global prevalence of diabetes in adults (aged 20-79 years) is currently 537 million, accounting for 10.5% of the world's population in the same age group. This number is projected to reach 643 million by 2030 and to 783 million by 2045. Additionally, the International Diabetes Federation (2021) reported that diabetes and its complications directly caused 6.7 million deaths in 2021. The situation has reached such a severe point that diabetes *mellitus* has been classified as an ‘’epidemic’’ by the Centre for Disease Control and Prevention, making it the only non-infectious disease to have been given the ‘’epidemic status’’ since 2002 (Booth et al., 2002).

Although the precise pathophysiology of diabetes remains unclear, genetic influences are evident from the higher concordance rates observed in monozygotic twins compared to dizygotic twins (Sanghera & Blackett, 2012; Poulsen et al., 2009). Furthermore, the cumulative risk of T2DM is significantly higher in first-degree relatives of T2DM patients than in the general population, indicating a 34% increase in incidence rate (Wu et al., 2014). Environmental factors such as physical activity resources, safety, amenities, walkability, green space and urban sprawl, have been identified as potential influences on lifestyle choices and exposure (Dendup et al., 2018), which may consequently lead to an increased risk of developing diabetes. Lifestyle factors known to be of great importance in the etiology of diabetes include physical inactivity (Hu et al., 2001), sedentary lifestyle (Zimmet et al., 2001), smoking (Manson et al., 2000) and alcohol consumption (Cullmann et al., 2012). These factors are typically cumulative, and, when combined with the primary risk factor associated with T2DM, obesity, can heighten the risk of developing the condition, as suggested by several epidemiological studies (Belkina & Denis, 2010). According to the report published by the World Health Organization (WHO, 2011) in 2011, approximately 90% of individuals diagnosed with T2DM can be attributed to obesity. Although obesity is a multi-faceted trait (Walley et al., 2006) affected by genetic, metagenomic and environmental influences, it is also modifiable (Thaker, 2017). This chapter will discuss (in section 1.3) various interventions that can be employed to reduce body weight by reducing body fat.

1.2.3 The pathophysiology of pre-diabetes to diabetes

Studies have demonstrated that healthy individuals maintain fasting glucose concentrations between 3.9 and 5.6 mmol/L (Abdul-Ghani, 2006) and rarely exceed 3

mmol/L increases post-prandially (Defronzo, 2009). However, with the development of the condition, maintaining homeostasis of glucose concentration becomes challenging. Several studies, which included measurements of glucose, insulin secretion and insulin resistance concluded that the progression from normal glucose tolerance (NGT) to type 2 diabetes is a continuous and progressive process (Ferrannini et al., 2004; Mason et al., 2007; Sattar et al., 2007). The initial stage of this development process is characterised by a compensatory period when IR sets with a concomitant augmentation of insulin production by enlarged ß-cells (Defronzo, 2009). Tabak and colleagues (Tabák et al., 2009) have illustrated that insulin sensitivity may diminish from up to 13 years prior to the emergence of diabetes in some individuals studied, with a steeper decline approximately 5 years pre-diagnosis.

Insulin resistance is characterised by the reduced ability of insulin-targeting tissues, such as the skeletal muscle and adipose tissue, to respond to high physiological insulin levels. While the precise mechanisms of insulin resistance are not yet fully understood, several theories have been proposed. Skeletal muscle, which plays a key role in insulin-stimulated glucose disposal, and the liver and adipose tissue, which are critical sites for glucose-induced insulin signalling, are considered crucial for unravelling the underlying mechanisms responsible for insulin resistance. Notably, as glucose consumption is largely conducted in the skeletal muscle, insulin resistance at this site can significantly impact overall metabolism in the body (Defronzo, 2009). Studies are suggesting that a decrease in insulin-stimulated glucose uptake is attributed to a faulty translocation of GLUT4 to the cell membrane in the skeletal muscle (Lee et al., 2022). Nevertheless, physical activity has been shown to promote GLUT4 translocation via AMPK regulation (O’Neill et al.,

2011; Wojtaszewski et al., 1999), suggesting that the issues may be related to the signalling pathway rather than the transport of GLUT4. Moreover, the activity of IRS-1 associated PI3K activity were found to be reduced in type 2 diabetic patients with insulin resistance in skeletal muscle (Fröjdö et al., 2009; X. Huang et al., 2018), suggesting that the defects may be, but not limited to the proximal level of insulin signalling. In addition to the skeletal muscle IR, the normal function of the liver to control glucose levels postprandially by stimulating deposition of glucose as glycogen, is also affected (Lewis et al., 2021), as noted in T2DM individuals, where hepatic gluconeogenesis is increased (Basu et al., 2005; Krssak et al., 2004). This is associated with defects in transcription factors in the liver, such as Forkhead Box O1 (FOXO1), as well as defects in lipolysis in the adipose tissue due to insulin's inability to complete one of its main functions in the adipose tissue (Rebrin et al., 1996).

The second stage in the progression of the disease is when the β-cells can no longer keep up the compensatory reaction to decreased insulin sensitivity, resulting in fasting and postprandial glucose concentrations that are not maintained in the normal range (Defronzo, 2009; Weir & Bonner-Weir, 2004).

The third stage, referred to as of 'unstable early decompensation period' is when the β-cells are unable to compensate for the level of IR, causing an immediate increase in glucose concentrations (Ferrannini et al., 2004). This phase bridged the gap between prediabetes and overt type 2 diabetes. The last 2 stages of diabetes (stable decompensation and severe decompensation), both associated with overt type 2 diabetes, are outside of the scope of this thesis (Tabák et al., 2012).

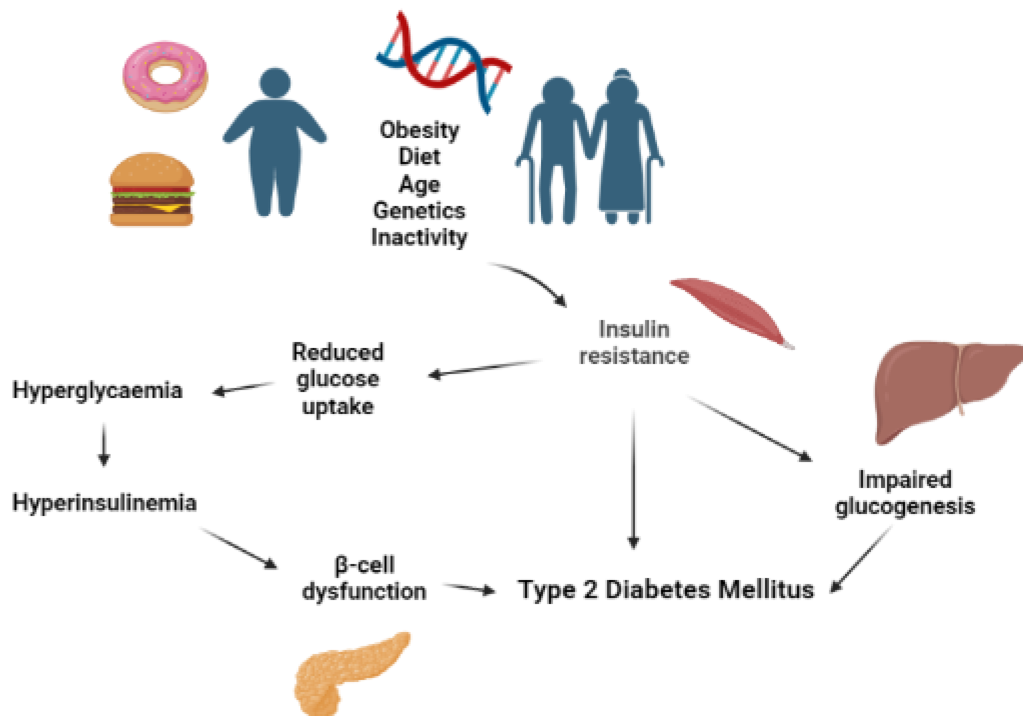

Figure 1.3 Visual representation of the pathophysiology of Type 2 Diabetes *mellitus* including some of the most important risk factors associated with the development of the condition

1.2.4 Diagnosing Type 2 Diabetes Mellitus

The criteria for diagnosing diabetes *mellitus* had been modified since the original guidelines provided by the World Health Organisation in 1985 by the Expert Committee on the diagnosis and Classification of Diabetes (2002). The revised criterion for diagnosing diabetes is presented in **Table 1.1**, yet the gold standard remains the Oral Glucose Tolerance Test (OGTT). This consists of ingesting a 75g anhydrous glucose solution and measuring blood glucose concentrations for 2 h post glucose solution ingestion. A diagnosis of diabetes is made when blood glucose concentrations are ≥11.1 mmol/l (200mg/dl) two hours after ingesting the glucose solution. Similarly, if blood

glucose concentration is between 7.2 mmol/l (130dl/l) and 11.1 mmol/l (200mg/dl) 2h post OGTT, the person will be diagnosed with glucose intolerance and deemed at increased risk of developing T2DM.

Table 1.1 Criteria for diagnosis of diabetes mellitus. Adapted from the expert Committee on the diagnosis and classification of diabetes mellitus, 2002 and supported by World Health Organisation (WHO, 2006)

Criteria
1. Symptoms of diabetes plus random plasma glucose concentrations ≥ 11.1mmol/l (≥200 mg/dl). Symptoms of diabetes include: polydipsia*, polyuria* and/or unexplained weight loss. Random is defined as any time during the day, disregarding the time since the last meal.
OR
2. Fasting* plasma glucose ≥7 mmol/l (≥126 mg/dl).
OR
3. 2h concentration of plasma glucose ≥11.1 mmol/l (≥200 mg/dl) during an OGTT.

Notes: *Polyuria is defined as excessive passage of urine; *Polydipsia as excessive intake of fluids and *Fasting as no caloric intake for at least 8 hours prior to testing.

1.3 Interventions to reduce and manage type 2 diabetes risk and impact

A successful diabetes management is thought to involve a series of interventions based on routine medical care support with individualised goals, life-style modifications, self-management education, mostly depending on the progression level of type 2 diabetes. Due

to the progressive nature of this metabolic condition, acting during the incipient phase of the condition development could significantly reduce the incidence and economic burden of the condition while maintaining quality of life.

Obesity (having a body mass index [BMI] ≥30) is a major risk factor for T2DM and it results from a chronic imbalance between energy intake and energy expenditure (Bellou et al., 2018; Bray et al., 2016; Carey et al., 1997) and several interventions aiming to tackle the progression of diabetes have its core in reducing obesity rates. While the complete mechanisms by which obesity leads to alterations of metabolic functions remains to be fully understood, there are several factors that have been identified as having an important role, involving both inter-organ communication systems, as well as cell-autonomous adaptations. Alterations to the endocrine and metabolic functions of adipose tissue are common in obese individuals and lead to an increased accumulation of inflammatory macrophages in the adipose tissue, which in turn activates pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin (IL)-6. This phenomenon is accompanied by high levels of plasma fatty acids and low lipid turnover (Sam & Mazzone, 2014). The progression of prediabetes to diabetes and the obesity pandemic have been a major concern in recent years, with multiple methods proposed to combat them.

These include the combination of diet and physical activity, alongside behaviour support and medication. This multi-faceted approach is believed to be the most effective means of tackling these issues. Two meta-analyses have demonstrated that the most effective way to halt the progression of the disease, in the context of obesity, is a significant weight loss (Merlotti, Morabito, & Pontiroli, 2014; Merlotti, Morabito, Ceriani, et al., 2014).

The next subsections of this chapter will focus on different interventions that involves a combination of pharmacological and lifestyle modifications associated with improvements in glycaemic control, weight loss and cardiovascular factors that are pertinent in patients with type 2 diabetes. The main focus of the thesis is on the impact of protein supplementation and physical activity in glycaemic control, which is further described in the next section.

1.3.1 Pharmacological interventions

Due to the intricate nature of obesity, resulting from a combination of genetic, metabolic, behavioural, and environmental factors, devising anti-obesity treatments is complex. In recent years, researchers have developed blood glucose lowering drugs, that have also been noted to have anti-obesogenic effects, such as sodium-glucose cotransporter (SGLT)-2 inhibitors and glucagon-like peptide-1 receptor agonists (GLP1-RA). SGLT2 are transporter proteins found in the proximal tubule of the kidneys and in the small intestine and they are responsible for facilitating the reabsorption of approximatively 90% of filtered glucose, functioning independently of insulin (Shubrook et al., 2015; Triplitt & Cornell, 2015). Studies have shown that this combined drug usage resulted in a reduction of approximately 2.8 kg (Bays et al., 2014) or 4.5 kg (Frías et al., 2016; Lundkvist et al., 2017) for individuals with obesity and without a diagnosis of diabetes. This is believed to be due to the effects of SGLT-2 inhibitors on glucose and sodium reabsorption in the kidneys, resulting in glucosuria, eliminating around 60-100g glucose per day in the urine and subsequently lowered plasma glucose concentrations, as well as the effects of GLP1-RAs on satiety. In a study examining the impact of a medication called semaglutide, which belongs to the GLP1-Ras class, on body weight, significant reductions in weight were

observed in the treatment group compared to the placebo group (with a decrease of over 5%). These findings highlight the efficacy of semaglutide as a promising approach in combating obesity (Smith et al., 2022). However, anti-diabetic drugs have also been noted to have pleiotropic effects with impact on cardiovascular disease risk. In addition, SLGT2 inhibitors are noted to change energy metabolism by increasing circulating ketone body levels, lipolysis and circulating hormones (Devenny et al., 2012; Tahara et al., 2013). The resulted increased mobilisation of stored fat was associated with an increase of low-density lipoprotein (LDL) cholesterol, which is known to increase the risk of heart disease and stroke.

Previous attempts to prevent diabetes through the use of pure anti-inflammatory agents have been unsuccessful (Everett et al., 2018). However, it has been acknowledged that metformin, a common drug used in diabetes treatment, has anti-inflammatory characteristics in addition to its pleiotropic effects. Consequently, it has been employed in the prevention and treatment of diabetes and metabolic syndrome (Cameron et al., 2016).

Although the beneficial impact of pharmacological agents on glycaemic control is undisputed, there are several side effects and issues that have been associated with glucose lowering agents that need to be considered by the medical team and patient before the drug therapy is implemented. Some of the side effects associated with metformin are lactic acidosis (frequency of 3 cases in 100.000 patients per year) (Cusi et al., 1996), abdominal discomfort and diarrhoea (Defronzo, 1999). In addition, and perhaps one of the most worrying side effects of glucose lowering therapies is the increased risk of hypoglycaemic episodes when using long-acting sulfonylurea treatment such as glyburide and

chlorpropamide. The proportion of patients that had one or more hypoglycaemic episodes was 17% for glibenclamide and 11% for chlorpropamide compared to 1.2% of patients treated by dietary interventions (Groop et al., 1987). Despite advancements in the management of pharmacotherapy for diabetes, it is important to note that sulfonylureas and meglitinides still carry a significant risk of hypoglycemia (Volke et al., 2022). In fact, the absolute risk of experiencing hypoglycemia is increased by 4-9% when compared to other glucose-lowering agents (Bolen et al., 2007) . Furthermore, when compared to a placebo for pramlintide, the risk of hypoglycemia is two to four times higher (Anderson et al., 2014; PA et al., 2003). The higher incidence in experiencing hypoglycaemic episodes has been attributed to a greater suppression of hepatic glucose production (Anderson et al., 2014). The additional complications associated with glucose controlling medication (anti-diabetic drug therapy), as well as the associated costs, make diet and targeted physical activity regimes the preferred options in managing glycaemic control in people with type 2 diabetes. In addition, the further benefits of associated with increased physical activity levels will be discussed in other sections of the thesis.

1.3.2 Bariatric surgeries

Individuals with obesity and diabetes have more difficulties losing weight and maintaining the reduced-weight state when compared to individuals with obesity only (Guare et al., 1995; Wing et al., 1987). Due to this, there has been an increasing need to find better options beyond the traditional medication treatment. As such, bariatric surgeries became the therapeutic option considered the most effective in achieving significant weight loss and maintenance and T2DM remission, as well as in decreasing the risk of T2DM (Celio & Pories, 2016) in individuals with obesity class III (BMI > 40)

or obesity class II (BMI between 35 and 39.9) with one or more obesity-related health issues (T2DM, hypertension or sleep apnea) (Buchwald et al., 2004) . This has been observed to happen through its pleiotropic effects on lipid metabolism regulation, intestinal physiology, bile acid metabolism, neuronal signalling, microbiome changes and hormone secretion (Buchwald, 2014). The most commonly performed bariatric gastric surgery are Roux-en-Y gastric bypass (RYGB), where a stomach pouch is created and attached to the jejunum through a Roux-en-Y alimentary limb and sleeve gastrectomy (SG) surgery (Ochner et al., 2011). RYGB surgeries have been reported to achieve T2DM remission in over 80% of the patients by 2 years (Buchwald et al., 2009), reducing the need for glycaemic control medication, as well as decrease the risk of diabetes related complications such as cardiovascular diseases and kidney disease (Pories & Dohm, 2012).

On the other hand, bariatric surgeries have been associated with potential risks and negative effects that have lifestyle and health implications. Patients who undergo bariatric surgeries are at risk of developing nutritional deficiencies due to reduced absorption of certain nutrients, which can lead to further complications such as anaemia, neuropathy or osteoporosis (Aills et al., 2008). They are also at risk of dumping syndrome (causing nausea, vomiting, diarrhoea and abdominal cramping) (van Beek et al., 2017), hernias (Jensen et al., 2016), gallstone formation (Shiffman et al., 1991) , as well as emotional and physiological effect that may lead to depression, anxiety, and other mental health problems (Mitchell et al., 2016).

1.3.3 Physical activity and dietary interventions

The prevalence of obesity has been gradually increasing over the last 50 years, and it has been associated with changes in human behaviour and lifestyle and decreased physical activity levels. One of these changes is related to an increased access and consumption of ultra-processed energy-dense foods, especially due to its increased palatability and accessibility (Swinburn et al., 2011). These foods, apart from providing a significant increase in energy intake, have also been inversely and significantly associated with the content of vitamin E, vitamins B12, vitamin D, niacin, iron, magnesium, zinc, selenium and fiber leading to micronutrient deficiencies, which has been associated with an increased risk of developing metabolic conditions (Louzada et al., 2015). Although further research is necessary to establish causation for most micronutrients, existing evidence suggests that deficiencies in vitamin D (Berridge, 2017) and selenium (Huang et al., 2022) are associated with an increased risk of diabetes.

Simultaneously, advancement in technology and urbanisation has led to increases in sedentary behaviour and decreases in physical activity levels, favouring activities performed while sitting or that have low energy expenditure levels, such as driving, working at a desk, or machine based (Dempsey et al., 2020; Marques et al., 2021). Furthermore, regular physical exercise training decreased around the world, with WHO reporting that 1 in 4 adults does not reach the recommended levels of physical activity levels that are 150-300 minutes of moderate-intensity aerobic training, or at least 75-100 minutes of vigorous-intensity exercise training (WHO, 2021).

Physical inactivity has been associated with an increased glucose concentration and exaggerated insulin response to a glucose load more than 45 years ago (Dolkas & Greenleaf, 1977). Evidence that IR develops quickly in the absence of physical activity was presented by Mikines and colleagues that observed an increased insulin response after seven days of bed rest in healthy individuals, which was associated with an attempt of the pancreatic β-cells to overcome peripheral insulin resistance (Mikines et al., 1989). Furthermore, by employing the hyperinsulinemic-euglycaemic clamp method, a study reported a reduction in insulin-mediated glucose disposal after 10 days of bed rest in healthy, well-trained volunteers (King et al., 1988). A subsequent study investigated the insulin-mediated glucose disposal reduction during physical inactivity and associated it with a ~16% decline in skeletal muscle glucose transporter type 4 (GLUT-4) concentration (Tabata et al., 1999). This suggests the profound impacts of physical inactivity (starting from as early as 7-10 days) on the content of GLUT-4 within skeletal muscle and subsequently on its ability to reduce insulin sensitivity in peripheral tissue that play a significant role in the development of T2DM.

Furthermore, physical exercise has been recognised as an essential non-pharmacological intervention effective in prevention and/or management of T2DM. A systematic review and meta-analysis of randomised controlled trials (RCTs) by Colberg and colleagues (Colberg et al., 2010) showed that exercise training improved HbA1c levels, insulin sensitivity, and glucose disposal in individuals with T2DM. The review also suggested that the combination of aerobic and resistance exercise may be more effective in improving glycemic control than either type of exercise alone. Another meta-analysis of

RCTs also reported improvements in HbA1c levels, IR, body weight, and lipid profile following aerobic exercise in people with T2DM (Umpierre et al., 2011).

Physical exercise improves insulin sensitivity by promoting glucose uptake in the skeletal muscle, reducing hepatic glucose production, increasing the number and activity of insulin receptors on the cell surface (Boulé et al., 2005; Perseghin et al., 1996) and increasing glucose transport and delivery to the working muscle via increased capillary perfusion (Sjøberg et al., 2011). Despite decades of research, the full mechanisms by which exercise promotes and induces benefits associates with health, including better glycaemic control, are still considered poorly understood. During exercise there is a profound increase in energy turnover due to energy demand increments and this leads to activation of AMP-activated protein kinase (AMPK), a key enzyme involved in metabolic regulation and adaptive processes of the cell to different energy demand levels by activating or inhibiting its downstream effectors (Spaulding & Yan, 2022). Its activation promotes glucose uptake and fatty acid oxidation (Hardie et al., 2016) and has implications in protein metabolism and other processes such as mitochondrial remodelling and autophagy. In skeletal muscle, AMPK phosphorylates and inhibits TBC1 domain family member 1 (TBC1D1) which activates the RAB-family G protein activity leading to activation and fusion of GLUT 4 to the plasma membrane, increasing glucose uptake (Spaulding & Yan, 2022).

AMPK activation by exercise also leads to alterations in the energy status of the cell and promotes increased gene transcription at the skeletal muscle level (McGee & Hargreaves, 2010). This promotes a cumulative effect of exercise training on increasing mRNA expression, which seem to be essential for intracellular adaptive responses, improving the

cell's function to alter the amount and type of protein. This means that, as an adaptation to contractile or other stimuli, the proteins turn over and synthesis rate increases, making the cells more capable of attaining a new steady state faster than cells that have not been stimulated (Hawley et al., 2014).

Nutritional interventions, including dietary pattern modifications have also been extensively studied due to their effects on diabetes risk and management and while its importance is undisputable, it is still one of the most difficult and controversial aspect in the management of the condition (Forouhi et al., 2018). There are several dietary approaches proposed by researchers to help decrease the risk of developing T2DM or to better control the glycaemic metabolism. One of them is the Mediterranean diet that emphasises the consumption of fruits, vegetables, whole grains, legumes, nuts and olive oil and was shown by a meta-analysis of 19 cohort studies that adherence to it was associated with a 23% decreased risk of the metabolic condition (Martín-Peláez et al., 2020). Another suggested approach is the DASH (Dietary Approaches to Stop Hypertension) eating plan, which apart from positively impacting blood pressure, it was shown to improve IR and hyperlipidaemia. Briefly, it refers to a balanced approach that promotes consumption of fat-free or low-fat dairy products, vegetables, fruits, fish, whole grains, nuts (Campbell, 2017).

High protein diets have also been proposed to reduce the risk of developing type 2 diabetes, especially due to their effect on body composition (Parker et al., 2002). However, the effect of increased protein intake on insulin sensitivity is more controversial and conflicting, requiring further research underpinning the physiological mechanistic

action. As a key component of this thesis, this topic is described into greater details within the next sections.

Chapter 2. Does high dietary protein intake contribute to the increased risk of developing prediabetes and type 2 diabetes?

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2.1 Abstract

Insulin resistance is a complex metabolic disorder implicated in the development of many chronic diseases. While it is generally accepted that body mass loss should be the primary approach for the management of insulin-resistance related disorders in overweight and obese individuals, there is no consensus among researchers regarding optimal protein intake during dietary restriction. Recently, it has been suggested that increased plasma branched-chain amino acid (BCAAs) concentrations are associated with the development of insulin resistance and T2DM. The exact mechanism by which excessive amino acid availability may contribute to insulin resistance has not been fully investigated. However, it has been hypothesised that mammalian target of rapamycin complex 1 (mTORC1) hyperactivation in the presence of amino acid overload contributes to reduced insulin-stimulated glucose uptake due to insulin receptor substrate (IRS) degradation and reduced Akt-AS160 activity. In addition, the long-term effects of high-protein diets on insulin sensitivity during both weight stable and weight loss conditions require more research. This review focusses on the effects of high-protein diets on insulin sensitivity and discusses the potential mechanisms by which dietary amino acids can affect insulin signalling.

Novelty bullets: Excess amino acids may over-activate mTOR, resulting in desensitisation of IRS-1 and reduced insulin-mediated glucose uptake.

Keywords: Type 2 diabetes, insulin resistance, mTOR/S6K1, high-protein diet, branched-chain amino acids.

2.2 Introduction

Dietary protein has a central role within a healthy eating pattern, as evidenced by the fact that protein is the only macronutrient often represented on educational food guides (e.g., USDA's MyPlate) and that a minimal amount in the diet in necessary for meeting the body's growth and regenerative requirements (National Academy of Sciences. The Institute of Medicine. Food and Nutrition Board, 2005). Moreover, the protein content of foods is often a consideration of consumers when shopping at grocery markets (Li & Dando, 2019). As such, it is crucial to not only consider the health benefits of eating a higher proportion of total daily energy intake from protein, but also consider the potential negative consequences on disease risk. From a clinical perspective, Type 2 diabetes (T2D) prevalence is predicted to reach 6.1% of the world's population by 2025 (Stumvoll et al., 2005a) (Stumvoll et al., 2005b) thus placing a major socio-economic burden on health care systems for decades to come. Indeed, T2D is a multifactorial disease characterised by impaired β-cell function, reduced insulin sensitivity, and increased hepatic glucose production (Kahn et al., 2014). There are a number of suggested causes of insulin resistance in skeletal muscle; including hyperinsulinaemia, hyperglycaemia & hyperlipidemia, all of which have been shown to decrease Akt protein activity and induce some form of hyperglycaemia (Chalkley et al., 1998; Frühbeck et al., 2001; Gonzalez-Castillo et al., 2015; Tremblay & Marette, 2001). The resulting hyperglycaemia increases the risk of secondary complications, including macrovascular and microvascular diseases (Stumvoll et al., 2005a). One of the most important lifestyle factors in controlling and preventing T2D is dietary manipulation. Many different protein centric diets have been proposed to improve metabolic health, such as diets high in protein content (>30% of total

energy intake) and low in carbohydrates. The popularity of controversial high-protein diets, such as Zone and Atkins diets, has revealed that high-protein diets may beneficially affect body composition (i.e., higher ratio of lean body mass to fat mass) and weight management by leading to a greater overall body weight loss (Anton et al., 2017). Moreover, high-protein diets have been advocated as an effective dietary intervention to improve insulin sensitivity. While the effect of carbohydrates and fats on glucose metabolism has been thoroughly investigated over the past three decades (Hollenbeck & Coulston, 1991; Jung & Choi, 2017; Kodama et al., 2009), the role of dietary proteins on glucose homeostasis and insulin sensitivity is yet to be established. Overall results from several meta-analyses and systematic reviews have indicated that the effect of acute or chronic high-protein diet on insulin resistance remains inconclusive (Ajala et al., 2013; Dong et al., 2013; Schwingshackl & Hoffmann, 2013; Yu et al., 2019). Moreover, in the long term, high-protein diets are thought to be linked to insulin resistance and increased risk of cardiovascular diseases and cancer (Song et al., 2004). (Sluijs et al., 2010) concluded, based on self-reported dietary assessments, that replacing 5 energy % from carbohydrate or fat with 5 energy % from protein increases diabetes risk by ~30%, but it was left unclear if the potential harmful effects were due to the protein content or other nutrients within the protein meals, such as iron. On the other hand, the Nurses' Health Study suggested that diets high in protein and fat from plant sources were associated with modestly reduced risk of T2D, when compared to animal sources (Halton et al., 2008). Clearly observational data and intervention studies do not reach the same conclusion. It is not clear whether high-protein diets are harmful, neutral, or beneficial for people with T2D. Therefore, this review will explore the mechanisms by which dietary proteins and

amino acids affect insulin resistance and glucose homeostasis, and discuss short-term and long-term effects from physiological and controlled studies.

2.3 High-protein diet and metabolic health

The phrase 'high-protein diet' can often be difficult to interpret as there is no standardized diet definition and simply refers to an eating pattern that emphasizes the consumption of protein dense foods (e.g., ≥1.0 g protein/kg/d or the upper range of the Acceptable Macronutrient Distribution Range at ≥20-35% of total daily energy intake from protein; see Table 1). Moreover, the term 'protein' in itself is non-descriptive and does not take into account that not all protein foods are created equal in terms of their ability to supply dietary requirements of essential amino acids (e.g., protein quality) (Phillips et al., 2015) or the energy intake required to meet the minimal essential amino acid requirement from various protein foods. Specifically, the ingestion of animal-based proteins are more efficient to achieve dietary essential amino acid requirement without the excessive ingestion of non-protein calories (Wolfe et al., 2018). In addition, animal products also vary in terms of their food matrices (nutrient-nutrient interactions), cooking preparation (grilled or deep fried, for example), home cooked vs. fast food, each of which can be manipulated to induce drastically different metabolic effects despite all falling under the same general umbrella of a high-protein diet. For example, on the other hand, plant-based protein sources, such as pulses or nuts, are also high in fibre and phytonutrients that may improve parameters of insulin sensitivity by themselves (Clark et al., 2018; Kim et al., 2017; Weickert & Pfeiffer, 2018). The fat content of a plant or animal based protein diet could also influence insulin sensitivity, as it known that n-6 polyunsaturated fats- the predominant dietary fatty acid being linoleic acid, and saturated fats, such as palmitic

acid, have divergent effects on insulin sensitivity (Bjermo et al., 2012; Summers et al., 2002). In summary, there are many factors that need to be considered when evaluating the effects of a high protein diet on tissue insulin sensitivity. In the next sections we will review the acute and long-term effect of high protein studied in the human literature.

Nonetheless, a number of studies have demonstrated key beneficial effects of eating a higher proportion of total daily energy intake from dietary protein, including fat mass loss coupled with lean mass retention and improved satiety, during energy restriction (Anton et al., 2017; Halton & Hu, 2004). Body mass loss is attributed to a higher satiety, preservation of resting metabolic rate, and perhaps even contribution from a greater thermic effect of high-protein diet compared with low-protein diet (Halton & Hu, 2004). Diet-induced thermogenesis is related to the energy required for the intestinal absorption, metabolism, and food storage during the postprandial period. Hence, a high-protein diet exerts a larger effect on energy expenditure (23-30%) compared with carbohydrates (5%-10%) and lipids (2%-3%) (Nair et al., 1983). This is thought to be explained by the high adenosine triphosphate (ATP) cost for protein synthesis, amino acid oxidation, and urea production (Robinson et al., 1990). Additionally, high-protein diets are linked to increased secretion of incretin peptides, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) which play an important role in postprandial insulin response (Fehse et al., 2005). Rizi and collegues (2018) reported that a high protein (51.4 % energy from protein) compared to a high carbohydrate meal induced a significantly lower postprandial response in ghrelin concentration and higher in GLP-1, which was maintained significantly higher 6 hours after the meal intake. This suggests that an increase in protein density of the meal will promote greater satiety

throughout the postprandial period. These key incretins potentiate glucose disposal by increasing insulin secretion from β-cells. GLP-1 also inhibits gastrointestinal motility and secretion thus augmenting satiety (Edholm et al., 2010). Other studies have shown that ingesting a protein-rich meal also increases GLP-1 and insulin levels leading to reduced postprandial hyperglycaemia and increased satiety (Akhavan et al., 2014; Blom et al., 2006). Numerous studies have shown that high-protein diets may also help to preserve lean body mass during weight loss in overweight and/or obese and T2D individuals (Bell et al., 2006; Larsen et al., 2010; Pignone, 2011) and correct accelerated protein catabolism (Bell et al., 2006; Gougeon et al., 2000). Hence, it is important to focus on 'high quality' weight loss, which is aimed at maintaining adequate skeletal muscle mass with a higher ratio of fat mass loss to ensure long-term weight management and physical performance.

2.4 Branched-chain amino acids and insulin resistance

Certain dietary proteins are more effective at providing target amounts of essential amino acids into circulation than others (animal based > plant based), especially the branched chain amino acids (BCAAs). BCAAs, which include leucine, isoleucine, and valine, are oxidised in peripheral tissues, mainly in skeletal muscle, and have diverse physiological and metabolic roles. In addition to their role as substrates for protein synthesis, BCAAs also act as anabolic signalling molecules, which are involved in signal transduction pathways essential in the regulation of protein synthesis and gene transcription (Dennis et al., 2011). Moreover, BCAAs are involved in lipolysis, lipogenesis, glucose metabolism and glucose transportation (Zhang et al., 2017).

BCAAs have emerged as a dietary component of interest in the development of insulin resistance due to their association with both insulin resistance and T2D (Nie et al., 2018). For example, (Newgard et al., 2009)'s study, using metabolomic approach, reported that obese people have increased catabolism of BCAAs, when compared to healthy weight controls, that was associated with insulin resistance. Similar findings were also reported in a cross-sectional study of subjects with metabolic syndrome (Huffman et al., 2009) and in cohorts of Chinese and Asian-Indian men (Tai et al., 2010). Wang (2011) reported that increased baseline plasma concentrations of BCAA and aromatic amino acids such as phenylalanine, tyrosine and tryptophan were associated with a higher chance of developing diabetes in the future. Likewise, systematic review and meta-analysis summarised current metabolomic studies and concluded that not only BCAA and aromatic amino acids are elevated in pre-diabetics and diabetics, but it is also suggested that elevated plasma BCAA concentrations are linked to higher risk of developing T2D (Guasch-Ferré et al., 2016), which could potentially be used as novel biomarkers. However, not all studies have shown that elevated plasma BCAA profiles are reliable predictors of T2D (Kouw et al., 2015) or insulin resistance (Beals et al., 2016, 2018). Moreover, habitual physical activity levels should be considered when evaluting the impact of blood BCAA concentrations and their link to disease risk.

While many clinical measures have been implicated as risk factors for T2D, including inflammatory cytokines, blood lipids and obesity, it is not known whether elevated plasma BCAAs are related to these factors. (Wang-Sattler et al. 2012) demonstrated that elevated BCAAs were predictive for T2D even after adjustment for BMI, physical activity, smoking and HDL cholesterol, indicating that BCAAs may contribute to insulin

resistance. Conversely, (Mahendran et al. 2017) suggested that high plasma BCAAs concentrations have no causal effect on insulin resistance and that it is the insulin resistance that is a causal factor for increased circulating fasting BCAA concentrations. However, this study was completed using associations between genetic risk scores calculated from fasting insulin levels and genetic variants associated with BCAA levels, without a detailed mechanism that may explain the link between the two. Furthermore, it has been reported that BCAAs clearance (Marchesini et al., 1991) and branched-chained alpha-keto acid dehydrogenase complex (BCKDC) activity are decreased in people with T2D (Adams, 2011). BCKDC catalyses an irreversible step in the catabolism of BCAAs to their respective ketoacids. BCKDC is inhibited by branched-chain α-ketoacid dehydrogenase kinase (BCKDK) and activated by the mitochondrial isoform of protein phosphatase 1K. The activity and the expression of these enzymes are affected in the obese and insulin resistance state thus contributing the BCAAs dysmetabolism (Bajotto et al., 2009).

Karusheva et al suggested that postprandial insulin sensitivity was improved during a 4 week intervention study, where the participants were randomly assigned to either have all BCAA or none in alternate weeks, as measured by mixed meal tolerance test but not during hypeinsulinemic-euglycaemic clamp, which suggests that whole body insulin sensitivity was not affected (Karusheva et al., 2019a). In support of this, 20g of BCAA for 4 weeks did not change insulin concentrations of people with prediabetes (Woo et al., 2019). Therefore, large differences in BCAA concentration might be required to meaningfully alter insulin sensitivity. It is not clear whether diet itself can induce the marked changes in BCAA even observed in these studies. Similarly, chronic consumption

of whey does not increase circulating BCAA in an 8-week weight loss trial (Piccolo et al., 2015), and neither did self-reported reduced or increased dairy protein did not change circulating free AA, including BCAA concentrations after a month (Prodhan et al., 2018). Weight loss itself alters the circulating amino acid profile, which is an added confounding factor (Tochikubo et al., 2016). Therefore, it is possible that while protein foods can increase BCAA acutely, but this effect does not persist over time.

Despite the evidence of elevated BCAAs level in obese and diabetic individuals, it is still not well understood whether high plasma BCAA concentrations are causally implicated in T2D or whether it is a consequence of pathophysiology of the disease. In summary, it is possible that increased level of BCAAs in the insulin-resistant state is a consequence of altered appearance and disappearance rate of BCAAs that is coupled with decreased activity of catabolic enzymes. Nevertheless, it has been proposed that high-protein diet, rich in BCAAs, can contribute to insulin resistance via hyperactivation of mammalian target of rapamycin or mechanistic target of rapamycin (mTOR, also known as RAPT, FRAP, RAFT) (Um et al., 2004). This is discussed in the following sections.

2.5 The role of mTOR in insulin signalling

mTOR is a highly conserved Ser/Thr protein kinase that is a central regulator of various cellular processes, such as cellular metabolism, cell growth and proliferation, protein synthesis, transcription and autophagy (Dobashi et al., 2011). mTOR is inhibited by rapamycin, which is known to induce anti-proliferative and immunosuppressive activities in cells (Janes & Fruman, 2009; Visner et al., 2003). mTOR integrates the input of upstream pathways, such as insulin, growth factors IGF-1, IGF-2 and amino acids. mTOR

assembles into two multi-component complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC2 is less sensitive to rapamycin and its stimulus is not well understood as only growth factors have been shown to induce mTORC2 activity. On the other hand, there is a greater understanding about the function of mTORC1 as it is nutrient sensitive. mTORC1 integrates several intracellular and extracellular signals and plays a key role in the activation of protein translation (Dobashi et al., 2011).

The role of mTORC1 in activating and regulating insulin signalling has been comprehensively investigated. Insulin signalling in skeletal muscle is initiated following binding of the insulin to insulin receptor (IR) that then binds to IR substrate (IRS-1/2). IR is a heterotetrametic bifunctional complex that belongs to the receptor tyrosine kinase superfamily and consists of two extracellular α and two transmembrane β subunits with tyrosine kinase activity (Knudsen et al., 2011). Insulin binding to IR stimulates structural changes to the α subunit that leads to autophosphorylation of a tyrosine kinase. Tyrosine phosphorylation leads to IRS-1 and -2 proteins interacting with the p85-regulatory subunit of phosphoinositide-dependent protein kinase-1 (PI3K). Activation of this enzyme results in the activation of the p110 catalytic subunit of PI3K and production of the second lipid messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 then binds to the pleckstrin homology domain of Akt [Also known as protein kinase B (PKB)] (Mackenzie & Elliott, 2014). Akt is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes, such as cell growth, proliferation, apoptosis, cell migration and glucose metabolism. There are three homologous isoforms of Akt that have been identified – Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ (Manning & Toker, 2017). Among

these isoforms, Akt2 plays a central role in insulin-stimulated glucose uptake in muscle tissues. Binding of PIP_3 to Akt results in conformational change in Akt that is then followed by its phosphorylation at Thr^{308} by PDK1, inducing about 10% of the kinase activity (Manning & Toker, 2017). Subsequently this leads to phosphorylation of specific serine residue (Ser^{473}) of the carboxyl-terminal hydrophobic motif of Akt by mTORC1 (Wick et al., 2000) (Fig. 1). This promotes full Akt activation and its translocation to the cytoplasm, mitochondria and nucleus where it phosphorylates its many substrates. Akt substrate 160 (AS160) is thought to be the most important signalling molecule for glucose uptake. Upon activation, AS160 is phosphorylated at Thr^{642} by Akt leading to reduction in Rab GTPase-activating proteins (Rab-GAP) activity and promoting glucose transporter-4 (GLUT4) translocation (Naufahu et al., 2018a; Sakamoto & Holman, 2008). GLUT4 is a glucose transporter containing 12-transmembrane domains, that is sequestered into specialised intracellular compartments known as GLUT4 storage vesicles (GSVs) in unstimulated cells. In response to insulin stimulation, GLUT4s are translocated from GSVs to the plasma membrane via targeted exocytosis to facilitate glucose uptake in muscle and fat tissue.

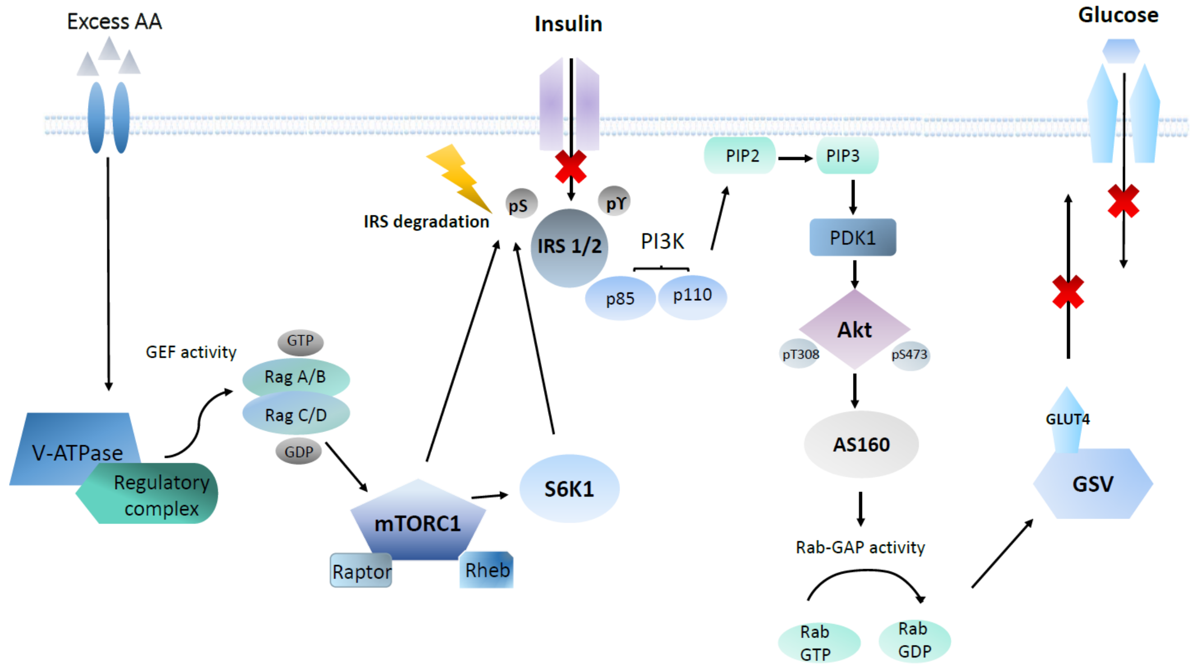

Figure 2.1 Potential mTOR/S6K1 signalling pathway in response to high intake of amino acids. These mechanisms are largely based on experimental models (e.g., *in vitro* and animal models) not commonly experienced in a healthy eating patterns such as meal combinations and protein dense foods.

Abbreviations: AA, amino acids; Akt, serine/threonine protein kinase; AS160, 160 kDa Akt substrate; GDP, Guanosine diphosphate; GEF, guanine nucleotide exchange factors; GLUT4, glucose transporter 4; GSV, GLUT4 storage vesicle; GTP, Guanosine-5'-triphosphate;IRS, insulin receptor substrate; PDK1, phosphoinositide-dependent protein kinase-1; PI3K, class IA phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Rab-GAP, Rab-GTPase-activating protein; Rab-GDP, guanosine-50-diphosphate-loaded Rab;Rab-GTP, guanosine-50-triphosphate-loaded Rab; Rag C/D, Rag heterodimer C/D; RagA/B, Rag heterodimer A/B; Raptor, Regulatory-associated protein of mTOR; S6K1, ribosomal protein S6 kinase beta-1; V-ATPase, vacuolar-type H^+-ATPase.

2.6 Amino acids and insulin resistance: proposed mechanisms

Dietary protein derived amino acids, primarily leucine, are potent activators of mTORC1 (Kimball et al., 2016; Son et al., 2019). Indeed, most of the evidence on the interaction between elevated postprandial dietary amino acid availability in circulation (or intracellularly) and the regulation of mTORC1 activation is based on free amino acid ingestion and/or isolated protein sources (Van Vliet et al., 2019). These methods usually lead to a rapid and transient pattern of aminoacidemia; neither of which are indicative of postprandial blood amino acid profiles after the ingestion of mixed meals or protein dense food sources (e.g., chicken, eggs, beef). In our hands, meal combinations and/or the ingestion of protein dense foods do not elicit such strong activation of mTORC1 or associated downstream targets (i.e., p70S6K or 4E-BP1) (Beals et al. 2016; Sawan et al. 2018; van Vliet et al. 2018; Van Vliet et al. 2019). In any case, based on *in vitro* culture models, intracellular amino acid availability regulates mTORC1 via the RAS-related GTP-binding protein (Rag) family of small GTPases: RagA, RagB (RagA/RagB) and Rag C, Rag D (RagC/RagD) (Han et al., 2012; Zoncu et al., 2011). In the presence of amino acids, regulator complex and vacuolar H^+ adenosine triphosphatase (v-ATPase) undergo conformational change resulting in stimulation of guanine nucleotide exchange factor (GEF) (Willoughby, 2015). GEF promotes GTP-nucleotide charging that activates Rag proteins resulting in the recruitment of mTORC1 to the lysosomal surface where mTORC1 is activated by Rheb (Kim et al., 2013). Activation of mTORC1 by amino acids is essential for many cellular functions, including anabolic process, cell growth and proliferation (Fig. 1). However, it has been hypothesised that mTORC1 hyperactivation, stimulated under non-physiological amino acid overload may contribute to insulin

resistance (Hay & Sonenberg, 2004; Tzatsos & Kandror, 2006; Um et al., 2004). *In vitro* studies have shown that chronic activation of the mTOR/S6K1 pathway by amino acids can promote insulin resistance in muscle cells via increased IRS-1 Ser^{307} phosphorylation and degradation thus leading to the impairment of PI3K stimulation (Haruta et al., 2000; Tremblay & Marette, 2001) (Fig. 1). PI3K is a key effector in insulin's metabolic action via the downstream activation of Akt, which is essential for GLUT4 translocation and glucose uptake. In addition, Um and colleagues reported that hyperactivation of mTORC1 has been shown to drive S6K1-mediated feedback inhibition of insulin signalling, thus reducing glucose uptake in skeletal muscles of obese rodents (Um et al., 2004). Other studies conducted in myotubes and isolated rat skeletal muscles also suggest that leucine may interfere with insulin signalling, given that leucine stimulates mTOR/S6K1 and IRS-1 Ser^{307} phosphorylation (Iwanaka et al., 2010; Tzatsos & Kandror, 2006). Collectively, this research suggests that over-activation of mTORC1 due to amino acid availability may contribute to insulin resistance in muscle. In contrast some research supports an opposing hypothesis that leucine improves insulin sensitivity and glucose control. Macotela (2011) demonstrated that mice fed a high-fat diet showed that, serine phosphorylation of IRS-1 was reduced, despite an increase in phosphorylation of S6K1, with chronic leucine supplementation for 8 weeks. In addition, leucine supplementation correlated with improved insulin sensitivity, a finding that is consistent with studies of obese rodents (Eller et al., 2013; Guo et al., 2010; X. Li et al., 2013). The differences in study design, including the metabolic phenotypes of obese rodents and protein supplementation dose and type, may explain some of the inconsistencies. While amino acids are potent activators of mTORC1, some suggest that amino acid associated activation of mTOR may

not be sufficient to induce insulin resistance (Houde et al., 2010; X. Li et al., 2013). It is possible that BCAAs may elicit different effects on glucose homeostasis, depending on the prevalence of anabolic and catabolic states of the organism (positive or negative energy balance) and thus making it difficult to determine the exact effect of BCAAs on insulin signalling (Bifari & Nisoli, 2017). Further detailed work examining amino acids and insulin resistance is required to determine key mechanistic pathways explaining the role of high-protein diet on glucose homeostasis.

Human studies examining the role of mTOR/S6K1 pathway in T2D are very limited. Bandyopadhyay (2005) reported that people with T2D have greater basal phosphorylation of IRS-1 at Ser307 that corresponded with lower insulin-stimulated IRS-1 tyrosine phosphorylation. Additionally, this study found that diabetics and obese individuals had lower PI3K and Akt activity compared with healthy participants. Similar findings were also reported in studies with prediabetics (Storgaard et al., 2001) and people with T2D (Karlsson et al., 2005; Kim et al., 1999; Krook et al., 2000). However, studies examining the effect of amino acids on mTOR/Akt and insulin resistance in human participants report inconsistent results. The effects of amino acids were shown to be associated with inhibitory phosphorylation of IRS-1 and inactivation of PI3K in healthy human participants (Tzatsos & Kandror, 2006). (Tremblay et al. 2005) studied healthy men and found that intravenous (IV) infusion of amino acids decreased insulin-stimulated glucose disposal, which the authors attributed to elevated IRS-1^{Ser312} phosphorylation and blunted PI3K, while failing to increase insulin-induced phosphorylation of Akt. Conversely, (Bassil et al. 2011) investigated the effects of a hyperinsulinaemic-hyperglycaemic clamp, starting with postabsorptive amino acid concentration then followed by infusion of amino

acids matching postprandial concentration in T2D individuals. This work showed that infusion of amino acids increased phosphorylation of Akt^{Ser473}, Akt^{Thr308} and $mTOR^{Ser2448}$, yet no changes were observed in the phosphorylation of IRS-$1^{Ser636/639}$ and IRS-$1^{Ser1101}$. Additionally, no changes were observed in glucose infusion rates, glucose disposal or in endogenous glucose production with IV amino acid infusion, suggesting that amino acid availability does not alter whole-body glucose control. Yet, with the oral ingestion of leucine and whey proteins, (Smith et al. 2015) showed that the positive effects of a weight loss diet with normal protein intake were blunted by an increase in protein intake, in obese, postmenopausal women. This was reflected in the decrease in glucose disposal and phosphorylation of Akt^{Ser473} when compared to the normal protein diet group. Such a finding is not consistent with the finding that there are no differences in postprandial muscle protein synthesis rates, an mTORC1 mediated event, after the ingestion of a meal-like amount of dietary protein between T2D patients vs. normoglycemic controls (Kouw et al., 2015). Taken together, these data suggest that excessive amino acids availability may contribute to insulin resistance via mechanisms yet to be determined, in humans with similar metabolic characteristics.

2.7 The effect of short and long-term high-protein diets on insulin resistance

The synergistically stimulating effect of protein and carbohydrate co-ingestion on insulin concentration in healthy adults was first reported in the 1960s (Pallotta & Kennedy, 1968; Rabinowitz et al., 1966). Van Loon and collegues (2003) demonstrated that amino acid ingestion with carbohydrates increased plasma insulin response 2-3-fold in patients with T2D. While an increase in the insulin concentration is itself sufficient to cause insulin resistance, this effect requires prolonged hyperinsulinemia (Del Prato et al., 1994).

However, studies examining long-term effect of high protein diets during energy restriction have reported a variety of beneficial effects, including medication reduction, better adherence and improvements in haemoglobin A1c (HbA1c) (Table 1). Most of the studies that have examined the effect of increasing dietary protein have done so in the context of energy restriction. While weight loss itself can improve insulin sensitivity, comparisons with the control group (provided that weight loss was matched) can provide insight into the metabolic effect of high protein on insulin sensitivity. In obese participants, energy restriction and high-protein diet have been shown to be more effective in reducing insulin resistance when compared to low protein diet (Claessens et al., 2009; De Luis et al., 2015; Farnsworth et al., 2003; Mateo-Gallego et al., 2017). On the other hand, some studies suggested that there are no differences between high-carbohydrate and high-protein diets (34% from total energy derived from protein and respectively 1.34 g protein/kg of body weight/day), and beneficial outcomes for insulin sensitivity are attributed to body mass reduction (Campos-Nonato et al., 2017; Noakes et al., 2005). In contrast, (Smith et al. 2016) reported that a high-protein diet (1.2 g protein/kg of body weight/day) may blunt beneficial effects of weight loss and prevent improvements in muscle insulin signalling through adverse effects on the metabolic pathways involved in oxidative stress, without any changes in p-mTOR^{Ser2448} or p-AMPK^{Thr172} in muscle. Similar disagreements about high-protein diet effect on insulin sensitivity are evident in studies with diabetic participants. While Sargrad and collegues (2005) reported no effect of high-protein diets on fasting insulin and glucose levels, (Gannon et al. 2003) demonstrated that high-protein diet decreased HbA1c after 5 weeks, with no associated weight change during the study. The discrepancy may be due to the fact that protein may

primarily affect postprandial insulin and glucose concentrations. It is also worth noting that all the foods within this study were provided by the researchers for the duration of the intervention, which is likely to have increased compliance to dietary protocol. Research that incorporated energy restriction during high-protein diet intervention reported a variety of beneficial outcomes, such as decreased fasting glucose and insulin requirements (Luger et al., 2013) as well as a reduction in HbA1c (Larsen et al., 2011). While high-protein diets are feasible and safe for individuals with T2D, they do not always provide superior long-term metabolic benefit over a standard protein intake during energy restriction. The inconsistent findings may be attributed to the confounding effects of differences in protein sources in diet (e.g. consumption of dairy and meat products versus plant proteins), differences in weight loss, participant characteristics, overall energy balance state and other lifestyle factors often cited in several systematic reviews and meta-analysis (Rietman, Schwarz, Blokker, et al., 2014; Schwingshackl & Hoffmann, 2013). There is also the issue that many long-term trials, which are included within meta-analysis, lack the internal validity required to assess physiological efficacy, unless they provide the intervention foods/diet and/or can objectively assess compliance. Studies that have used oral or IV amino acids can provide mechanistic insight that may overcome some of these limitations, and therefore inform the food-based literature.

Table 2.1 Overview of high-protein diets on insulin resistance in human studies

Participants	Duration	Dietary intervention	Main outcomes	References
Obese and overweight adults	12 weeks	≈30% restriction of total energy HP: 30 % of energy as protein	↔Fasting glucose ↓Fasting insulin ↓iAUC glucose	Farnsworth et al. 2003
Obese females	12 weeks	≈ 1300kcal per day HP: ≈34% of energy as protein	↓Fasting insulin ↓Fasting glucose	Noakes et al. 2005
Obese and overweight adults	12 weeks	500kcal per day for 5 weeks followed by normal ad libidum for 7 weeks HP: 25% of energy as protein	↔HOMA-IR ↔HbA1C	Claessens et al 2009
Obese adults	9 months	1050 cal/day, HP:34% of energy as protein	↓HOMA-IR ↓Fasting insulin ↓Fasting glucose	De Luis et al. 2015
Obese woman	6 months	Calorie restriction to induce 10% weight loss HP: 1.2 g protein/kg/day	↔Glucose R_a and R_d	Smith et al. 2016
Obese and overweight adults	6 months	Caloric restriction of 500 kcal less than the RMR HP: 1.34 g/kg body weight protein intake	↔HOMA-IR ↔HbA1c	Campo-Nonato et al.2017

Obese women	6 months	Caloric deficit of 600 kcal/day HP: 35% of energy as protein	↓HOMA-IR ↔HbA1c	Mateo-Gallego et al. 2017
Adults with T2D	5 weeks	No specific energy restriction HP: 30% of energy as protein	↓iAUC glucose ↔iAUC insulin	Gannon et. al. 2003
Adults with T2D	8 weeks	No specific energy restriction HP: 30% of energy as protein	↔Fasting insulin ↔Fasting glucose	Sargrad et al. 2005
Adults with T2D	12 weeks	No specific energy restriction HP: 30% of energy as protein	↓Fasting glucose ↓Insulin dose	Luger et al. 2013

Abbreviations: Basal glucose rate of appearance (R_a) and glucose rate of disappearance (R_d); HbA1c, glycated haemoglobin; HOMA2-IR, homeostasis model assessment of insulin resistance index 2 to assess insulin resistance; HP, high-protein diet; iAUC, the incremental area under the curve; RMR, resting metabolic rate.

2.8 Mechanistic studies with oral or intravenous amino acids

Manders and collgues (2005) reported that co-ingestion of amino acid mixture with carbohydrates during hyperglycemic clamp resulted in 3-4- fold greater insulin response that improved postprandial glucose disposal and lowered plasma glucose concentration in patients with T2D. However, studies with healthy individuals reported a decrease in whole-body glucose disposal and greater insulin resistance in response to IV amino acid

infusion during euglycaemic-hyperinsulinemic clamp (Flakoll et al., 1992; Krebs et al., 2002; Pisters et al., 1991; Tremblay et al., 2005). It is important to note that during hyperinsulinemic-euglycemic clamp, insulin-mediated decrease in amino acid concentration results in greater glucose disposal compared to when amino acids are maintained at their basal level, and they do not represent normal physiological concentration. As a result, glucose disposal during amino acid IV infusion is compared to a physiological condition where plasma amino acid concentrations are below the baseline levels. This could potentially explain a greater glucose disposal in 'control' compared to amino acid infusion. (Everman et al. 2015) designed a study in which BCAAs concentrations were maintained in the control group in a way that was not different from baseline/ postabsorptive levels during insulin infusion. In this way, they were able to investigate the role of increased plasma BCAA concentration on glucose disposal. The study concluded that short-term increase in plasma BCAAs does not modify insulin sensitivity of glucose metabolism.

2.9 Conclusion

Glucose homeostasis involves a complex interplay and cross-talk between pancreatic β-cells and insulin sensitive peripheral tissues. Amino acids are nutrient signals that can induce a variety of direct and indirect effects at the cellular and organismal level. Despite the potentially favorable effects of high protein intake, controversy exists regarding the findings from short (< 6 month) and long-term interventional studies and the differences in participants' characteristics. Moreover, there is a debate among nutrition experts on what the optimal amount of dietary protein is for those with T2D (Hamdy & Horton, 2011). In contrast, it has been proposed that 'excessive' amino acids may hyperactivate

the mTOR/S6K1 axis, which may present an inhibitory effect on insulin-mediated PI3K/IRS-1 activation, reducing insulin-stimulated glucose uptake and increasing insulin resistance. Indeed, understanding the molecular basis underpinning a macro-nutrient related disease risk is relevant to have a complete understanding of a recommendation providing dietary guidance; however, it is also important to consider the experimental model is not always indicative of a healthy eating pattern and thus dietary context is key for accurate interpretation. It is possible that a high-protein diet may contribute to hyperinsulinemia, yet, as a dietary strategy, it could be helpful for reducing body weight and subsequently increasing insulin sensitivity. Nevertheless, there is a need for more research exploring the effects of high-protein diets on both plasma BCAAs concentrations and intracellular signalling in insulin-sensitive tissue, which could potentially indicate whether high BCAAs concentrations contribute to insulin resistance or are a consequence of the disease.

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Chapter 3. The effects of acute moderate- and high-protein meal ingestion on insulin signalling in skeletal muscle and insulin sensitivity in individuals with obesity

3.1 Introduction

Type 2 diabetes (T2D) is a widespread metabolic disorder characterised by chronic hyperglycaemia, resulting from ineffective insulin activity and defective β-cell function (NICE UK, 2015). This condition has been identified as a leading public health problem, with serious implications for mortality and morbidity around the world (WHO, 2014). Aetiopathology of T2D can be arise from genetic abnormalities and predisposition, epigenetic mechanisms, lifestyle, and environmental factors. Diet and physical activity have been identified as modifiable lifestyle factors that can be used to manage, treat, or postpone the onset of T2D. Nutrition is viewed as a fundamental element in the management of T2D, with implications for glycaemic control and weight management, ultimately helping to prevent or delay complications related to hyperglycaemia (American Diabetes Association, 2016). One of the diet models proposed to manage glycaemic control is high-protein (HP) diets, rich in branched-chain amino acids (BCAAs; i.e., isoleucine, leucine, and valine). Research has demonstrated benefits of HP diets on parameters associated with T2D and obesity, such as body mass, glucose and lipid metabolism, and muscle protein synthesis (Lynch & Adams, 2014). However, whether HP diets can prevent metabolic disease is a highly contentious issue and currently a topic of fervent debate within the scientific community. Notably, a crucial aspect that has drawn attention in this context is the potential relationship between high protein diets and insulin resistance (IR) as described into more details further.

IR is a metabolic state characterised by reduced ability of insulin-responsive tissues to take up glucose despite exposure to physiologically normal concentrations of insulin, and is considered a pathogenic drive in the development of various health issues including

metabolic syndrome and T2D (Lee et al., 2022). The relationship between high concentrations of circulating BCAAs and IR remains unclear, as it is not yet understood whether elevated levels of circulating BCAAs contribute to IR or are a consequence of impaired insulin action and are not a direct consequence of high protein diets. Yet, elevated levels of plasma BCAAs have been linked to IR and could be a predictor of the development of T2D up to 12 years beforehand (Alfaqih et al., 2018; Bloomgarden, 2018; White et al., 2021) and further research is required to confirm or disprove the suitability of high protein diets as a lifestyle intervention to reduce the risk or progression of T2D.

The oxidation of BCAAs and other amino acids follows a similar pathway, although there are some differences in the process. BCAAs are primarily oxidised in peripheral tissues, such as skeletal muscle (Holeček, 2018), and regulated by the availability of amino acids (Brosnan & Brosnan, 2006), whereas other amino acids (AA) can be catabolised in several tissues such as the liver, kidney and intestines and are mainly regulated by the energy state of the cell (Holeček, 2018; Zhang et al., 2007). This means that BCAAs are potent activators of the protein synthesis pathway in peripheral tissue via the activation of mechanistic target of rapamycin complex 1 (mTORC1), in both resting and post-exercised skeletal muscle (Karlsson et al., 2006).

mTORC1 is a serine-threonine protein kinase that acts as a crucial regulator of cell growth and metabolism, including lipid and protein synthesis, energy storage, and mitochondrial biogenesis, and dysregulation of this pathway has been implicated in numerous diseases, including cancer and metabolic conditions (Saxton & Sabatini, 2017). In the presence of persistent hyperaminoacidaemia, overactivation of mTORC1 leads to the phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and the subsequent deactivation of

phosphatidylinositol 3-kinase (PI3K) (Um et al., 2006). PI3K has an essential role in insulin-dependent glucose uptake via the activation of Protein Kinase B (Akt), along with phosphorylation of its downstream target Akt substrate of 160kDa (AS160), which promotes glucose transporter-4 (GLUT-4) translocation to the cell membrane, thereby facilitating glucose disposal by skeletal muscle (R. W. A. Mackenzie & Elliott, 2014). This negative feedback loop on PI3K/Akt/AS160 results in decreased GLUT-4 translocation and glucose uptake, contributing to the pathogenesis of IR and T2D (Tremblay et al., 2005).

Hyperaminoacidaemia is defined as abnormally high concentrations of amino acids in the blood, with the normal concentration range varying by individual amino acid between 50-1000 µmol/L. Hyperaminoacidaemia can occur in several situations, such as in individuals with inherited disorders of amino acid metabolism or consuming high amount of dietary protein (Elango et al., 2012). However, as the amount of protein a person would need to ingest in order to become hyperaminoacidaemic depends upon a range of factors, such as an individual's body mass, activity level, and overall health status, as well as the source and quality of the protein ingested (Brosnan & Brosnan, 2006), it is difficult to have a general definition of what 'high-protein' means. While acute hyperaminoacidaemia is generally considered safe in healthy individuals, it can have various consequences in individuals with obesity, particularly when coupled with IR and other metabolic dysfunctions commonly observed in obesity. *In vitro* study shows that high physiological levels of amino acids inhibit early stages of insulin action in myotube cells decreasing glucose disposal (Patti et al., 1998). A study investigated the effects of chronic hyperaminoacidaemia on glucose metabolism in obese Zucker rats. The authors

found that the hyperaminoacidaemic rats exhibited impaired glucose tolerance, decreased insulin sensitivity, and increased hepatic glucose production compared with control rats (She et al., 2007). *In vivo* human studies also show a decrease in insulin sensitivity during amino acid infusions, especially BCAA (Flakoll et al., 1991; M. M. Robinson et al., 2014). Firstly, it is important to note that intravenous amino acid infusion bypasses the digestive system and the complex hormonal and neural signals involved in nutrient sensing and metabolism regulation that occur during normal dietary protein digestion. Therefore, the metabolic responses observed with intravenous AA infusion may not fully reflect the dietary protein intake. Secondly, the amino acid solution used in the study was a standardised mixture that does not account for differences in AA composition and bioavailability of dietary proteins.

Therefore, in order to overcome some of the AA infusion studies' limitations, this current study aimed to investigate the effects of moderate- (50 g) and high-protein (100 g) meals, composed of chicken breast, on glucose metabolism and proteins involved in the insulin-stimulated glucose uptake pathway and protein synthesis in skeletal muscle in humans with obesity. Comparisons were made between the trials to assess if the high protein dose induced intracellular dysregulation causing a downregulation in insulin signalling when compared with the moderate dose. As the energy content of the high-protein meal was double that of the moderate-protein meal, we also tested the effect of ingesting a moderate-protein meal containing added fat, that matched the energy content of the high-protein meal, to investigate whether differences with the higher protein dose were dietary protein-specific or were attributable to the energy content of the meal. Additionally, the

study aimed to assess the changes in plasma amino acid concentration for up to 4 hours following these meals.

3.2 Material and methods

3.2.1 Study design

This study employed a randomised order, double-blinded design with a 14-day washout period with 3 conditions differentiated by the meal provided. Participants either had a chicken meal containing 50 grams of protein [moderate protein dose (MPD)], 100 grams of protein [high protein dose (HPD)] or 50 grams of protein with added fat [moderate protein dose and added fat (MPDAF)] to match the energy content of the HPD meal.

3.2.2 Ethical approval and subject recruitment

Ethical approval for the experimental design and procedures was granted by the University of Roehampton Ethics Committee (reference number LSC 189/235) prior to the start of data collection. All experimental procedures were conducted in accordance with the World Medical Association's revised declaration of Helsinki for Medical Research Involving Humans (World Health Organisation Declaration of Helsinki, 2000). Participants were recruited through advertisements placed locally and online and through university-wide group emails.

3.2.3 Inclusion/ exclusion criteria for participants

In this study, inclusion and exclusion criteria were defined to identify suitable participants. To be included in the study, individuals needed to meet the following criteria: a non-diabetic state, as assessed by fasting blood glucose concentration below 7 mmol/L

(<126 mg/dL) or random plasma glucose below 11.1 mmol/L (<200 mg/dL); a Body Mass Index (BMI) equal to or greater than 30 kg/m^2; and an age range of 30-65 years.

On the other hand, several exclusion criteria were applied to exclude certain individuals from participation in the study. Exclusion criteria included: pregnant women or those taking hormone contra conception therapy; individuals with secondary complications of metabolic syndrome (such as neuropathy, nephropathy, cardiovascular diseases, stroke, or hypertension); individuals with a known diagnosis of active cancer; current smokers; individuals requiring insulin or any other glycaemia-altering medication; individuals diagnosed with arthritis, rheumatism, or gout spondylitis; and individuals unable to mobilise independently. These criteria were implemented to ensure a specific target population and maintain the safety and validity of the research findings.

3.2.4 Informed consent

A written participant information sheet (available in Appendix A) and familiarisation visits were used to comprehensively inform each volunteer about the objectives, possible risks, and time commitments associated with the present study. The volunteers were also informed that they have the right to withdraw from the study at any time without providing a reason. All information collected during the study was kept on password-protected computers or stored in locked cabinets. All personal data remained pseudoanonymous following data release into the public domain. All volunteers in the study provided written informed consent.

3.2.5 Participants and preliminary measurements

Nine individuals with obesity (n males=5, n females=4) were recruited for participation in this study. Subjects' clinical characteristics are presented in Table 1. Participants were required to attend the Metabolic Investigation Suite (MIS) at the University of Roehampton. The first visit was a screening and familiarisation session in which the volunteers attended the laboratory for around 3 hours. The first hour was used to describe the research study to the participants and gain written informed consent for participation in the study.

This first visit was also used to obtain preliminary data such as body fat percentage and body mass (BodPod, Life Measurement, Inc., Concord, California) as described in (Kosicka et al., 2013) by using non-invasive air displacement plethysmography. Briefly, the subject was wearing minimal clothing, such as tight-fitting shorts and t-shirt, and sat in the Bod Pod which measured the amount of air displaced by the individual sitting in the chamber. Once the body volume was determined, the individual's body density was calculated by dividing the body mass (measured before the test) by body volume. The Bod Pod then calculates body fat percentage using the Siri equation which takes into account age, sex, and body density. The Bod Pod was calibrated on the morning of each test according to the manufacturer's instructions.

Height was measured to the nearest 0.1 cm using a Seca 213 stadiometer with subjects standing barefoot. Blood pressure was monitored using an automated oscillometric blood pressure monitor (Omron Healthcare, Hamburg, Germany).

Capillary whole blood samples were collected for the determination of glycated haemoglobin ($HbA1_c$) values (NycoCard Reader II, Abbott) and glucose concentrations. A small amount of blood (5 µL) was collected from the volunteer's finger using a lancet and fingerstick device. The blood sample was then transferred to a disposable cuvette containing a lyophilized reagent that reacts with $HbA1_c$ in the blood. The cuvette was inserted into the NycoCard Reader II, which automatically measures the HbA1c concentration in the sample using a reflectance photometry method. The NycoCard Reader II was calibrated using quality control procedures as per manufacturer's recommendations.

Blood glucose concentrations were determined using Biosen C-Line (EKF Diagnostics, UK) by placing 20 µL of blood into the pre-filled reaction cups with 1 mL of haemolysis solution. The cup was then closed and gently inverted for mixture of the solution. The sample was inserted into the analyser which then measured the glucose concentration in the solution and provided a value. The Biosen C-Line was calibrated using calibrators and quality control procedures as per manufacturer's recommendations.

Waist and hip circumferences were measured using a measuring tape. The waist was considered the narrowest part of the torso, while the hips were the widest part of the gluteal region. The tape was positioned parallel to the floor without compressing the skin.

For participants involved in the study post start of the Covid-19 pandemic (data collection after March 2020), skin temperature was measured using a non-contact infrared thermometer (Brannan, Fisher Scientific, U.K.).

3.2.6 Experimental protocol

Participants attended the laboratory for the experimental protocol at ~ 9:00 am on three occasions, having fasted for 12 hours prior, with each visit separated by 14-30 days. To allow for administration of glucose solution during the intravenous glucose tolerance test (IVGTT), a cannula was placed into the antecubital vein of one arm. A separate cannula was placed into a dorsal hand vein, in a retrograde direction, for frequent sampling of arterialised-venous blood, achieved through use of a thermoregulated hot box (~ 60°C) (McGuire et al., 1976). Increasing hand temperature allowed arterialised blood (~98% arterialised) to be sampled from a vein without the dangers of direct arterial sampling. This physiological mechanism is explained in a different section (**3.2.7**).

The sampling cannula was connected to a 3-way tap to allow for the administration of sterile saline (0.9% NaCl, Baxter Healthcare Ltd, Thetford, UK) to prevent blockages. A baseline blood sample and a muscle biopsy were collected as described later in the chapter (**3.2.8**. and **3.2.9**).

Following the baseline blood sample and muscle biopsy, participants were provided a chicken meal containing 50 grams of protein, 100 grams of protein or 50 grams of protein with added fat to match the energy content of the HPD meal. The fat that was added in the MPDAF consisted of 25 g of vegetable oil, which was the equivalent of 231 kcal. The software Dietplan 7 (Forestfield Software Ltd, United Kingdom) was used for food composition analysis in order to determine the amount of baked chicken required (**Table 3.1**).

Following the meal, a sterile labelled glucose solution was administered via the antecubital vein cannula. The injection was given over 1-2 minutes and the line was then flushed with 10 mL of saline solution. A total of 25 blood samples (~ 5 mL each) were drawn after the intravenous glucose solution administration at the following time points: 2, 3, 4, 5, 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 60, 70, 80, 100, 120, 140, 160, 180, 210, and 240 min, using a 5-mL syringe (BD Medical, Singapore).

A second muscle biopsy was collected at 240 minutes following the finish of the controlled meal, with participants in a supine position using the conchotome method (Dietrichson et al., 1987), as described into more details in a different section.

3.2.7 Arterialised blood sampling

The concentration of certain metabolites, including glucose, is lower in peripheral venous blood than peripheral arterial blood, as skeletal muscle takes up some of it when the blood passes through the tissue. In order to gain the most accurate measurement of the concentration of hormones and metabolites in peripheral blood, in most cases it is advised that blood be sampled from arteries rather than veins. However, this would involve placing an arterial line, a procedure that can cause discomfort to the participant and increase the risk of harm to them. By exposing the blood sampling arm to temperatures >60°C, the arterioles start to dilate and increase blood flow through the skin to increase heat loss via radiation and convection. This physiological reflex in humans diverts blood along anastomoses and bypasses the tissues that would take up metabolites from arterial blood (McGuire et al., 1976).

3.2.8 Muscle biopsy procedure and lysing

The muscle biopsies were taken from the musculus vastus lateralis by trained individuals deemed competent in this procedure. Briefly, the volunteer lay in a supine position and antiseptic solution (Videne, Ecolab, United Kingdom) was applied on the skin to the sampling site of the thigh. Local anaesthetic (2% lidocaine) was injected in the subcutaneous fat and the fascia (maximum dose of 3 mg/kg or 120 mg lidocaine in total). An approximate 1-cm incision was made with a sterile scalpel. Then a forceps biopsy needle was used to remove a muscle sample from the vastus lateralis. Immediately after collection, muscle samples were washed in ice-cold saline, with visible fat removed before being frozen in liquid nitrogen and transferred to −80°C until analysis.

The lysing buffer was prepared with protease and phosphatase inhibitor cocktail (Thermo Fisher, UK) to preserve protein phosphorylation and added in an Eppendorf tube with the muscle sample and sonicated until the solution was homogenous. The samples were centrifuged at 4°C for 15 min at 6000 RPM (2000 g) and the pellet was separated from the supernatant by pipetting out the supernatant.

3.2.9 Blood centrifugation and storage

All blood samples were immediately dispensed into two different vacutainers; one containing lithium heparin and the other containing ethylenediaminetetraacetic acid (EDTA). The vacutainers were inverted 4-5 times to ensure that the blood was mixed with the additive, then placed on ice until the end of the experimental protocol (max 4 hours). The vacutainers were next centrifuged at 4°C, 6000 RPM (2000 *g*), for 10 minutes and

the resulting plasma was aliquoted into 1.5-mL Eppendorf tubes that were stored at -80°C until further analysis.

3.2.10 Controlled meal preparation

All meals were prepared at the University of Roehampton, Whitelands College in the Food Laboratory. The meals consisted of baked chicken which was prepared on the same day of the trial. The chicken was baked in the oven at 180°C for 30 min and it was blended before served. For the MDPAF trial (50 grams of protein plus fat added to match the energy content of the high-protein trial), vegetable oil was added to the blended chicken until the energy content of the HPD meal was matched.

Table 3.1 Meal composition analysis for the 3 trials completed in the present study

Trial	Protein (g)	Chicken (g)	Added fat (g)	Energy (kcal)
MPD	50	156	0	231
HPD	100	312	0	462
MPDAF	50	156	25	462

Abbreviations: MPD, moderate protein dose; HPD, high protein dose; MPDAF, moderate protein dose and added fat

3.2.11 Infusate preparation for IVGTT

All infusates were prepared on the morning of the trial and administered under sterile conditions. After the powdered stable isotope labelled compound was weighted, it was mixed with 10 mL sterile saline solutions (0.9% NaCl, Baxters, UK) by using a sterile syringe. Once this has fully dissolved, the mixture was passed through a 0.22 µm Millipore filter (Bedford, MA) for infusion. The isotope administration to the volunteers

consisted of powdered labelled glucose; 28.4 mg/kg of body mass [$6,6^2H_2$] Glucose (product code: 389374, D-Glucose$^{-13}C_6$, Sigma-Aldrich, Merck, Darmstadt, Germany) and 250 mg/kg of body mass of unlabelled glucose (50% glucose, Baxters, United Kingdom).

3.2.12 Blood analysis

Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA), (EIA-2935, DRG Instruments GmbH, Germany) with an assay range of 1.76 to 100 μIU/mL and coefficients of variations (CV): the intra-assay CV was 2.6%, and the inter-assay CV was 2.9%. This consists of a 96-well solid-phase two-site enzyme immunoassay based on the direct sandwich technique. 25 μL of each of the six insulin standards (0, 6.25, 12.5, 25, 50, and 100 μIU/mL) were added to the plate in duplicate to produce a calibration curve of concentration against absorbance. Plasma samples were defrosted and vortexed, then 25 μL of each EDTA-plasma sample was added in duplicate to the remaining wells. 25 μL of anti-insulin conjugate (biotin conjugated mouse) was added to each of the wells and then incubated at room temperature for 30 minutes. After the incubation period, the wells were washed 3 times with 350 μL of washing solution that was prepared as per the manufacturer instructions. Following the final wash, the plate was inverted and tapped on absorbent paper to remove any remaining washing solution and then 50 μL of streptavidin peroxidase enzyme complex was added to each well and was incubated for another 30 minutes at room temperature. After the incubation period, the plate was washed as described above and then 50 μL of peroxidase substrate was added to each well. The reaction was stopped 15 minutes later by adding 50 μL of stop solution to each well, creating a yellow colourimetric endpoint. The absorbance

of light was measured within 10 minutes of adding the stop solution - at a wavelength of 450 nm - using a microplate reader (Thermo Scientific Multiscan EX, United Kingdom) and Ascent Software, version 2.6 (ThermoFisher, United Kingdom).

Plasma amino acid concentrations were determined by using Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) technology with the following method. In the experimental procedure, 10 µL of human plasma was transferred to an Eppendorf tube. To this, 10 µL of the Internal Standard (Glycine-2-^{13}C, Sigma-Aldrich; #279439) at a concentration of 30.4 µg/uL was added. Protein precipitation was carried out by mixing the sample with 40 µL of cold isopropanol containing UPLC-MS grade, 1% formic acid (v/v). The mixture was vortexed for 30 seconds and incubated at -20 °C for 20 minutes. Subsequently, the samples were centrifuged at 13,000 g (4°C) for 10 minutes. From the resulting supernatant, 10 µL was used for amino acid derivatisation, as described in the following paragraph.

For the derivatisation step, 1 mL of UPLC-MS grade acetonitrile was added to the AccQTag Ultra reagent powder and mixed by vortexing for 30 seconds. The mixture was then heated at 55 °C to dissolve the reagent. Next, 70 µL of borate buffer was added to the samples, followed by the addition of 20 µL of AccQTag Ultra derivatizing reagent solution. The samples were vortexed for 30 seconds, and the derivatisation reaction was carried out by heating at 55 °C for 10 minutes.

The UHPLC-MS/MS analysis was conducted using an Acquity UPLC binary system (Waters) connected to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters TQ-S Micro). Electrospray ionisation (ESI) in positive ion mode was employed for MS/MS detection using multiple reaction monitoring (MRM) for compound quantification. The

source conditions were set as follows: capillary voltage, 3.5 kV; source offset, 30 V; desolvation temperature, 450 °C; source temperature, 150 °C; desolvation gas flow, 650 L/h; cone gas flow, 150 L/h; nebulizer gas, 7.0 bar; collision gas, 0.15 mL/min.

The chromatographic separation was achieved using reversed-phase gradient chromatography on an ACQUITY UPLC® HSS T3 1.8-µm column (Waters). The mobile phase consisted of 0.1% formic acid in LC-MS grade water (v/v) (A) and 0.1% formic acid in LC-MS grade acetonitrile (v/v) (B). The column temperature was maintained at 45 °C, and a linear gradient elution was performed at a flow rate of 0.65 mL/min. The gradient started with 4% of mobile phase B for 0.5 minutes, followed by an increase to 10% B over 2 minutes, then to 28% B over 2.5 minutes, and finally to 95% B over 1 minute.

3.2.13 Muscle analysis

Muscle tissue homogenates were used for quantification of relative total muscle protein content via Lowry's method (Bio-Rad DC protein assay). Then, standard immunoblotting techniques were used by loading the same amount of total protein (25 µg) into 7.5% precast polyacrylamide gels before being transferred via the semidry method to nitrocellulose membranes (Bio-Rad, United Kingdom).

Membranes were blocked in Tris-buffered saline-Tween (TBS-T)20 containing 2-5% bovine serum albumin (BSA) or dry skimmed milk for one hour at room temperature. Membranes were incubated with primary polyclonal antibodies overnight at 1:1000 dilution in 2-5% BSA or skimmed milk at 4°C. The primary antibodies used were: anti-total Akt2 (no. 3063 Cell Signalling Technology) (Raun et al., 2018), $pAkt^{S473}$ (no. 9271,

Cell Signalling Technology) (Karusheva et al., 2019b), pAkt^{T308} (no. 9275, Cell Signalling Technology) (Raun et al., 2018), Anti-IP6K1 antibody (ab129595, Abcam), recombinant anti-S6K1 (ab32359), pS6K1^{Thr389} (no. 9234, Cell Signalling Technology), pAMPKα1^{Ser485}/AMPKα2^{Ser491} (no. 4185, Cell Signalling Technology), anti AMPKα (no. 2532, Cell Signalling Technology). Membranes were then washed in TBS tween and incubated in goat anti-rabbit secondary antibody (ab216773, Abcam) at 1:10,000 dilution in 0.5% to 5% BSA or skimmed milk for one hour at room temperature. After being washed in TBS-T, membranes were scanned and quantified with Odyssey® Fc Imaging System (LI-COR).

3.2.14 Data modelling

Western blots signal was normalised to total protein measurement using stain-free imaging technology (Bio-Rad) (Colella et al., 2012). To establish a baseline for each target protein in each participant, the average baseline signal was calculated. Subsequently, the signal observed after the meal was normalised to the respective participant's average baseline signal. This normalisation approach allowed for comparisons and assessment of changes in protein expression levels post-meal.

Blood glucose and plasma insulin concentrations during IVGTT were used to model the following parameters:

Glucose effectiveness (S_G) represents the ability of glucose to disappear from the blood without any change in insulin concentrations and it quantifies the fractional rate of glucose utilisation by the central nervous system and red blood cells and the rest of the insulin-independent tissues (Ader et al., 1985). S_G was calculated from a minimal model of insulin

and glucose after an IVGTT. The model also provides insulin sensitivity (S_I) calculations, which is defined as the ability of insulin to enhance glucose disappearance and inhibit glucose production (Ahrén & Pacini, 2021). The mathematical procedure for minimal model parameter calculations is explained by previous studies (Pacini et al., 2009; Pacini & Bergman, 1986). The calculations for this study for S_G and S_I were completed using Simulation Analysis and Modeling (SAAM II) software, version 2.3.3, Nanomath LLC, Washington, USA.

Early insulin response (insulinogenic index) was calculated as $(I_{30}-I_0)/(G_{30}-G_0)$, where I_{30} and G_{30} are insulin and glucose concentrations at min 30, I_0 and G_0 are insulin and glucose concentrations at min 0; Matsuda index (insulin sensitivity) was calculated as $\frac{10000}{\sqrt{G0*I0*Gmean*Imean}}$, where G_0= glucose at 0 min, I_0= insulin at 0 min, G_{mean}= average glucose concentration during IVGTT, I_{mean}= average insulin concentration during IVGTT; Disposition index (DI) = insulinogenic index * Matsuda index; Peak insulin/fasting insulin (I_p/I_0); Modified β cell index (MBCI) as $(G_0 \times I_0) / (G_2 + G_1 - 7)$, where G_0, G_1, and G_2 denote blood glucose concentrations (mmol/L) at 0, 1, and 2 h, respectively, and I_0 denotes insulin concentration (μU/mL) at 0 h (Zheng et al., 2015). Area under the curve of IVGTT was calculated for insulin and glucose concentrations as described in (Bravata et al., (2003). Additionally, the acute insulin response to glucose (AIR_g) was calculated using the AUC of insulin concentration in the first 10 minutes of the IVGTT (Park et al., 2021) using the well-established trapezoidal rule, a numerical integration technique that approximates the AUC by dividing the curve into small trapezoids and summing their areas (Bravata et al., 2003).

Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated with the following formula: fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5] and homeostasis model of percent β-cell function (HOMA β%) as follows: 20 × fasting insulin (μU/mL)/(fasting glucose – 3.5 (mmol/L)] (Uwaifo et al., 2002).

Amino acid concentrations were normalised to the Internal Standard (Glycine-2-13C). Furthermore, the area under the curve (AUC) was calculated as described above (Bravata et al., 2003) .

Plasma amino acid values are given in arbitrary units (A.U.) as the integrated area count for each individual BCAA within a sample was divided by the integrated area count of the internal standard (2-^{13}C-glycine) within the same sample. This method provides normalisation of each BCAA to a known concentration of added glycine.

3.2.15 Statistical analysis

All statistical analysis tests were carried out using the statistical analysis software IBM SPSS Statistics (version 28.0.1.1.) (IBM, Illinois, USA). Differences between the conditions were evaluated using multi-way analysis of variance (ANOVA). Differences between BCAA over time and between conditions were completed using repeated measures two-way ANOVA. Correlations between parameters were evaluated using Pearson correlation coefficients. Data are expressed as mean (SEM), unless otherwise specified. Statistical significance was accepted at $P<0.05$.

3.3 Results

Table 3.2 Subjects' baseline characteristics. Data presented as mean (± SEM)

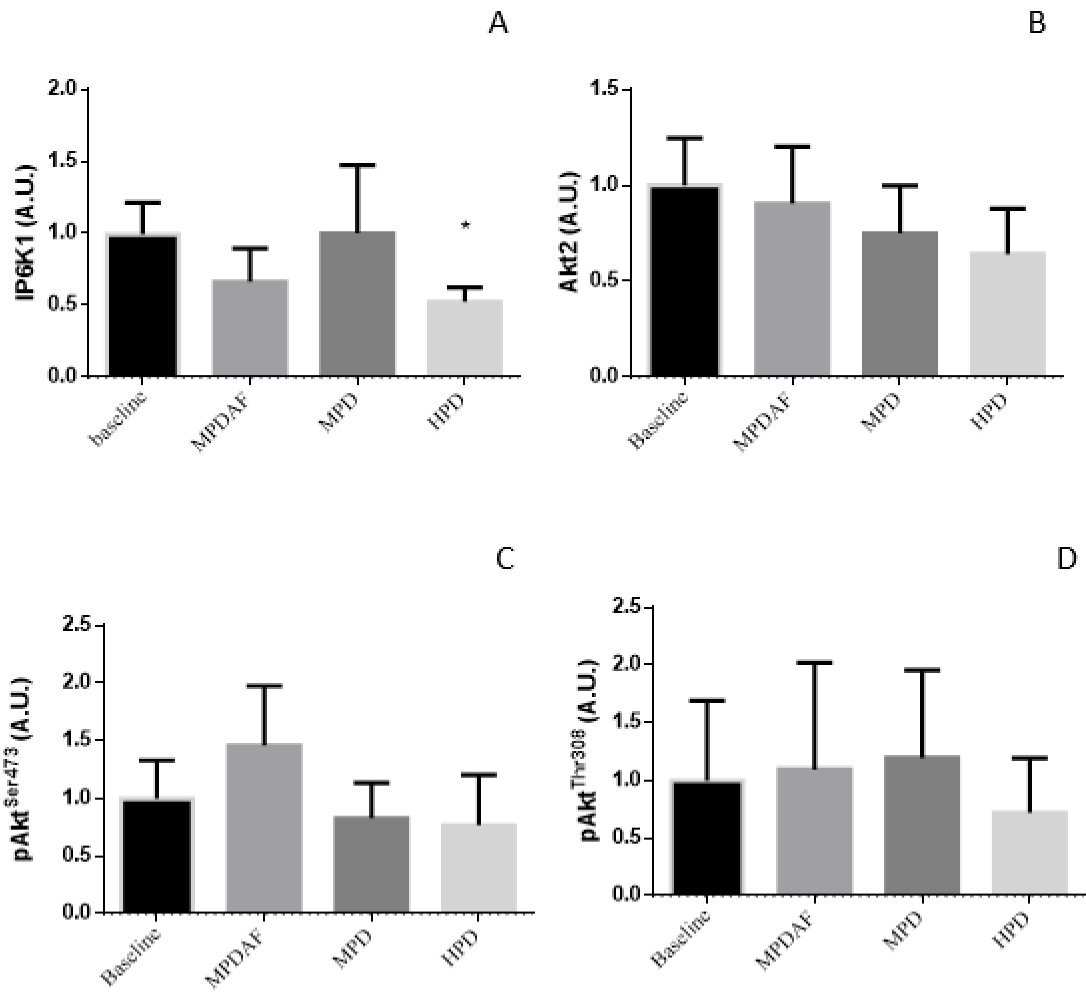
Variable	Participants (n=9)
Age (y)	46.1 (9.7)
Height (m)	1.68 (0.09)
Body mass (kg)	94.9 (9.3)
BMI (kg/m^2)	33.2 (2.0)
Waist circumference (cm)	102.2 (7.5)
Hip circumference (cm)	115.3 (4.8)
Waist:hip ratio	0.88 (0.06)
Body fat %	43.5 (10.2)
Lean mass (kg)	53.1 (13.9)
Fat mass (kg)	44.1 (7.2)
Systolic blood pressure (mm Hg)	126.1 (11.1)

Diastolic blood pressure (mm Hg)	78.1 (7.2)
Fasting blood glucose (mmol/L)	4.5 (0.4)
HbA1$_c$ (%)	5.4 (0.1)

3.3.1 Skeletal muscle analysis

We investigated total and phosphorylated states of proteins involved in insulin-stimulated glucose transport in skeletal muscle, immediately before and 4 hours after controlled meal ingestion in nine participants with obesity.

From western blot analysis, protein content of IP6K1 (**Figure 3.1A**) decreased in the 4 hours following the HPD when compared with baseline (p=0.048), with no significant differences in the MPD (p=0.891), nor in the MPDAF (p=0.327) for the same comparison (i.e. against averaged baselines within trial). pAkt^{Ser308}/Akt2 (**Figure 3.1F**) increased from baseline to the 4-hour sample in the HPD trial (p=0.046). Finally, AMPK (**Figure 3.1J**) decreased from baseline in the HPD trial (p=0.006), with no significant differences between the groups. No significant differences were observed between the conditions for Akt2 (**Figure 3.1B**), pAkt^{Ser473}(**Figure 3.1C**), pAkt^{Thr308} (**Figure 3.1D**), pAkt^{Ser473}/total Akt2 (**Figure 3.1E**), total S6K1 (**Figure 3.1G**), pS6K1^{Thr389} (**Figure 3.1H**), pS6K1^{Thr389}/total S6K1(**Figure 3.1I**), pAMPK$_{\alpha1}{}^{Ser485}$/AMPK$_{\alpha2}{}^{Ser491}$ (**Figure 3.1K**), (pAMPK$_{\alpha1}{}^{Ser485}$/AMPK$_{\alpha2}{}^{Ser491}$)/ total AMPK (**Figure 3.1L**).


A
B
IP6K1 (A.U.)
2.0
1.5
1.0
0.5
0.0
*
baseline
MPDAF
MPD
HPD
Akt2 (A.U.)
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD
C
D
pAktSer473 (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD
pAktThr308 (A.U.)
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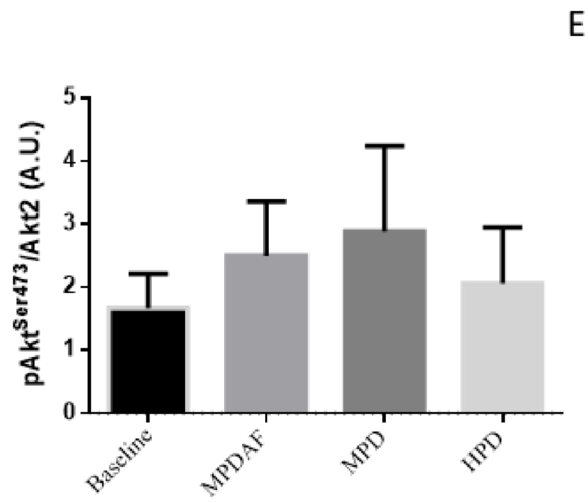
2.0
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD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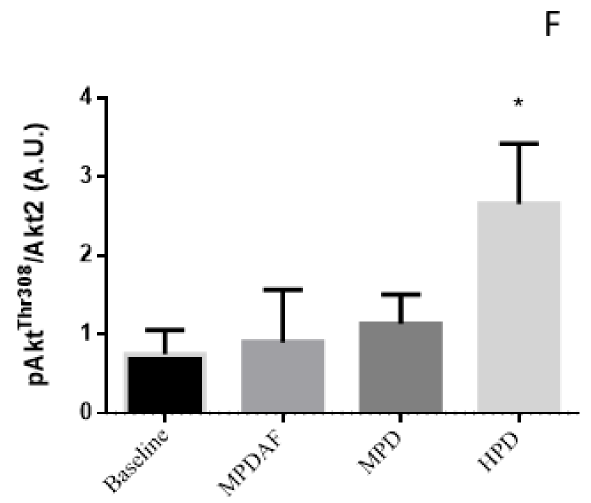
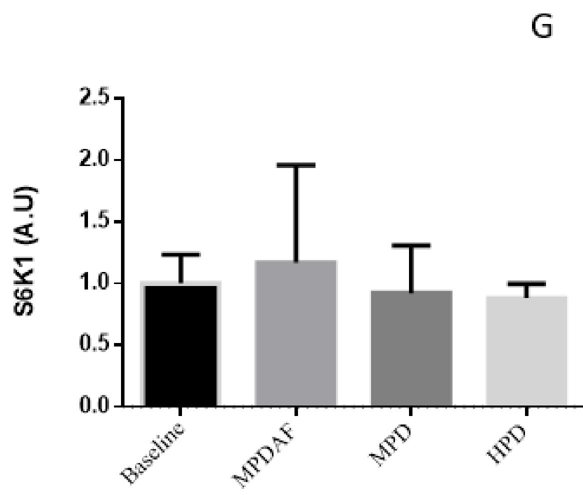
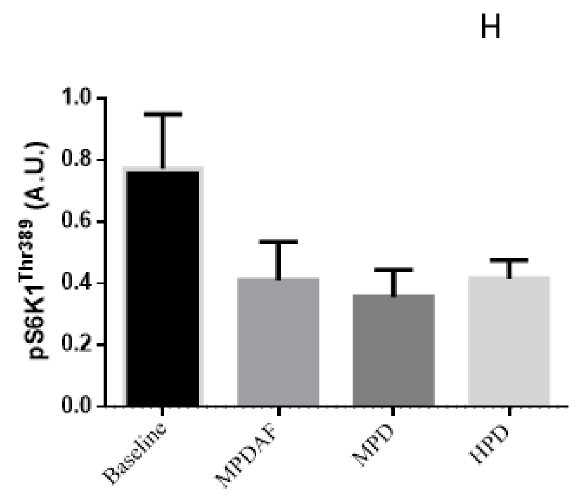
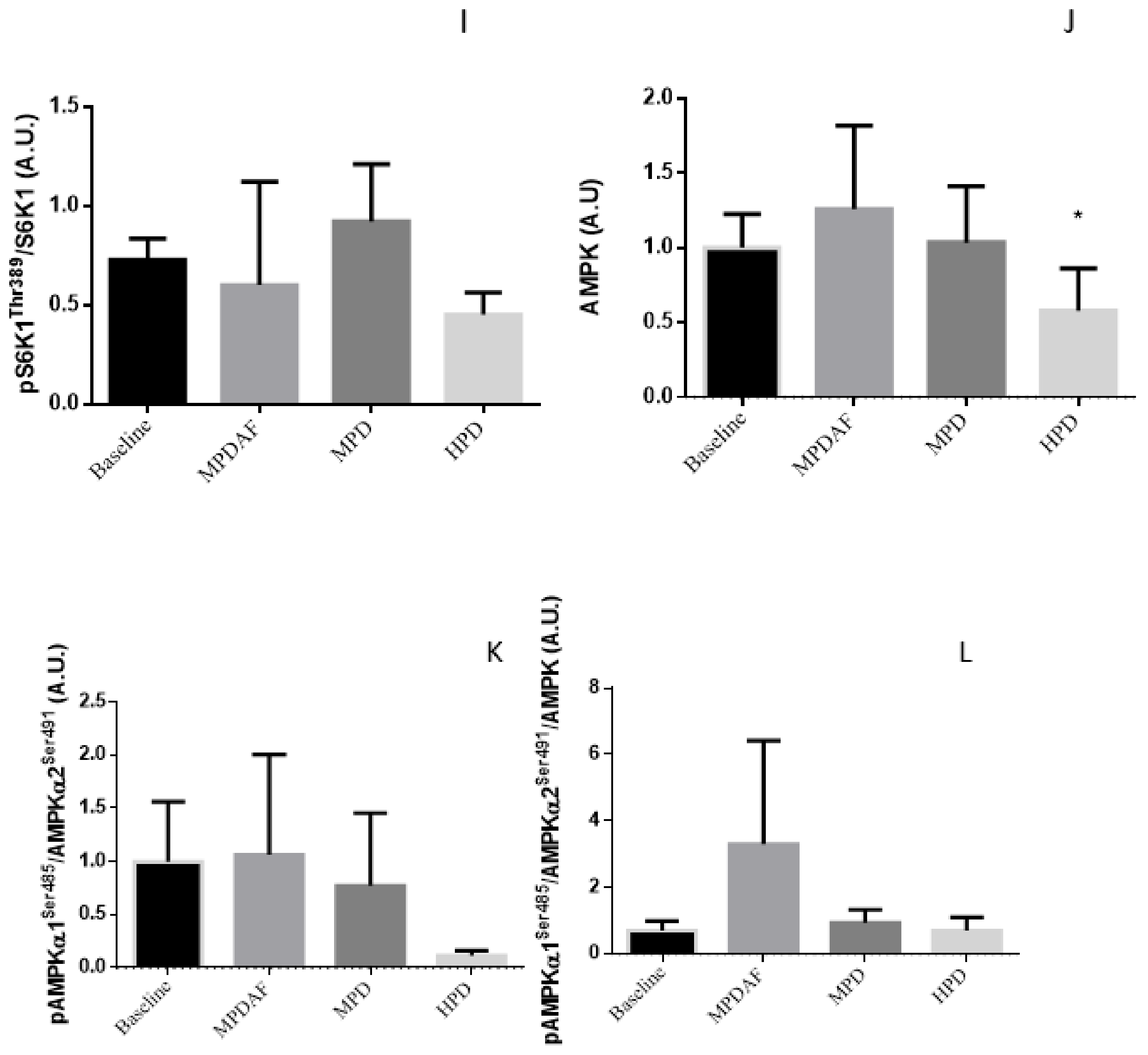
pAkt^{Ser473}/Akt2 (A.U.)
5
4
3
2
1
0
Baseline
MPDAF
MPD
HPD


F
pAkt^{Thr308}/Akt2 (A.U.)
4
3
2
1
0
*
Baseline
MPDAF
MPD
HPD


G
S6K1 (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD


H
pS6K1^{Thr389} (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
MPDAF
MPD
HPD


I
pS6K1^Thr389/S6K1 (A.U.)
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD
J
AMPK (A.U)
2.0
1.5
1.0
0.5
0.0
*
Baseline
MPDAF
MPD
HPD
K
pAMPKα1^Ser485/AMPKα2^Ser491 (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
MPDAF
MPD
HPD
L
pAMPKα1^Ser485/AMPKα2^Ser491/AMPK (A.U.)
8
6
4
2
0
Baseline
MPDAF
MPD
HPD

M

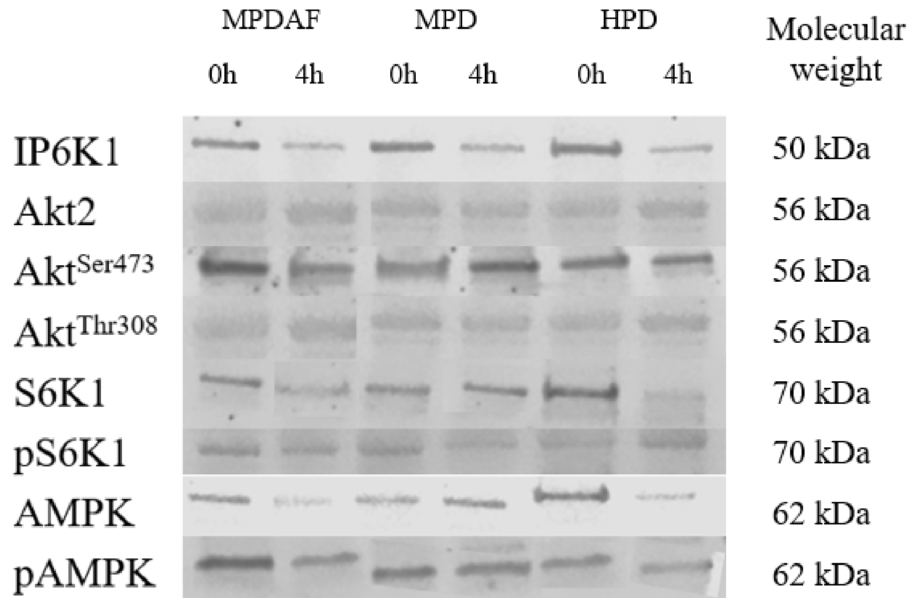


Figure 3.1 Skeletal muscle protein content for IP6K1 (A), Akt2 (B), pAkt^{Ser473}(C), pAkt^{Thr308} (D), pAkt^{Ser473}/Akt2 (E), pAkt^{Thr308}/total Akt2 (F), total S6K1 (G), pS6K1^{Thr389} (H), pS6K1^{Thr389}/total S6K1 (I), Total AMPK (J), pAMPKα1^{Ser485}/AMPKα2^{Ser491} (K), (pAMPK$_{α1}^{Ser485}$/AMPK$_{α2}^{Ser491}$)/ total AMPK (L), Western blot representative expression of all targets for baseline and 4 hours post meal ingestion (M). * denotes significant differences from baseline, $P<0.05$. n baseline=9, n MPDAF= 8, n MPD=7, n HPD=8. Data expressed as mean ± SEM. Abbreviations: MPDAF, moderate protein dose and added fat; MPD, moderate protein dose; HPD, high protein dose. Data for 4 h post meal have been normalised to averaged baseline for the 3 conditions.

3.3.2 Plasma analysis

From glucose effectiveness and insulin sensitivity calculations from a minimal model of insulin and glucose following IVGTT, this study found no significant differences between the groups (MPDAF, MPD and HPD) (**Figure 3.2**).

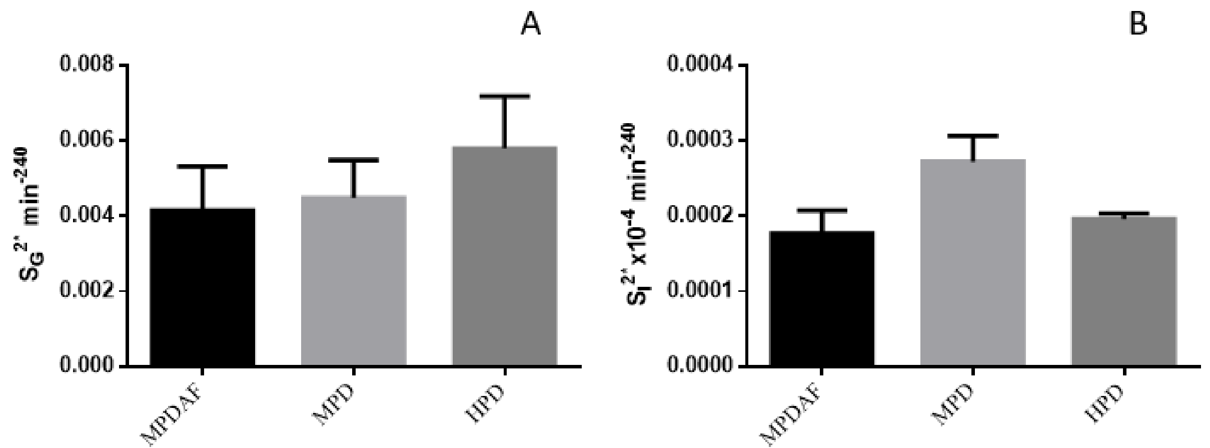
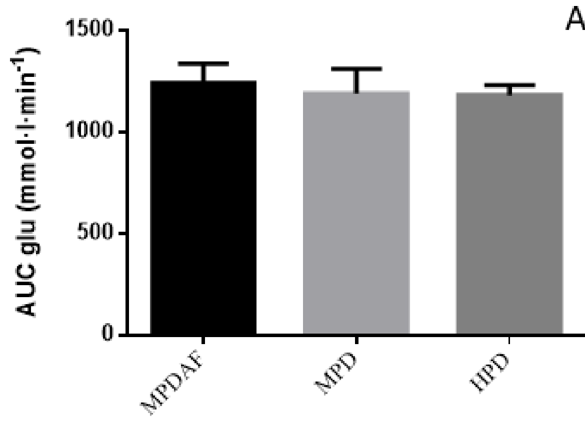
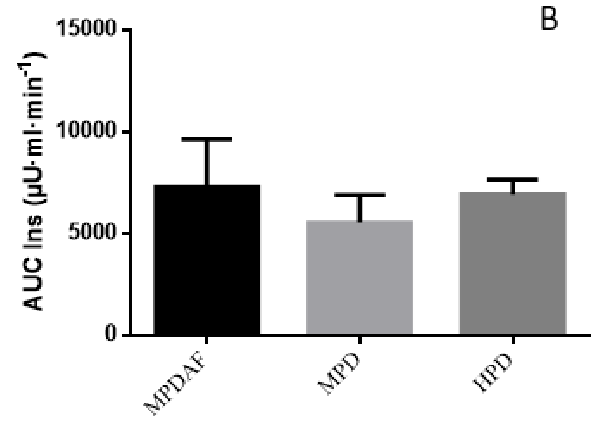
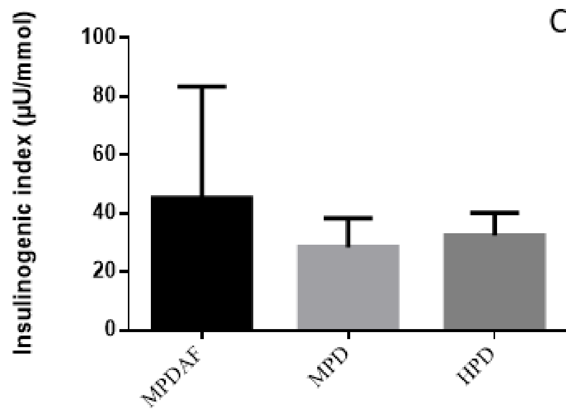


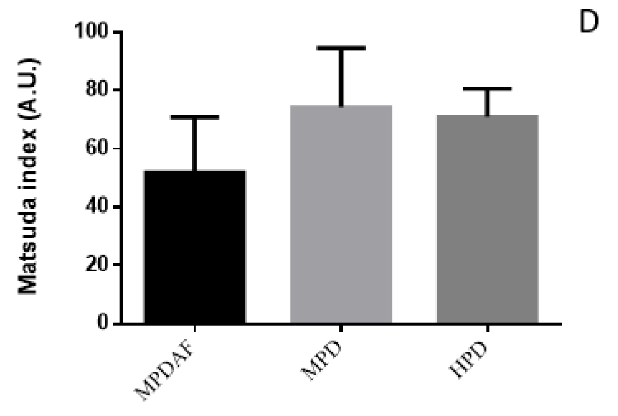
Figure 3.2 Glucose effectiveness (S_G) (A) and Insulin sensitivity (S_I) (B) calculated for each of the trials: MPDAF, MPD and HPD. n=9 for each group. Data expressed as mean ± SEM. Abbreviations: MPDAF, moderate protein dose and added fat; MPD, moderate protein dose; HPD, high protein dose. Data expressed as mean ± SEM.

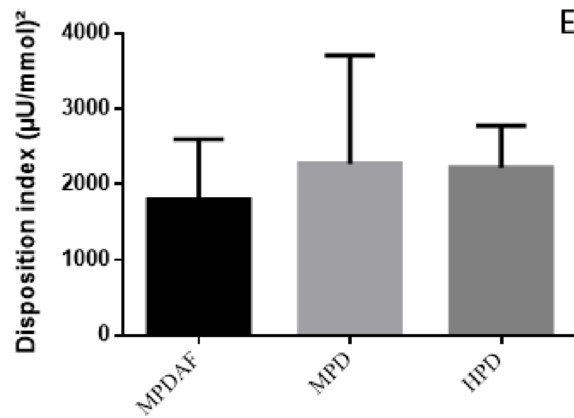
From plasma analysis and further calculations, this study found no statistical differences between the 3 conditions (HPD, MPD and MPDAF) for AUC_{glu}, AUC_{ins}, insulinogenic index, Matsuda index, Disposition index, Ratio between peak insulin concentration and fasting insulin concentration, Modified β cell function index (MBCI), or Acute insulin response to glucose (**Figure 3.3**).

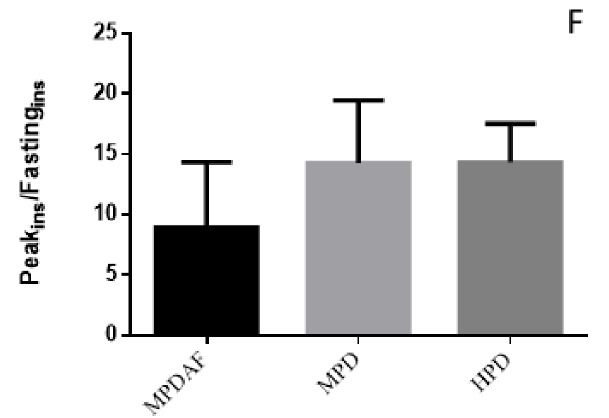

A
AUC glu (mmol·l·min^{-1})
1500
1000
500
0
MPDAF
MPD
HPD


B
AUC Ins (μU·ml·min^{-1})
15000
10000
5000
0
MPDAF
MPD
HPD


C
Insulinogenic index (μU/mmol)
100
80
60
40
20
0
MPDAF
MPD
HPD


D
Matsuda index (A.U.)
100
80
60
40
20
0
MPDAF
MPD
HPD


E
Disposition index (μU/mmol)²
4000
3000
2000
1000
0
MPDAF
MPD
HPD


F
$Peak_{ins}/Fasting_{ins}$
25
20
15
10
5
0
MPDAF
MPD
HPD

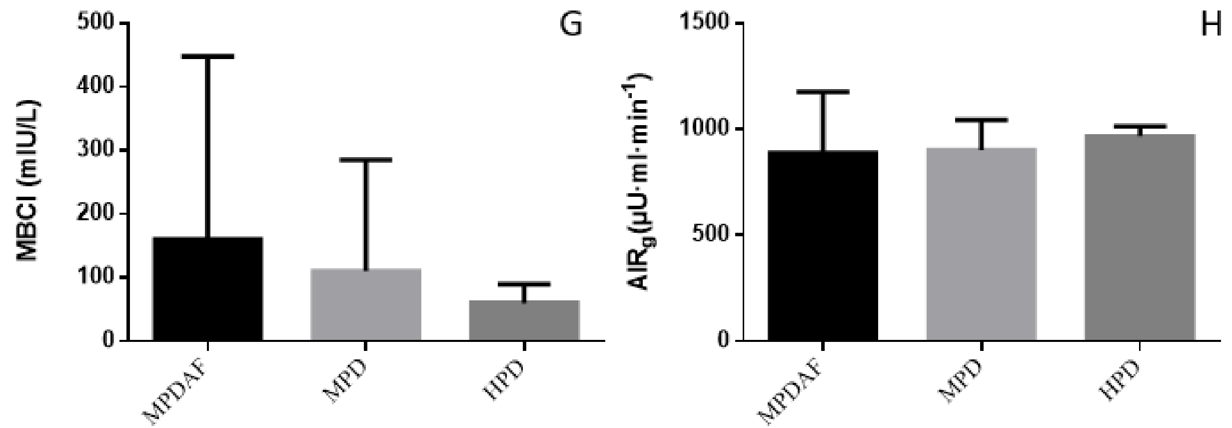


Figure 3.3 Area under the curve for glucose concentration during IVGTT (A); Area under the curve for insulin concentrations during IVGTT (B); Insulinogenic index (early insulin response) (C); Matsuda index (insulin sensitivity) (D); Disposition index (E); Ratio between peak insulin concentration and fasting insulin concentration (F); Modified β cell function index (MBCI) (G); Acute insulin response to glucose (AIR_g) (H). n= 9 for each group. MPDAF = moderate protein dose added fat (50 g protein and 25 g fat), MPD = moderate protein dose (50 g), HPD = high protein dose (100 g). IVGTT= intravenous glucose tolerance test. Data expressed as mean ± SEM.

The HOMA-IR (**Figure 3.4A**) was calculated as an estimation of insulin sensitivity, with no statistically significant difference found between groups (p=0.806). Also, HOMA- β% (**Figure 3.4B**) was calculated as a measure of insulin secretion by the β-cells in the pancreas and provides an estimate of fasting β-cell function, with no significant difference found between groups (p=0.641).

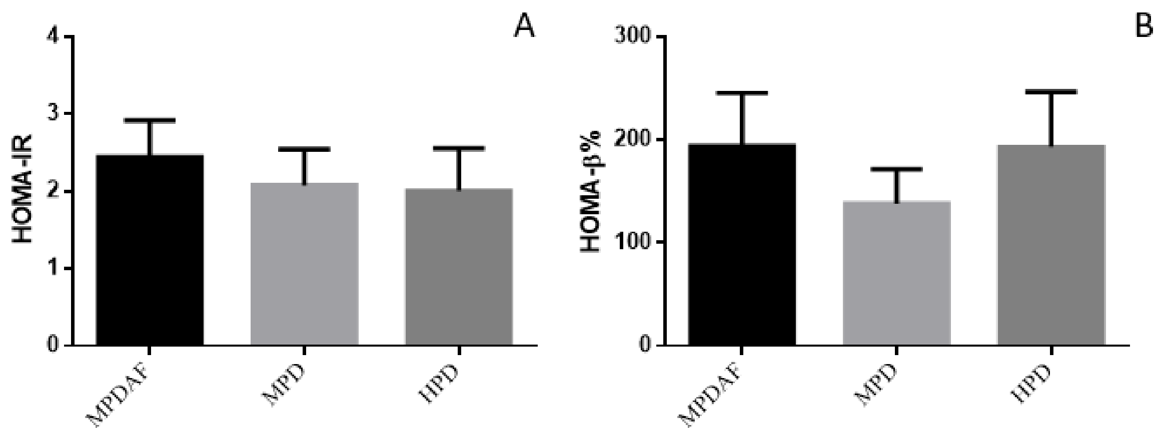
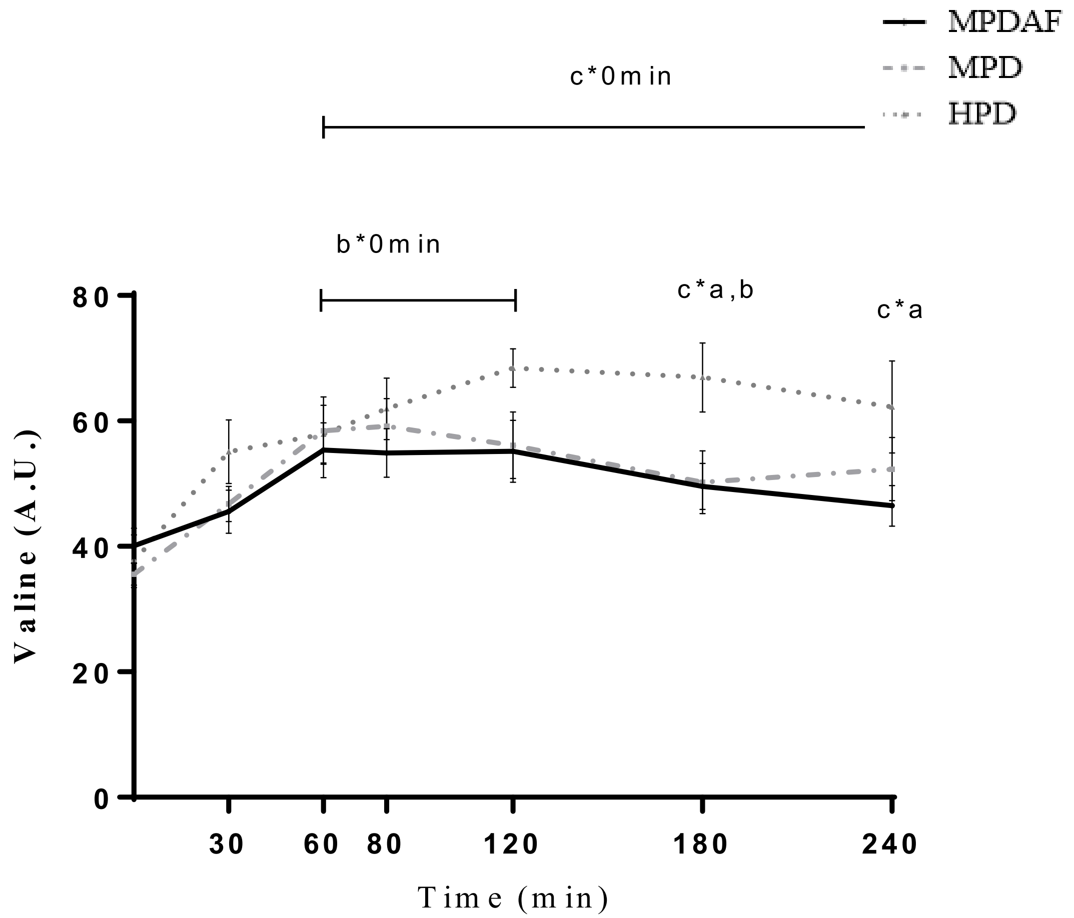


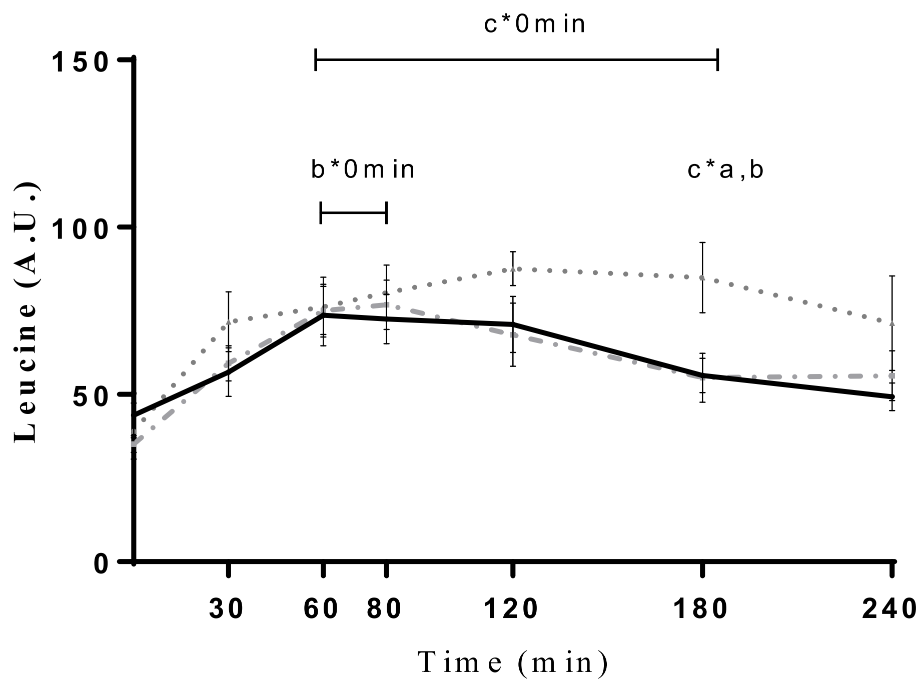
Figure 3.4 The homeostasis model assessment of insulin resistance (HOMA-IR) (A) and homeostasis model assessment of percent β-cell function (HOMA-β%) (B) calculated for each of the trials: MPDAF, MPD and HPD. Data expressed as mean ± SEM. n= 9 for each group. Abbreviations: MPDAF, moderate protein dose and added fat; MPD, moderate protein dose; HPD, high protein dose. Data expressed as mean ± SEM.

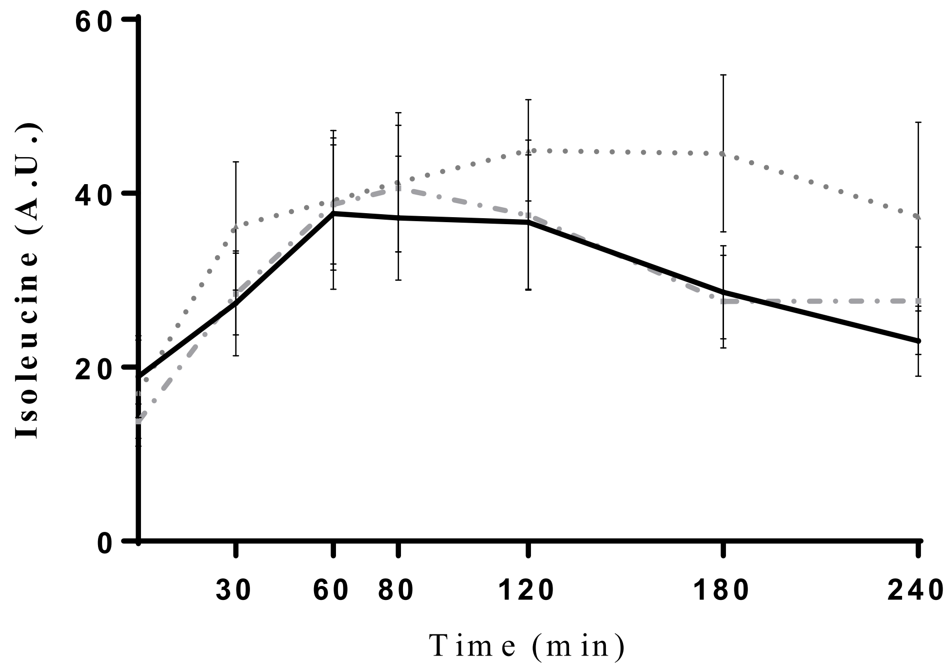
From ultra-high-performance liquid chromatography-MS/MS analysis, we observed an increase in some BCAA over time (**Figure 3.5**). More specifically, within the HPD trial valine concentrations significantly increased from baseline from minute 60 post meal ingestion and have been maintained significantly increased for the remaining duration of the trial (up to 4 hours). In a similar manner, valine concentrations were also increased from baseline in the MPD trial from minute 60 until 120-minute post meal ingestion. Within the MPDAF trial, we see no significant changes compared to baseline at any time point following meal ingestion. In addition, valine's plasma concentration was significantly higher in the HPD trial compared to MPD and MPDAF at 180-minute post meal (p=0.032 and 0.012 respectively) and different compared to MPDAF at 240-minute post meal (p=0.029).

Leucine concentrations were significantly higher compared to baseline from minute 60 to minute 180 post meal ingestion within the HPD, whereas in the MPD trial they were significantly higher than baseline only at minute 60 and 80 post meal ingestion, with no significant increases noted in the MPDAF trial. Additionally, leucine concentrations were higher in the HPD trial when compared to the other 2 conditions (MPDAF and MPD) at 180-minute post meal ingestion ($p=0.015$ and $p=0.031$ respectively).

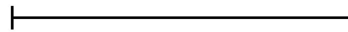
While no changes were seen in isoleucine concentrations over time post meal or between the conditions (MPDAF, MPD and HPD) at any time point (30-240 minute), total BCAA concentration was significantly higher compared to baseline from minute 80 to minute 180 post meal ingestion in the HPD trial, whereas in MPD it was increased compared to baseline from minute 60 to minute 80, with no significant changes compared to baseline seen in MPDAF. In addition, total BCAA concentrations in the HPD trial was significantly higher when compared to MPDAF concentrations at 180-minute post meal ingestion ($p=0.035$).


MPDAF
MPD
HPD
c*0min
b*0min
c*a,b
c*a
80
60
40
20
0
Valine (A.U.)
30
60
80
120
180
240
Time (min)


c*0min
b*0min
c*a,b
150
100
50
0
Leucine (A.U.)
30
60
80
120
180
240
Time (min)



c*0min



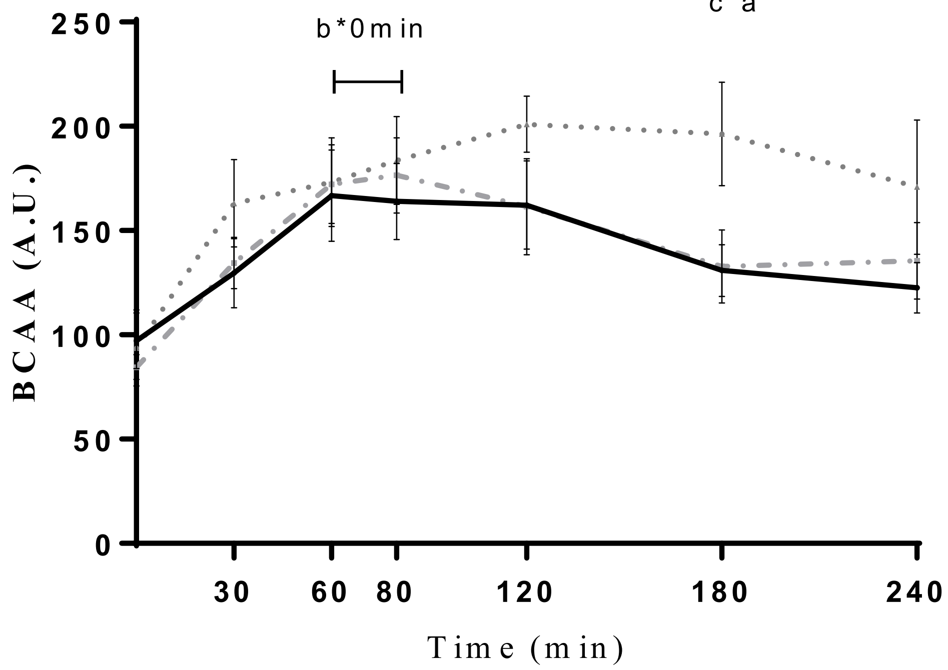
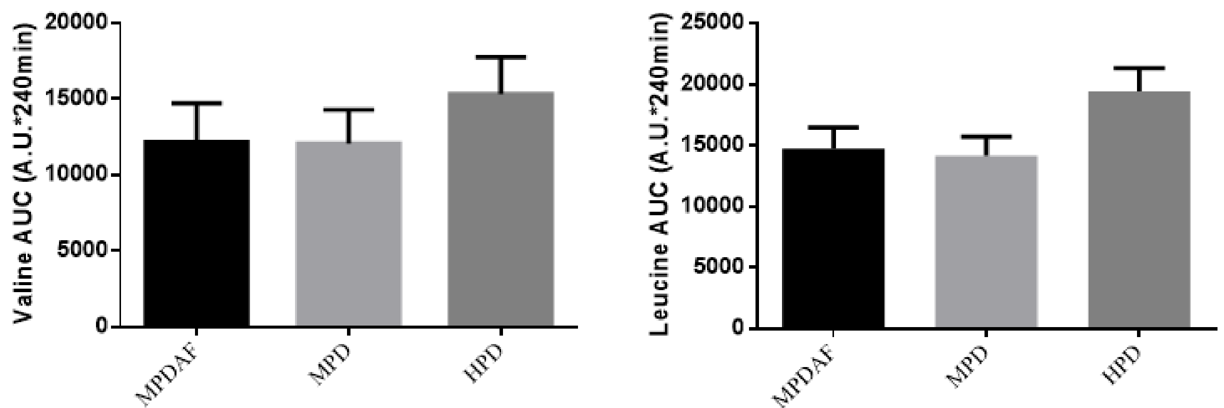


Figure 3.5 Plasma amino acid concentrations of individuals with obesity after ingestion of different protein-containing meals for individual Branched-chain amino acids (BCAAs) or combined, Abbreviations: MPDAF (a), moderate protein dose and added fat; MPD (b), moderate protein dose; HPD (c), high protein dose. n= 9 for each group. Data expressed as mean ± SEM. Trial*0min represents significant difference in the specified time interval compared to baseline values (0 min). Trial*trial represents significant difference between the specified trials at the marked time point.

No differences between conditions (MPDAF, MPD and HPD) were seen in plasma AUC calculations for valine, leucine, isoleucine and total BCAA concentrations (**Figure 3.6**).



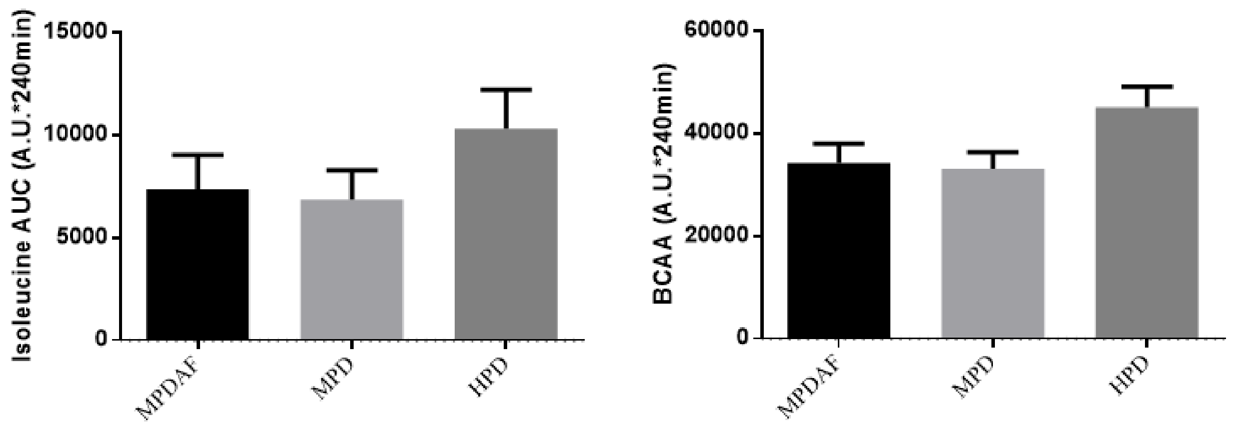


Figure 3.6 Plasma BCAA AUC of individuals with obesity after ingestion of different protein-containing meals for individual Branched-chain amino acids (BCAAs) or combined, Abbreviations: MPDAF, moderate protein dose and added fat; MPD, moderate protein dose; HPD, high protein dose. n= 9 for each group. Data expressed as mean ± SEM.

3.4 Discussion

The current study investigated the effects of different protein doses on the cellular mechanisms that govern insulin-stimulated glucose uptake pathway and protein synthesis in skeletal muscle in individuals with obesity. Specifically, we assessed the effects of moderate-protein (50 g, MPD) versus high-protein (100 g) (HPD) meal, as well as versus a meal of moderate protein dose with added fat (MPDAF), which matched the energy content of the high protein dose. We assessed muscle samples before (fasted) and 4-hour post-meal samples. To assess the whole-body response to the interventions, four-hour intravenous glucose tolerance test (IVGTT) was administrated to determine the acute effects of the protein meals on glucose control. Additionally, we assessed the acute effect

of different-sized protein meals on plasma branched-chain amino acid concentrations as high plasma concentrations have been linked with IR and progression towards T2D.

How did differently sized protein meals influence glucose control in an acute manner?

The present investigation, despite variations in protein intake, did not reveal any statistically significant distinctions in either glucose effectiveness or insulin sensitivity among the three distinct conditions (HPD, MPD, and MPDAF). The rationale behind the anticipated alterations in insulin sensitivity attributed to increased protein consumption has been thoroughly elucidated in **Chapter 2** of this thesis. Moreover, although excess dietary fat has been linked to dyslipidemia and heightened risk of insulin resistance and TT2D development (Barber et al., 2021), the incorporation of 231kcal from fat sources from the MPDAF trial falls within the standard range of a balanced diet encompassing all macronutrients. Hence, we anticipated no adverse effects on insulin sensitivity. This expectation is corroborated by the absence of alterations in any of the glucose control parameters measured between the MPD and MPDAF groups.

Glucose effectiveness holds a fundamental position within the domain of glucose metabolism and assumes a crucial role in the maintenance of steady blood glucose concentrations. It pertains to glucose's inherent capacity to facilitate its uptake and utilisation across various tissues, including skeletal muscle, independently of insulin's actions (Ahrén & Pacini, 2021) Additionally, it encompasses the ability to suppress gluconeogenesis, signifying the liver's ability to curtail its glucose release into the bloodstream, thereby further stabilising blood glucose levels and it is estimated to account for ≈70% of glucose disposal after an intravenous glucose administration (Hu et al., 2021).

Although dietary proteins are recognised for their insulinotropic effect, which stimulates insulin production (Rietman, Schwarz, Tomé, et al., 2014), the current study did not uncover any discrepancies in insulin concentrations, as assessed by the insulin area under the curve, among the different experimental conditions. However, as the present study did not assess rates of insulin clearance or C-peptide production, a good indicator correlated with insulin secretion (Venugopal et al., 2023), it remains uncertain whether there are significant differences in insulin secretion among the three distict trials.

Furthermore, HOMA-IR is used to characterise the relationship between fasting glucose and insulin concentrations, and it reflects the balance between hepatic glucose output and insulin secretion as a result of the feedback loop between the β-cells and the liver with ranges below 1.0 considered normal and values above these values may indicate insulin resistance (Levy et al., 1998). In the current study, the mean values of HOMA-IR indicate the presence of insulin resistance. On the other hand, HOMA-β% is an indicative measure of β-cell function with values ranging from 0% to over 200%, with values of 100% and above considered normal, while values below may indicate impaired β-cell function (Matthews et al., 1985; Wallace & Matthews, 2002). Notably, the mean HOMA-β% in our study falls within the normal range, indicating that β-cell function remains preserved in this group of individuals with obesity.

Our results demonstrate the presence of fasting insulin resistance in individuals with obesity, while β-cell function remains intact in the fasted state. Moreover, the absence of significant differences in HOMA-IR and HOMA-β% between the trials reinforces that the participants were in similar metabolic states.

How did differently sized protein meals influence IP6K1 in an acute manner (4 hours post meal ingestion)?

In the current study, we observed that HPD led to a decrease in IP6K1 content when compared to baseline fasting samples, while no significant changes were observed in the other trials (MPD or MPDAF). These results suggest that in an acute manner, 4 hour post meal ingestion, IP6K1 content is downregulated by dietary protein intake only after a certain intake threshold, as no differences were seen in the MPD trial, or when energy intake was matched in the MPDAF. The regulation of IP6K1 in skeletal muscle is of significant interest due to its role in the modulation of glucose metabolism and insulin signalling (Chakraborty, 2018; Mukherjee et al., 2020). Several studies have suggested that elevated levels of IP6K1 in skeletal muscle may contribute to the development of insulin resistance and type 2 diabetes (Barclay et al., 2020; Ghoshal et al., 2022; Naufahu et al., 2018b). Therefore, the ability of high-protein meals to decrease IP6K1 levels in skeletal muscle in an acute manner (post meal) may have important implications for the prevention and management of these metabolic disorders. It has been suggested that the high amino acid content of protein may stimulate insulin secretion and enhance insulin signalling (Claessens et al., 2009; Farnsworth et al., 2003), leading to improved glucose uptake and utilisation in skeletal muscle by activating Akt (Chakraborty et al., 2010).

Was the reduction in IP6K1 following a high-protein meal mediated by Akt?

The current study also investigated the effects of the different-sized protein meals on Akt2 expression and activation, a protein with critical roles in metabolism and insulin-stimulated glucose uptake, hence aiding in elucidating the precise underlying mechanisms of Akt and downstream of it that is crucial for prevention or management of IR. We did

not observe any significant changes in total Akt2 expression following moderate- or high-protein meal ingestion. However, we observed an elevation in the ratio between phosphorylated Akt at Thr^{308} over total Akt2 in the HPD, indicating an activation of Akt. Akt activity is tightly regulated by phosphorylation at the Thr^{308} site, which is a prerequisite for its full activation, and at Ser^{473}, which enhances Akt activity (Chog et al., 2005). This finding is in agreement with the established mechanism of Akt activation in skeletal muscle, as insulin activated Akt is stimulated by protein intake and leads to increased protein synthesis rates and glucose uptake (Haydon et al., 2002; Tremblay & Marette, 2001).

The heightened availability of BCAAs, particularly leucine, subsequent to high-protein meals, is acknowledged for its potential to activate mTOR and its downstream targets, including S6K1. In certain in vitro studies, such activation has been linked to reduced insulin's capacity to activate Akt, thereby impairing insulin signalling (Patti et al., 1998; Yip et al., 2010). This notion is supported by a human in vivo study where amino acid infusion (with a 25% BCAA composition) resulted in an abrupt rise in IR and S6K1 activity (Tremblay et al., 2005). Although we noted significantly elevated levels of BCAA, as well as valine and leucine following the HPD trial, which outlasted those seen at lower doses of protein (MPDAF and MPD), substantial alterations in S6K1 were not seen. Consequently, distinctly from the intravenous infusions studies where the digestion system is circumvented, in the meal-induced context, the increased ratio of Akt^{Thr308} to Akt2 seen following the HPD meal could potentially be attributed to IP6K1 modulation rather than S6K1 activation. The changes in IP6K1 content are supported by a previous study that also noted Akt activation in response to high-protein intake correlated with

change in IP6K1 content. (Kim et al., 2019). Furthermore, IP6K1 and Akt have a shared path involving phosphatidylinositol bisphosphate (PIP2). While IP6K1 catalyses the conversion of PIP2 into Ins (1,4,5) P3, PIP2 also contributes to Akt 2 activation through the generation of phosphatidylinositol-3,4,5-triphosphate (PIP3)- a lipid membrane that bind to Akt's plekstrin homology (PH) domain (Ancu et al., 2021). This study suggests that a decline in IP6K1 muscle content could result in an increased PIP2 availability, consequently increasing Akt activity. More specifically, as phospholipids bind specifically to the PH domain of Akt, that would mean that PIP3 could either promote the activation of the kinase that phosphorylates Akt on Thr^{308} or to bind to Akt itself and change its conformation to allow phosphorylation to occur at Thr^{308}, and not at Ser^{473} (Stokoe et al., 1997; Wei et al., 2019). This is supported by the lack of changes noted in phosphorylation of Akt at Ser^{473} site, when compared to Thr^{308}. In line with the lack of changes in phosphorylation at Ser^{473}, a different study found that in skeletal muscle cell line incubated with leucine, insulin has a 50% decreased ability to phosphorylate Akt on Ser^{473} (Saha et al., 2010).

How is AMPK mediated by different-sized protein meals?

Anabolic stimuli and activation of the protein synthesis pathway are known to inhibit AMPK activity by 36 to 64%, as shown in different tissues, such as the liver and skeletal muscle in animal tissue or *in vitro* studies (Gamble & Lopaschuk, 1997; Valentine et al., 2014; Witters & Kemp, 1992). Therefore, it has been hypothesised that the HPD will lead to an increase in its inhibitory phosphorylation sites (as expanded upon subsequent paragraph), resulting in a reduction of its overall AMPK content. The results of the current study are partially in line with that, as we see a decrease in total AMPK expression

in the four hours post-HPD ingestion. Although we did not directly measure glycogen content in our study, it is important to note that the glucose load administered intravenously to the participant four hours prior to the muscle biopsy is expected to result in an increase in muscle glycogen content. We have taken this into account when analysing our findings. Furthermore, we have considered the inverse relationship between AMPK activation and muscle glycogen content that has been reported in previous human (Wojtaszewski et al., 2003) and rodent studies (Derave et al., 2000; Wojtaszewski, Jørgensen, et al., 2002) and summed up in a more recent review (Kjøbsted et al., 2018). This inverse relationship serves as a potential explanation for the decrease in total AMPK content observed in our study, given the anticipated increase in muscle glycogen content due to the glucose load.

Apart from total AMPK content, we have also analysed AMPK phosphorylation four hours post-meal. Although AMPK phosphorylation at Thr^{172} is the main activation site of the enzyme, studies show that Akt-related AMPK inhibition that is caused by anabolic signals, such as insulin, is primarily mediated through the phosphorylation of AMPK inhibitory site $Ser^{485/491}$ on α1/α2 in different tissues such as adipocytes (Berggreen et al., 2009), smooth muscle cells (Ning et al., 2011), cardiomyocytes (Horman et al., 2006; Soltys et al., 2006), and skeletal muscle (Valentine et al., 2014). These studies have reinforced this by inhibiting Akt, which consequently resulted in an attenuation of inhibiting AMPK by phosphorylating $Ser^{485/491}$ on α1/α2, which meant that AMPK maintained its activity (Horman et al., 2006; Kovacic et al., 2003; Ning et al., 2011). Hence, we hypothesised that the changes in AMPK would be Akt-related and analysed this specific phosphorylation site, $Ser^{485/491}$ on α1/α2. However, in this study, we did not

note any changes in the phosphorylation of AMPK site $Ser^{485/491}$ on $\alpha1/\alpha2$, which may suggest that the inhibition of AMPK may be caused by a different, independent pathway not involving Akt, as also suggested by studies completed in cultured hepatocytes (Valentine et al., 2014); or that Akt may phosphorylate other as yet unknown sites on AMPK. The involvement of an independent of Akt or insulin pathway in AMPK inhibition is also supported by the lack of significant difference in the area under the curve of insulin between trials.

Is S6K1 total content and phosphorylation increased after protein-based meals?

While activation of mTORC1/S6K1 is essential for different cellular functions, including cell growth and proliferation, it has been hypothesised that overstimulation of this pathway by nutrient overload, such as amino acids, may be an additional factor in the development of insulin resistance via increased IRS-1 tyrosine phosphorylation and degradation (Tremblay & Marette, 2001; Um et al., 2006). This finding has been investigated through a comprehensive methodology employing a hyperinsulinaemic-euglycaemic clamp coupled with amino acid infusions leading to an approximately 2.5-fold increase in blood amino acid concentrations. Consequently, there was a substantial 3.7-fold elevation observed in skeletal muscle S6K1 protein content after 180 minutes of low peripheral hyperinsulinemia and 30 minutes of prandial-like hyperinsulinemia clamp infusions compared to baseline sample (Tremblay et al., 2005) . Building upon these insights, the present study sought to examine the alterations in S6K1 content before and four hours following the consumption of different sized protein meals, hereby providing insights into the physiological dynamics of S6K1 postprandially. Intriguingly, no significant changes were observed in either the total S6K1 levels or its phosphorylated

state at Thr^{389} during this time period for any of the conditions, indicating that the influence of plasma amino acid levels on S6K1 may be notably attenuated compared to infusion studies.

Plasma AA responses following different sized protein meals

The effects of branched-chain amino acids (BCAA), namely leucine, valine and isoleucine, on metabolic health have been described in the literature with conflicting effects depending on the context (Bishop et al., 2022). Elevated concentrations of BCAAs have been observed in the circulation of individuals with obesity and type 2 diabetes (Bassil et al., 2011; Newgard et al., 2009; Tremblay et al., 2007). This phenomenon, known as the "BCAA signature" (Bishop et al., 2022), has also been observed in genetically obese mouse models, where it has been linked to abnormalities in amino acid catabolism (Zhou et al., 2019).

In our study, we observed that in the HPD trial, valine concentrations remained significantly elevated compared to baseline from the 60th minute up to the 240th minute post meal ingestion. In contrast, in the MPD, this elevation was observed only until the 120th minute. These findings suggest that a higher protein meal results in prolonged increase in plasma amino acid level. Furthermore, we observed that 3 hours after meal ingestion, plasma valine concentrations remained significantly higher in the HPD group compared to both the MPD and the moderate-protein diet with added fat (MPDAF) groups. However, at the 4-hour mark after a high-protein meal, valine concentrations were significantly higher only in comparison to the MPDAF trial, not the MPD trial. This indicates that the combination of dietary fat and protein may contribute to a more pronounced decline in valine levels at the 4-hour time point. While we did not detect any

significant changes in isoleucine concentration in the HPD trial over time or in comparison to the other two conditions, the increase in valine concentration can potentially lead to the production of toxic intermediates during BCAA catabolism. These intermediates, particularly from valine and isoleucine, have been associated with promoting conditions such as atherosclerosis and inducing type 2 diabetes (Fiehn et al., 2010) ,

In a C2C12 skeletal muscle cell line, valine has been shown to be metabolised into 3-hydroxy-isobutyrate (3-HIB), which can enhance fatty acid uptake by skeletal muscle (Jang et al., 2016). This phenomenon has also been supported by animal models, which demonstrated that increased levels of 3-HIB can lead to lipid accumulation due to incomplete lipid oxidation products, ultimately causing insulin resistance in skeletal muscle (Giesbertz et al., 2015; Jang et al., 2016) . Additionally, in the liver, excess valine is believed to indirectly contribute to insulin resistance through BCAA-related metabolites, specifically branched-chain keto acids (BCKA) (Newgard, 2012) . These metabolites can impair the Krebs cycle by accumulating acylcarnitines, ultimately leading to oxidative stress and insulin resistance. Despite these observed changes in valine concentration and its potential metabolic consequences, it is important to note that we did not observe any detrimental effects on glucose control. Therefore, despite the negative associations linked to increased valine levels, our study suggests that the consumption of a high-protein meal, containing 100g of protein, out of which 17.4g BCAA (8.1g leucine, 4.7g valine and 4.6 isoleucine) (Kim et al., 2017) does not appear to have adverse effects on glucose control.

Leucine concentrations exhibited a notable increase, remaining significantly elevated for a duration of up to 180 minutes following the consumption of a high-protein meal. These elevated levels were notably distinct when compared to the MPDAF and MPD trials. This indicates that the ingestion of a high-protein meal sustains higher circulating plasma amino acid levels for an extended period after eating. Despite this increase in leucine levels, our study did not reveal any significant alterations in insulin sensitivity across any of the trial groups. This finding can be elucidated by considering that leucine concentrations did not reach the threshold required to initiate changes in insulin signalling. This threshold is discussed in (Jang et al., 2016), where it is noted that it is only when leucine concentrations are increased by 80-150% that they inhibit glucose uptake and induce insulin resistance.

Intriguingly, as it has been shown that infusion of amino acids mixture resulted in not only plasma amino acids increase, but also hyperinsulinemia and activation of muscle S6K1 (Tremblay et al., 2005). Additionally, in rat muscle, it has been shown that infusing mixture of amino acids and glucose (with 8.9/L leucine) impaired insulin-stimulated glucose disposal and also insulin-mediated suppression of glucose production, which was associated with a decrease in AMPK content (Saha et al., 2010). In the present study, a decrease in AMPK was observed in the HPD trial, in contrast to the other two trials (MPDAF and MPD), where no significant changes were noted, despite all three trials employing an identical glucose load. AMPK is a well-known energy sensor, and it is recognised that an excess of nutrients such as glucose or amino acids can diminish AMPK activity(Coughlan et al., 2015). Notably, in the current study, only the combination of a higher protein intake (100g) and a glucose solution led to a decrease in AMPK. This

suggests that the elevated protein intake, compared to the moderate dose, may be the causal factor behind the AMPK decrease.

As previously mentioned, it was expected that S6K1 would increase concurrently with the decrease in AMPK, as part of the amino acid-mediated mTORC1-S6K1 activation pathway (Melnik, 2012). This activation can play a role in negatively regulating IRS-1 phosphorylation, potentially leading to amino acid-induced insulin resistance (Tremblay et al., 2005). However, contrary to expectations, elevated amino acid levels in our current study did not lead to an overactivation of S6K1.

3.5 Conclusion

In conclusion, this study sheds valuable light on the potential of different-sized protein meals as a dietary intervention for enhancing metabolic health and glycemic control. While none of the meals tested (MPD, MPDAF, or HPD) induced significant alterations in insulin sensitivity or glucose effectiveness, the consumption of a high-protein meal (containing 100 grams of protein) did result in a notable reduction in muscle IP6K1 levels. This observation holds promise as it suggests a potential association with a decreased risk of developing Type 2 Diabetes. Additionally, these changes coincided with an increase in the ratio between phosphorylated Akt^{Thr308} and total Akt2 activity, a finding that could hold positive implications for glycemic control by potentially amplifying insulin-stimulated glucose uptake in muscle.

However, it is important to note that we also observed a decrease in AMPK in the HPD trial, which we initially hypothesised to be Akt-related. Nevertheless, this study did not

reveal any changes in the phosphorylation of AMPK site Ser485/491 on $\alpha1/\alpha2$, raising the possibility that the inhibition of AMPK might be attributed to a distinct and independent pathway not involving Akt.

In summary, during the acute postprandial period, although higher protein doses do not appear to directly impact insulin sensitivity, they may exert favorable effects by influencing proteins within the insulin signalling pathway. This highlights the complexity of dietary interventions and their multifaceted effects on metabolic processes, further highlighting the need for continued exploration in this field to elucidate the intricate interplay between diet, metabolic health, and glucose regulation.

Chapter 4. The effects of one-legged resistance exercise training, with/without protein supplementation, on the insulin-stimulated glucose uptake pathway in skeletal muscle

4.1 Statement of contribution

The work within this chapter stems from a collaboration with the research group of Professor Francis Stephens at the University of Exeter. Data collection was performed at the University of Exeter and other outcome measures from the same research design and data collection have been published in the American Journal of Physiology - Endocrinology and Metabolism (PMCID PMC8897029, doi: 10.1152/ajpendo.00328.2021). The present paper investigated the effects of one-legged resistance exercise training with/without protein supplementation on glucose control, the insulin-stimulated glucose uptake pathway in skeletal muscle, and fasting plasma branched-chain amino acid concentrations in healthy humans. The researcher, with her direct supervisory team, generated the research hypotheses, and the skeletal muscle and plasma sample analysis plan for this chapter. The experiments for the skeletal muscle and plasma analyses presented in this chapter were performed by the applicant in the laboratories at the University of Roehampton. Finally, the applicant wrote the manuscript below that is in preparation for publication and will therefore be the first author.

4.2 Introduction

Insulin resistance (IR) is a complex metabolic disorder implicated in the development metabolic syndrome and many chronic diseases, type 2 diabetes mellitus (T2DM) and is defined as a state of reduced responsiveness to high insulin concentrations in tissues that are normally insulin-sensitive, such as skeletal muscle (Zimmet, Alberti, and Shaw, 2001). Obesity is a key risk factor in IR development and progression, primarily due to

the accumulation of lipids within skeletal muscle fibres. This lipid build-up is closely associated with elevated concentrations of plasma fatty acids. Among the lipids affected, ceramide is notably overproduced as a result of the increased concentration of fatty acids (Wymann & Schneiter, 2008). The presence of ceramide triggers the activation of protein phosphatase-2A (PP2A), which subsequently leads to the inactivation of protein kinase B (Akt) and ultimately diminishes the insulin response (Summers, 2006). Interventions that reduce body fat mass by using a combination of dietary changes and/or increased total energy expenditure through exercise training have been shown to reduce obesity and improve insulin sensitivity and management of metabolic syndrome (Petersen et al., 2012; Straznicky et al., 2014; Weickert, 2012).

Dietary protein is an important component of a healthy lifestyle due to its roles in body protein remodelling, with inadequate protein ingestion being linked to malnutrition, even when associated with adequate energy intake. The recommended dietary allowance (RDA) for the general population are 0.75 (COMA, 1991) and 0.8 g protein/kg body mass/d in the United Kingdom and in the United States of America, respectively (Jäger et al., 2017), with those engaging in regular exercise requiring up to 1.2 g protein/kg/d (Antonio, 2019; Kreider & Campbell, 2009), or as high as 1.6 g protein/kg/d for people engaging in intense exercise (Phillips, 2012; Tarnopolsky, 2004). These greater needs in dietary protein are associated with exercise-induced protein synthesis, immune and endocrine responses, as well as mitochondrial biogenesis (Kreider & Campbell, 2009; Lemon, 1991). In their study, Philips et al. observed a notable increase in the fractional synthesis rate (FSR) of muscle protein following a single bout of exercise (Phillips et al., 1997). Specifically, they found that the muscle protein FSR was elevated by 112% from

resting levels 3 hours after exercise and remained significantly increased up to 48 hours post-exercise, with a 34% increase compared to the baseline resting value. Additionally, the researchers observed an increase in the fractional breakdown rate (FBR) of muscle protein up to 24 hours following the exercise intervention when compared to the baseline measurement, highlighting the increased need for dietary protein during exercise.

Replacement of some dietary carbohydrate with protein in low-fat diets (<30%) has been shown to enhance body mass loss (Skov et al., 1999), with favourable changes in body composition in several short-term (≤ 6 months) controlled studies (Brinkworth et al., 2004; Layman et al., 2003; Parker et al., 2002). The body fat loss seen when increasing the protein content within an isoenergetic diet has been attributed to three main mechanisms. First, proteins are more satiating than carbohydrate or fat and may increase the sensation of fullness, perhaps leading to a reduction in the overall energy intake (Leidy et al., 2007; Noakes, 2008). Second, proteins have a higher thermic effect of food when compared with fat and carbohydrate, leading to an increased energy expenditure (Halton & Hu, 2004). Third, during body mass loss and energy-restricting diets, there is an increased risk of losing skeletal muscle mass along with fat tissue, and increased dietary protein intake preserves lean tissue mass and contributes to the long-term improvements in body composition (Layman et al., 2003; Mettler et al., 2010).

Nevertheless, the impact of high-protein diets on insulin sensitivity is a controversial topic and requires additional research and understanding. While dietary proteins promote insulin secretion due to their insulinotropic effect (Rietman, Schwarz, Tomé, et al., 2014), leading to increased glucose clearance from the bloodstream in the shorter term, longitudinal studies have suggested a link between high dietary protein intake and

increased plasma branched-chain amino acid (BCAA) concentrations, which have been further linked with an increased risk of developing IR, metabolic syndrome, and T2DM (Pounis et al., 2010; Ricci et al., 2011; Sluijs et al., 2010; Song et al., 2004). However, the current literature does not provide a clear answer as to whether high-protein diets and high circulating concentrations of BCAAs contribute to IR or are a consequence of a blunted inhibitory effect of insulin on proteolysis (Halvatsiotis et al., 2002; Welle & Nair, 1990) or due to dysfunctions in mitochondrial metabolism of BCAA (Adams, 2011; She et al., 2007; Zhou et al., 2019).

The exact mechanism by which excessive amino acid availability may contribute to insulin resistance has not been fully understood. However, it has been hypothesised that mechanistic target of rapamycin (mTOR) complex 1 hyperactivation in the presence of amino acid overload contributes to reduced insulin-stimulated glucose uptake due to insulin receptor substrate (IRS) degradation and reduced Akt-AS160 activity (Ancu et al., 2021). However, this is a notion that is relatively novel and requires greater scrutiny through human studies. In addition, it is not clear if exercise training can alleviate any potential defects associated with amino acid (AA) ingestion, as the AAs may be utilised to fulfil the demands resulting from resistance exercise-induced increases in muscle myofibrillar protein breakdown as explained in Tipton's comprehensive review paper (Tipton, 2011). A systematic review including 11 studies reported that exercise training programmes are effective strategies to improve glucose control and insulin sensitivity in people with T2DM (Sampath Kumar et al., 2019), and we have hypothesised that exercise training will lead to reduced fasting plasma BCAA concentrations due to insulin's role in regulating the uptake and utilisation of BCAAs by skeletal muscle. Another possible

mechanism for the reduction in BCAA concentrations with exercise is that during physical training, uptake and utilisation of BCAAs by the muscles is increased for use as an energy source (Shimomura et al., 2004). In contrast, during a 12-week exercise training regime involving endurance and resistance training in overweight individuals, BCAA concentrations were not reduced, while insulin sensitivity (IS) improved (Glynn et al., 2015). Furthermore, similar outcomes in BCAA concentrations were observed in individuals with T2DM or non-alcoholic fatty liver (NAFL) and in a cohort of BMI-matched control participants (Vanweert et al., 2021).

Based on the strong associations between plasma BCAAs and IR, and given that physical activity is considered a strong tool in improving glucose control and lowering T2DM risk, we investigated the effects of dietary protein supplementation on glucose metabolism and on skeletal muscle proteins involved in the insulin-stimulated glucose uptake and protein synthesis pathways. Additionally, within the scope of this investigation, the protein content was combined with pomegranate or tart cherry extract, known for its abundance in phytochemicals recognised for their positive impact on well-being. This extract has been documented to enhance muscular function, attributable to its anti-inflammatory (Du et al., 2019) and antioxidant properties (Amri et al., 2017). Moreover, evidence has indicated its efficacy in ameliorating diet-induced insulin resistance among rat models subjected to high fat dietary regimens (Zhang et al., 2022).

Through a one-legged exercise model, comparisons were able to be made between exercised and non-exercised skeletal muscle within the same individuals, with either protein-polyphenol supplementation or placebo supplementation, to assess if protein supplementation had detrimental effects on glucose uptake relative to placebo, and if

exercise provided a protective effect against a possible AA-induced intracellular insulin dysregulation.

Furthermore, we assessed the involvement of inositol hexakisphosphate kinase 1 (IP6K1), a little-studied protein that regulates a variety of cellular processes, including insulin signalling, glucose metabolism, and inflammation, and has been noted to be involved in the pathogenesis of several diseases, including T2DM. Studies have shown that IP6K1 can inhibit the insulin signalling pathway by negatively regulating the activity of Akt, a critical mediator of insulin's effect on glucose metabolism (Chakraborty et al., 2010). Inhibition of Akt leads to impaired insulin signalling, reduced glucose uptake, and increased hepatic glucose production, contributing to the development of IR. This may lead to a decrease in BCAA catabolism leading to an accumulation of BCAA in the plasma, which was previously correlated with IR. However, this potential mechanism has not been tested to date.

In addition to its effect on insulin signalling, IP6K1 reduces cellular energy levels and can inhibit AMPK activity (Zhu et al., 2016), leading to an increase in glucose production and decrease in glucose uptake. Modulating its activity may represent a potential therapeutic strategy (Ghoshal et al., 2022; Herlea-Pana et al., 2021). One way of modulating it is though exercise training; high-intensity exercise was noted to decrease skeletal muscle IP6K1 content post-exercise in people with pre-diabetes and in *in vitro* work which was correlated with improvements in insulin sensitivity (Naufahu et al., 2018b).

No data are currently available to characterise IP6K1 in the context of protein supplementation and exercise, so the present study investigated the effect of exercise

training with protein-polyphenol or placebo supplementation on IP6K1 content. Finally, we aimed to investigate the changes in fasting plasma branched-chain amino acid concentrations following exercise training with and without protein-polyphenol supplementation.

This study hypothesised that protein supplementation may elicit a reduction in insulin sensitivity within the non-exercised leg relative to the exercised leg. Additionally, we expected that IP6K1 would exhibit diminished activity irrespective of protein supplementation subsequent to the exercise intervention. Finally, we postulated an increase in individual and combined plasma BCAA concentrations in the PPB group and not in the PLA group.

4.3 Materials and methods

4.3.1 Participants

Ethical approval for the experimental design and procedures was granted by the University of Exeter's Sport and Health Sciences Research Ethics Committee (reference number 71206/B/09) prior to the start of data collection. All individuals participating in the study provided written consent at least 24 h after receiving verbal and written explanations of the experimental procedures.

Sixteen healthy, recreationally active men and women volunteered to take part in the present study (as a subset of participants from (Pavis et al., 2022). Before the start of the training programme, height, body mass and habitual dietary intake records were collected. General participant characteristics have been adapted from the original study and are described in **Table 4.1**.

Several exclusion criteria were established to ensure a homogeneous participant group and minimise confounding factors. The following were exclusion criteria: previous diagnosis of metabolic or cardiovascular conditions; self-reported habitual dietary protein intake below 0.8 g/kg body mass/day or above 1.6 g/kg body mass/day; musculoskeletal injuries that could potentially impact the ability to perform exercise or impair exercise performance; engaging in more than two sessions per week of resistance training or more than six hours per week of endurance training within the six months prior to the study; or currently taking anti-inflammatory medicines or nutritional supplements. By excluding people who met these criteria, the study focussed on a specific population, with the intention that the responses to study interventions would not be influenced by relevant pre-existing health conditions, inadequate or excessive habitual protein intake, recent pre-study exercise behaviours, or recent medication/supplement use.

4.3.2 Human experimental protocol

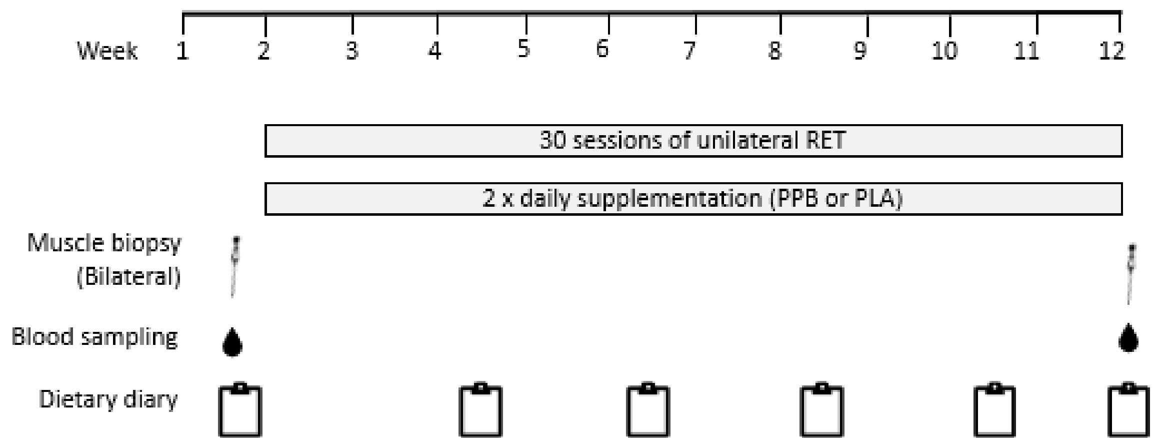


Figure 4.1 Graphical representation of the experimental protocol. A leg was randomly assigned for each participant to undergo 30 sessions of resistance exercise

training (RET), completed in 10-11 weeks (3 sessions/week). Participants consumed post-exercise session and pre-bed protein-polyphenol beverages (PPB, n=8) or maltodextrin placebo (PLA, n=8). Muscle biopsies (from both legs) and blood samples were collected pre and post the testing period. Habitual dietary intake was recorded before, during, and after the testing period.

Participants were randomly assigned to consume either protein-polyphenol beverages (PPB) or maltodextrin placebo beverages (PLA) using a double-blind, placebo-controlled, parallel group study design. Volunteers undertook two familiarisation visits during which they were familiarised with the isokinetic dynamometer (Biodex System 3, Biodex Medical Systems, Inc., Shirley, NY) and individual settings were determined to be used during the training programme. Considering leg dominance counterbalance, one leg was randomly allocated to undergo the 30 sessions of resistance exercise training, whereas the other leg was assigned as the control, non-training leg (refer to **Figure 4.1** for graphical representation of the experimental protocol).

Bilateral muscle biopsies from musculus vastus lateralis and a 10-mL venous blood sample were taken before and at the end of the training programme. Participants arrived in the laboratory in the morning following an overnight fast and 48 hours of prior abstinence from exercise and alcohol consumption.

Exercise training programme

All participants performed 30 sessions of resistance type exercise under supervision during the resistance training programme (RET), performing around 3 sessions/week. If one training session was missed, it was performed at the end of the 10-week training programme in a maximum of 11 weeks in total. Each training session consisted of five

sets of 30 maximal isokinetic knee extensor contractions, alternating between eccentric and concentric types of contraction (2 sets of eccentric contractions and 3 sets of concentric contractions per training session). Participants were provided with verbal encouragement during each set and had 120 s of passive rest between sets (Pavis et al., 2022). Duration for the RET programme was not different between groups ($p>0.05$) and mean values were 73±3 and 73±2 days for PPB and PLA groups, respectively.

Nutritional intervention

The nutritional supplementation began after the first training session and continued until the completion of the exercise training programme, in a double-blind fashion. Beverages were made up from coded sachets containing powders identical in appearance. They either contained a commercially available supplement (Beachbody Performance Recover, Beachbody LLC, Santa Monica, California, U.S.A.), consisting of 20 g of protein (mix of whey, pea, and casein; of which 2.4 g was leucine), 10 g carbohydrate, and 650 mg pomegranate extract (PPB treatment), or an isoenergetic, taste- and colour-matched maltodextrin placebo beverage containing 30 g carbohydrate (PLA treatment), each made in 225 mL of water. After finishing the drinks, an extra 50 mL of water was added to the container and consumed, increasing ingestion of any residual supplement powder within the container.

All volunteers received additional sachets to consume at home in the same manner on the morning of a non-exercising day. Furthermore, all participants were provided with a different sachet to prepare a pre-bed beverage that was consumed no more than 30 minutes before going to bed. The pre-bed beverage for the PPB treatment group consisted of 18 g of protein (1.9 g of which is leucine from micellar casein) and 480 mg of tart cherry

extract, or an isoenergetic, taste- and colour-matched maltodextrin beverage for the PLA treatment group. No adverse side-effects were reported by the participants, with good tolerance. Adherence to the nutritional intervention was 99±0% in the PPB group and 98±1% in the PLA group and was assessed by using a diary to record the time of consumption. No difference in adherence was noted between groups ($p>0.05$).

Habitual dietary intake

Participants completed a 3-day weighed food and drink diary to assess habitual dietary intake, including two weekdays and one weekend day, before and after the RET programme. Furthermore, they completed a 2-day food and drink diary approximately every 2 weeks to assess dietary intake throughout the training programme. Energy intake and macronutrient composition were assessed from the food and drink diaries using online software (Nutritics, Swords, Dublin, Ireland).

4.3.3 Immunoblotting

Muscle tissue homogenates were used for quantification of relative total muscle protein content via Lowry's method (Bio-Rad DC protein assay). Then, standard immunoblotting techniques were used by loading the same amount of total protein (25 μg) into 7.5% precast polyacrylamide gels before being transferred via a semi-dry method to nitrocellulose membranes (Bio-Rad).

Membranes were blocked in Tris-buffered saline (TBS)-Tween 20 containing 2-5% bovine serum albumin (BSA) or dry skimmed milk for one hour at room temperature. Membranes were incubated with primary polyclonal antibodies overnight at 1:1000 dilution in 2-5% BSA or skimmed milk at 4°C. The primary antibodies used were: anti-

total Akt2 (no. 3063 Cell Signalling Technology) (Raun et al., 2018), $pAkt^{S473}$ (no. 9271, Cell Signalling Technology) (Karusheva et al., 2019b), $pAkt^{T308}$ (no. 9275, Cell Signalling Technology) (Raun et al., 2018), Anti-IP6K1 antibody (ab129595, Abcam), recombinant anti-S6K1 (ab32359), $pS6K1^{Thr389}$ (no. 9234, Cell Signalling Technology), $pAMPK\alpha1^{Ser485}/AMPK\alpha2^{Ser491}$ (no. 4185, Cell Signalling Technology), anti AMPKα (no. 2532, Cell Signalling Technology). Membranes were then washed in TBS tween and incubated in goat anti-rabbit secondary antibody (ab216773, Abcam) at 1:10,000 dilution in 0.5% to 5% BSA or skimmed milk for one hour at room temperature. After being washed in tris-buffered saline (#1706435, Bio-Rad, United Kingdom) with 0.1% tween 20 detergent (#1610781, Bio-Rad, United Kingdom), membranes were scanned and quantified with Odyssey® Fc Imaging System (LI-COR). Blots were normalised to total protein measurement using stain-free imaging technology (Bio-Rad, United Kingdom) (Colella et al., 2012).

4.3.4 Blood analysis

Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA), (EIA-2935, DRG Instruments GmbH, Germany). This consists of a 96-well solid-phase two-site enzyme immunoassay based on the direct sandwich technique. 25 µL of each of the six insulin standards (0, 6.25, 12.5, 25, 50, and 100 µIU/mL) were added to the plate in duplicate to produce a calibration curve of concentration against absorbance. Plasma samples were defrosted and vortexed, then 25 µL of each EDTA-plasma sample was added in duplicate to the remaining wells. 25 µL of anti-insulin conjugate (biotin conjugated mouse) was added to each of the wells and then incubated at room temperature for 30 minutes. After the incubation period, the wells

were washed 3 times with 350 µL of washing solution that was prepared as per the manufacturer instructions. Following the final wash, the plate was inverted and tapped on absorbent paper to remove any remaining washing solution and then 50 µL of streptavidin peroxidase enzyme complex was added to each well and was incubated for another 30 minutes at room temperature. After the incubation period, the plate was washed as described above and then 50 µL of peroxidase substrate was added to each well. The reaction was stopped 15 minutes later by adding 50 µL of stop solution to each well, creating a yellow colourimetric endpoint. The absorbance of light was measured within 10 minutes of adding the stop solution - at a wavelength of 450 nm - using a microplate reader (Thermo Scientific Multiscan EX, United Kingdom) and Ascent Software, version 2.6 (ThermoFisher, United Kingdom).

Plasma amino acid concentrations were determined by using Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) technology with the following method. In the experimental procedure, 10 µL of human plasma was transferred to an Eppendorf tube. To this, 10 µL of the Internal Standard (Glycine-2-^{13}C, Sigma-Aldrich; #279439) at a concentration of 30.4 µg/uL was added. Protein precipitation was carried out by mixing the sample with 40 µL of cold isopropanol containing UPLC-MS grade, 1% formic acid (v/v). The mixture was vortexed for 30 seconds and incubated at -20 °C for 20 minutes. Subsequently, the samples were centrifuged at 13,000 g (4°C) for 10 minutes. From the resulting supernatant, 10 µL was used for amino acid derivatisation, as described in the following paragraph.

For the derivatisation step, 1 mL of UPLC-MS grade acetonitrile was added to the AccQTag Ultra reagent powder and mixed by vortexing for 30 seconds. The mixture was

then heated at 55 °C to dissolve the reagent. Next, 70 µL of borate buffer was added to the samples, followed by the addition of 20 µL of AccQTag Ultra derivatizing reagent solution. The samples were vortexed for 30 seconds, and the derivatisation reaction was carried out by heating at 55 °C for 10 minutes.

The UHPLC-MS/MS analysis was conducted using an Acquity UPLC binary system (Waters) connected to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters TQ-S Micro). Electrospray ionisation (ESI) in positive ion mode was employed for MS/MS detection using multiple reaction monitoring (MRM) for compound quantification. The source conditions were set as follows: capillary voltage, 3.5 kV; source offset, 30 V; desolvation temperature, 450 °C; source temperature, 150 °C; desolvation gas flow, 650 L/h; cone gas flow, 150 L/h; nebulizer gas, 7.0 bar; collision gas, 0.15 mL/min.

The chromatographic separation was achieved using reversed-phase gradient chromatography on an ACQUITY UPLC® HSS T3 1.8-µm column (Waters). The mobile phase consisted of 0.1% formic acid in LC-MS grade water (v/v) (A) and 0.1% formic acid in LC-MS grade acetonitrile (v/v) (B). The column temperature was maintained at 45 °C, and a linear gradient elution was performed at a flow rate of 0.65 mL/min. The gradient started with 4% of mobile phase B for 0.5 minutes, followed by an increase to 10% B over 2 minutes, then to 28% B over 2.5 minutes, and finally to 95% B over 1 minute.

Plasma IP6K1 concentrations were determined using a commercially available sandwich ELISA kit (HUFI03803, Assay Genie, Ireland) by following manufacturer's instructions.

4.3.5 Data modelling

Homeostasis Model Assessment 2 of Insulin Resistance (HOMA2-IR), β-cell function (%B) and insulin sensitivity (%S) were obtained by HOMA Calculator v2.2.3 (Unit, 2004). Delta (Δ) values were calculated to determine the change in each variable from baseline to post-intervention within each participant. For glucose, insulin, HOMA2-IR, % β cell function (%B) and % insulin sensitivity (%S), valine, leucine, isoleucine, and total BCAAs, the Δ values were calculated as follows: Δ = value after study intervention minus value at baseline visit (before exercise training and nutritional supplementation) (Morimoto et al., 2014). Percentage change (%Δ) was calculated as follows: (Δ minus baseline value) *100.

Plasma amino acid values are given in arbitrary units (A.U.) as the integrated area count for each individual BCAA within a sample was divided by the integrated area count of the internal standard (2-^{13}C-glycine) within the same sample. This method provides normalisation of each BCAA to a known concentration of added glycine.

4.3.6 Statistical analyses

All statistical analyses were carried out using SPSS [version 28.0.1.1. Chicago, Il, USA (15)]. Baseline characteristics and Δ values were compared between groups using Student's independent-samples *t*-test. A two-way mixed model analysis of variance (ANOVA) was used to investigate changes in habitual diet during the study (time and group factors). Data from Western blot analysis was not normally distributed, according to Kolmogorov-Smirnov tests of normality and it has been transformed to parametric values due to data using square root transformation. Differences over time and between

the conditions were evaluated using two-way repeated measures ANOVA (diet and exercise factors) for proteins from skeletal muscle analysis and plasma parameters. Data are expressed as mean (± SEM). Statistical significance was accepted at $P<0.05$.

4.4 Results

4.4.1 Participant characteristics

There were no differences in characteristics between the groups at baseline (shown in **Table 4.1**), nor in the self-reported habitual diet, assessed by dietary records as explained in more detail in Pavis et al., (2022).

Table 4.1 Participant characteristics

	PPB (n=8)	PLA (n=8)
Age (y)	24 (1)	25 (2)
Body mass (kg)	65.5 (3.5)	67.6 (2.5)
Height (m)	1.70 (0.03)	1.68 (0.03)
BMI (kg/m^2)	22.3 (0.7)	23.9 (1.0)

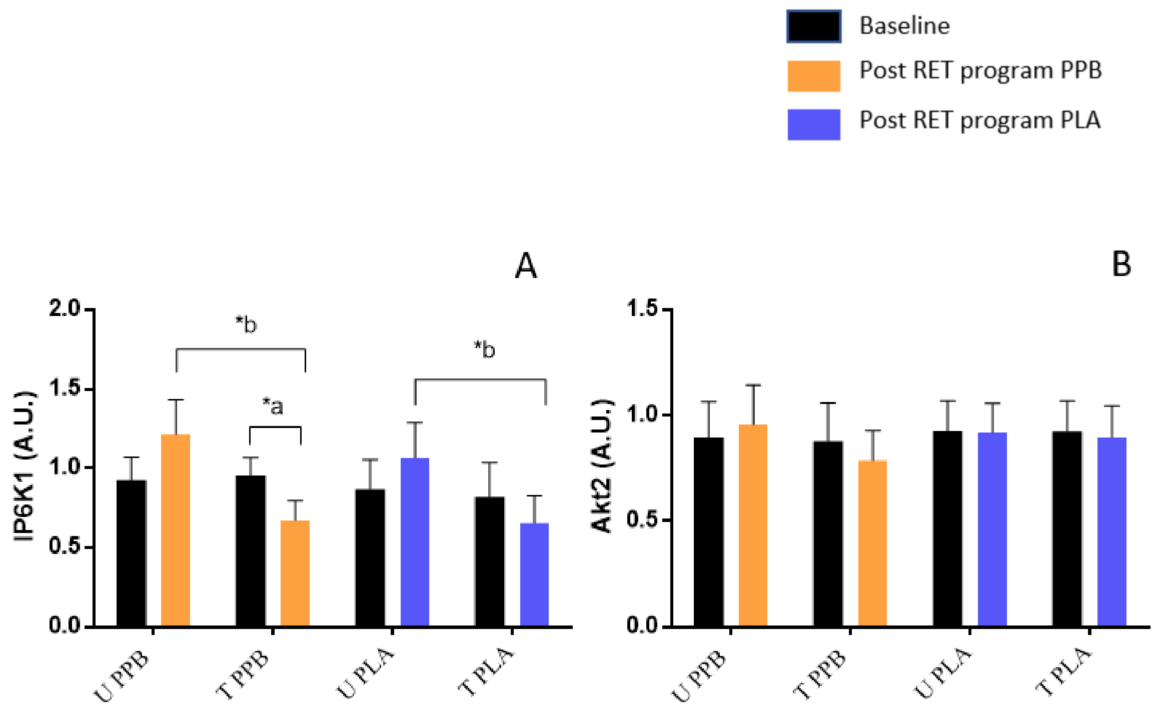
Values are expressed as mean (± SEM). Abbreviations: PPB, protein polyphenol beverage group; PLA, maltodextrin placebo group; BMI, Body Mass Index.

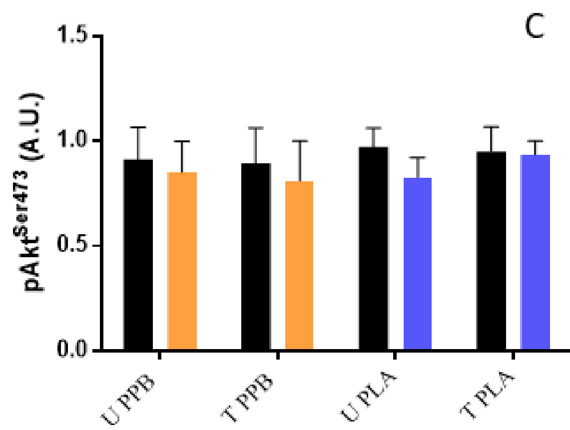
4.4.2 Skeletal muscle analysis

We investigated total and phosphorylated states of proteins involved in insulin-stimulated glucose transport in muscle, before and after ten weeks of unilateral exercise training in eight participants in the protein-polyphenol beverage group (PPB) and eight participants in the maltodextrin-placebo group (PLA), in the exercised leg, as well as the non-exercised leg.

From Western blot analysis, protein content of muscle IP6K1 (**Figure 4.2A**) was observed to be decreased in the exercised leg when compared with baseline (P=0.025) in the PPB group, suggesting a potential association with the intervention, with no significant change within the PLA group (P=0.231). IP6K1 content was significantly higher in the non-exercised leg when compared to the exercised leg with PPB supplementation (P=0.019) post exercise intervention. Additionally, within the PLA group, IP6K1 content was higher in the non-exercised leg compared to exercised leg post exercise intervention (P=0.046). Akt^{Ser473}/ Akt2 (**Figure 4.2E**) was observed to be lower in the non-exercised leg from the PPB group post exercise training, when compared to baseline (P=0.046), but not in the PLA (P>0.05). Total AMPK (**Figure 4.2J**) was observed to be lower in the exercised leg of the PPB group post exercise training when compared to baseline (P= 0.044), but not in the PLA group (P>0.05). Also, total AMPK was lower post exercise training in the exercised leg compared to the non-exercised leg in the PPB group (P=0.002), but not in the PLA group (P>0.05). Similarly to total AMPK, $pAMPK\alpha1^{Ser485}/AMPK\alpha2^{Ser49}1$ (**Figure 4.2K**) was also observed to be lower post exercise training in the exercised compared to the non-exercised leg in the PPB group (P=0.004), but not in the placebo (P>0.05). Additionally, $pAMPK\alpha1^{Ser485}/AMPK\alpha2^{Ser491}$ was lower in the exercised leg

from the PPB group when compared to PLA post exercise training (P=0.013), but not in the non-exercised leg (P>0.05). No significant differences were observed in protein content between the conditions or over time for total Akt2 (**Figure 4.2B**), $pAkt^{Ser473}$(**Figure 4.2C**), $pAkt^{Thr308}$ (**Figure 4.2D**), $pAkt^{Thr308}$/total Akt2 (**Figure 4.2F**), total S6K1 (**Figure 4.2G**), $pS6K1^{Thr389}$ (**Figure 4.2H**), $pS6K1^{Thr389}$/total S6K1(**Figure 4.2I**), ($pAMPK_{\alpha1}^{Ser485}$/$AMPK_{\alpha2}^{Ser491}$)/total AMPK (**Figure 4.2J**), ($pAMPK_{\alpha1}^{Ser485}$/$AMPK_{\alpha2}^{Ser491}$)/ total AMPK (**Figure 4.2L**).

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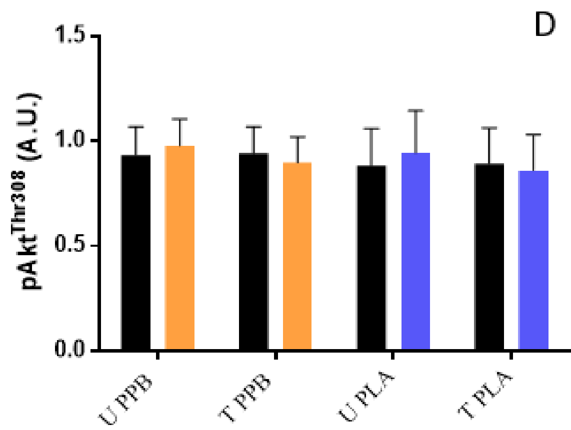
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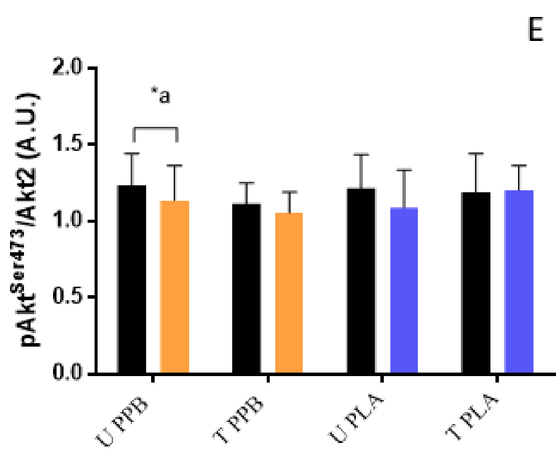
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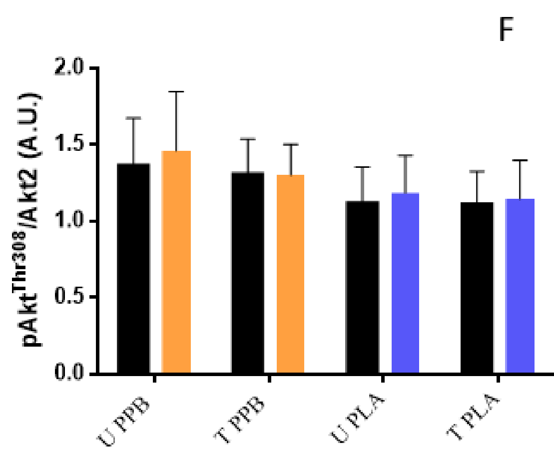
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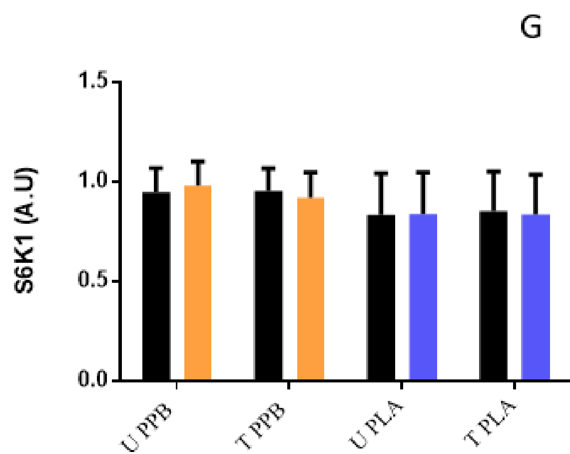
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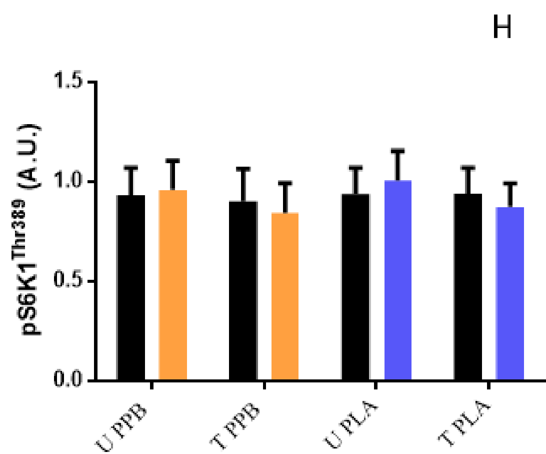
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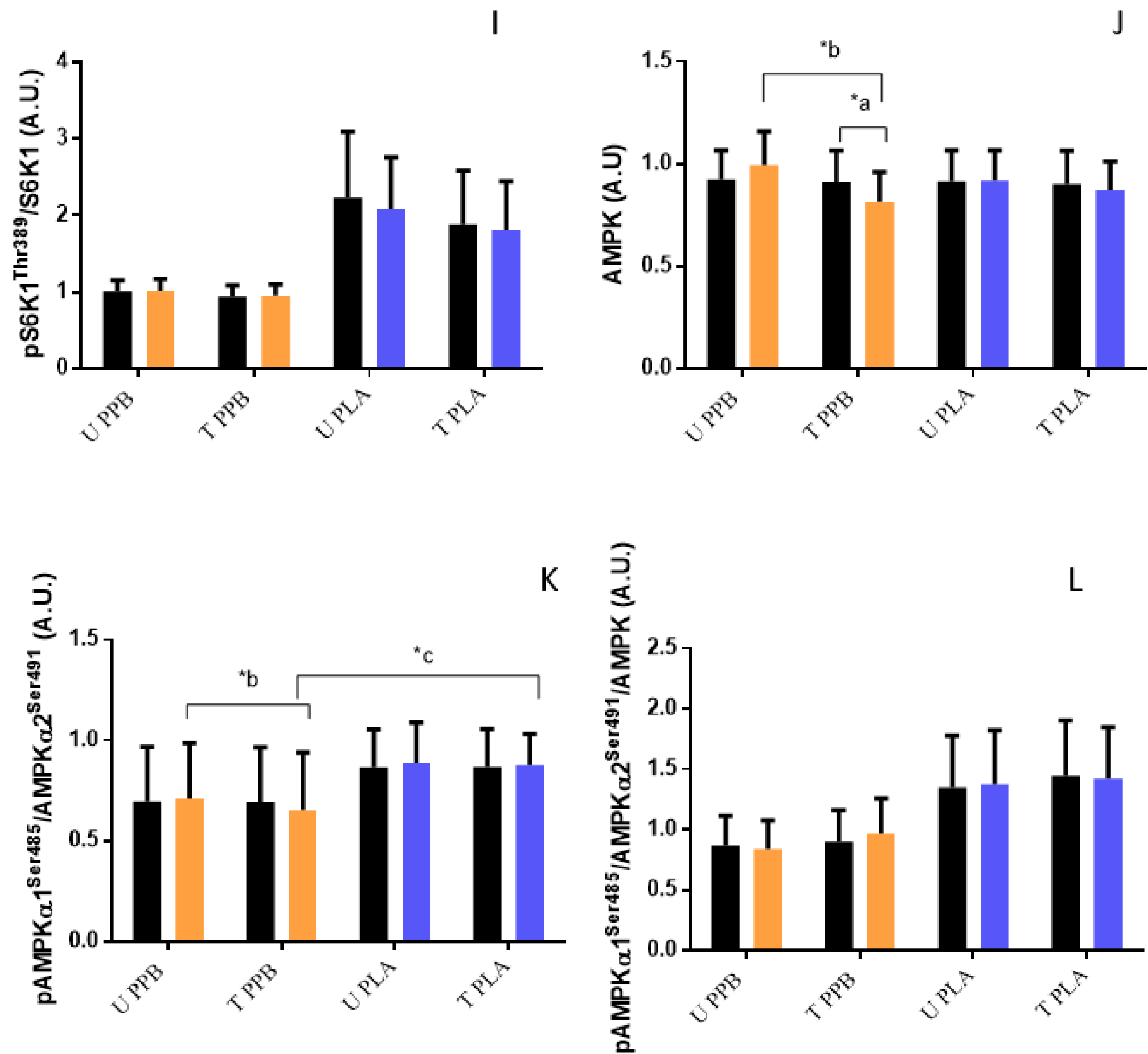

Figure 4.2 Skeletal muscle protein content in protein-polyphenol beverage (PPB) and maltodextrin placebo (PLA) groups (n=8, n=8 respectively), separated between trained (T) and control untrained leg (U), before (Baseline) and post 30 sessions of unilateral resistance-type exercise training for IP6K1 (A), total AKT2 (B), pAkt^{Ser473}(C), pAkt^{Thr308} (D), pAkt^{Ser47}3/total Akt2 (E), pAkt^{Thr308}/total Akt2 (F), total S6K1 (G), pS6K1^{Thr389} (H), pS6K1^{Thr389}/total S6K1 (I), total AMPK (J), pAMPKα1^{Ser485}/AMPKα2^{Ser491}, (K) (pAMPK$_{α1}^{Ser485}$/AMPK$_{α2}^{Ser491}$)/ total AMPK (L). Nutritional supplementation was provided twice daily (post-exercise or in the morning of non-exercising days, and pre-bed). Black columns represent baseline values (pre nutritional and exercise interventions), orange and blue columns

represent post intervention values for PPB and PLA groups, respectively. *a denotes significant differences over time, *b denotes significant differences between legs (T and U) within the same group (either PPB or PLA), *c denotes significant differences between groups in the same leg (T), Significant difference was accepted at P<0.05. Data expressed as mean (± SEM).

4.4.3 Plasma analysis

We investigated the change between baseline and post 10 weeks intervention by calculating delta (Δ) values (**Figure 4.3**) for glucose, insulin, % β cell function, and % insulin sensitivity, Δ HOMA2-IR and noted a significant increase in Δ insulin (**A**) and Δ HOMA2-IR (**B**) for the PLA group when compared with PPB (p=0.039 and p=0.029 respectively). No statistically significant changes were seen for Δ glucose (p= 0.162), Δ %B (p= 0.697) or Δ %S (p= 0.130). Additionally, we assessed changes from baseline within each group and noted a significant increase in HOMA-2IR (p=0.004) and a decrease in % insulin sensitivity (p=0.026) in the PLA group, with no significant changes in the PRO group (p=0.751 and p=0.659 respectively).

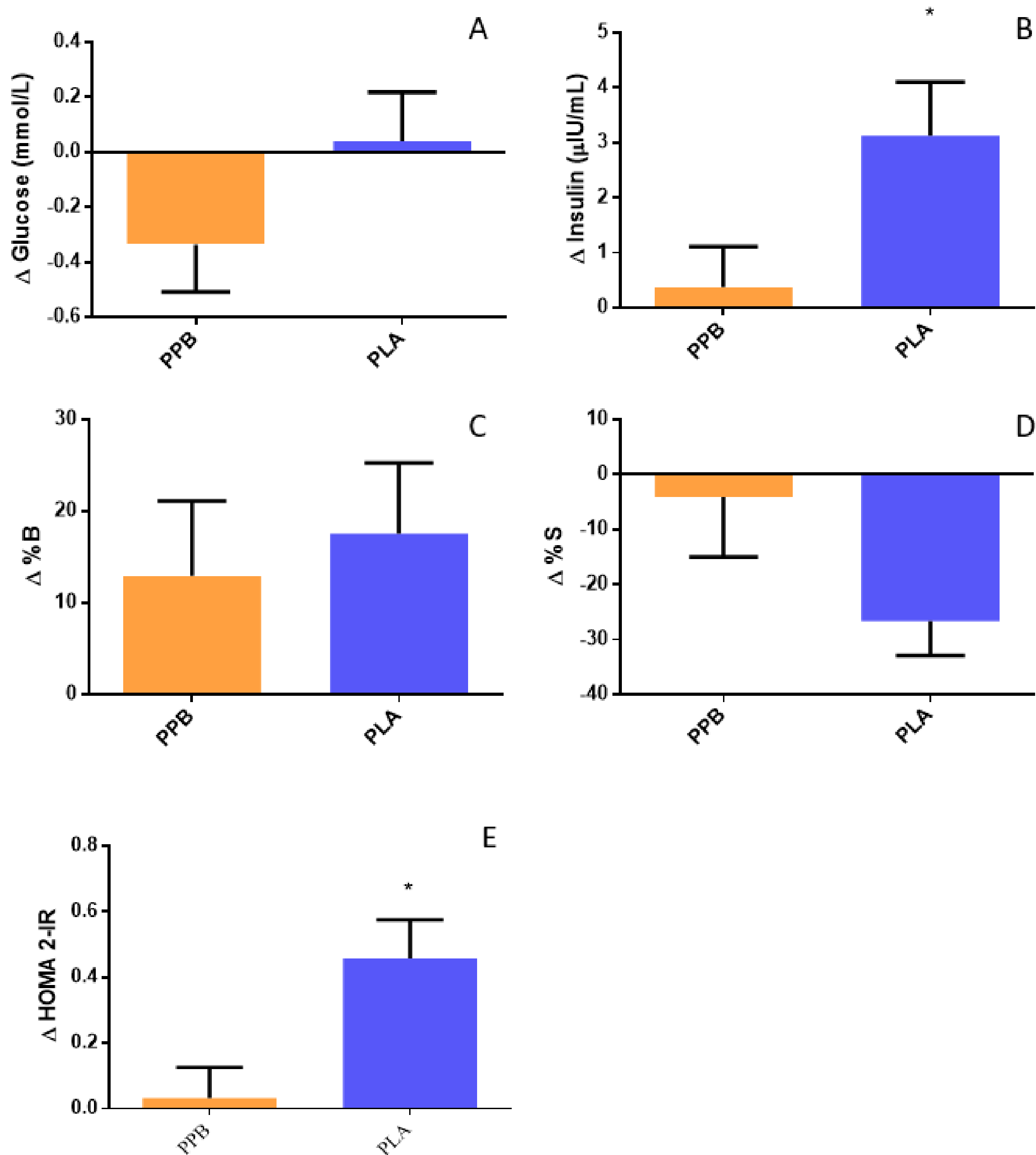

Figure 4.3 Delta (Δ) values for glucose (A), insulin (B), %B (C), %S (D) and HOMA2-IR (E) in the PPB (n=8) and PLA (n=7) groups. Orange columns represent values for the PPB group and blue columns represent values for the PLA group, * denotes significant differences between the groups, P<0.05. Data expressed as mean (± SEM). Abbreviations: % B: % β-cell function; % S: % insulin sensitivity; HOMA2-IR: The homeostasis model assessment 2 of insulin resistance.

We also investigated fasting plasma concentrations (**Figure 4.4**), absolute difference (Δ, **Figure 4.5**) and percentage change (%Δ, **Figure 4.6**) between post-exercise intervention and baseline for individual and total branched-chain amino acids, between PPB and PLA groups. No significant differences were noted in plasma concentrations for any of the BCAAs or the sum of them either between conditions (PPB and PLA) or over time ($P>0.05$), however, we observed a significant increase in Δ valine, Δ leucine and Δ total BCAAs for the PPB group when compared to PLA ($p=0.029$, $p=0.038$, and $p= 0.036$ respectively). Although a similar trend was noted for Δ isoleucine, the p value for the comparison was not statistically significant ($p=0.058$). For percentage change, we noted a significant pre-to-post intervention increase in valine, leucine, and total BCAAs for the PPB group when compared with PLA ($p= 0.034$, $p= 0.041$ and $p= 0.041$ respectively), with a similar trend for isoleucine ($p=0.061$).

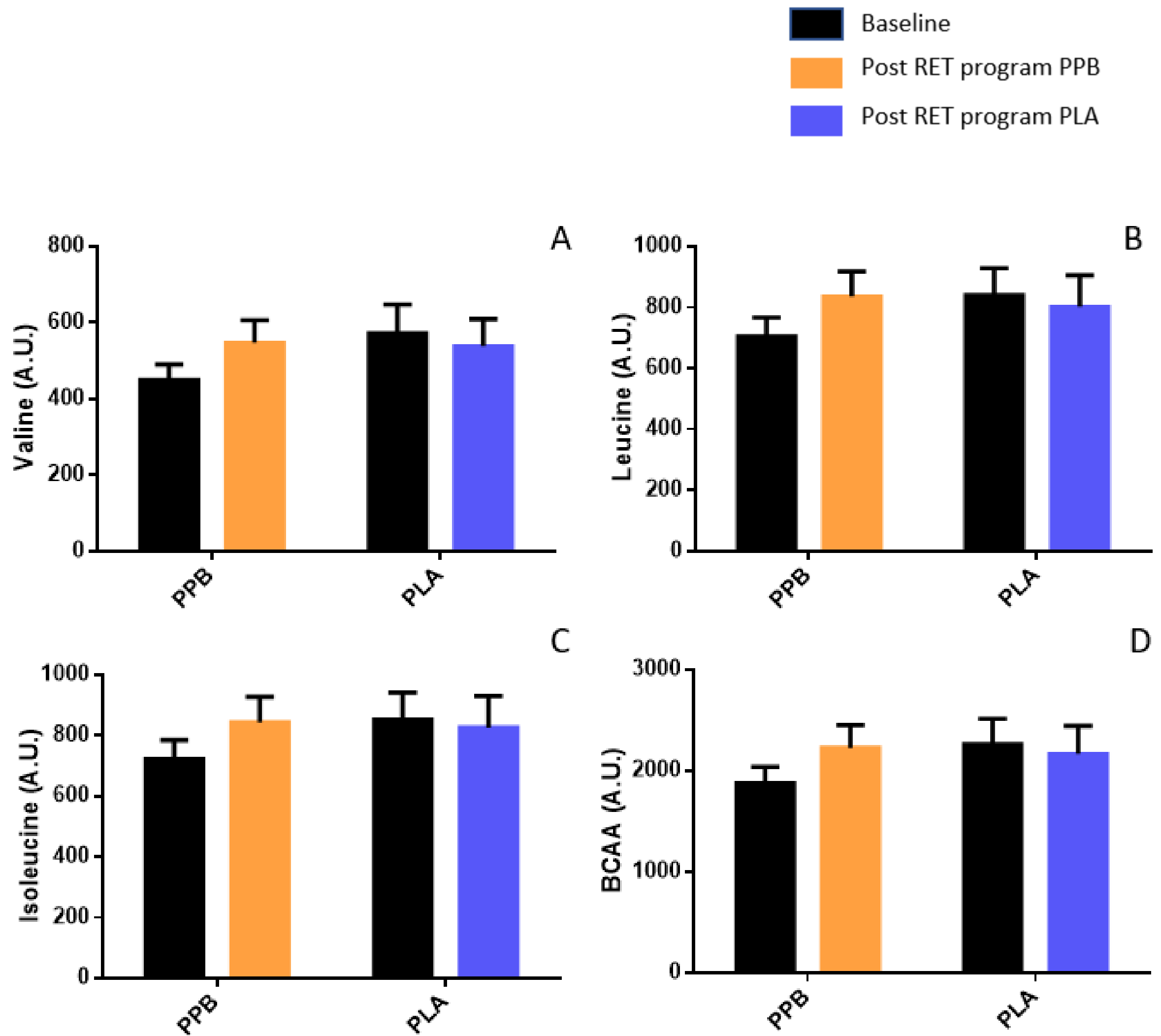

Figure 4.4 Plasma branched-chain amino acid concentration before (Baseline) and post 30 sessions of unilateral resistance-type exercise in the PPB (n=8) and PLA (n=7) groups. Black columns represent baseline values (pre-nutritional and exercise interventions), orange and blue columns represent post-intervention values for PPB and PLA groups, respectively. BCAA (D) represents the sum of the 3 BCAAs.

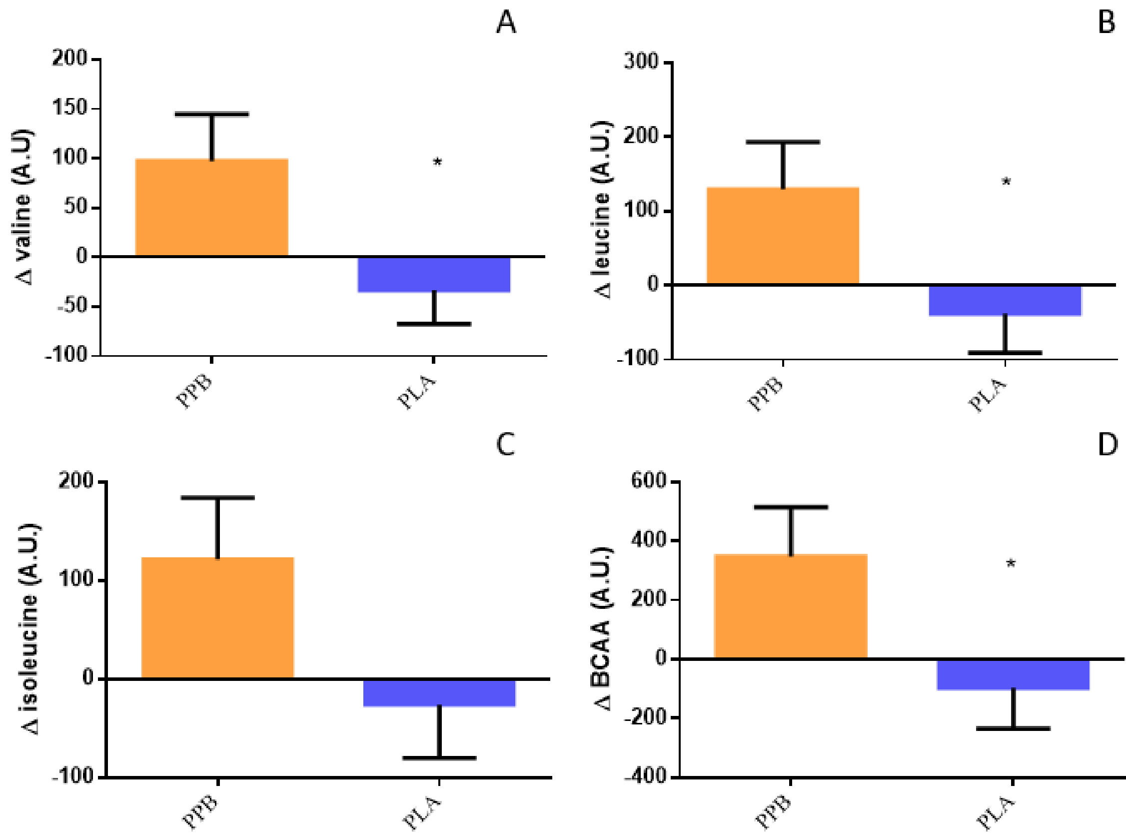

Figure 4.5 Absolute differences (Δ) between post-exercise intervention and baseline for plasma branched-chain amino acid concentrations individually (A valine, B leucine, C isoleucine) and total (D) (data expressed as mean ± SEM), * denotes significant differences between PPB (n=8) and PLA (n=7) groups, P<0.05.

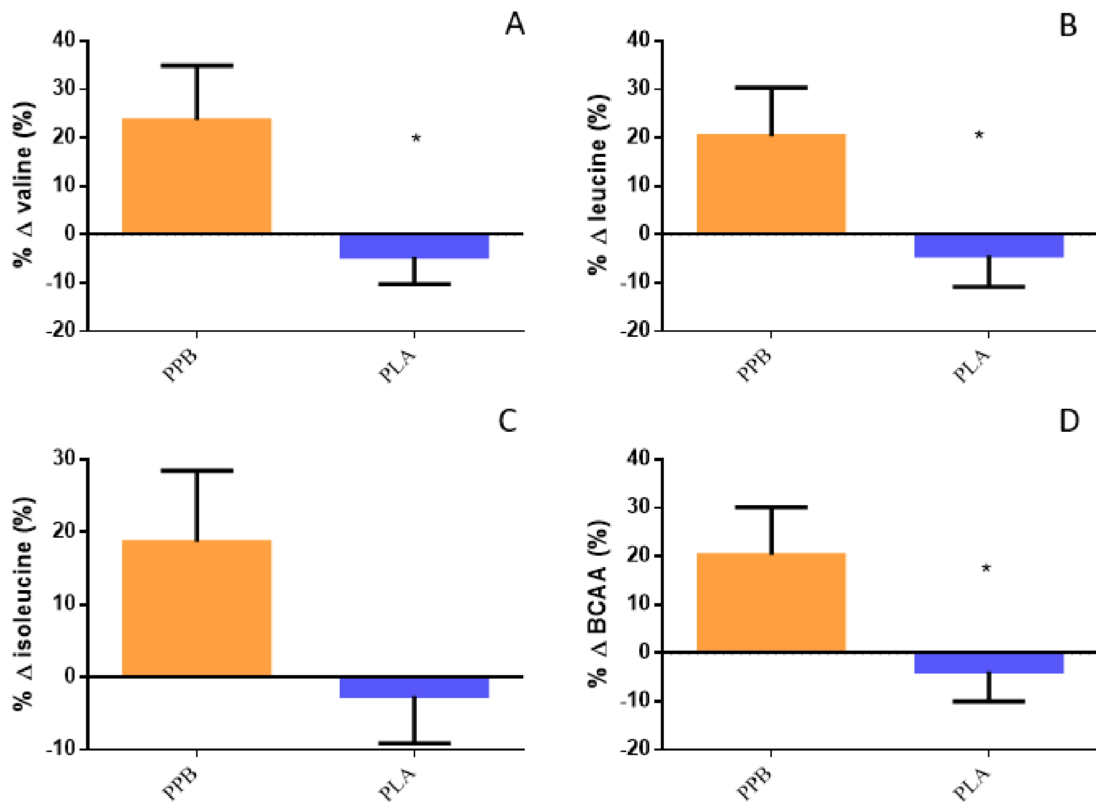

Figure 4.6 Percentage change (% Δ) between post exercise intervention and baseline for plasma branched-chain amino acids individually (A valine, B leucine, C isoleucine) and total (D) (data expressed as mean SEM). * denotes significant differences between PPB (n=8) and PLA (n=7), P<0.05.

Plasma IP6K1 concentration did not differ between the baseline and post-intervention time point for the PPB group (p= 0.226), or the PLA group (p= 0.857), nor was there a difference between the groups at the post-intervention time point (p=0.916) (**Figure 4.7**). We also did not note any significant difference between the groups for Δ plasma IP6K1 (p= 0.435).

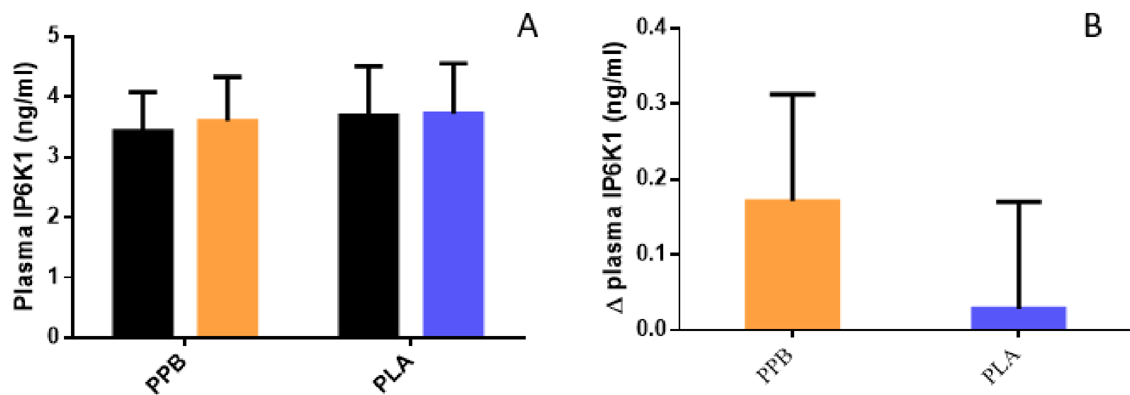

Figure 4.7 Plasma concentrations for inositol hexakisphosphate kinase 1 (IP6K1) before (baseline) and post 30 sessions of unilateral resistance-type exercise training in the PPB (n=8) and PLA (n=7) groups (A) and absolute difference between post intervention and baseline values (B). Black columns represent baseline values (pre nutritional and exercise interventions), orange and blue columns represent post intervention values for PPB and PLA groups, respectively.

4.5 Discussion

The present study assessed the effects of protein-polyphenol or placebo supplementation combined with one-legged resistance exercise training on glucose metabolism and insulin-stimulated glucose uptake and protein synthesis pathways at a cellular level in skeletal muscle. This analysis was performed by comparing exercised and non-exercised skeletal muscle samples in healthy individuals. Secondly, this study determined, for the first time, the effects of protein supplementation on the relatively novel protein, IP6K1, in the muscle, with or without exercise training and in plasma. Lastly, we aimed to assess the changes in plasma branched-chain amino acid concentrations following 10 weeks of unilateral exercise training with protein-polyphenol or placebo supplementation. Skeletal

muscle has an important role in mediating whole body glucose metabolism and a better understanding of its important regulators can lead to more targeted therapies for improving insulin sensitivity in muscle.

The present study found a statistically significantly greater increase in Δ HOMA2-IR and Δ fasting insulin in the PLA group compared with the PPB group. These findings align with existing knowledge that insulin acts to inhibit AMPK through phosphorylation at the inhibitory site $\alpha 1^{Ser485}$. Our study observed an increase in $pAMPK_{\alpha 1}^{Ser485}/pAMPK_{\alpha 2}^{Ser491}$ in skeletal muscle in the PLA group compared with the PPB group following 10 weeks of resistance training. However, no significant difference in total AMPK was observed between the PPB and placebo groups. This discrepancy might be attributed to AMPK activation by high-intensity muscle contractions, as reported in other studies (O'Neill, 2013; Richter & Ruderman, 2009), which could have mitigated a potential AMPK deactivation caused by increased insulin levels. More on the AMPK signalling further down in the discussion.

The increased insulin resistance, as assessed by HOMA2-IR observed in the placebo group after the resistance exercise training intervention may have several underlying reasons. Resistance muscle contractions are known to elevate muscle breakdown rates and stimulate muscle synthesis rates (Smeuninx & McKendry, 2016). When supported by protein supplementation, the damages associated with resistance training may be attenuated (Pearson et al., 2022) with a further increase in myofibrillar protein synthesis rates (Hartono et al., 2022). Data published elsewhere by the authors indicate a 33% increase in myofibrillar protein synthesis rates in the PPB group compared to placebo after an acute bout of exercise at the study's outset (Pavis et al., 2022). Despite both types

of supplementation (PLA or PPB) leading to an approximately 5% increase in muscle volume, the absence of the same level of nutritional support in the placebo group might have resulted in an elevated demand for insulin to maintain glucose metabolism and support muscle protein synthesis due to its anabolic effect (Chow et al., 2006; Fluckey et al., 2006). A comprehensive review paper examining the role of insulin in the regulation of muscle protein synthesis and breakdown revealed that insulin primarily stimulates muscle protein synthesis (MPS) rates in the presence of elevated amino acid (AA) plasma levels (Abdulla et al., 2016) . However, it also exhibits the capacity to reduce muscle protein breakdown (MPB) rates regardless of AA availability. Consequently, the elevated insulin levels observed in the placebo (PLA) group may represent a compensatory mechanism, facilitating similar increases in muscle volume as the protein-supplemented group (PPB), irrespective of AA availability. This suggests that the placebo group may rely on insulin-mediated processes to maintain muscle growth after resistance exercise training program, highlighting the adaptability of muscle metabolism in response to variations in nutrient availability and insulin signalling.

Moreover, an increase in exercise-induced inflammatory response, though not directly measured in our study, could potentially contribute to the observed insulin resistance. Inflammatory responses following exercise are known to negatively influence insulin sensitivity.

On the other hand, our results show no change in HOMA2-IR following the 10 weeks of resistance exercise in the PPB group which is supported by Andersen and colleagues, as measured at 16 and 52 weeks during exercise training (Andersen et al., 2016), which may reflect that the volunteers had a baseline HOMA-2IR value close to the ideal level

(average HOMA2 -IR=1.37) suggesting normal glucose metabolism (Bermúdez et al., 2014).

How was Akt regulated by exercise and protein-polyphenol supplementation?

In the present study, we also investigated the effects of exercise training with protein or placebo supplementation on Akt2, a key signalling molecule involved in insulin-stimulated glucose uptake in skeletal muscle. Our findings showed no significant changes in total Akt_2 in skeletal muscle following the exercise intervention, regardless of the type of supplementation used. These results are consistent with previous studies (Coffey et al., 2006; Deshmukh et al., 2006; Eliasson et al., 2006). On the contrary, some research groups have reported that exercise interventions have either increased or decreased the activity of Akt (Blomstrand et al., 2006; Creer et al., 2005; Dreyer et al., 2006; Terzis et al., 2008). The inconsistent results of these studies may be attributed to various factors, such as exercise mode, type and time, the nutritional status and metabolic state of the participants involved and ingestion of macronutrients that has been demonstrated to indirectly regulate the activity of Akt through its action on insulin secretion (Terzis et al., 2008). Understanding the mechanisms and regulation of Akt and its phosphorylation in skeletal muscle is essential for the development of therapeutic strategies aimed at treating metabolic and muscle disorders.

While no changes were noted in total Akt_2 across the conditions , we noted a decrease in the ratio between phosphorylation of Akt at Ser^{473} and total Akt_2 in the untrained leg in the PPB compared to baseline, which indicates a lower activity at Akt^{473} compared to the activity of Akt, suggesting a reduction in total Akt activity, which is associated with impaired insulin signalling and metabolic dysfunction (Miao et al., 2022). Akt

phosphorylation at Ser^{473} is a critical regulatory event that enhances Akt activity and downstream signalling. In skeletal muscle, Akt^{Ser473} phosphorylation is stimulated by insulin and exercise and is associated with increased glucose uptake, glycogen synthesis, and protein synthesis (Haydon et al., 2002; Tremblay & Marette, 2001). Therefore, changes in the ratio of phosphorylated Akt^{Ser473} to total Akt levels can be used as a marker of Akt activation and possibly cellular insulin sensitivity, as supported by some studies (Albers et al., 2015; Tonks et al., 2013) but not all (Ramos et al., 2021) as explained by (Sylow et al., 2021). Furthermore, while it is known that Akt is downregulated in insulin resistant muscle, Sylow and colleagues showed that the Akt pathway only partially decreased insulin stimulated glucose transport by ~60%, attributing the remaining ~40% to inhibition of Ras-related C3 botulinum toxin substrate1 (Rac1) and that a simultaneous inhibition of Akt and Rac1 led to a completely blocked insulin-stimulated glucose transport pathway in mice models via distinct parallel pathways (Sylow et al., 2014) .

It is important to note that full activation of Akt is tightly controlled by phosphorylation at both Thr and Ser sites (Chog et al., 2005). The activation of Akt at both phosphorylation residues is required for altering the conformation phosphorylation of a different key regulator of protein synthesis which is p70 ribosomal S6 kinase (S6K1) (Camera et al., 2010). This was the case in the current study as we did not note any changes in phosphorylation of Akt at Thr^{308} and subsequently we did not observe any changes in total S6K1, nor in its phosphorylation at Thr^{389} site. It was also shown that other Akt inhibitors, such MK-2206, inhibits insulin-stimulated glucose metabolism and are also associated with inhibition of insulin-induced S6K1 phosphorylation, as well as Akt downstream targets such as Akt substrate of 160 kDa (AS160) (Lai et al., 2012).

Why is AMPK downregulated by exercise training and protein-polyphenol supplementation?

The results of the current study show that total AMPK was not changed by exercise alone, as no changes in total AMPK content was noted in the placebo group between exercised and non-exercised leg post exercise intervention. However, it was decreased by the combination of exercise and protein-polyphenol supplementation, as shown by a decrease in total AMPK content compared to baseline in the trained leg of the PPB group, but not solely by protein-polyphenol intake as no differences were noted over time in the untrained leg of the PPB group. Interestingly, the combination of exercise and protein supplementation seems to have led to a cumulative inhibitory effect, as shown by the decrease in total protein content in the trained leg compared to the untrained leg in the PPB. It is known that AMPK activation leads to stimulation of catabolic pathways, such as fatty acid oxidation and inhibition of anabolic pathways such as lipid and protein synthesis (Mihaylova & Shaw, 2011) .

We hypothesised that the reduction in total AMPK activity would be due to phosphorylation of inhibitory sites $Ser^{485/491}$. The literature notes that exercise changes pancreatic insulin secretion in a unique, intensity dependent manner, with moderate and high intensity exercise noted to increase insulin secretion when adjusted for changes in IR at the skeletal muscle level (Malin et al., 2016) . Moreover, as described in a review paper by Rietman and collegues, the insulinotropic effect of dietary proteins also promotes insulin secretion (Rietman, Schwarz, Tomé, et al., 2014). It is also known that insulin inhibits AMPK through phosphorylation of the inhibitory sites $Ser^{485/491}$ on the $\alpha1/\alpha2$ in a variety of tissues such as cardiomyocytes (Soltys et al., 2006), vascular smooth skeletal

muscle (Ning et al., 2011), adipocytes (Berggreen et al., 2009), and skeletal muscle by reducing AMPK's activity by 40-70% (Valentine et al., 2014). However, this was not the case, as we note significant reduction in the inhibitory phosphorylation sites pAMPKα1^{Ser485}/AMPKα2^{Ser491} in the trained leg compared to untrained leg in the PPB group and also when compared to the trained leg in the PLA group. Also, we did not notice any differences between conditions, nor exercised vs non exercised leg in the ratio between phopspho-AMPK Ser$^{485/491}$ and total AMPK. In addition, we did not notice any changes in fasting insulin concentrations between baseline and post intervention in the PPB group. Taken together, it may suggest that the reduction in AMPK in the present study may be insulin independent.

Another hypothesis for the changes seen in AMPK is related to IP6K1 content, a possible connection that was observed by other studies too; it was concluded from mouse models that IP6K1 is involved in reducing whole-body energy expenditure, reducing the body's metabolism by upregulating AMPK (Ghoshal et al., 2022; Zhu et al., 2016). We noted that IP6K1 was downregulated in the exercised leg compared to the non-exercised leg independently of protein supplementation, as explained into the next section, while AMPK was decreased by exercise training in the context of protein supplementation only, 48 post last exercise session and in a fasted state, when the muscle biopsy was taken.

Did the protein-polyphenol supplementation influence muscle IP6K1 content following exercise training?

Previous research indicates that IP6K1 is involved in disruptions to insulin sensitivity and glucose metabolism and its deletion protects mice from high-fat diet induced obesity and insulin resistance (Ghoshal et al., 2022). Moreover, it was also shown to modulate

pancreatic β-cell function (Chakraborty, 2018; Mukherjee et al., 2020; Ray, 2007), and inhibiting this kinase may mitigate metabolic dysfunctions and delay or prevent the onset of metabolic conditions. In the current study, a significant decrease in IP6K1 muscle content was observed in the exercised leg of the PPB group after ten weeks of unilateral exercise training, while no significant differences were observed within the PLA group. Naufahu and colleagues found that skeletal muscle IP6K1 was acutely reduced, and two-compartment modules of insulin sensitivity were improved following high intensity exercise in glucose intolerant individuals, suggesting that a downregulation of IP6K1 may have a positive role on glucose control in humans, at least at the muscle level (Naufahu et al., 2018b). The current study shows that IP6K1 is also downregulated by exercise in a chronic manner, as shown by the decrease in the exercised leg following 10 weeks of training, even 48 hours after the last training session, when the muscle biopsy was taken. Additionally, our results suggest that the protein-polyphenol supplementation may have had an additive positive effect on reducing IP6K1 content in the exercised muscle, as no changes were noted over time in the exercised leg within the placebo control group.

Interestingly, the significant increase in the non-exercised leg compared to the exercised leg post exercise intervention in both the PPB and PLA groups, suggest that exercise training may have a different effect on IP6K1 expression, impacting muscle tissue that was not directly exercised, by seeing a local increase in IP6K1 expression. Furthermore, it is plausible that protein-polyphenol supplementation alone may have a negative impact on insulin signalling and glucose uptake in the absence of exercise. This is also supported by a previous study who demonstrated that IP6K1 muscle content was increased in individuals with obesity 120 min post prandially lean meat compared to the basal value

(Barclay et al., 2020). In addition, previous studies have indicated that elevated levels of IP6K1 can impair insulin signalling by inhibiting Akt (Chakraborty et al., 2010). Although we did not notice a significant decrease in total Akt_2 or $pAkt^{473/308}$ between the exercised and non-exercised legs, we did observe a decline in estimated Akt activity over time in the non-exercise leg in the PPB group, as noted by the difference in the ratio between total Akt to phosphorylated Akt, as explained above. This is also in line with the formation and metabolism cascade of inositol polyphosphates in human cells which reveals that the process is initiated by the enzyme phospholipase C (PLC) which catalyses the conversion of phosphatidylinositol bisphosphate (PIP_2) into Ins (1,4,5) P3, indicating a requirement for PIP_2 for IP6K1 production (Desfougères et al., 2019; Shah et al., 2017). Moreover, PIP_2 is part of the activation process of Akt by insulin, as PIP_2 and activation of phosphoinositide-dependent protein kinase-1 (PI3K) leads to formation of the lipid messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3) which then binds to the plekstrin homology domain of Akt, eventually leading to its activation (Ancu et al., 2021). These findings suggest that IP6K1 may not directly inhibit Akt activation, but its formation from PIP_2 may be a contributing factor, supported elsewhere (Chakraborty et al., 2010) and further explained by Mackenzie and Elliott (2014).

The reduction in IP6K1 content observed in the exercised leg of the PPB group over time provides evidence for the potential benefits of protein-polyphenol supplementation on insulin signalling and glucose uptake in skeletal muscle when coupled with exercise training. However, the significant increase in IP6K1 content in the non-exercised leg compared to the exercised leg with protein-polyphenol supplementation post-exercise

intervention emphasises the critical role of exercise as a crucial factor in mediating this potential positive effect in insulin-signalling.

Why phospho-Akt did not increase when muscle IP6K1 decreased?

Contrary to our initial hypothesis, the present study did not observe an increase in phospho-Akt$^{473/307}$ associated with a decrease in IP6K1 in the exercised leg, as supported by previous in vitro research conducted on C2C12 mouse myoblasts (R. Barclay & Mackenzie, 2018), as well as animal studies that found enhanced Akt signalling upon IP6K1 knocking out in skeletal muscle (Chakraborty, 2018; Chakraborty et al., 2010). This lack of changes in phospho-Akt in our study may be attributed to the influence of the circulating blood IP6K1 concentration on Akt phosphorylation, which could outweigh the impact of muscle IP6K1. This new hypothesis is supported by the significant variations in IP6K1 content between the exercised and non-exercised legs in both supplementation groups. Consequently, as exercise affects IP6K1 content differently in the exercised and non-exercised muscle, it appears that phospho-Akt is also influenced differently in one-legged exercise interventions compared to previous exercise studies that have reported enhanced Akt activity.

How was plasma IP6K1 influenced by resistance exercise training with PPB or PLA supplementation?

To investigate this new hypothesis, we examined plasma IP6K1 concentrations and found no changes between baseline and post intervention values in either group or between PPB and PLA at the post intervention value time point. The absence of significant changes in plasma IP6K1 concentrations following an exercise intervention, as observed in the study,

raises intriguing questions regarding the connection between plasma and muscle IP6K1 levels. While the study's findings indicate that localised changes in muscle IP6K1 occurred in response to the exercise intervention, the lack of corresponding alterations in plasma IP6K1 levels challenges our understanding of the relationship between these two compartments. The study by Naufahu et al. (2018) previously demonstrated a correlation between plasma IP6K1 and HOMA-IR, suggesting a potential role for plasma IP6K1 in insulin resistance (Naufahu et al., 2018b).

The current study's results suggest that muscle-specific changes in IP6K1 do not necessarily translate to similar changes in the circulation, indicating a possible compartmentalisation of IP6K1 regulation. Further investigation is warranted to elucidate the mechanisms underlying this discrepancy and to determine whether muscle-derived IP6K1 has a distinct physiological function or if there are factors governing its release into the bloodstream. One possibility that warrants consideration is the existence of plasma-derived IP6K1. It is conceivable that the source of plasma IP6K1 may not solely be muscle tissue but could originate from other organs or tissues. This raises questions about the dynamics of IP6K1 secretion, its transport, and potential modulation. These findings have implications for our understanding of IP6K1's role in insulin resistance and metabolic health, potentially opening avenues for novel therapeutic strategies.

Plasma BCAA responses following protein supplementation

The fasting concentrations of individual branched-chain amino acids (BCAAs) as well as their combined total value were examined between baseline and post-exercise intervention in both groups: PPB and PLA. No significant changes were observed in BCAA levels within each group or between the groups at the post-intervention level. This

suggests that the exercise intervention, regardless of protein supplementation, did not elicit a noticeable overall effect on fasting BCAA levels. These findings align with some previous studies that reported no association between short intervention studies on BCAA intake (Cavallaro et al., 2016; Wang et al., 2011), as assessed by dietary recall, and fasting BCAA levels, while contrasting with other studies that found a weak correlation (Jennings et al., 2016; Tai et al., 2010). To present a more comprehensive overview of the changes between the PPB and PLA groups, the calculation of Δ and %Δ indexes was employed for a better observation of individual changes from baseline to post intervention value.

Considering the observed increase in HOMA-2IR in the PLA group compared to no change in the PPB group, and the potential role of BCAA in interorgan crosstalk (Gancheva et al., 2018), it prompted us to explore the possibility that the HOMA-2IR changes may be related to dysregulation of BCAA catabolism and the role of exercise intervention in this process. During exercise, the rate of muscle protein breakdown increases (Phillips et al., 1997), and adequate protein intake is essential to support this process. When protein demand is not met, such as possibly in the case of the PLA group, the observed increased insulin production may act as a compensatory mechanism (Abdulla et al., 2016; Chow et al., 2006), leading to metabolic dysfunction and increased rates of IR as seen in the current study (Thomas et al., 2019), as explained in previous sections.

On the other hand, given that our colleagues have observed an increase in the myofibrillar synthesis rate in the PPB group (Pavis et al., 2022), compared to the PLA group, it is plausible that this may have led to increased BCAA uptake and utilisation, resulting in higher levels of BCAA in the blood and explaining the difference between the groups

without changes over time between baseline and post-intervention within each group. In conclusion, the significant increase in Δ valine, Δ leucine and Δ BCAA for the protein supplementation group compared to the placebo group suggests that protein supplementation had a notable impact on BCAA metabolism compared to PLA during the intervention period. This effect may be related to enhanced muscle protein synthesis and the specific amino acid composition of the protein supplement. Moreover, considering the HOMA-2IR changes in the placebo group, we can infer that protein supplementation, coupled with resistance exercise training, leads to better glycaemic control compared to placebo supplementation.

In addition, while some studies in patients with type 2 diabetes have shown high levels of BCAA in the skeletal muscle and increased activation of S6K1 (Tremblay et al., 2005), this current study did not observe an increase in total or phosphorylated S6K1 in the PPB group, nor an increase in insulin resistance. Thus, the protein supplementation coupled with resistance exercise appears to be more beneficial than placebo supplementation by showing no detrimental effects on IR measured by HOMA-2IR and no increases in fasting levels of BCAA which have been associated with insulin resistance and impaired glucose metabolism, which in turn are key contributing factors to the pathogenesis of type 2 diabetes.

4.6 Conclusions

In conclusion, this study provides significant insights into the potential effects of protein or placebo supplementation, in combination with 10 weeks of unilateral resistance exercise training, shedding light on protein supplementation potential as a dietary

intervention for enhancing metabolic health and improving glycaemic control. The combination of exercise and PPB supplementation lead to decreases muscle IP6K1 content, which may be associated with a reduced risk of developing type 2 diabetes. Conversely, exercise without protein supplementation led to a localised muscle increase in IP6K1 when compared to the exercised muscle, as well as increase in fasting insulin and insulin resistance, measured by HOMA2-IR post 10 weeks of exercise training in this group. Furthermore, notable negative effects of protein supplementation were observed in the non-exercised muscle, with a reduction in the ration between phosphorylated Akt^{Ser473} and total Akt2 activity, which could have adverse implications for glycaemic control by potentially decreasing insulin-stimulated glucose uptake in the non-exercised muscle.

The combination of exercise and protein supplementation offers promise as a strategy to mitigate the risk of type 2 diabetes through the observed decrease in muscle IP6K1 content. Further investigation is required to examine if muscle IP6K1 can impact plasma IP6K1 in longer and perhaps full body exercise interventions.

However, caution is warranted when considering protein supplementation in the absence of exercise. The localised increase in muscle IP6K1 content, coupled with elevated fasting insulin and insulin resistance, suggests potential negative effects on glucose metabolism. The observed negative impact of protein supplementation on Akt activity in the non-exercised muscle further raises concerns regarding the potential disruptions in insulin-stimulated glucose uptake, highlighting the importance of exercise as a key factor in modulating the metabolic effects of protein supplementation. These findings collectively emphasise the complexity of dietary and exercise interactions and their implications for

metabolic health. While protein supplementation appears to have beneficial effects when combined with exercise, its isolated use without exercise may not yield the same favourable outcomes.

Chapter 5. General discussion

This doctoral thesis explores the influence of dietary protein supplementation and resistance exercise training on the development of insulin resistance (IR) and type 2 diabetes (T2D). The study investigates the impact of protein availability on glucose metabolism and the proteins associated with insulin-stimulated glucose uptake and protein synthesis pathways in the skeletal muscle. To address specific research questions, this research investigated AA availability in two distinct populations: obese individuals (Chapter 3) and individuals of healthy weight (Chapter 4).

Insulin resistance is characterised by a diminished capacity of insulin responsive tissues primarily encompassing the liver, muscle, and adipose tissue to effectively respond to the biological actions of insulin and it stands as a critical determinant in the pathogenesis of metabolic disorders, notably T2D (Lee et al., 2022). The precise mechanisms leading to insulin resistance in these tissues types remain elusive, but it is firmly established that skeletal muscle plays a central role in insulin-stimulated, by accounting for over 80% of glucose disposal postprandially (Merz & Thurmond, 2020). Unravelling these intricate molecular mechanisms promises valuable insights into the development of insulin resistance that may shape future life-style habits and / or provide future pharmaceutical targets.

Both dietary proteins, especially BCAAs, and exercise interventions are known to activate the muscle-contraction-stimulated and insulin-stimulated glucose uptake pathways. However, elevated BCAA concentrations have also been linked to IR, although it remains unclear whether high plasma BCAAs contribute causally or result from this condition (Nie et al., 2018). It is worth noting that numerous studies examining the impact of protein supplementation on glucose metabolism rely on amino acid infusion studies. While these

types of studies are important for elucidating the mechanism of actions of the activation pathway, they are known to significantly increase the bioavailability of amino acids compared to the bioavailability following intake of more natural dietary protein. In addition, currently there are limited studies that provide comprehensive data examining the impact of whole food dietary sources, particularly in terms of their ability to stimulate muscle protein synthesis rate and glucose uptake, when compared to isolated protein sources. This is particularly important as there is a prevailing tendency for individuals to primarily derive their dietary protein from whole foods during most meals. Beyond the provision of essential amino acids, protein-rich whole foods come bundled with a diverse array of non-protein components, including lipids, micronutrients, and various bioactive compounds, intricately interwoven within their food matrix (Burd et al., 2019). These multifaceted constituents have the potential to engage in intricate interactions that may significantly influence the regulation of muscle protein synthesis rates.

To overcome some of these limitations of previous published studies, Chapter 3 investigated the immediate effects of moderate- (50 g) and high-protein (100 g) meals, both featuring chicken breast as the protein source. A third trial was incorporated to this study to examine the impact of a moderate-protein meal with added fat, matching its energy content with the high-protein meal. The inclusion of this control trial played a pivotal role in our study by enabling it to dissect whether the observed differences between trials were primarily attributable to the increased protein intake or potentially influenced by an augmented energy intake. Furthermore, the control trial served as a valuable tool in investigating the impact of dietary fat supplementation on amino acid absorption when compared to the other experimental conditions. This comprehensive

approach facilitated a more nuanced understanding of the complex interactions between macronutrients and amino acid absorption. Our initial hypothesis suggested that a high-protein meal consumption would down-regulate key proteins involved in the insulin signalling cascade. It was further hypothesised that if these molecular proteins were altered by AA ingestion, would this have a negative effect on whole-body insulin sensitivity. However, Chapter 3's findings provided some evidence of improvements of insulin signalling with no measurable differences in insulin resistance. In Chapter 4, our focus shifted to the extended effects of 10-week protein supplementation, either with or without a concurrent exercise intervention. In this context, we hypothesised that protein supplementation would negatively affect whole body glucose control and proteins associated with insulin-stimulated glucose uptake. Our hypothesis centered around the possibility of an overstimulation of the protein kinase B - mechanistic target of rapamycin (Akt-mTOR) pathway driving ribosomal protein S6 kinase beta-1 (S6K1) -mediated feedback inhibition of insulin signalling (Ancu et al., 2021). Additionally, we anticipated that the introduction of an exercise intervention might positively counteract these potential negative effects.

The evidence presented in this thesis aims to not only contribute to our understanding of these topics but also to provide insights into future research avenues and the clinical implications of protein supplementation and exercise interventions.

5.1 High-protein intake, glucose control and exercise

Numerous studies have demonstrated the health benefits of increasing the proportion of daily energy intake derived from dietary protein, especially during energy restricting weight loss diets (discussed in Chapter 2). These benefits encompass the preservation of

lean mass, and an elevated thermic effect compared to low-protein diets (Antonio, 2019; Halton & Hu, 2004). However, the findings within this thesis indicate that acute ingestion of 100 g of chicken-based protein does not induce significant changes in insulin sensitivity compared to lower protein doses. This conclusion stems from assessments of insulin sensitivity and glucose effectiveness in the four hours following meal consumption using the intravenous glucose tolerance test (IVGTT).

Over the long term, after a 10-week period of one-legged resistance exercise training and placebo supplementation, fasting insulin levels and insulin resistance increased from baseline as presented in Chapter 4. This highlights the importance of protein intake, particularly with resistance exercise regimens. The increase of skeletal muscle protein breakdown rates (MBRs) and muscle protein synthesis rate (MPS), likely occurring in response to the exercise stimulus necessitates an increased dietary protein intake to provide a positive protein balance during the increased protein turnover (Pearson et al., 2022). Within this study, we observed a potential compensatory mechanism of insulin secretion. Specifically, there was evidence of elevated insulin secretion, as indicated by the elevated fasting insulin concentration observed in the PLA (placebo) group post intervention vs baseline values. One of the anticipated outcomes of resistance exercise training is muscle hypertrophy. Interestingly, the data also showed that both types of supplementations (protein and placebo) resulted in a similar increase in muscle volume, approximately 5%, with no significant disparity between the two groups (Pavis et al., 2022). This implies that the muscle exhibits an adaptability to potentially inadequate dietary protein levels through insulin-mediated processes. These processes appear to be compensatory mechanism that promote increased muscle mass growth by reducing

muscle protein breakdown rates (Abdulla et al., 2016). However, this adaptative response appears to have adverse effects on glucose metabolism. It is associated with an increase in the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), coinciding with elevated insulin levels in the placebo group.

While we did not observe improvements in the HOMA-IR within the protein supplementation and exercise group, this lack of change may be attributed to the participants' initially normal levels of insulin sensitivity at the study's outset. In other words, 10 weeks of one-legged resistance exercise training without appropriate ingestion of protein, considering the increase in MBR and MPS rates, leads to increase in IR, whereas protein supplementation leads to no changes in the normal glycaemic control noted in that group.

5.2 The effects of protein ingestion and exercise training on IP6K1 level

Inositol hexakisphosphate kinase 1 (IP6K1) remains a relatively novel protein, with emerging significance in various cellular processes affecting glucose metabolism and insulin signalling. Specifically, it exerts its influence by inhibiting Akt, a critical protein involved in the insulin signalling cascade, thereby reducing insulin-stimulated glucose uptake and increasing hepatic glucose production (Chakraborty et al., 2010).

The present study reveals intriguing insights. In an acute context (chapter 3), we observe a reduction in muscle IP6K1 protein content following the consumption of a high-protein meal (100 grams), just four hours post-ingestion in an obese population. While this reduction is concomitant with an increase in the Akt^{Thr308} to total Akt2 ratio, showing increased Akt activation, which would create a molecular environment to facilitate increased glucose uptake, there are no discernible alterations in total Akt2 protein content.

Furthermore, in our longitudinal investigation (chapter 4), we note that ten weeks of combined exercise training with protein supplementation resulted in a significant decrease in skeletal muscle IP6K1 protein content. Notably, exercise training alone does not yield the same effect, underscoring the role of protein supplementation in diminishing muscle IP6K1 levels in the context of exercise. We attribute these changes in skeletal muscle IP6K1 and Akt to their shared origin from phosphatidylinositol bisphosphate (PIP2). In essence, the formation of IP6K1 from PIP2 may be a contributing factor to the observed inhibition of Akt, consistent with prior research findings (Chakraborty et al., 2010; Naufahu et al., 2018a) .

Furthermore, we detect disparities between the trained and untrained legs, irrespective of protein supplementation in Chapter 4. Specifically, in the post-exercise intervention sample, we observed a significant increase in muscle IP6K1 content in the untrained leg compared to the trained leg. This suggests that the trained leg acts locally to decrease IP6K1 compared to the untrained leg and perhaps that the untrained leg is less insulin sensitive, driven, at least partly by the effects of IP6K1 on Akt signalling as discussed in sections 3.4 of Chapter 3 and 4.5 of Chapter 4.

To gain deeper insights into the impact of exercise on IP6K1, we extended our analysis to plasma IP6K1 in Chapter 4. Intriguingly, we found no significant changes between baseline and post-exercise intervention in either the protein or placebo supplementation groups. This observation indicates that the one-legged exercise intervention employed in this study may not be sufficient to induce alterations in plasma IP6K1, which has established correlations with insulin sensitivity (Naufahu et al., 2018b). These results

further suggest a compartmentalisation of IP6K1 regulation, and it is conceivable that the source of plasma IP6K1 may not be solely muscle, but other organs or tissues. This is discussed in more detail in Section 4.5 of Chapter 4 of this thesis.

In summary, our findings suggest that high protein intake can lead to a reduction in muscle IP6K1 content in the short term. However, for more sustained and significant reductions over a period of 10 weeks, the combination of resistance exercise and protein supplementation appears to be the most effective approach for reducing muscle IP6K1 concentrations.

5.3 Plasma BCAA concentration

Elevated concentrations of plasma branched-chain amino acids (BCAA) have been consistently observed in individuals diagnosed with T2D or obesity, with notable associations to perturbations in amino acid catabolism elucidated through genetically obese mouse models.

As expanded upon in Chapter 3 of this doctoral dissertation, our investigations has generated data in line with our initial hypothesis. Following the ingestion of meals containing varying protein quantities, we detected noteworthy increases in key BCAA concentrations.

In particular, the high-protein dosage elicited a substantial and sustained elevation in valine, leucine, and total BCAA concentrations relative to baseline, when compared to the two other experimental conditions—moderate protein intake and moderate protein intake with an added fat component. Notably, in the case of valine, we observed a swifter decline in plasma concentrations at the 240-minute mark post-meal ingestion, a phenomenon attributed to concurrent fat ingestion alongside protein, as supported by a

different study showing that fat co-ingestion with either of the other 2 macronutrient slows down digestion time (Dangin et al., 2002). This finding is highlighted by the significant difference in valine concentrations of the high protein dose when compared to the moderate protein dose alone, but not when compared to the moderate protein dose with added fat at the 240-minute post meal ingestion.

Furthermore, it is noteworthy that in the context of the MPDAF trial, the simultaneous ingestion of fats and protein did not result in significant alterations compared to baseline values for any of the BCAAs at any time point up to 4 hours. Importantly, within the same trial, it is imperative to highlight that we did not observe improvements in muscle proteins associated with insulin resistance, such as IP6K1. This observation highlights the intricate complexity of the interplay between macronutrient intake and the modulation of key physiological markers. While our findings suggest that the lack of increases in plasma BCAA post-prandially may not necessarily translate into the same positive effects on skeletal muscle signalling associated with glucose uptake, the multifaceted nature of these interactions necessitates a more comprehensive understanding.

Additionally, as outlined in Chapter 4, our study extended over a 10-week period and was complemented by resistance exercise training. Intriguingly, protein supplementation did not induce significant alterations in fasting concentrations of BCAA, including valine, leucine, and isoleucine, nor did it significantly affect their cumulative levels after 10 weeks of supplementation within the context of a daily exercise regimen. It is worth noting that this observation is coupled with an increase in insulin resistance, as measured by the HOMA-2IR index, within the placebo group following the 10-week period of placebo supplementation.

To sum up, based on the findings from these investigations, it is reasonable to infer that, in the context of exercise training, the incorporation of protein supplementation exerts beneficial effects when compared to placebo supplementation. The significant increase in Δ valine, Δ leucine and Δ BCAA for the protein supplementation group when compared to the placebo cohort suggests that protein supplementation had a notable impact on BCAA metabolism compared to PLA during the interventional phase of the longitudinal study. This effect may be related to enhanced muscle protein synthesis facilitated by the specific amino acid composition in the protein supplement. Notably, the absence of an increase in insulin resistance within the protein supplementation cohort, unlike the placebo cohort, further highlights the potential advantages of protein supplementation in ameliorating insulin resistance under the conditions of exercise training. This is expanded upon in section 4.5 of Chapter 4.

Notably, though not within the scope of this thesis, it is worth highlighting the intricate role played by myokines, which are released by skeletal muscle in response to the dynamic contractions induced by exercise. Myokines have emerged as fascinating molecular messengers that exert a range of beneficial metabolic effects, not limited to the confines of skeletal muscle but extending their influence systemically by fostering an anti-inflammatory milieu and promoting insulin sensitivity (Das et al., 2020; Huh, 2018; Pedersen, 2019; Pedersen & Febbraio, 2012). While the precise importance of myokine production by skeletal muscle remains a subject of ongoing investigation, we do possess insights into the synthesis of L-β-Aminoisobutyric acid (BAIBA), a natural metabolite

originating from the catabolism of L-valine catalysed by transaminase enzymes (Tanianskii et al., 2019).

It is of particular interest to note that BAIBA serves as a noteworthy example of a myokine with distinctive properties. BAIBA emerges as a byproduct from valine secreted by myocytes in response to the dynamic stimuli of exercise in a reaction by the mitochondrial enzyme 4-aminobutyrate aminotransaminase (Audzeyenka et al., 2023). Significantly, its levels exhibit an inverse association with the risk of metabolic conditions (Kammoun & Febbraio, 2014; Roberts et al., 2014) and improving insulin sensitivity (Audzeyenka et al., 2023). Therefore, within the purpose of this investigation into the effects of protein supplementation coupled with a longitudinal exercise regimen, the absence of statistically significant differences between baseline and post-intervention values for BCAA, particularly valine, may be attributed, at least in part, to the intricate interplay of muscle-derived factors production, including myokines. This is supported by a different study that suggests that muscle contraction during exercise can promote the catabolism of valine into BAIBA, therefore inhibiting any potential BCAA-induced IR (Shou et al., 2019).

This novel and evolving topic requires further exploration to comprehensively unravel the benefits it offers and, in particular, to elucidate the intricate relationship between protein supplementation and myokine production and its potential implications for optimising health and exercise interventions.

5.4 Thesis limitations

Chapter 3 of this study necessitated participants to arrive at the laboratory in the morning while in a fasted state and subsequently consume their regular diets prior to engaging in the research study. This dietary intake, which varied among participants, could potentially

have influenced their post-absorptive metabolism, as noted in previous research (Robertson et al., 2002). The ingestion of varying quantities of fat and carbohydrates has been associated with the potential to disrupt anabolic signalling pathways and foster disparities between the experimental trials. In retrospect, it may have been beneficial to implement strategies aimed at standardising the composition of participants' evening meals in an effort to mitigate the impact on skeletal muscle metabolism during the post-absorptive phase. This precaution could have contributed to enhanced control and minimised potential confounding factors in the study's outcomes.

Another noteworthy limitation of this study is related to the measurement of plasma BCAA concentrations, which were assessed in arbitrary units rather than the more universally recognised international system unit of mmol/L. The utilisation of Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) technology for measuring plasma amino acid concentrations represented a novel approach within the laboratory at the University of Roehampton. This cutting-edge technique was developed through collaboration with an in-house UHPLC-MS/TS expert. Unfortunately, due to the unavailability of specific BCAA standards, our ability to perform calculations necessary for expressing concentrations in mmol/L was limited.

To circumvent this challenge, an in-house labeled glycine standard was employed to normalise each BCAA value by comparing it to the known concentration of the added labeled glycine standard. While this approach allowed us to make relative comparisons within the study, it prevented us from directly investigating and comparing baseline plasma BCAA levels between the two distinct populations examined in the study: obese individuals with elevated insulin resistance (as assessed by the HOMA-2IR) in Chapter 3

and normal-weight individuals in Chapter 4. Future research investigations may benefit from access to specific BCAA standards, facilitating a more comprehensive investigation of these intriguing associations.

Another limitation of this study is related to increased variability in measurements of parameters associated with glucose metabolism. Prior research conducted within the laboratory of the thesis director of studies (Mackenzie et al., 2011; Ancu et al., 2021) has shown considerable variability in parameters associated with glucose metabolism within groups characterised by glucose intolerance, including individuals suffering with pre-diabetes or obesity. This observation is supported in a separate study by (Vague & Nguyen, 2001), further reinforcing the trends of high metabolic variability. Consequently, this substantial variability might obscure clinically significant insights by potentially downplaying the treatment effect. This, in turn, accentuates the challenge posed by the relatively modest sample size employed in Chapter 3, constituting an additional limitation of this thesis. In adhering to ideal standards, the sample size was determined to achieve adequate statistical power, balancing the ability to detect meaningful effects while minimising the risk of Type I and Type II errors. However, pragmatic constraints, including resource limitations and accessibility to the target population, influenced the final sample size. Despite potential limitations in statistical power, efforts were made to recruit an appropriate cohort given the constraints. Moreover, the interpretation of results should not solely rely on p-values but also take into account effect sizes, acknowledging their importance in assessing the practical significance of findings. Therefore, future studies should seek to recognise the variability within the populations affected by glucose

intolerance and address these challenges while aiming for robust and well-powered research designs.

5.5 Future research directions

This thesis provides insights into the modulation of muscle IP6K1 in response to anabolic stimuli, specifically examining the effects of protein ingestion and resistance exercise training in both acute and longitudinal contexts (spanning a 10-week duration). While Chapter 4 briefly examined the impact of these stimuli on plasma IP6K1, the discussion remains limited in its exploration of this aspect. An intriguing direction for future research lies in investigating how distinct metabolic states might influence plasma IP6K1 levels. Additionally, it is essential to explore the mechanistic underpinnings that drive the observed increase in muscle IP6K1 compared to non-stimulated muscle, particularly focusing on the production of IP6K1 from PIP2 as an alternative mechanism to the activation of Akt within the muscle cell. Such investigations will further our understanding of the intricate interplay between anabolic stimuli, metabolic states, and IP6K1 regulation, potentially uncovering novel pathways with implications for muscle physiology and overall metabolic health.

In addition, this study only investigated the effects of protein and resistance exercise training on BCAA, however the literature also shows that elevated circulating concentrations of the aromatic amino acids, namely tyrosine, phenylalanine and tryptophan are consistently linked to IR and the progression of T2D (Arjmand et al., 2022; Magnusson et al., 2013; Wang et al., 2011; Yamada et al., 2015; Yamakado et al., 2015), demonstrating robust associations with impaired insulin sensitivity in overweight

and obese individuals (Huffman et al., 2009). The observed positive correlations between phenylalanine, tyrosine, and insulin resistance suggest a potential compensatory mechanism wherein these amino acids stimulate insulin secretion to counteract early stages of insulin resistance (Bi & Henry, 2017).

Elevated levels of tryptophan have been associated with an increased risk of developing diabetes, but it remains unclear whether this association is solely due to high plasma levels or if it involves changes in tryptophan metabolism during the early stages of insulin resistance (Chen et al., 2016). Conversely, studies have demonstrated that supplementation with l-tryptophan can suppress the rise in glucose concentrations and preserve insulin secretion in diabetic rats (Inubushi et al., 2012). These beneficial effects may be attributed to the ability of tryptophan, as a precursor, to increase serotonin synthesis (Broderick & Jacoby, 1988; Young, 2007). Serotonin has been shown to directly improve insulin sensitivity through serotonylation in skeletal muscle (Al-Zoairy et al., 2017), influencing the insulin signalling cascade, as well as indirectly through its role in regulating mood and appetite, which impacts satiety, food intake, and body weight (De Matos Feijó et al., 2011). Although the role of serotonin in glucose uptake requires further investigation, it is known that serotonin impacts the insulin signalling cascade via the small GTPase Rab4 which becomes serotonylated and therefore activated leading to translocation of GLUT4 to the cell membrane and glucose uptake into the cell increases.

However, several factors should be taken into consideration, as they can influence the associations between tryptophan and the risk of diabetes. One such factor is the competition of tryptophan with tyrosine and other amino acids for transport across the blood-brain barrier (Worobey, 2022). Additionally, the availability of other amino acids

can also affect tryptophan's actions. It is important to note that serotonin synthesised in the brain specifically influences emotional status, underscoring the significance of its transport across the blood-brain barrier (Bektaş et al., 2020) .

In situations where amino acid levels increase following a high-protein meal, the availability of tryptophan in the brain may be limited, which may explain why supplementation of tryptophan by itself yields positive results rather than as part of a complete protein meal. Furthermore, it should be acknowledged that serotonin synthesis and the association between elevated tryptophan levels and insulin sensitivity are influenced by various factors, necessitating further investigation. For instance, the effects of tryptophan supplementation in conjunction with other amino acids or in isolation require more extensive exploration.

5.6 Conclusions

In conclusion, our findings suggest that increased dietary protein intake has the capacity to modulate muscle IP6K1, presenting a promising treatment for both obese and normal-weight populations. In the acute context, we have observed a significant reduction in muscle IP6K1 levels following high-protein meal ingestion, signifying a potential acute mitigation of IP6K1's inhibitory effects on insulin signalling. This acute response highlights the immediate benefits of protein intake in potentially lowering the risk of T2D.

However, our longitudinal study highlights the importance of a holistic approach that combines exercise and protein supplementation. Over a 10-week period, the combined intervention yielded a significant decrease in skeletal muscle IP6K1 levels, representing a potential strategy for mitigating T2D risk by reducing insulin resistance. This

observation underscores the significance of adequate dietary protein intake within the context of exercise training regimens.

It is imperative to employ caution when considering protein supplementation in the absence of exercise over the long term. Our study revealed increased localised muscle IP6K1 in the non-exercised leg, indicating that protein supplementation alone may not provide the same benefits as when combined with exercise. Additionally, the increase in insulin resistance, as measured by the HOMA-2IR following 10 weeks of intervention without exercise, raises concerns about the potential consequences of isolated protein supplementation. Hence, within the clinical context of metabolic conditions, the concentration of IP6K1 may be utilised in conjunction with an array of biomarkers to enhance the precision of early detection and forecasting of T2D development. They could also serve as a means to evaluate the efficacy of diverse strategies in mitigating the risk of diabetes, such as exercise regimens or dietary interventions.

Moreover, the reduction in muscle Akt activity, as evidenced by the ratio between phosphorylated Akt^{Ser473} and total Akt2 in the untrained leg with protein supplementation over 10 weeks, as expanded upon in Chapter 4 of this thesis, adds another layer of complexity to this picture. This reduction in Akt activity may have adverse implications for glycemic control, emphasising the importance of a comprehensive understanding of the interplay between protein supplementation and exercise.

While promising avenues for mitigating T2D risk have emerged, a nuanced approach is essential. Future research studies should further explore the intricate mechanisms at play, ultimately informing personalised strategies for the prevention and management of T2D and insulin resistance in diverse populations.

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Appendix

Appendix A – Participant consent form (double click on first page to open pdf document)

PARTICIPANT CONSENT FORM

Title of research project: The effects of high protein meals on glucose control in obese or pre-diabetic adults.

Brief description of research project:

You are invited to take part in the study about the effects of high protein meals on obese and pre-diabetic adults. Please take time to read the following information carefully as it is important for you to understand why the research is being done and what it will involve.

What is the purpose of the study?

Previous studies have indicated that a diet high in proteins (HP) can help control blood sugar levels in people with type 2 diabetes. Although many studies have investigated the effect of HP diets on metabolic function, mechanistic action and overall effect on glucose control remain inconclusive. Therefore this study will assess if the acute consumption of proteins will affect blood glucose and insulin concentrations in obese or pre-diabetic participants.

Who can take part?

We are looking for:
- Pre-diabetic OR obese individuals (Body Mass Index $\geq$ 30 kg/ m^2)
- Pre-diabetes criteria: Glycated haemoglobin (HbA1c) values between 6- 6.4% OR fasting plasma glucose levels between 6.1- 7.0 mmol/L
- Non-diabetic
- Age 30-65 years old
- Blood pressure $<$140 mmHg systolic/ $<$90 mmHg diastolic

Individuals suffering from any complications (i.e. neuropathy, nephropathy, retinopathy, vascular diseases, strokes, hypertension, cardiovascular disease), those with anaemia, those who are pregnant, have high blood pressure $>$140 mmHg systolic/ $>$90 mmHg diastolic, current smokers, individuals requiring insulin or any other glycaemic altering medication and consuming high protein diets are unfortunately unable to take part in this study. Health questionnaire will be carried out to confirm your eligibility for the study.

Do I have to take part?

It is entirely up to you if you wish to participate in the study as taking part in this study is voluntary. Therefore, you can withdraw from the study at any time without giving a

Appendix B -Ethics Application (double click on first page to open pdf document)

NAME: DEPARTMENT:	ETHICS REFERENCE:

ETHICS
APPLICATION FORM
(Staff and Research Students)
Sept 2017

PLEASE CHECK THE RELEVANT BOX

(NB. double click on the check box and select 'checked')

MEMBER OF STAFF ☐ RESEARCH STUDENT ☒ (MPhil, PhD, EdD, PsychD)

EXTERNAL INVESTIGATOR ☐ STUDENT (Other)** ☐

*If you are a transfer student or conducting collaborative research you may not need to complete this form: please see Section 2.2. of the Guidelines. **If you are on a taught course you do not need to complete this form unless your project is worth more than 50% of your total credits or you have been asked to do so by your supervisor*

SECTION 1: PERSONAL DETAILS *Please complete the header with your name and Department*	
Name (lead):	Oana Ancu
Other investigators:	Monika Mickute Dr Nicholas Hurren Dr Richard Mackenzie
Correspondence address:	1076 Parkstead House, Whitelands
Telephone no:	+44 (0) 20 8392 3652
Email: *(all correspondence will be sent by email unless otherwise requested)*	ancuo@roehampton.ac.uk
FOR STUDENTS ONLY:	
Programme of Study & Department:	PhD, Life Sciences
Mode of study (full-time/part-time)	Full-time
Director of Studies & Supervisor: (If you are on a taught course please just give the name of your supervisor)	Director of Studies: Supervisor: Dr Richard Mackenzie
FOR EXTERNAL INVESTIGATORS ONLY (please see Section 4.5 of the Ethical Guidelines):	
Name of Academic Assessor:	

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