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Insulin Resistance in Adult Type-2 diabetic Skeletal Muscle: The Effects of Exercise and Dietary-Protein Induced Skeletal Muscle Plasticity controlling Microvascular Blood Flow and Glucose Transport

A Thesis

Presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

(Sport and Exercise Science)

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GENERAL ABSTRACT

Introduction: Insulin-stimulated skeletal muscle glucose uptake is impaired in Type-2 Diabetes Mellitus (T2DM). Insulin resistance leads to reduced skeletal muscle microvascular function and insulin signalling. The purpose of the thesis was to evaluate and compare the effect of chronic intake of a novel keratin-derived protein (WDP) and whey protein, in conjunction with exercise training, on glucose homeostasis and skeletal muscle glucose uptake in T2DM.

Methods: In a randomized, double-blinded clinical trial, thirty-five men and women with T2DM completed a 14-week exercise intervention but were randomly assigned to ingest either post-exercise and evening supplements of 20 g WDP-whey protein blend (WDP, n = 11), whey protein (WHEY, n = 12) or isocaloric maltodextrin (CON, n = 12). Before and after the intervention, fasting HbA_1c and glucose clearance rate (GCR) during a hyperinsulinaemic isoglycaemic clamp were measured. Insulin-stimulated skeletal muscle blood flow and volume were measured during the clamps via near - infrared spectroscopy. Muscle from the *m. vastus lateralis* was harvested prior to and at 1-h into the clamps to determine skeletal muscle insulin signalling proteins.

Results: Substantially bigger improvements in WDP compared to WHEY or CON were found for GCR, insulin-stimulated GLUT4 translocation and insulin-stimulated blood flow. Fasting $eNOS^{ser1177}/eNOS$ possibly increased in WDP and WHEY compared to CON. Capillarization improved in all groups with unclear differences between groups.

Conclusion: WDP-whey blend ingestion during 14 weeks of exercise training improved skeletal muscle plasticity and some processes involved in insulin-stimulated glucose uptake to a greater magnitude compared to whey protein or an exercise-only group in T2DM. WDP protein holds the potential to be an additional therapy to exercise as a treatment in T2DM.

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STATEMENT OF CONTRIBUTIONS

Study conception and design was by Prof. David Rowlands, Dr. Martin Gram, Dr. Jonathan Cornwall and Prof. George Dias. Ethical proposal was written by Dr. Martin Gram and Prof. David Rowlands. Participants were recruited and study co-ordinated by Dr. Martin Gram. Supplements were designed by Dr. Martin Gram and Prof. David Rowlands and prepared for double-blinded matters by Mr. Brandon Woolley. Exercise training sessions were supervised by Wouter Peeters and Dr. Martin Gram. Research assistants helped with planning and assisting exercise sessions, data collection and analysis.

CHAPTER 4:

Hyperinsulinaemic isoglycaemic clamps were supervised by Dr. Martin Gram, Wouter Peeters and one of three general practitioners, Nick Oscroft, Patricia Whitfield or Anne-Julie Oxbøll with assistance from Prof. David Rowlands, Melissa Black and Shanggari Venugopal. Muscle biopsies were conducted by a general practitioner with assistance from Wouter Peeters and Dr. Martin Gram. Near-infrared spectroscopy methods were developed by Adam Lucero and data was collected during clamps by Martin Gram and Shanggari Venugopal. Wouter Peeters and Prof. Phil Sheard developed the immunofluorescence protocol. Wouter Peeters and Dr. Kirsty Danielson developed the Western blot protocol. Wouter Peeters analysed immunofluorescence and Western blot data. Prof. David Rowlands and Wouter Peeters performed statistical analysis.

CHAPTER 5:

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LIST OF ABBREVIATIONS

1RM	1 Repetition Maximum
AIT	Aerobic interval training
Akt	Protein kinase B
AMPK	5' AMP-activated protein kinase
Arp2/3	Actin-related protein 2/3
AS160	Akt-substrate of 160 kDa
BCAA	Branched-chain amino acids
C/F_i	Capillaries per fibre
CC	Capillary contacts
CFPE	Capillaries-to-fibre perimeter exchange index
CRP	C-reactive protein
DAG	Di-acyl glycerol
EE	Early endosomes
eNOS	Endothelial nitric oxide synthase
ERC	Endosome recycling compartment
GCR	Glucose clearance rate
GLUT	Glucose transporter
GSK	Glycogen synthase kinase
GSV	Glucose storage vesicle
HbA_1c	Glycated haemoglobin
HIIT	High-intensity interval training
HK	Hexokinase

HOMA-IR	Homeostatic model assessment of insulin resistance
IL	Interleukin
IRAP	Insulin-regulated aminopeptidase
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
WDP	Keratin-derived protein
L-NAME	N-nitro-L-arginine-methyl ester
L-NMMA	L-NG-monomethyl arginine
mBF	Muscle blood flow
mBV	Muscle blood volume
MMIT	Mixed-mode interval training
mTOR	Mammalian target of rapamycin
Myo1C	Myosin 1C
MyoV	Myosin V
NIRS	Near-infrared spectroscopy
NO	Nitric oxide
NOX2	Nicotinamide-adenine-dinucleotide phosphate (NADHP)-oxidase
PCC	Pearson's correlation coefficient
PDK	Phosphoinositide-dependent protein kinase-1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
Rac1	Ras-related C3 botulinum toxin substrate 1
RET	Resistance exercise training

ROS	Reactive oxygen species
SF	Sharing factor
SNAP23	Synaptosomal-associated protein of 23 kDa
SNARE	Soluble NSF Attachment Protein
TEM	Transmission electron microscopy
T2DM	Type-2 Diabetes Mellitus
TGN	Trans-Golgi network
TNF-α	Tumour necrosis factor α
VAMP2	Vesicle associated membrane protein 2
VEGFA	Vascular endothelial growth factor A

CHAPTER 1

GENERAL INTRODUCTION

1.1. Type-2 Diabetes Mellitus

Type-2 Diabetes Mellitus (T2DM) is a chronic non-communicable disease of which the primary phenotype is having chronically elevated blood glucose concentrations (hyperglycaemia). T2DM has reached epidemic proportions over the last couple of decades. In 2010, Shaw et al. {, 2010 #361} estimated that by 2030, 7.7% of the global population would be diagnosed with type-2 diabetes. A World Health Organization report, using data collected in 2014, indicated the estimated prevalence of all types of diabetes was 8.5%, and rapidly increasing (WHO, 2016). T2DM has a large economic burden as estimates show that diabetes costs the world US$ 827 billion per year (Seuring et al., 2015). Moreover, T2DM leads to other health complications such as increased risk in cardiovascular diseases and overall risk in mortality, with 2.2 million worldwide deaths as a result of impaired blood glucose homeostasis, the key feature in diabetes (WHO, 2016). Taken together, the increasing prevalence, economic burden and increased risk in co-morbidities that accompany diabetes, urgently require effective preventative and treatment strategies.

Hyperglycaemia indicates a disturbance in glucose homeostasis; the regulation of blood glucose concentrations which normal concentrations range between 70-90 mg·dL^{-1} or ~3.9-5.5 mmol·L^{-1} (Abdul-Ghani et al., 2006). The disturbance partially originates from the development of insulin resistance in peripheral tissues, including skeletal muscle, which plays a large role in insulin-stimulated glucose uptake (Katz et al., 1983). Insulin resistance in skeletal muscle results in an impaired ability to take up glucose in response to insulin, thereby failing to restore blood glucose concentrations as observed in T2DM (DeFronzo et al., 1985; Ferrannini et al., 1988). Therefore, interventions should target ways to overcome skeletal muscle insulin resistance and improve the uptake of glucose into skeletal muscle.

1.2. Treatments to improve glucose homeostasis and insulin sensitivity

Exercise has been a well-established treatment for T2DM. Both a single bout (Mikines et al., 1988) and regular endurance exercise training improve glucose homeostasis (Bird et al., 2016).Therefore, the American Diabetes Association (ADA) and American College of Sports Medicine (ACSM) recommend that exercise or physical activity of both aerobic and strength signature should be included as a therapy to prevent and manage T2DM (Colberg et al., 2010).

Additionally, interventions on nutrition could be considered as an adjunct therapy to exercise to further manage T2DM (Bantle et al., 2008). The 2008 report stated that there was insufficient evidence to modify usual protein intake (15%-20% of energy) in diabetes management. However, later evidence linked dietary protein intake to glucose homeostasis and insulin sensitivity with higher protein intake resulting in improved glycaemic control compared to lower protein diets in T2DM (Dong et al., 2013). Moreover, in a meta-analysis, Wycherley et al. (2012) reported that high-protein diets (25%- 35% of total daily energy intake) mitigate losses in muscle mass under energy-restricted conditions compared to standard-protein diets (12% - 18% of total energy intake).

Apart from increasing total daily protein intake, the protein source (Ouellet et al., 2007), optimizing timing of intake (Kouw et al., 2017) and combining exercise training with protein intake (Wycherley et al., 2010a) are additional factors that might determine the effect of protein intake as a treatment for T2DM. Currently, there are very limited studies on the effect of combined exercise and protein intake as an intervention in T2DM (Francois et al., 2017; Gaffney et al., 2017) and many variables on the type of protein are yet to be investigated.

1.3. Keratin-derived protein

Keratin is a protein that is present in nails, feathers and hair of mammals, and contains relatively high concentrations of thiol-containing amino acids cysteine and other amino acids including arginine and glycine (Crum et al., 2018). However, due to the insoluble disulphide bonds the protein is considered to be poorly digestible. Hydrolysis and chemical methods have been developed to break the disulphide bonds (Shorland et al., 1970), thereby improving digestibility (Crum et al., 2018).

Recently, a technique was developed to manufacture edible dietary protein that was derived from sheep wool (S. Dias et al., 2016). Keratin is one of the main components of wool, hence the reference to keratin-derived protein (WDP). WDP contains relatively high concentrations of the amino acids cysteine, glycine and arginine, which isolated supplementation has been shown to improve insulin sensitivity (Sekhar et al., 2011).

There is an economic benefit to the use of WDP as well. The costs of manufacturing and producing wool-derived protein was demonstrated to be less than half that of production of whey protein. New Zealand has a large wool industry and if WDP would be demonstrated to be effective in improving health status in T2DM, this could have a large economic impact on the wool industry in New Zealand. Moreover, wool is a renewable source (by shearing) and has no animal welfare issues associated with the production, thereby making it a sustainable and environmentally friendly source of protein.

1.4. Aims and hypothesis of the thesis

In summary, insulin-stimulated skeletal muscle glucose uptake is one of the key processes in the ability of the body to regulate glucose homeostasis. People with T2DM show peripheral resistance to the action of insulin which results in impaired glucose

homeostasis with chronically elevated blood glucose concentrations. Combined exercise and dietary protein intake might be a beneficial intervention to improve insulin sensitivity in T2DM, but the amino acid composition of the protein might be an important modulator to further improve exercise-induced improvements in insulin sensitivity. Therefore, a clinical trial was designed to target the aim of the thesis, which is to investigate whether timed ingestion of a novel keratin-derived protein combined with supervised exercise training leads to improvements in insulin sensitivity in people with T2DM compared to whey protein or a placebo. The aims of the thesis can be broken down into the following structure:

1) Investigate the effect of fourteen weeks of exercise and protein supplementation on glucose homeostasis and skeletal muscle GLUT4 translocation.

2) Investigate the effect of fourteen weeks of exercise and protein supplementation on skeletal muscle microvascular functioning.

3) Develop and validate a novel method to quantify GLUT4 translocation.

The *a priori* hypothesis of the thesis was that combined exercise and post-exercise protein ingestion leads to superior improvements in glucose clearance rates, insulin-stimulated GLUT4 translocation and skeletal muscle microvascular function, compared to exercise only. Moreover, we hypothesized that because of the unique amino acid composition of the keratin-derived protein supplement, this protein would induce superior improvements in glucose clearance rates, insulin-stimulated GLUT4 translocation and skeletal microvascular function compared to whey protein.

1.5. Outline of the thesis

Chapter 2 analyses the literature on the mechanisms of skeletal muscle glucose uptake, the pathophysiology of insulin resistance and T2DM and the current evidence on combined exercise training and co-ingestion of dietary protein as an intervention to improve insulin sensitivity

Chapter 3 outlines the design and methods used in the clinical trial to investigate the effect of fourteen weeks of exercise training and post-exercise protein ingestion in humans diagnosed with T2DM.

Chapter 4 describes the effect of fourteen weeks of exercise and supplementation of either a novel keratin-derived protein-whey protein blend, whey protein or a placebo control on insulin-stimulated glucose disposal, HbA_1c, microvascular haemodynamics and enzymatic activity of proteins controlling NO bioavailability (eNOS and NOX2) and capillary density in skeletal muscle. GLUT4 translocation, using immunofluorescence, in T2DM.

Chapter 5 describes the attempt to develop and validate a novel method to quantify GLUT4 translocation and compare this to a previously used method that quantifies GLUT4 translocation. The novel approach uses immunoelectron microscopy, a combination between immunohistochemistry and electron microscopy, to quantify GLUT4 translocation in the plasma membrane with higher spatial resolution compared to a previously used method, immunofluorescence, which uses widefield microscopy that reaches lower magnifications and therefore results in lower spatial accuracy.

Chapter 6 summarizes the presented findings in the thesis, discusses limitations of the work and provides novel insights on the use of protein in conjunction with exercise as a treatment for T2DM in order to provide recommendations for future research.

CHAPTER 2

THE REGULATION AND DYSREGULATION OF SKELETAL MUSCLE GLUCOSE UPTAKE IN TYPE 2 DIABETES MELLITUS: EXERCISE AND DIETARY PROTEINS AS POTENTIAL TARGETS FOR IMPROVING INSULIN SENSITIVITY

Literature Review

2.1. Introduction

The reduced wellbeing and health care cost associated with increased rates of obesity and Type 2 diabetes mellitus (T2DM) make finding effective ways to treat and prevent chronic metabolic diseases of high importance (Ogurtsova et al., 2017; Seidell et al., 2015). Chronically elevated blood glucose concentrations is the primary phenotype observed in T2DM. Accordingly, interventions that improve the mechanisms to lower blood glucose concentrations, such as increasing physical activity and reducing dietary calorie intake are recommended by the World Health Organization to treat and prevent the development of T2DM and obesity (Klein et al., 2004).

The chronically elevated blood glucose concentrations associated with T2DM are partially the result of a reduced postprandial glucose disposal response that originates from the resistance of peripheral tissues to the action of insulin (Abdul-Ghani et al., 2010). Of the peripheral organs, skeletal muscle is the primary site for glucose disposal (Katz et al., 1983), and therefore is an important component of glucose homeostasis. Chapter 2 will provide an evaluation of the role of skeletal muscle in glucose homeostasis and describes the process of glucose uptake in response to insulin. Furthermore, the chapter describes what factors contribute to the development of insulin resistance in T2DM, physiological impairments in the glucose uptake pathway and the effects of exercise and dietary protein as interventions to improve insulin sensitivity.

2.2. Organs involved in glucose homeostasis

Glucose homeostasis describes the regulation of blood glucose concentrations, which ranges between 70-90 mg·dL^{-1} or ~3.9-5.5 mmol·L^{-1} (Abdul-Ghani et al., 2006). However, glucose homeostasis is challenged continuously. The most common challenge is the response to a rise in blood glucose concentrations, which happens after

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consumption of a carbohydrate-rich meal. In this postprandial state, beta cells in the pancreas detect the rapid rise in blood glucose concentrations and react by secretion of the hormone insulin. The subsequent increase in plasma insulin concentration results in a cascade of physiological events in order to counteract the increase in blood glucose concentration. Insulin suppresses hepatic glucose output, thereby preventing a further increase in blood glucose concentration (Felig et al., 1976). Additionally, insulin stimulates the uptake of glucose to different peripheral insulin-sensitive tissues, including skeletal muscle, liver and adipose tissue, where it is stored in the form of glycogen or converted into lipids, thereby restoring normal blood glucose concentrations. During the post-absorptive state, or when insulin has lowered blood glucose concentrations back within the normal range, the pancreatic hormone glucagon regulates and stimulates hepatic glucose output in order to prevent blood glucose concentrations to drop into dangerously low concentrations of < 2.8 mmol·L^{-1} or 50 mg·dL for healthy adults or < 3.9 mmol·L^{-1} for diabetic adults (NIDDK, 2016). These mechanisms with a negative feedback loop ensure tight control of glucose homeostasis. When the tight control is interrupted, the body is exposed to potentially lethal risks. Low blood glucose concentrations, hypoglycaemia, can result in organ dysfunction, including the brain which can result in loss of consciousness and death. In contrast, a chronic high blood glucose concentration, hyperglycaemia as observed in T2DM, is toxic and can cause physical tissue damage over time, such as cardiovascular damage, kidney damage and nerve damage (C. G. Lee et al., 2011; Park et al., 2004). In the WHO 2016 diabetes report, estimations were provided that 35% of adults with T2DM have retinopathy, that 80% of end-stage renal disease cases are caused by T2DM, that having diabetes doubles or triples the risk of a cardiovascular event and that rates of

lower limb amputation are 10 to 20 times higher in diabetes compared to non-diabetic populations (WHO, 2016)

2.3. The role of skeletal muscle in glucose homeostasis

Skeletal muscle is the primary site for postprandial glucose disposal. An early study from Katz et al. (1983) showed that in young healthy adults, leg glucose uptake was increased several-fold after oral ingestion of 92 g glucose and researchers concluded that around ~70% of the glucose load was taken up by skeletal muscle. Later studies found that 30% of starch ended up in skeletal muscle after consumption of a mixed meal in healthy humans (Capaldo et al., 1999). Additionally, during exercise at 75% of maximal exercise capacity, blood glucose contributes ~20% to energy production and over ~50% of energy is derived from muscle glycogen (van Loon et al., 2001). Collectively, glucose transport during feeding and exercise highlights the importance of the role of skeletal muscle in maintaining glucose homeostasis by providing the muscle with fuel for energy production, whilst keeping blood glucose concentrations within acceptable ranges. An efficient mechanism is needed to transport glucose to skeletal muscle in order to rapidly restore postprandial blood glucose concentrations via insulin secretion and to supply skeletal muscle with glucose during rapid changes in energy demand. Two mechanisms that are proposed to be the rate limiting steps in insulin-stimulated skeletal muscle glucose uptake are the delivery of glucose and insulin to the skeletal muscle fibre via the cardiovascular system (Barrett et al., 2009) and the transport of glucose across the sarcolemma (Wasserman et al., 2011).

2.4. Delivery of insulin and glucose to the skeletal muscle in the postprandial state

After ingestion, digestion and absorption of glucose, the detection of a rise in blood glucose concentration results in the pancreatic secretion of insulin. Once insulin is

secreted, it travels through the circulatory system, binds to endothelial cells of the blood vessels and activates different mechanisms at different levels of the microvascular bed with the overall goal to enhance nutrient and insulin supply and subsequently to control glucose homeostasis (Barrett et al., 2011). From observations in *in vivo* skeletal muscle of humans using positron emission tomography, supra-physiological concentrations of insulin induce a rapid vasodilatory response in terminal arterioles, which enhances local blood flow (volume per time) and perfusion (volume per amount of tissue) and results in increased delivery of nutrients and insulin (Baron et al., 1990; Raitakari et al., 1995). However, under physiological concentrations of insulin, no significant or delayed increases in local blood flow were observed (Bonadonna et al., 1998; Vollenweider et al., 1993), questioning the relevance of increased total microvascular blood flow under representative physiological conditions. Subsequent research demonstrated that insulin increased microvascular perfusion by recruitment of previously unperfused capillaries (Vincent et al., 2002). The increase in recruited capillaries enhances the total surface area (Gudbjornsdottir et al., 2003) available for trans-endothelial transport of nutrients and insulin (Barrett et al., 2011).

The microvascular response to insulin is suggested to be dependent on nitric oxide (NO) bioavailability. NO synthesis is catalysed by the enzyme endothelial nitric oxide synthase (eNOS), converting nitrate or the amino acid L-arginine into NO. Steinberg et al. (1994) infused the eNOS inhibitor L-NG-monomethyl arginine (L-NMMA) into the femoral artery of healthy people and showed reductions in insulin-mediated leg blood flow and leg glucose uptake (Steinberg et al., 1994). Complementary animal data from Vincent et al. (2003) showed that inhibition of eNOS with *N*-nitro-L-arginine-methyl ester (L-NAME) blocked limb blood flow, microvascular recruitment and subsequent

blunted glucose uptake in rats, highlighting the role of NO in insulin-mediated glucose uptake.

When insulin binds to the insulin receptor on the endothelial cell membrane, it initiates a signalling cascade involving insulin receptor substrate (IRS) - phosphoinositide 3-kinase (PI3K) – protein kinase B (Akt) to activate eNOS and subsequently increasing the bioavailability of NO (Muniyappa et al., 2007). Specifically, phosphorylation of the serine 1177 residue in eNOS (eNOS^{ser1177}) is involved in the activation of eNOS and subsequent synthesis of NO (Montagnani et al., 2001). At the level of resistance blood vessels and terminal arterioles, NO triggers smooth muscle relaxation and vasodilation. At the capillary level, NO is suggested to be involved in regulating trans-endothelial transport of insulin (**Figure 2.1**). NO is involved in the development of caveolin-like vesicles that take up insulin from the capillaries and transport it across the endothelial cell to the muscle interstitium (H. Wang et al., 2013; H. Wang et al., 2011). Thus from the above, a working model has been suggested that insulin stimulates its own uptake and delivery across the endothelial cell in the skeletal muscle capillaries (Barrett et al., 2011).
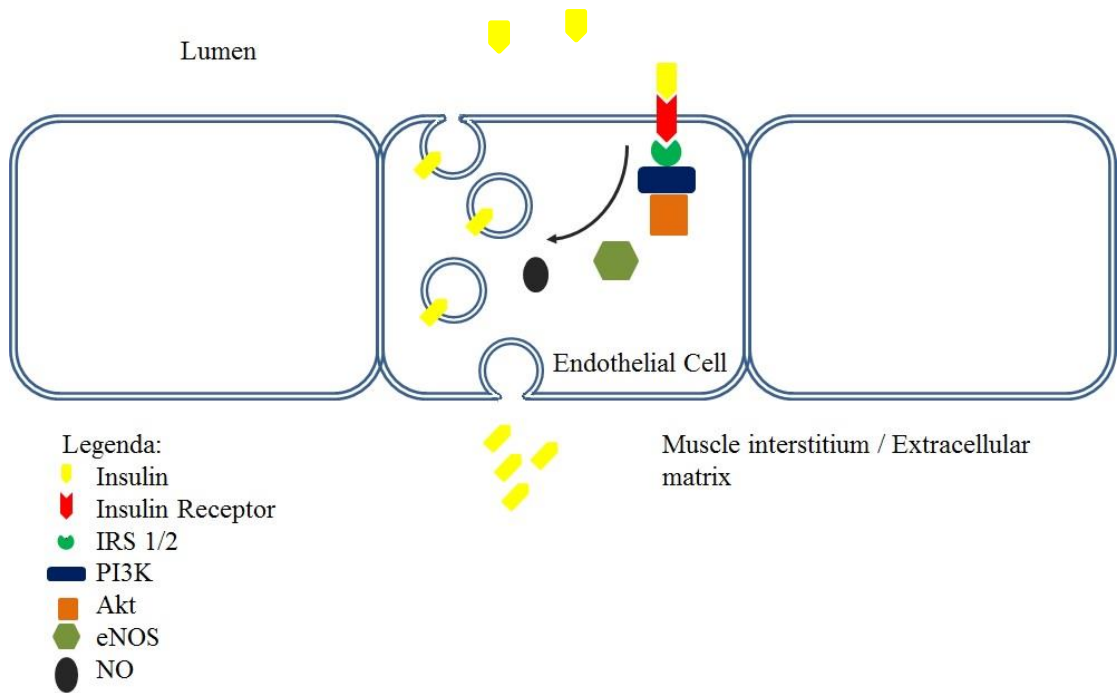
Figure 2.1 – A simplified version of the trans-endothelial transport of insulin. Insulin triggers its own transport across endothelial cells in skeletal muscle capillaries by activating an IRS/PI3K/Akt signalling cascade which activates eNOS. eNOS increases NO availability which stimulates the formation of caveolin-like vesicles that absorb insulin and release it into the muscle interstitium where it can action on the skeletal muscle plasma membrane. The information in this figure is based on references (Barrett et al., 2011) and (Muniyappa et al., 2007).

2.5. Skeletal muscle glucose uptake in the postprandial state

For glucose transport across the skeletal muscle fibre, the rate limiting step is the translocation of glucose transporter 4 (GLUT4) to the plasma membrane which is initiated by insulin dependent and independent (exercise) pathways (Klip, 2009). Here, the insulin-dependent pathway is described (**Figure 2.2**).

After being transported from the capillaries into the muscle interstitium or the extracellular domain of the skeletal muscle fibre, insulin binds to the insulin receptor

(IR) present in the plasma membrane, causing phosphorylation of the receptor that changes the structure and thereby opening up docking sites for insulin receptor substrate (IRS) (De Meyts, 2000; White, 1998). After the docking of IRS to the insulin receptor, IRS-1 undergoes tyrosine phosphorylation, activating downstream pathways. Phosphatidylinositol-3-kinase (PI3K) binds to IRS1 and-2, which leads to the activation of phosphoinositide-dependent protein kinase-1 (PDK1) (Shepherd et al., 1998). Subsequently PDK1 phosphorylates Akt (also known as protein kinase B, PKB) and Akt-substrate of 160 kDa (AS160), undergoes threonine and serine phosphorylation at several sites (Kane et al., 2002; Sano et al., 2003) which then activates Rab GTPases, which are involved in vesicle trafficking, more specifically, vesicle trafficking of GLUT4 containing vesicles (GSV) towards the plasma membrane (Klip et al., 2014). Furthermore, downstream products of the insulin-signalling cascade also initiate different processes, for example regulation of glycogen synthesis via glycogen synthase kinase 3 (GSK3) activation regulated by Akt stimulation (Cohen et al., 2001).

2.5.1. Vesicle trafficking:

GLUT4 concentration in the plasma membrane available for glucose transport is dependent on the process of endocytosis and exocytosis. Most evidence of these processes come from rat adipocytes, 3T3-L1 mouse adipocytes and cell cultures of rat L6 skeletal muscle cells and is extensively reviewed by Foley et al. (2011). In the fasted state, GLUT4 continuously moves at a slow rate between the plasma membrane and intracellular compartments packed in vesicle-like structures, with the majority of GLUT4 stored at the perinuclear region and low concentrations in the plasma membrane (Ploug et al., 1998). In *in vivo* rat skeletal muscle, stimulation with either contraction or insulin produced a rapid redistribution of GLUT4 from intracellular compartments to the plasma membrane (Ploug et al., 1998). Identified intracellular

compartments containing GLUT4 are: early endosomes (EE), endosome recycling

compartment (ERC), trans-golgi network (TGN) and GLUT4 storage vesicles (GSV)

(Foley et al., 2011). Each type of these compartments can be distinguished from another

by its composition, and size (Foley et al., 2011). Endosomal structures appear to be

200-300 nm in diameter and contain a unique protein called transferrin receptor

(Lemieux et al., 2000). GSVs are smaller in diameter (50-70 nm based on isolated rat

adipose tissue (Malide et al., 2000; Ploug et al., 1998)) and characterized by the

presence of proteins like vesicle-associated membrane protein 2 (VAMP2) and insulin-

regulated aminopeptidase (IRAP) (Foley et al., 2011; Ramm et al., 2000), which are

required for tethering, docking and fusion of the GSV with the plasma membrane.

Based on these differences in vesicle composition, studies were able to separate

processes of endocytosis and exocytosis of GLUT4 upon stimulation, which has led to

the observation that insulin and exercise trigger GLUT4 translocation from different

intracellular pools. Insulin stimulation increases glucose uptake primarily via GLUT4

exocytosis whereas exercise both stimulates GLUT4 exocytosis and inhibits GLUT4

endocytosis (Lemieux et al., 2000). This evidence is accompanied by the additive effect

of glucose uptake when exercise and insulin stimulation are combined (Douen et al.,

1990).

Upon insulin stimulation, Ras-related C3 botulinum toxin substrate 1 (Rac1), a GTPase

belonging to the rho family proteins, is activated via Akt2 by GTP loading *in vivo*

skeletal muscle cells of mice (Takenaka et al., 2014) which initiates remodelling and

polymerisation of the cytoskeleton for the movement of GSVs towards the plasma

membrane (Chiu et al., 2011). Rac1 activates its downstream target p-21-activated

kinase (PAK1) via threonine 423 phosphorylation which induces actin remodelling.

Disruption of Rac1 – PAK1 leads to reduced GLUT4 translocation and glucose uptake

(Tsakiridis et al., 1994) and is dysregulated in insulin resistant human skeletal muscle (Sylow et al., 2013). Also downstream of Rac1, the Arp2/3 complex is activated, which also catalyses the remodelling of actin filaments. The previously mentioned Rab GTPase proteins then initiate the activation of Myosin V (MyoV), a motor protein, which transports GSV along actin filaments towards the plasma membrane (Klip et al., 2014). Myo1c is another molecular motor that is involved in the tethering of GSV once arrived at the membrane site. However, Myo1c does not seem to act upon Rac of Akt, but rather passively tethers incoming GSV with the plasma membrane (Klip et al., 2014). Once tethered, GSV's have to dock and fuse with the plasma membrane. This requires a delicate interaction between Soluble NSF Attachment Receptor (SNARE) proteins. A link has to be made between the intracellular vesicle and the plasma membrane. Intracellular vesicles contain v-SNARE (vesicle-SNARE) proteins and the plasma membrane contains t-SNARE (targeting-SNARE) proteins. Docking and fusing of a vesicle at the plasma membrane requires the connection of these two types of SNARE proteins. For GLUT4 translocation, VAMP2 is a v-SNARE proteins that is involved in docking and fusing and is highly expressed in GSV's (Kristiansen et al., 1996; Randhawa et al., 2000). t-SNARE proteins involved in GLUT4 translocation are Synaptosomal-associated protein 23 (SNAP23) and syntaxin 4 which has been shown in human skeletal muscle cells (Strauss et al., 2016).
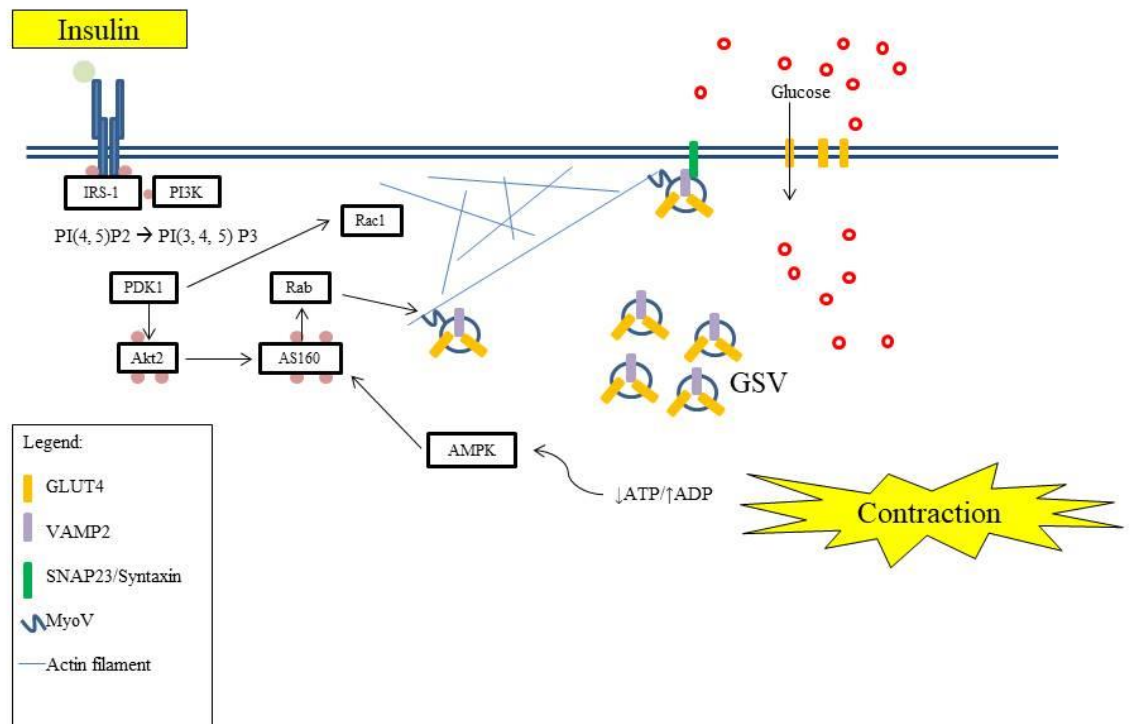
Figure 2.2 – GLUT4 translocation to the skeletal muscle plasma membrane. GLUT4

translocation is both activated by insulin and muscle contraction. Proximal signalling is

different between the two stimuli, however, later in the cascade, the two show

similarities for GLUT4 translocation. Insulin binding to the insulin receptor induces a

signalling cascade that bifurcates, one arm activating remodelling of the cytoskeleton

via Rac1, the other arm phosphorylating AS160, which activates motor proteins (MyoV)

which translocate GLUT4 storage vesicles (GSV) towards the plasma membrane.

Muscle contraction changes the energy status of the muscle fibre, which activates AMP-

activated protein kinase (AMPK) and phosphorylates AS160. The figure is the PhD's

own work and based on references in Section 2.5.

2.6. Candidate mechanisms in the development of skeletal muscle insulin resistance and T2DM

Despite carrying a genetic component, the risk of developing T2DM is strongly linked

to obesity (Colditz et al., 1995). Obesity, and thus risk of T2DM, is a consequence of a

chronic positive energy balance due to chronic periods of physical inactivity and/or

excess calorie intake, which results in skeletal muscle maladaptations. Physical

inactivity and increased calorie intake have several physiological consequences that are

associated with the development of insulin resistance as discussed below (**Figure 2.3**).
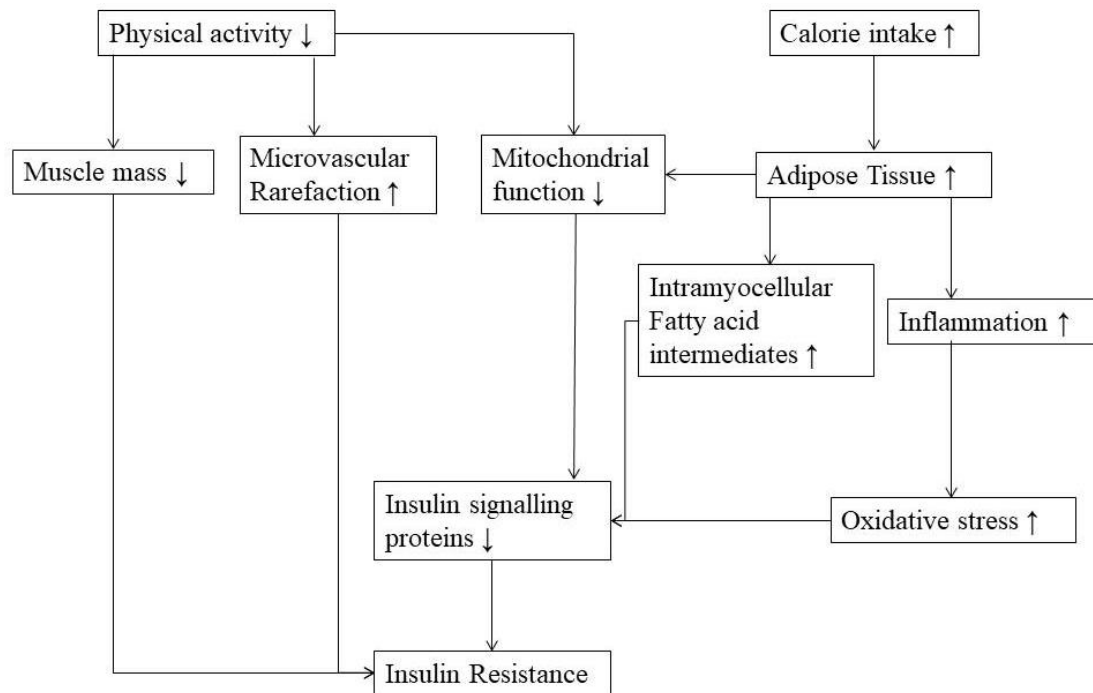
Figure 2.3 – Pathways in the development of insulin resistance. A decrease in physical

activity and an increase of calorie intake lead to maladaptations in skeletal muscle

which ultimately induce insulin resistance. The pathway is the PhD's own work.

2.6.1. Muscle mass

Several studies have provided evidence that skeletal muscle mass is positively

associated with insulin sensitivity (C. G. Lee et al., 2011; J. S. Lee et al., 2010).

Moreover, a reduction in muscle mass as a result of physical inactivity or disuse results

in a reduction in insulin sensitivity (Dirks et al., 2016). One study has shown that two

weeks of leg immobilization in healthy young men decreased lean leg mass by 3% and

quadriceps cross-sectional area by 8.4%, with significant decreases observed after only

five days (Wall et al., 2014). These observations stress the importance for regular

physical activity on muscle mass and insulin sensitivity. On top of the loss in skeletal

muscle during disuse and physical inactivity, aging is accompanied with losses in

muscle mass, defined as sarcopenia. After the age of forty and when remaining inactive,

skeletal muscle mass declines by approximately 1% per year (Janssen et al., 2000), with

decrements accelerating with age (Lexell et al., 1988). Moreover, older adults with

T2DM had 3% lower leg and appendicular muscle mass and the age-related loss in

muscle mass and an 8% reduction in leg strength compared to age-matched

normoglycaemic humans, likely due to a combination of atrophic signalling induced by

inactivity and age-related sarcopenia (Leenders et al., 2013). This demonstrates that in

T2DM, age-related loss in muscle mass is even higher in T2DM compared to non-

diabetics.

2.6.2. *Microvascular dysfunction*

Insulin-stimulated response in blood flow during a hyperinsulinaemic euglycaemic

clamp has been showed to be impaired and 30% lower in T2DM compared to lean

individuals resulting in slower delivery of glucose and insulin (Laakso et al., 1992;

Reynolds et al., 2017). Additionally, microvascular density is important for insulin-

mediated glucose uptake, as it determines the surface area that is available for insulin

and nutrient transport to the muscle (Lillioja et al., 1987). Compared to obese and

T2DM, lean humans had higher capillary density which was positively associated with

insulin sensitivity (Gavin et al., 2005; Groen et al., 2014). Lower capillary density or

microvascular rarefaction is likely the result of a decrease in physical activity, as

Klausen et al. (1981) showed that detraining caused a reduction in capillary density in

healthy men.

As previously mentioned, the microvascular response to insulin is dependent on nitric oxide bioavailability that is produced by the activation of the enzyme eNOS via phosphorylation of the serine residue 1177 (eNOS^{ser1177}). Although no studies have directly investigated the effect of inactivity, skeletal muscle eNOS content was reduced in overweight women, a likely consequence of inactivity, compared to lean women (Hickner et al., 2006). Moreover, people with T2DM have a lower ratio of phosphorylated eNOS^{ser1177} to total eNOS content (eNOS^{ser1177}/eNOS) as a measure of eNOS enzyme activity, compared to lean controls in a basal state (Reynolds et al., 2017). Interestingly, eNOS^{ser1177}/eNOS content remained unchanged after insulin stimulation in both lean and obese T2DM humans, despite observed increases in femoral blood flow only in lean humans (Reynolds et al., 2017).

2.6.3. Chronic low-grade inflammation

A chronic positive energy balance results in fat storage and expanding visceral and subcutaneous adipose tissue. It has been shown that expanding adipose tissue secretes pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and this is linked with skeletal muscle insulin resistance (Hotamisligil et al., 1993). T2DM and obesity are associated with increased circulating levels of TNF-α, but also other pro-inflammatory cytokines including interleukin-6 (IL-6), interleukin-1beta (IL-1β) and C-reactive protein (CRP) that originate from adipose tissue (Herder et al., 2005; Spranger et al., 2003; Wei et al., 2008). The inflammatory cytokines interfere with the insulin-signalling pathway at the skeletal muscle site. TNF-α activates c-Jun N-terminal kinase (JNK) that inhibits tyrosine phosphorylation and increases serine phosphorylation of IRS-1 in skeletal muscle which results in impaired insulin-mediated glucose uptake (Bouzakri et al., 2007; Hotamisligil et al., 1994; Hotamisligil et al., 1996).

2.6.4. Altered skeletal muscle lipid metabolism

The positive energy balance and the subsequent increase in adipose tissue also cause the rise in circulating lipids and intramyocellular lipids. Most lipids are transported through the circulation packed as triglycerides in vesicles or as free fatty acids. When dietary lipids enter the cell, they can be stored as intramyocellular lipid droplets or used immediately for energy production via beta-oxidation. Large intramyocellular lipid droplets stored at the subsarcolemmal region are negatively associated with insulin sensitivity and elevated in T2DM (Daemen et al., 2018).

Additional to the localization of lipids in the cell, handling of the lipids induces insulin resistance. One of the intermediate steps in the breakdown of triglycerides to free fatty acids for subsequent energy production is the formation of diacylglycerol (DAG) and acyl CoA. In the presence of excess fat and oversupply of lipid influx into the skeletal muscle cell, DAG accumulates in the cytosol leading to lipotoxicity (Amati, 2012; Kitessa et al., 2016). Here, DAG activates PKC that decreases the tyrosine phosphorylation activity of IRS-1 and PI3K and thus insulin sensitivity, leading to insulin resistance (Erion et al., 2010). Other lipids that could initiate insulin resistance are ceramides. Ceramides are classed as sphingolipids and have a role in membrane structure, cell differentiation, proliferation and apoptosis (Woodcock, 2006). However, their other role as signalling molecules is activating PKC, inducing insulin resistance via a similar mechanism as DAG. Although there is evidence that supports this theory, there are also studies that did not find an association between skeletal muscle DAG and ceramide content and insulin sensitivity (Skovbro et al., 2008; Sogaard et al., 2016). However, there is evidence to indicate that only specific types of DAG induce insulin resistance, as it has been shown that endurance athletes have higher skeletal muscle DAG content compared to obese people with T2DM (Amati et al., 2011)

2.6.5. Mitochondrial dysfunction

The role of mitochondria in the development of insulin resistance currently is a topic of debate. Some analysis of muscle biopsies has revealed that skeletal muscle of humans with T2DM has impaired mitochondrial function compared to skeletal muscle from lean humans. Experiments demonstrate lower intermyofibrillar mitochondrial content in insulin resistant and T2DM skeletal muscle (Amati et al., 2011), reduced enzyme activity (Kelley et al., 2002) and respiration capacity when normalized for mitochondrial content (Mogensen et al., 2007). Additionally, offspring of T2DM humans demonstrated lower mitochondrial activity compared to insulin-sensitive non-offspring humans (Petersen et al., 2004). The authors suggested that a mitochondrial dysfunction compared to healthy offspring, defined as reduced ATP turnover and the capacity to oxidize fat in both resting fasted and insulin stimulated states, is an early event in the development of T2DM and likely to be causal to insulin resistance. It could be argued that impaired mitochondrial function results in the inability to use lipids for energy production, leading to further accumulation of intramyocellular lipids and lipid intermediates that interfere in the insulin-signalling cascade.

In contrast, there is also evidence that mitochondrial function is not impaired in T2DM and that fat oxidation is even increased in T2DM (Holloszy, 2013). However, later studies showed that, when normalized for mitochondrial content, no differences were detected for the O_2 influx needed to produce ATP, between healthy and T2DM skeletal muscle (Boushel et al., 2007). This indicates that the impaired mitochondrial function and the subsequent risk of developing insulin resistance and T2DM is a consequence of reduced mitochondrial content in T2DM that is likely a result of an inactive, sedentary lifestyle, rather than a causal link between general mitochondrial dysfunction in offspring of people with T2DM (Wagenmakers, 2005). This argument is further

strengthened by the observation that physical fitness-matched T2DM and obese adults had similar mitochondrial respiratory function and that aerobic exercise training improved mitochondrial function in both groups (Hey-Mogensen et al., 2010). Therefore it was suggested that the additional insulin resistance as observed in T2DM compared to obese humans is not a result of impairments in mitochondrial function, but more likely a result of increased reactive oxygen species production in T2DM compared to obese adults (Hey-Mogensen et al., 2010).

2.6.6. Oxidative stress

Energy production in the mitochondria produces reactive oxygen species (ROS) that are needed for normal cellular function. However, excess production of ROS can result in cellular damage. T2DM is accompanied by an increase in ROS production and is positively correlated with insulin resistance (Anderson et al., 2009; Nourooz-Zadeh et al., 1997). ROS activates intracellular kinases such as MAPK, JAK/STAT and JNK that cause serine phosphorylation of IRS-1, Akt and therefore interfere in the insulin-signalling cascade resulting in reduced insulin-mediated glucose uptake (Wei et al., 2008). Additionally, ROS production leads to increased pro-inflammatory cytokine expression, including TNF-α that further exacerbates inflammation and increases insulin resistance (Wei et al., 2008).

Not only is there an increase in the production of oxidants, the antioxidant defence mechanism is also impaired in T2DM. For instance, Calabrese et al. (2012) showed that the antioxidant glutathione is lower in plasma of T2DM compared to healthy humans. Glutathione is a tripeptide, and includes the amino acid cysteine, which contains a thiol group. This thiol scavenges oxidants, thereby maintaining cellular redox homeostasis.

Oxidative stress is also present in the skeletal muscle microvasculature in obese humans (Silver et al., 2007). Free radicals including superoxide anions ($\cdot O_2^-$) are generated by the NADPH-oxidase enzymes, of which NOX2 (gp91phox) is a component. Upon superoxide anion generation, they can react with NO and forming peroxynitrite, thereby scavenging and reducing NO bioavailability. NO bioavailability promotes microvascular blood flow via microvascular vasodilation and capillary recruitment (Steinberg et al., 1994). Therefore a decrease in NO bioavailability leads to impaired microvascular function. In isolated skeletal muscle arterioles from healthy humans, flow-mediated dilation under hyperinsulinemia was reduced whilst NOX2 was elevated, suggesting increased production of $\cdot O_2^-$ (Mahmoud et al., 2017).

2.7. Exercise as an intervention to improve insulin sensitivity

Exercise training is widely accepted as a powerful tool to improve glucose homeostasis in T2DM. In a meta-analysis, Boule et al. (2001) demonstrated that both endurance and resistance exercise training for more than 8 weeks improved HbA_1c compared to a resting control group. Moreover, ten weeks of one-legged aerobic or high-intensity interval training improved whole-body glucose clearance (Dela et al., 1995) and skeletal muscle glucose clearance compared to the non-exercised leg (Dela et al., 2018). Both endurance-type and resistance-type exercise have been demonstrated to improve HbA_1c without one being more effective than the other (Yang et al., 2014). Hence, both WHO (WHO, 2016) and American Diabetes Association (Colberg et al., 2010) recommend combined resistance-type and endurance-type exercise as a primary treatment to improve glycaemic control in T2DM. An extensive body of literature has provided evidence on the physiological mechanisms by which exercise improves glucose homeostasis and skeletal muscle insulin sensitivity in T2DM via reversing the maladaptation to decreased physical inactivity and increased nutrient intake as

displayed in **Figure 2.3** (Bogdanis et al., 2013; Egger et al., 2013; Hayashino et al., 2014; Meex et al., 2010). For the purpose of the thesis, skeletal muscle microvascular and muscle fibre signalling adaptations will be discussed. Most adaptations to exercise training are the result of accumulated signals from acute single exercise bouts (Bird et al., 2016).

2.7.1. Microvascular adaptations

Exercise acutely leads to a rapid redistribution of blood flow and perfusion towards skeletal muscle to supply the muscle with oxygen and nutrients for energy production. The exercise-induced increase in blood flow velocity elevates blood shear stress in microvessels and results in the expression of skeletal muscle vascular endothelial growth factor A (VEGFA) (Hoier et al., 2014). VEGFA stimulates skeletal muscle angiogenesis, the formation of new capillaries and thereby increases the capillary surface area available for insulin and nutrient transport. Some (Hepple et al., 1997; Holloway et al., 2018), but not all (Cocks et al., 2014) evidence showed that in healthy young and elderly humans, resistance and endurance exercise training increases measures of capillarization in a fibre-type specific manner. Skeletal muscle fibres comprise of different types. Type I fibres, or slow-twitch fibres have higher aerobic capacity, whereas type II fibres have more contractile elements and are able to generate more force. Moreover type II fibres undergo hypertrophy in response to (resistance) exercise training, resulting in a larger cross-sectional fibre diameter. When measuring capillary density (capillaries•mm^2) after resistance exercise training, increases in muscle fibre diameter might mask changes in microvascular adaptations unless fibre-type specific analysis is conducted. In T2DM, 10 weeks of aerobic exercise training, but not high-intensity interval training, has been shown to increase capillaries per fibre (Mortensen et al., 2018) but without increases in capillary density (Lithell et al., 1985).

Strength training for 6 weeks in T2DM did not increase capillary density; however, no fibre-type specific measures were conducted, which might have caused wrongful inference as the diameter of type II fibres increased (Holten et al., 2004). In T2DM, VEGFA expression was increased after 12 weeks of exercise training and was accompanied with improved insulin sensitivity, which still underlines the importance of microvascular function on insulin sensitivity (Wagner et al., 2016).

Enzymatic activity of eNOS is influenced by a single bout of exercise showing increased phosphorylation at eNOS^{ser1177}, but not total eNOS in skeletal muscle microvascular capillaries (Cocks et al., 2013). eNOS^{ser1177} phosphorylation increases NO availability which acutely results in increased local blood flow and perfusion that is needed to enhance substrate delivery. In a series of experiments, Cocks and colleagues showed that six weeks of exercise training, i.e. sprint interval training and endurance training increased microvascular capillary density and eNOS content in both young sedentary males (Cocks et al., 2013) and obese males (Cocks et al., 2016a) which was accompanied with improvements in insulin sensitivity. Additionally, in T2DM, high-intensity interval training, but not aerobic training increased microvascular eNOS expression in skeletal muscle (Mortensen et al., 2018)

2.7.2. Skeletal muscle insulin signalling adaptations
Exercise acutely increases glucose uptake in the muscle by the activation of insulin-independent pathways, such as the AMPK pathway, that results in increased GLUT4 translocation (**Figure 2.2**) to take up glucose that can be used for immediate energy utilization. However, exercise also has positive effects on subsequent insulin-stimulated glucose uptake (Holloszy, 2005). Experiments in both insulin resistant animals (Castorena et al., 2014; Ropelle et al., 2006) and humans (Devlin et al., 1987; Perseghin

et al., 1996) show that insulin-stimulated glucose uptake is improved after an acute bout of exercise because of enhanced insulin sensitivity (Cartee, 2015). Moreover, a single bout of cycling exercise increased GLUT4 mRNA expression and GLUT4 translocation in T2DM (Hussey et al., 2012; Kennedy et al., 1999).

Exercise training, defined as repeated bouts of exercise over multiple days or weeks, improved insulin sensitivity in T2DM (Dela et al., 1995; Vind et al., 2011). Exercise training for seven days (O'Gorman et al., 2006) to ten weeks (Dela et al., 1994) showed both increased GLUT4 mRNA expression and GLUT4 protein expression levels. Regulators for increased GLUT4 expression are suggested to be increased AMPK activity and elevated levels of AS160 phosphorylation and total content (Vind et al., 2011).

2.8. Dietary protein intake as an intervention to improve insulin sensitivity

Intake of dietary proteins have been linked with improved glycaemic control and insulin sensitivity. In a meta-analysis, Dong et al. (Dong et al., 2013) found that high-protein diets (> 25% of total energy intake) in T2DM humans resulted in improved glycaemic control compared to low to moderate protein diets (<20% of total energy intake). Next to the quantity of protein intake, the composition of the consumed protein is proposed to elicit beneficial effects on insulin sensitivity. For example, consumption of cod protein, depleted of omega-3, has been shown to improve skeletal muscle insulin sensitivity in insulin resistant rats (Tremblay et al., 2003) and humans (Ouellet et al., 2007). Proteins are comprised of amino acids, of which there are 20. Each amino acid has its unique chemical structure and therefore potentially a different physiological function. The effects of dietary protein on skeletal muscle insulin resistance might differ depending on the amino acid composition of the protein.

2.8.1. Microvascular adaptations

As described in **section 2.4**, insulin-stimulated glucose disposal is dependent on NO bioavailability in the microvascular system for the increase in microvascular recruitment and trans-endothelial transport of insulin. One amino acid that can be converted into NO is arginine and therefore holds potential as a nutritional component to improve insulin sensitivity. In fact, L-arginine supplementation for one month has been demonstrated to increase insulin sensitivity by improved forearm blood flow and glucose disposal during a hyperinsulinaemic euglycaemic clamp in T2DM compared to a placebo (Piatti et al., 2001). Monti et al. (2013) has shown beneficial effects of L-arginine enriched biscuits on insulin sensitivity and endothelial function in healthy and metabolic syndrome subjects.

When investigating the effect of whole-intact proteins, there is some limited evidence that consumption of milk-derived proteins improves endothelial function under hypertensive conditions. Sanchez et al. (2011) showed that daily consumption of casein hydrolysate for 6 weeks reduced the development of hypertension in spontaneously hypertensive rats compared to a placebo group, which was accompanied by higher eNOS expression in aortic tissue. Additionally, Fekete et al. (2016) reported improved vascular function via increased flow-mediated dilation in people with prehypertension after 8 weeks of whey consumption compared to placebo. Although these studies provide some evidence for the role of protein consumption to improve endothelial function, caution has to be warranted to translate and link the evidence to potential improvements via microvascular adaptations in insulin sensitivity in a diabetic population, even though hypertension is often prevalent in T2DM (Tatsumi et al., 2017).

2.8.2. Skeletal muscle fibre adaptations

Another amino acid that acts directly on the skeletal muscle fibre is the amino acid leucine (Moore et al., 2009). Leucine has been identified as a nutritional activator of the mammalian target of rapamycin complex 1 (mTORC1) that initiates the translation process of muscle protein synthesis (Anthony et al., 2000). Prolonged activation of muscle protein synthesis could result in a net positive protein balance that could lead to skeletal muscle hypertrophy. However, a positive net protein balance is more potent when protein feeding is combined with an exercise stimulus (Biolo et al., 1997) (see **section 2.9**). Additionally, leucine, together with valine and isoleucine, are classed as branched-chain amino acids (BCAA) and are essential amino acids. D'Antona et al. (2010) has reported that BCAA supplementation resulted in increased mitochondrial biogenesis and upregulated ROS defence system in middle-aged mice and is dependent on mTORC1 activity. Additionally, infusion of a mix of amino acids has been demonstrated to increase skeletal muscle mitochondrial protein synthesis in healthy subjects (Bohe et al., 2003). However, in elderly T2DM humans, leucine ingestion for six months did not improve glycaemic control and oral glucose insulin sensitivity (Leenders et al., 2011).

Two amino acids that are of interest for increasing skeletal muscle plasticity are cysteine and glycine. These amino acids are suggested to play a role in the synthesis of glutathione, an antioxidant that is reduced in T2DM (Calabrese et al., 2012; Sekhar et al., 2011). Indeed, supplementation of cysteine and glycine in uncontrolled T2DM resulted in a significant increase in glutathione in plasma, suggesting a decrease in systemic oxidative stress (Sekhar et al., 2011). The majority of studies investigate glutathione concentrations in erythrocytes or liver tissue (El-Hafidi et al., 2018; Sekhar et al., 2011), however, the role of glutathione in skeletal muscle might be important as

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in T2DM there is an increase in oxidative stress by formation of ROS which interferes with insulin signalling (Anderson et al., 2009; Wei et al., 2008). Increasing skeletal muscle glutathione synthesis could assist in neutralizing ROS and thereby improve insulin sensitivity; however, the effects of cysteine and glycine supplementation on skeletal muscle glutathione are currently unknown.

2.8.3. Keratin-derived Protein

Keratin is a protein that is present in nails, feathers and hair of mammals, and contains relatively high concentrations of thiol-containing amino acids cysteine and other amino acids including arginine and glycine (Crum et al., 2018). Intake of these amino acids have been associated with improved glycaemic control as outlined in **section 2.8.1 and 2.8.2.**, making keratin a potential candidate as a nutritional supplement for T2DM treatment. Keratin is insoluble and considered to be poorly digested due to the disulphide bonds in cysteine, however hydrolysis methods have been developed to increase digestibility via breaking the disulphide bonds (Crum et al., 2018; Shorland et al., 1970). Subsequently, Houltham et al. (2014) showed that daily ingestion of 40 grams of wool-derived keratin is tolerable in adults without serious side effects on the gastrointestinal system compared to casein protein, except for an increase in flatulence. Some limited evidence has found a positive effect of chronic keratin ingestion, derived from chicken feathers, on lean mass in trained cyclists (Crum et al., 2018) and haematocrit and haemoglobin in rat (Wolber et al., 2016) compared to casein protein. However, the effect of chronic keratin supplementation on insulin sensitivity and glycaemic control in T2DM have not been investigated yet.

2.8.4. Whey Protein

Whey protein is, together with casein, the most abundant protein in milk, with a ratio of 20%/80% respectively. Whey protein has been rated as a high-quality protein, based on the protein digestibility-corrected amino acid score (PDCAAS) (Schaafsma, 2012) and digestible indispensable amino acid score (DIAAS) (Wolfe et al., 2016). These scores are based on the relative and total amount of essential amino acids or indispensable amino acids in relation to the required daily intake of these amino acids. Whey protein is more rapidly digested and absorbed compared to casein (Pennings et al., 2011a) and promotes muscle protein synthesis to a greater extent than casein and soy protein (Tang et al., 2009), attributed to its high leucine content. As described in **section 2.8.2.** leucine promotes remodelling of skeletal muscle, making whey protein a potential treatment in T2DM.

The role of whey protein as a treatment for T2DM has not yet been fully established. Ingestion of whey before a meal has been demonstrated to acutely reduce postprandial hyperglycemia (Clifton et al., 2014; Jakubowicz et al., 2014; Ma et al., 2009), and twelve weeks of whey-enriched breakfast consumption reduces HbA_1c in T2DM (Jakubowicz et al., 2017). This effect is attributed to the insulinotropic properties of protein. Secretion of insulin is impaired in T2DM in response to carbohydrate ingestion, but is intact in response to amino acid and protein ingestion (Manders et al., 2014; Manders et al., 2005). Hence, a pre-load of whey before a meal increases the release of insulin in T2DM, which helps with reducing postprandial blood glucose concentrations.

However, acute whey protein ingestion has also been demonstrated to induce insulin resistance and reduce insulin-stimulated glucose uptake during a hyperinsulinaemic euglycaemic clamp (Smith et al., 2015). Insulin resistance in the presence of elevated

plasma amino acids, and thereby reducing skeletal muscle glucose disposal, has been attributed to the activation of the mTORC1 and overactivation of downstream S6 kinase 1, which suppresses insulin signalling via IRS-1 serine 312 phosphorylation (Tremblay et al., 2005). There is emerging evidence that BCAA metabolism is disrupted in T2DM, and high plasma concentrations of BCAA are associated with an increased risk in T2DM (Lynch et al., 2014). Therefore, whey protein, with a central role for the BCAA leucine, as a treatment in T2DM remains debatable and warrants further investigation.

2.9. Combined exercise and dietary protein intervention

Providing the suggested evidence that exercise and dietary protein intake have independent beneficial effects on insulin sensitivity, combining exercise and post-exercise dietary protein ingestion could potentially lead to additive adaptations and superior improvements in insulin sensitivity compared to exercise or dietary protein intake only. Exercise and post-exercise protein ingestion has been shown to be an effective strategy to increase skeletal muscle mass in both young and elderly healthy men (Cermak et al., 2012). Given the potential link between skeletal muscle mass and insulin sensitivity (Matta et al., 2016), nutritional and exercise interventions to increase lean mass are a proposed treatment strategy (Witard et al., 2018).

2.9.1. Systematic search

To review the literature for existing studies on the effect of combined exercise and protein ingestion in T2DM on glycaemic control, a systematic search was performed in August 2017 (**Appendix A**). Five studies were identified that met inclusion criteria (**Figure 2.4**). Study details are summarized in **Table 2.**1. The duration of the intervention ranged from 10 weeks to a year. Two studies used resistance exercise training (RET) (Wycherley et al., 2010b), one study combined resistance-exercise

training (RET) with aerobic interval training sessions (AIT) (Tay et al., 2015), one study used high-intensity interval training (HIIT) (Francois et al., 2017) and one study used mixed-mode interval training (MMIT) (Gaffney et al., 2017). All studies implemented at least 3 non-consecutive days of exercise per week where Gaffney et al. (2017) implemented 5 exercise sessions per week. Three studies investigated the effect of exercise and dietary protein modifications under hypocaloric conditions creating a moderate energy deficit of ~500 – 1000 kcal (Tay et al., 2015; Wycherley et al., 2010a, 2010b), whereas two studies had unrestricted calorie guidelines (Francois et al., 2017; Gaffney et al., 2017). Protein sources used for supplementation or to increase protein intake were whey (Gaffney et al., 2017), milk protein (Francois et al., 2017; Wycherley et al., 2010b) or were not specified (Tay et al., 2015; Wycherley et al., 2010a). Wycherley et al. (2010b), Francois et al. (2017) and Gaffney et al. (2017) provided ~20 grams of protein in close proximity to the exercise session as reported by the literature to be the optimal dose to stimulate muscle protein synthesis (Moore et al., 2009; Witard et al., 2014). Wycherley et al. (2010b) specifically compared whether timing of protein ingestion resulted in differences in glycaemic control and body composition by comparing protein ingestion prior to, or two hours post-exercise. Tay et al. (2015) compared a high-carbohydrate with a low-carbohydrate diet where the high-carbohydrate diet derived 17% of total energy from proteins and the low-carbohydrate derived 28% of total energy from protein, which converted to a daily protein intake of ~0.7 $g \cdot kg^{-1} \cdot day^{-1}$ and ~1.0 $g \cdot kg^{-1} \cdot day^{-1}$ respectively. A second study by Wycherley et al. (Wycherley et al., 2010a) compared a daily protein intake of ~0.7 $g \cdot kg^{-1} \cdot day^{-1}$ (moderate protein intake) with ~1.2 $g \cdot kg^{-1} \cdot day^{-1}$ (high protein intake).

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│ Articles identified n = 327     │
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│ were removed n = 202        │      │ through reference checking n = 4│
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│ Records screened n = 202    │─────►│ Excluded n = 167                │
└─────────────────────────────┘      │  • Non-English (23)             │
                │                     │  • No appropriate study design  │
                │                     │    (123)                        │
                │                     │  • No human population (4)       │
                │                     │  • No appropriate human         │
                │                     │    population (6)               │
                │                     │  • Intervention (9)             │
                │                     │  • No full text available (1)   │
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│ Full text articles assessed │─────►│ Excluded n = 30                 │
│ for eligibility n = 35      │      │  • No measure of insulin        │
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                │                     │    control (16)                 │
                │                     │  • Glycaemic baseline values    │
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│ Studies included in final   │
│ analysis n = 5              │
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Figure 2.4 – Flowchart of paper selection throughout the review process.

2.9.2. Effect of exercise and protein ingestion on glycaemic control

Measures of insulin resistance and glycaemic control were obtained from fasting blood

samples to calculate homeostatic model assessment for insulin resistance (HOMA-IR),

glycosylated haemoglobin (HbA$_1$c), during 24 h of continuous glucose monitoring or a

hyperinsulinaemic euglycaemic clamp (**Table 2.2**). Wycherley et al. (2010b) and Tay et

al. (2015) observed reductions in HOMA2-IR values of ~0.7 and Gaffney et al. (2017)

reported a 14.1% decrease in HOMA-IR when exercise training was combined with

whey supplementation, but no differences were observed between groups in all three

studies. HbA$_1$c (%) decreased by ~1% in three studies that implemented a hypocaloric

diet (Tay et al., 2015; Wycherley et al., 2010a, 2010b) whereas no change was found by

Francois et al. (2017) who added 20 grams of skimmed milk powder in addition to a

free living diet. Mean 24 hours blood glucose concentration was reduced by 0.5 ± 1.1

mmol•L^{-1} after 12 weeks of HIIT and skimmed milk supplementation, however no

difference was observed with the control group who only performed HIIT (Francois et

al., 2017).

2.9.3. Effect of exercise and protein ingestion on body composition

Body composition changed significantly in the studies (**Table 2.3**). Overall, body

weight decreased by ~10% (~10 kg) under hypocaloric dietary conditions (Tay et al.,

2015; Wycherley et al., 2010b). Wycherley et al. (2010a) reported greater reductions in

body weight and fat mass when a high protein diet (1.2 g·kg^{-1}·day^{-1}) was combined with

resistance exercise training compared to a high protein diet only (-12.7% vs. -8.7%

body weight respectively). Timing of protein intake around exercise had no effect on

magnitude of loss in body weight (Wycherley et al., 2010b). Francois et al. (2017)

reported a small but significant decrease in bodyweight (-0.9 ± 3.9 kg) after twelve

weeks of HIIT and ingestion of 20 g protein post-exercise under unrestricted energy

intake. Reductions in body weight were accompanied by significant reductions in fat mass (Francois et al., 2017; Tay et al., 2015; Wycherley et al., 2010a), irrespective of supplement timing (Wycherley et al., 2010b). Interestingly, studies under hypocaloric conditions reported losses in fat free mass (muscle mass) of ~2 kg, even with daily protein intakes of 1.2 $g \cdot kg^{-1} \cdot day^{-1}$ (Tay et al., 2015; Wycherley et al., 2010b). In unrestricted energy conditions and ingestion of 20 g milk protein after HIIT, there was significant increase in fat free mass of 1.07 ± 2.76 kg, however daily protein intake was not reported in $g \cdot kg^{-1} \cdot day^{-1}$ (Francois et al., 2017).

Table 2.1 – Individual study details from included studies[1]

	Subject Details					Intervention Details			
	Population	Sex (Female)	Age (yr)	Length (wk)	Exercise type (frequency d/w)	Protein Intervention (% of total energy in diet)	Protein source	Control group	Energy restricted
Francois et al. (2017)	T2DM	53 (34)	40-75	12	HIIT (3)	~20 g	Milk protein	Exercise only	-
Wycherley et al. (2010b)	T2DM	34 (NR)	56.6 ± 7.1	16	RT (3)	25 g pre-exercise	Skimmed milk powder	25 g post-exercise	Intake: M: 7000 kJ/d F: 6000 kJ/d
Wycherley et al. (2010a)	T2DM	83 (NR)	56.1 ± 7.5	16	RT (3)	Normal protein (19%) High protein (33%)	NR	High-protein only	Intake: M: 7000 kJ/d F: 6000 kJ/d
Tay et al. (2015)	T2DM	115 (49)	57 ± 7	52	AET + RT (3)	High protein (28%)	NR	Normal protein (17%)	Deficit: 500 – 1000 kcal/d
Gaffney et al. (2017)	T2DM	24 (0)	55.7 ± 5.6	10	MMIT (5)	20 g	Whey	Exercise only	N

[1]Individual details from studies regarding population characteristics, intervention duration, type and frequency of exercise, details of protein supplementation, control group and energy restriction

during the intervention. RT, resistance exercise training; AET, aerobic exercise training; HIIT, high-intensity interval training; MMIT, mixed mode interval training; NR, not reported.

Table 2.2 – Outcomes of glycaemic control for the studies[1]

	Measure	Time		Group	Interaction
Francois et al. (2017)	HbA$_1$c	-0.22 ± 0.39% mmol/mol	P < 0.01	NR	P = 0.92
	CGM 24h	-0.5 ± 1.1 mmol/l	P = 0.01	NR	P = 0.74
Wycherley et al. (2010b)	HbA$_1$c	-1.1 ± 0.1% mmol/mol	P < 0.001	P0: -1.0 ± 0.9% P2: -1.1 ± 0.6%	P = 0.53
	HOMA2-IR	-0.8 ± 0.8	P < 0.001	P0: -0.7 ± 0.7 P2: -0.8 ± 0.9	P = 0.58
Wycherley et al. (2010a)	HbA$_1$c	NR	P < 0.001	CON: -1.1 ± 0.6% HP: -1.8 ± 1.6% CON + RT: -1.1 ± 0.7% HP + RT: -1.1 ± 0.7%	P = 0.21
Tay et al. (2015)	HbA$_1$c	NR	NR	LOW-CARB 95%CI (-1.2, -0.7%) HIGH-CARB 95%CI (-1.3, -0.8%)	P = 0.65
	HOMA-IR	NR	NR	LOW-CARB 95%CI (-0.9, -0.5) HIGH-CARB 95%CI (-0.9, -0.4)	P = 0.69
Gaffney et al. (2017)	HOMA-IR	Control 95%CI (-30.1 – 28.3) Whey 95% CI (-25.3 – 1.08)	Unclear Benefit possible	Control-Whey 95%CI (-34.2, 25.4)	Unclear
	GDR	Control 95%CI (-5.4 – 64.8) Whey 95% CI (1.2 – 60.7)	Benefit Likely Benefit very likely	Whey-Control 95% CI (-28.0, 44.8)	Unclear

[1]Outcomes of the studies for insulin sensitivity or glycaemic control. All studies used null-hypothesis for statistical significance unless stated otherwise. P-values and 95% confidence intervals are provided when reported in the study. HbA$_1$C, Glycosylated Haemoglobin; CGM, continuous glucose monitoring; HOMA-IR, homeostatic model assessment of insulin resistance; GDR, Glucose disposal rate; NR, not reported.

Table 2.3 – Outcomes of body composition for studies[1]

	BW (kg)				FM (kg)				FFM (kg)			
	Time		Group	Interaction	Time		Group	Interaction	Time		Group	Interaction
Francois et al. (2017)	−0.9 ± 3.9	P = 0.03	NR	P = 0.46	NR		NR		+1.07 ± 2.76	P = 0.01	NR	P > 0.05
Wycherley et al. (2010b)	−11.9 ± 6.1	P < 0.001	P0: −11.3 ± 5.9 P2: −12.6 ± 6.5	P = 0.52	−10.0 ± 4.4	P < 0.001	P0: −9.4 ± 4.6 P2: −10.6 ± 4.3	P = 0.45	−1.9 ± 3.1	P < 0.001	P0: −1.8 ± 3.2 P2: −2.0 ± 3.1	P = 0.85
Wycherley et al. (2010a)	NR	P < 0.001	CON: -8.6 ± 4.6 HP: -9.0 ± 4.8 CON + RT: -10.5 ± 5.1 HP + RT: -13.8 ± 6.0	P = 0.04	NR	P < 0.001	CON: -6.5 ±3.7 HP: -7.1 ± 4.0 CON + RT: -8.1 ± 3.8 HP+RT: -11.4 ± 3.9	P = 0.006	-2.0 ± 2.3	P < 0.001	CON: -2.2 ± 1.9 HP: -1.9 ± 1.5 CON + RT: -2.4 ± 2.5 HP + RT: -2.4 ± 3.1	P = 0.91
Tay et al. (2015)	NR	P < 0.05	LOW-CARB -9.8 (-11.7, -7.9) HIGH-CARB -10.1 (-12.0, -8.2)	P = 0.18	NR	NR	LOW-CARB 95%CI (-9.0, -6.0) HIGH-CARB 95%CI (-10.5, -6.8)	P = 0.09	NR	NR	LOW-CARB 95% CI (-2.3, -1.2) HIGH-CARB 95% CI (-2.2, -1.0)	P = 0.67
Gaffney et al. (2017)	NR				NR				NR			

[1]Outcomes of body composition. P-values and confidence intervals are provided when reported in the study. BW, body weight; FM, fat mass; FFM, fat-free mass; HP, high protein; RET, Resistance-exercise training; CON, control; NR, Not reported;

Thus far, only five studies have examined the combined effect of exercise and dietary protein intake on glycaemic control in humans diagnosed with T2DM. These studies have demonstrated improved glycaemic control after an exercise protein co-intervention, however combining exercise with dietary protein intake did not show any further benefit compared to isolated exercise or protein intake. A possible explanation for why the addition of protein intake to exercise has not been found more beneficial compared to exercise alone could be the type of dietary protein. Most experiments used 20 grams of a milk-derived protein source. Justification for the use of this type and quantity comes from evidence that milk and whey are quickly digestible proteins, containing high concentrations of essential amino acids, including leucine. Evidence indicates that 20 grams of these proteins maximally stimulate protein synthesis after exercise (Witard et al., 2014).

However, as described in **section 2.8**, different proteins might elicit different responses and a response is potentially dependent on the amino acid profile of the dietary protein. No difference in improvements in insulin sensitivity between endurance and resistance exercise training are observed (Yang et al., 2014). Moreover, comparison of different types of dietary protein show that skeletal muscle remodels at faster rates after both types of exercise (Moore et al., 2009; Rowlands et al., 2015). These observations suggest that it is likely that the type of protein consumed is a determining factor to further improve insulin sensitivity compared to exercise alone. This requires further investigation to analyse the effect of exercise and comparing the intake of various dietary proteins (**section 2.8.3, 2.8.4.**) after exercise on glycaemic control and insulin sensitivity in humans with T2DM.

2.10. Techniques to measure GLUT4 translocation

Over the last decades, many techniques have been developed to measure GLUT4 translocation with the aim to quantify GLUT4 incorporated in the plasma membrane. In the early discovery phase of glucose transporters, studies used the fungal toxin Cytochalasin-B, which binds to glucose transporter sites (Wardzala et al., 1978). Evidence that cytochalasin-B specifically binds to glucose transporters comes from studies that show that transport of D-glucose into cells is inhibited in the presence of cytochalasin-B, indicating competitive binding (Wardzala et al., 1981). The technique allows measuring the exact number of glucose transporters in the plasma membrane and was therefore able to show that there was a 4-fold increase of plasma-bound glucose transporters in response to insulin stimulation (Wardzala et al., 1978). However, the disadvantage of cytochalasin-B for measuring glucose transport is that it is not specific (Biolo et al., 1997). This means that cytochalasin-B does not distinguish between GLUT4 and GLUT1, based on evidence that cytochalasin-B also binds to erythrocytes which do not contain GLUT4, but GLUT1 transporters (Lin et al., 1974). Therefore, initially it remained uncertain whether the 4-fold increase in glucose transporters in response to insulin was due to an increase of GLUT1 or GLUT4 to the plasma membrane.

Subcellular fractionation is another technique towards measuring GLUT4 translocation. The technique uses differential centrifugation speeds to separate cell fractions based on their molecular weight and density. The method is therefore able to isolate the cellular membrane fraction from the rest of cellular components. Next, the membrane fraction can be assayed for GLUT4 concentration that is incorporated in the plasma membrane with methods including Western blots. However, to isolate sufficient plasma membrane fraction for acquiring high sensitivity, this technique requires large amounts of muscle

tissue. This amount of muscle tissue can be obtained in animal experiments, collecting a whole muscle group of an anaesthetized animal. However, in percutaneous Bergstrom needle technique used for human skeletal muscle tissue collection ~100 mg is at the upper yield of the amount that can be collected (Tarnopolsky et al., 2011). There are human studies that used the subcellular fractionation technique to purify skeletal muscle plasma membrane fractions, however the collection of the muscle was performed with an open biopsy to harvest one gram of skeletal muscle which is not a preferable option (Goodyear et al., 1996; Kennedy et al., 1999). On top of that, fractionation techniques are sensitive to contamination with other cell fractions, causing misinterpretation of the results.

Western blotting and qualitative PCR are two widely used techniques in biomedical research. These techniques measure protein expression and gene expression of an encoded protein respectively and they have been used to analyse total GLUT4 protein content and GLUT4 mRNA expression in experiments. Besides the large amount of tissue that is needed and the risk of contamination, these methods do not measure translocation of GLUT4. mRNA is only a precursor for de novo protein synthesis and GLUT4 protein might not be produced in the endoplasmatic reticulum or trans-golgi network as post-translational steps might suppress the production of the protein (Koussounadis et al., 2015; Vogel et al., 2012). Total GLUT4 protein only reflects the total amount of protein in the cell, but not the specific location of the protein i.e. translocated with the plasma membrane or stored intracellularly.

Immunofluorescence can be used to investigate whether GLUT4 is incorporated in the plasma membrane. Studies have used L6 skeletal muscle and transgenic mice that expressed GFP-GLUT-HA in cardiomyocytes and skeletal muscle (Fazakerley et al.,

2009; Lizunov et al., 2012). With genetic modification, a green fluorescent protein (GFP) and a hemagglutinin (HA) tag were expressed with GLUT4 protein. Since the HA is tagged on the first exofacial loop of GLUT4, antibodies bind to this tag to the epitope only when GLUT4 is exposed to the external surface i.e. when it is fully incorporated in the plasma membrane and thus able to transport glucose into the cell. Although these experiments show significant increases in GLUT4 translocation after insulin and contraction-like stimuli (Fazakerley et al., 2009), this method cannot be applied in human research, since genetic modification cannot be performed in humans for obvious ethical reasons.

Other studies have applied immunofluorescence as well to quantify GLUT4 translocation and showed increased trafficking of GLUT4 towards the plasma membrane. However, these studies lack a clear plasma membrane marker and thus assume that GLUT4 that seems to be close to what appears to be the plasma membrane is incorporated in the plasma membrane (Ploug et al., 1998; W. Wang et al., 1996). Recently, Bradley et al. (2014) introduced the use of dystrophin as a plasma membrane marker. Dystrophin is continuously distributed a few nanometers sub-sarcolemmal and therefore has been shown to be a valid marker for the plasma membrane (Byers et al., 1991). Using co-localization as an analytical tool, studies have shown that glucose ingestion and endurance exercise results in a significantly increased co-localization of GLUT4 with dystrophin in healthy human skeletal muscle, which indicates increased GLUT4 translocation (Bradley et al., 2015). However, the disadvantage of immunofluorescence is its spatial resolution when using a widefield light microscope for image capturing. As described in **Section 2.5.1.**, GSV are 50-70 nm in diameter, microscopes have spatial resolutions of ~100 nm. Additionally, fluorescent areas that are not in the plane of focus, produce blurred spots on the captured image. When co-

localization is applied, this blur might result in higher areas of co-localization where there is none, which induces error in the interpretation whether GLUT4 is incorporated or in close proximity to the plasma membrane.

A solution to the spatial resolution is using a microscope with higher resolution. The transmission electron microscope (TEM) in combination with immunocytochemistry has been used in several studies investigating GLUT4 translocation (Ploug et al., 1998; Slot et al., 1991a; Slot et al., 1991b; W. Wang et al., 1996). TEM can produce images on a nanometre scale and is therefore able to visualize the plasma membrane and to distinguish it from intracellular regions. Immunogold is a technique, which uses a gold particle as a secondary antibody. This gold particle produces a high contrast when visualized with TEM, thereby increasing the spatial accuracy of the targeted protein. With this technique it has been shown that insulin results in a sevenfold increase in GLUT4 translocation in the skeletal muscle of rats and even a fourteen-fold increase when insulin and exercise is combined (Ploug et al., 1998). Only one study has applied the method to human tissue (Friedman et al., 1991). Interestingly, they did not find GLUT4 in the plasma membrane in either basal or insulin-stimulated conditions, but rather showed the presence of GLUT4 in the transverse tubules and terminal cisternae. Unfortunately, due to technical difficulties that comes with the method and the introduction of cheaper and more convenient techniques, this method has disappeared as a way to analyse GLUT4 translocation, despite overcoming limitations that have been addressed with other techniques. Moreover, because of the high spatial resolution, multiple images are required to include sufficient plasma membrane surface to assure external validity in respect to whole skeletal muscle mass, whereas confocal and widefield microscopes can visualize multiple cells in a single image, increasing external

validity. Therefore, between visualization tools, there is a trade-off between spatial resolution and external validity.

2.11. Summary

Skeletal muscle plays a pivotal role in glucose homeostasis by increasing insulin-stimulated glucose uptake in the postprandial state. In T2DM, insulin resistance, a consequence of reduced physical activity and increased calorie intake, impairs the insulin-stimulated glucose uptake in skeletal muscle, which leads to chronically elevated blood glucose concentrations. Exercise and dietary protein manipulations have been demonstrated to independently reduce insulin resistance and it has been proposed that combined exercise and protein ingestion could further improve insulin sensitivity and glucose homeostasis in T2DM with potentially a role for specific amino acid profiles. Improvements might be multifactorial and might include: enhanced insulin-signal transduction in skeletal muscle with increased insulin-stimulated GLUT4 translocation and improved skeletal muscle microvascular function via increased capillary density and increased phosphorylation activity of $eNOS^{ser177}$.

In the following chapters of the thesis, an intervention was designed to study the hypothesis that fourteen weeks of post-exercise keratin-derived protein ingestion further improves insulin sensitivity compared to exercise only or exercise combined with whey protein ingestion. Proposition for this hypothesis comes from the argument that this novel protein has relatively high concentrations of cysteine, glycine and arginine. These amino acids have been proposed to improve skeletal muscle insulin sensitivity via increased glutathione availability which attenuates cellular oxidative stress, improving insulin-signalling, GLUT4 translocation and via improving microvascular function. We also test the effectiveness of WDP compared to whey protein and thereby further

elaborate on previous research that investigated the role of post-exercise whey ingestion in T2DM.

Furthermore, the thesis describes the attempt to use and validate immunogold electron microscopy to measure GLUT4 translocation as current techniques have considerable limitations.

CHAPTER 3

GENERAL METHODS

3.1. Participants

Men and women of all ethnicities, aged 35-70 years and diagnosed with T2DM were recruited to participate in the trial (**Appendix B/C**). Inclusion criteria were having a HbA1C > 48 mmol/mol as T2DM diagnosis criteria during the last 12 months and not meeting current American College of Sport Medicine (ACSM) activity guidelines of 150 min of moderate/vigorous intensity exercise per week. For safety concerns, exclusion criteria were the requirement of insulin medication, the use of β-blockers and a history of cardiovascular incidents. If participants were taking hypoglycaemic medication (e.g. metformin, pioglitazone or gliclazide), they were asked to maintain their medication throughout the study period. Before volunteering in the trial, participants were provided information regarding the risks and dangers about the study before they signed an informed consent (Appendix B). After giving informed consent participants underwent a maximal exercise test on a cycle ergometer. Participants were also screened for cardiac abnormalities during this test via 12 lead electrocardiogram. **Figure 3.1** displays the recruitment numbers for the intervention. The study was approved by the Central Health and Disability Ethics Committee, New Zealand and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12614001197628).

3.2. Study design

This study was a double-blinded, parallel-group, randomized controlled trial, which consisted of 14 weeks of exercise training and dietary protein supplementation. Participants all completed the same exercise training regime over the fourteen weeks but were randomly assigned to ingest either a blend of keratin-derived protein and whey (WDP), whey protein (WHEY) or a placebo supplement (CON). Participants were encouraged to maintain dietary and medication habits throughout the intervention period.
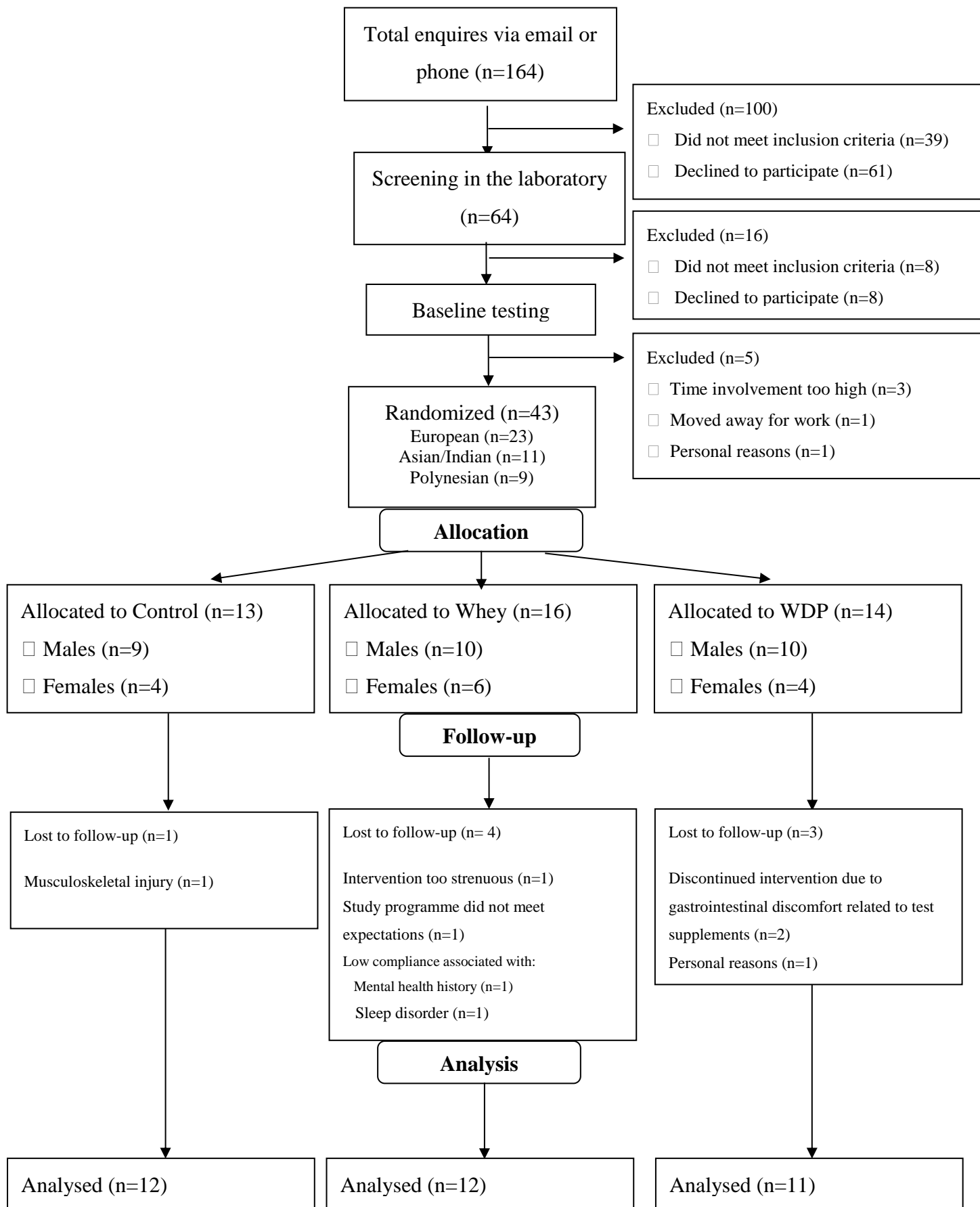
Figure 3.1 – Consort-style recruitment flowchart for the study intervention.

Participants were randomized into one of the three conditions using the method minimization as recruit (Hopkins, 2010) via the published spreadsheet (http://sportsci.org/2010/wghminim.htm). Participants were stratified based on age, maximal wattage output (W_{max} as a proxy for fitness) and glucose clearance rates at baseline to minimize the interference of these parameters on primary outcomes (Coon et al., 1992; Yates et al., 2002). Supplements were ingested twice a day, immediately after training and in the evening. The timing of physiological and performance tests is shown in **Figure 3.2**. Before starting the intervention, participants were familiarized with the equipment and performance tests.
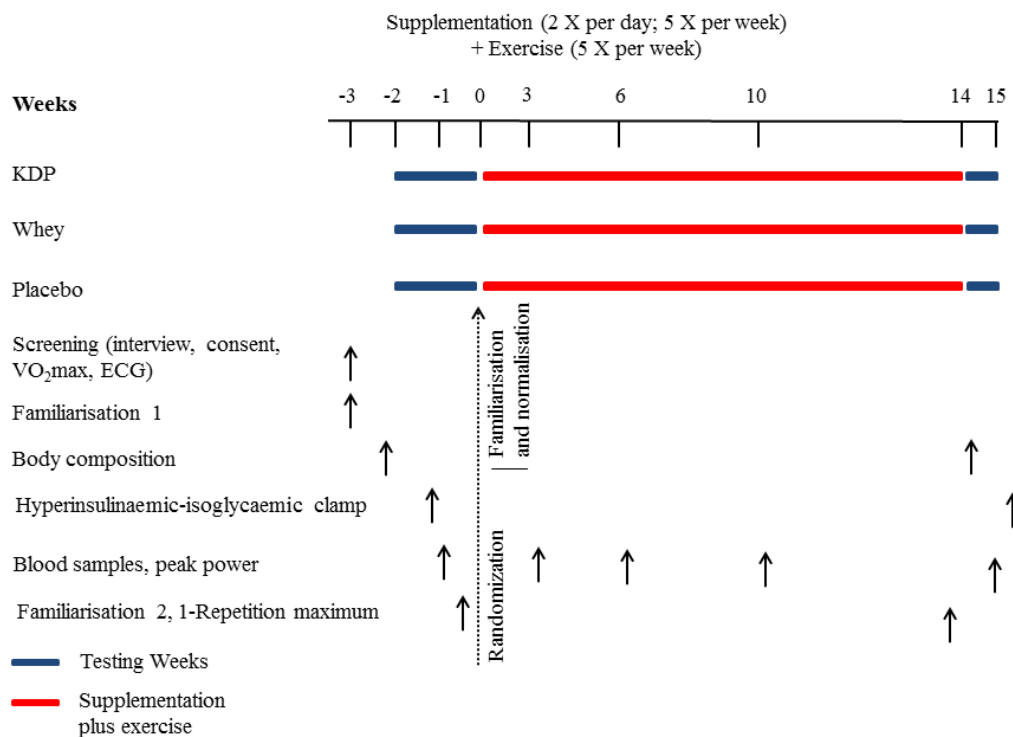
Figure 3.2 – Outline of the intervention study

3.3. Training protocol

3.3.1. Preliminary Testing

Before the start of the intervention, participants were familiarized with the equipment before undergoing maximal strength capacity test for bench press, leg press, lateral pulldown and hip thruster exercises using one repetition maximum (1RM). After a brief warming up, participants performed working sets of 8 repetitions with increasing load until failure to complete 8 repetitions after which estimates of 1RM were calculated using Brzycki method (Brzycki, 1993). Between every set, participants had 2 minutes of recovery. Maximal endurance capacity (wattage output (W_{max})) was measured during an incremental ramp protocol on an indoor cycle ergometer (VeloTron RacerMate, Seattle, WA). After a warming-up of three minutes at 40 W, the resistance of the ergometer increasing with 1 W per 3 seconds and participants cycled until volitional fatigue was reached, defined as the inability to maintain a cadence >70 revolutions/min.

3.3.2. Training during the intervention

The fourteen weeks of exercise training consisted of five training sessions per week with a progressive increase in workload of 1-2% every week. Four of the five sessions were endurance training and the fifth session was resistance training. All training sessions were executed in the morning in a fasted state followed by the ingestion of the supplement. The endurance training was performed on an indoor cycle ergometer (Velotron RacerMate). The order of training sessions was consistent every week. Session 1: cycling consisting of five peaks with durations of 1-2-3-2-1 minutes at 75% of W_{max} separated by 1-2-3-2-1 minutes of active recovery. Session 2: resistance training consisted of 3 sets of 10-12 repetitions at 55% of 1RM for bench press, leg press, lateral pulldown and hip thruster and was finished with two sets of 300 m maximal rowing. Session 3 and 5: cycling consisting of 3 x 10-12 minute intervals at

70

60% of W_{max} separated with one minute of active recovery. Session 4: cycling consisting of 10 x 1 minute intervals at 90% of W_{max} separated by two minutes of active recovery.

3.4. Supplement composition

Several different feeding scenarios were tested in order to optimize the palatability and logistical handling of the supplements. In the end, the supplements were in the form of a 70 g baked muffin (Appendix D) ingested twice daily on exercise days on top of the habitual diet as well as 10 capsules size "0" (23 x 8 mm), which were ingested after the exercise session. Supplements were administered in a double-blinded manner. Supervision of administration after each exercise session ensured compliance to the supplement intake. To ensure compliance to the evening supplement, automated text messages were sent every evening and participants were asked the following morning on their compliance. Total daily added protein doses of the treatments were 1) WDP group 17 g of WDP + 23 g whey (WPI-A895; Fonterra, Auckland, New Zealand) 2) WHEY group 40 g whey and 3) CON ingested no protein but an isocaloric amount of 50% maltodextrin and 50% low protein gluten free flour. Amino acid composition is outlined in **Table 3.1.**

Table 3.1 – Macronutrient and amino acids composition for the three different treatment supplements in addition to participants' habitual diet[1].

MACRONUTRIENTS	CON[a]	WHEY	WDP[b]
Protein (g)	3.6	42.7	40.2
Carbohydrate (g)	66.8	30.8	29.8
Fat (g)	26	26	26
Energy (kJ)	1958	1978	1951
AMINO ACIDS			
Alanine	0.1	2.0	1.7
Arginine	0.1	0.9	1.9
Aspartic Acid	0.2	4.0	2.8
Cysteine	0.0	1.0	2.0
Glutamic Acid	0.3	6.8	5.8
Glycine	0.1	0.7	1.2
Histidine	0.0	0.7	0.5
Isoleucine	0.1	2.5	2.0
Leucine	0.1	4.0	3.4
Lysine	0.1	3.6	2.5
Methionine	0.0	0.9	0.6
Phenylalanine	0.1	1.2	1.3
Proline	0.1	2.3	2.3
Serine	0.1	1.8	2.1
Threonine	0.1	2.6	2.3
Tyrosine	0.1	1.3	1.6
Valine	0.1	2.3	2.2
Units	g.day^{-1}	g.day^{-1}	g.day^{-1}

[1] Amino acid analysis was performed using a standard hydrochloric acid hydrolysis followed by RP HPLC separation using AccQ Tag derivatization. Cysteine/methionine were analysed using performic acid oxidation (AOAC 994.12). [a] Amino acids present in CON are explained by the presence of protein in the other supplement ingredients. Tryptophan was not measured. [b] Amino acid intake in WDP is derived from the combination of 17 g WDP powder + 23 g Whey protein powder.

3.5. Hyperinsulinaemic isoglycaemic clamp

Before and after the intervention, participants underwent a two hour hyperinsulinaemic isoglycaemic clamp with insulin infusion rate of 80 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} (**Figure 3.3**). Participants reported to the laboratory in the morning after an overnight fasted state. After obtaining height, weight and a urine sample the participant laid in a supine position. A cannula was inserted in a medial cubital vein for infusions of insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) and glucose (25%, Baxter, Illinois, USA) using an insulin (Carefusion, Alaris CC Plus) and glucose (Carefusion, Alaris GP Plus) pump. Insulin was prepared as a 50-mL infusate using 3 mL of the subjects own whole blood and set at a constant infusion of 15 mL/hour.

A second cannula was inserted in the dorsal hand vein of the contralateral arm and placed in a hotbox (˜60˚C) to obtain arterialized blood samples. Before the start of the clamp, a fasted blood sample was collected in an EDTA-containing tube (BD Vacutainer® EDTA) and analysed for HbA$_1$c. Additional blood samples were collected at t = 0, 60, 90 and 120 min in spray-coated silica tubes (BD Vacutainer® Plus tube containing spray-coated silica) for assessment of serum insulin concentrations. Starting glucose infusion rate was 0.25 x kg body weight. Blood glucose levels were maintained at individuals isoglycaemia, determined from fasting blood glucose concentrations, throughout the clamp by measuring whole blood glucose concentration from a 1 mL blood draw every five minutes using a blood glucose analyser (YSI 2300 stat plus, YSI Life Sciences) with subsequent adjustment of the glucose infusion rate. During the post-intervention clamp, glucose concentrations were clamped at the average blood glucose concentrations during the last 20 min of the pre-intervention clamp. Before the clamp was started, a baseline skeletal muscle biopsy was obtained. An insulin-stimulated skeletal muscle biopsy was taken at t = 60 min. The participants diet was repeated the

day before the clamp by replication of the diet that was recorded in a recorded diet diary the day before their first clamp day. The post-intervention clamp was performed 48 hours after the last exercise session.

Glucose infusion rates were transformed to glucose clearance rates to allow a comparison between the participants who were clamped at different glucose values. The glucose clearance rate was calculated by dividing the glucose infusion rate with the prevailing whole blood glucose concentration over the final 20 minutes of the clamp by:

$$GCR \ (mg \cdot min - 1 \cdot kg - 1)$$

$$= \frac{\text{Glucose infusion rate } (mg \cdot min - 1 \cdot kg - 1)}{\text{Blood glucose concentration } (mg \cdot 100mL - 1)} \cdot 100$$

As blood glucose concentrations might not be perfectly stable in the steady-state phase, a space correction (DeFronzo et al., 1979) was applied to the glucose infusion rate during the last 20 min of the clamp to correct non-steady state glucose concentrations using the following formula: Space correction = (G2-G1) x 0.095 where G2 and G1 are the glucose concentrations at the end and the beginning of the time period between two measures.
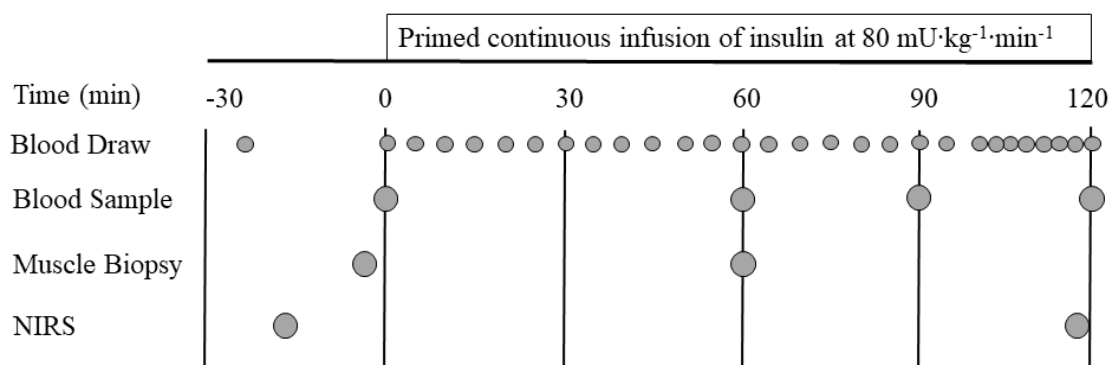
Figure 3.3 – Schematic representation of the hyperinsulinaemic isoglycaemic clamp protocol. NIRS: Near-infrared Spectroscopy.

3.6 Blood analysis

Samples for HbA_1c analysis were left on ice for 30 min before being analysed using Variant Turbo Ion Exchange high-performance liquid chromatography (Bio-Rad D100, Wellington SCL lab, Wellington, NZ). The tubes for insulin assay were left to clot for 30 min at room temperature before being centrifuged at 2000 rcf for 10 min at 4°C. Next, serum was aliquoted and stored in -80ºC until further analysis by double antibody radioimmunoassay (Nutrition lab, Massey University, Palmerston North, NZ).

3.7. Near infrared spectroscopy (NIRS)

Fasting and insulin-stimulated muscle blood flow (mBF) and perfusion (mBV) were measured during the hyperinsulinaemic isoglycaemic clamp using NIRS protocols as previously described and validated (Lucero et al., 2018). Briefly, a NIRS probe (PortaLite; Artinis Medical Systems BV, Elst, The Netherlands) was secured over the belly of the right *m. vastus lateralis* muscle two-thirds from the origin of the muscle and parallel to the muscle fibres (**Figure 3.4A**). Wavelengths were emitted from LEDs at 760 and 850 nm and collected at 10 Hz to detect relative changes in the concentration of oxygenated haemoglobin $[HbO_2]$ and deoxygenated haemoglobin [HHb], respectively, as well as the haemoglobin concentration in the total blood volume ([tHb] = $[HbO_2]$ + [HHb]).With an inter-optode distance of 3.5 cm for the LEDs, the theoretical penetration distance of the signal was 1.75 cm (Chance et al., 1992). Accordingly, muscle and subcutaneous adipose tissue thickness was determined using B-mode ultrasound (Terason; United Medical Instruments Inc., San Jose, CA, USA) to optimise the depth of photon projection and participants with an adipose tissue thickness of > 2 cm were excluded from the analysis to ensure measurements derived from muscle tissue only. mBV was determined from the average total haemoglobin (t[Hb]) concentration (µM) over 5 minutes, at rest before (fasting, 10 minutes before the start of insulin

infusion) and 120 minutes after insulin infusion (insulin-mediated) and expressed as a
surrogate measure of capillary recruitment. mBF was determined from the slope of the
t[Hb] signal during a 15-second venous cuff occlusion (70-80 mmHg), and converted to
mL·min^{-1}·100 mL^{-1} using the equation:

$$mBF = \frac{\Delta t[Hb] \cdot 60}{([Hb] \cdot 1000)/4} \cdot 100$$

where [Hb] was determined from a resting blood draw, and $\Delta t[Hb]$ averaged over 5
measurements taken 1 minute apart (**Figure 3.4B**).
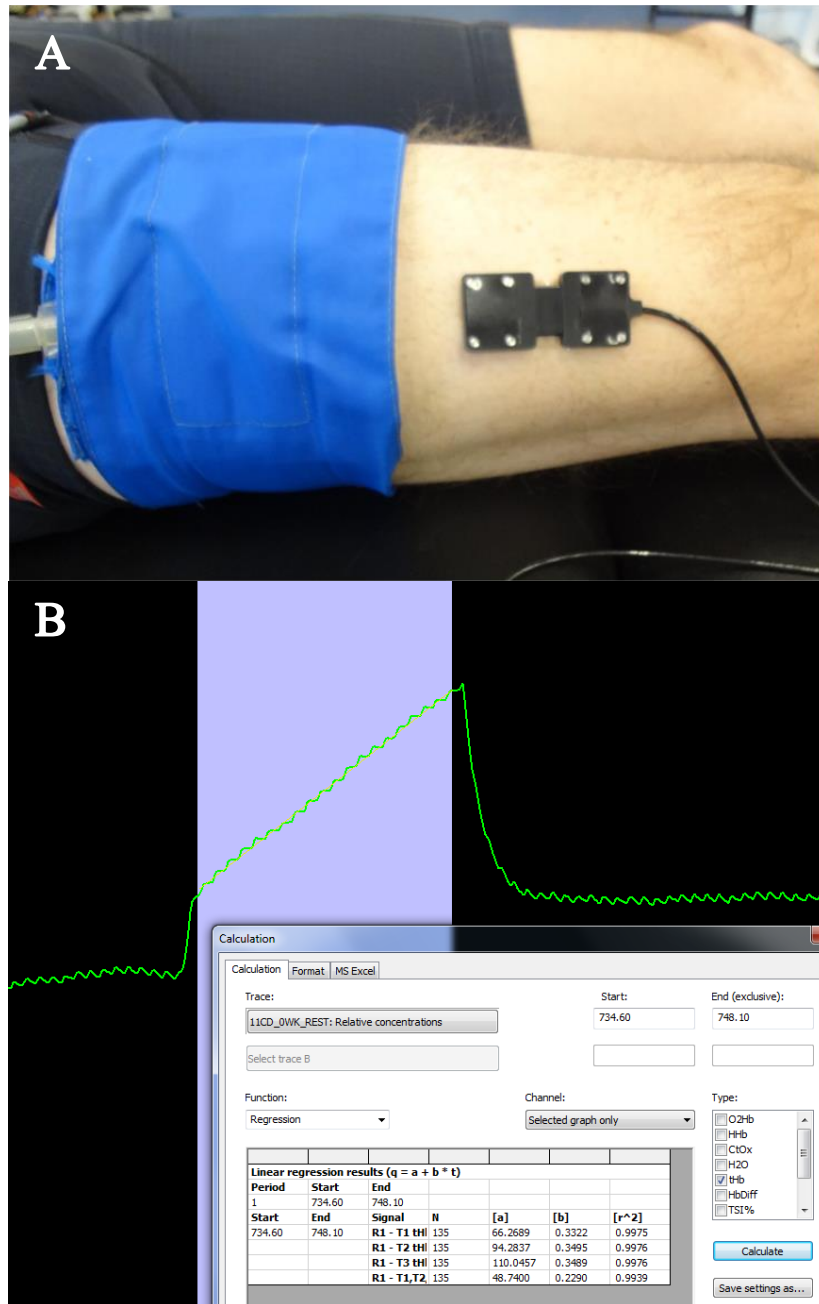
Figure 3.4 – Placement of the NIRS probe and cuff inflation (A) and representative screenshot for a single measurement of muscle blood flow using near-infrared spectroscopy (B). The green line represents total haemoglobin concentration t[Hb]. After initiation of cuff inflation, venous blood return is blocked and arterial, oxygen-containing haemoglobin enters the local muscle area resulting in an linear increase in t[Hb] dependent on blood flow rate. The gradient of the regression slope represents the blood flow rate. Figure 3.4A is the courtesy of Dr. Kim Gaffney.

3.8. Human skeletal muscle collection

Skeletal muscle tissue (~150 mg wet weight) was obtained using the suction-assisted percutaneous Bergstrom needle technique (Bergstrom, 1975). After applying local anaesthesia (1% Xylocaine), a small incision was made in the skin of the left leg to access the *m. vastus lateralis* muscle. The *m. vastus lateralis* was chosen as muscle site since the exercise intervention predominantly involved leg exercises (cycling) and therefore physiological changes are most likely to occur here. Moreover, the muscle site is easily accessible, is not located near major arteries and nerves and has routinely been used in human muscle physiology research. Samples were immediately freed from any visible fat and blotted dry to remove excess blood. Next, the samples were divided for different types of muscle analysis. Samples for immunofluorescence microscopy were embedded in Tissue Tek OCT (Sakura Finetek, the Netherlands), immediately frozen in liquid nitrogen-cooled isopentane and stored at -80°C until further analysis. Samples for immuno-gold electron microscopy were processed in two different ways. Samples were put in 0.1 M cacodylate buffer containing 3% paraformaldehyde and were left at room temperature for two hours until samples were transferred into 0.1 M sodium cacodylate buffer and stored at 4°C until further analysis. For the second process protocol, samples were fixed in 24 mg procaine dissolved in Krebs buffer for five minutes, transferred to Zamboni solution (PBS containing 22 g/L paraformaldehyde, 15% saturated picrid acid) for 30 minutes at room temperature, and put in fresh Zamboni for an additional 3.5h at 4∘C. Next, muscle samples were dissected in bundles of single muscle fibres in 0.1 M PBS, transferred to 50/50 PBS/glycerol and placed in the fridge for 24h before storing it at -20°C until further analysis. Remaining tissue was snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

3.9. Immunofluorescence protocol

Samples were cut in 7 µm thick sections in transverse orientation using a microtome cryostat (Leica CM1850, Wetzlar, Germany). The temperature in the cryostat was maintained at -20⁰C. Sections were put onto superfrost microscope slides (Sigma-Aldrich, Z692255), air-dried for one hour and put into 1x TBS, pH 7.6 when stained immediately or were stored at -80⁰C until staining. In case of later analysis, slides were left to dry at room temperature for one hour before starting the staining protocol. The staining protocols were optimized by testing different antibody dilutions in order to get optimal images. Starting antibody dilutions were based on manufacturers advice and adjusted if required. (**Appendix E**).

3.9.1. Skeletal muscle fibre

3.9.1.1. GLUT4

Before starting antibody incubation, samples were incubated with 1% bovine serum albumin (BSA) for 30 min to reduce non-specific antibody binding. Samples underwent a mild wash consisting of a 3x 5 min was in 1X TBS. Next, samples were incubated overnight at 4⁰C in 1:250 GLUT4 anti-rabbit polyclonal primary antibody (Abcam, UK, Abcam654) and 1:500 dystrophin anti-mouse polyclonal antibody (Sigma-Aldrich, D8168) diluted 1x TBS containing 1% BSA, 0.01% Triton-X and 0.02% Tween-20. Then, samples were washed thoroughly consisting of 3x5 minutes with 1x TBS, 3x5 minutes with 2x TBS and 3x5 minutes with 1x TBS. Samples were incubated with appropriate secondary antibodies. GLUT4 was targeted with 1:500 goat anti-rabbit IiG488 (Alexafluor488, Invitrogen, A-11034) and dystrophin was targeted with 1:500 goat anti-mouse IiG594 (Alexafluor594, Invitrogen, A-11005) in antibody diluent at room temperature for 2 hours. Samples were washed thoroughly and incubated for 3 min with 1:200 TrueBlack (Lab Supply, NZ, 23007), dissolved in 70% ethanol to block

autofluorescence created by lipofuscin. Next, samples were mounted in 30/70 TBS/glycerol and a coverslip was placed on top of the sample.

3.9.1.2. IRS-1 serine 312 phosphorylation

Samples were incubated overnight at 4℃ in 1:250 IRS-1^{ser312} anti-rabbit polyclonal primary antibody (Invitrogen, PA1-1054) diluted 1x TBS containing 1% BSA, 0.01% Triton-X and 0.02% Tween-20. Then, samples were washed thoroughly consisting of 3x5 minutes with 1x TBS, 3x5 minutes with 2x TBS and 3x5 minutes with 1x TBS. Samples were incubated with secondary antibodies, 1:200 goat anti-rabbit IiG594 (Alexafluor594, Invitrogen, A-11005) in antibody diluent at room temperature for 2 hours. After a thorough wash, samples were incubated for one hour at room temperature with 10 µg/mL *Ulex europaeus*-FITC conjugate (UEA-I-FITC, Sigma-Aldrich, L9006) and 50 µg/mL wheat germ agglutinin (WGA-350, Invitrogen, W11263) in 1x TBS to visualize endothelial cells and skeletal muscle plasma membrane respectively. After samples were washed thoroughly and incubated for 3 min in 1:200 TrueBlack (Lab Supply, NZ, 23007) dissolved in 70% ethanol , slides were mounted with 30/70 TBS/glycerol and a coverslipwas placed on top of the sample. Representative images are displayed in **Figure 3.5**.
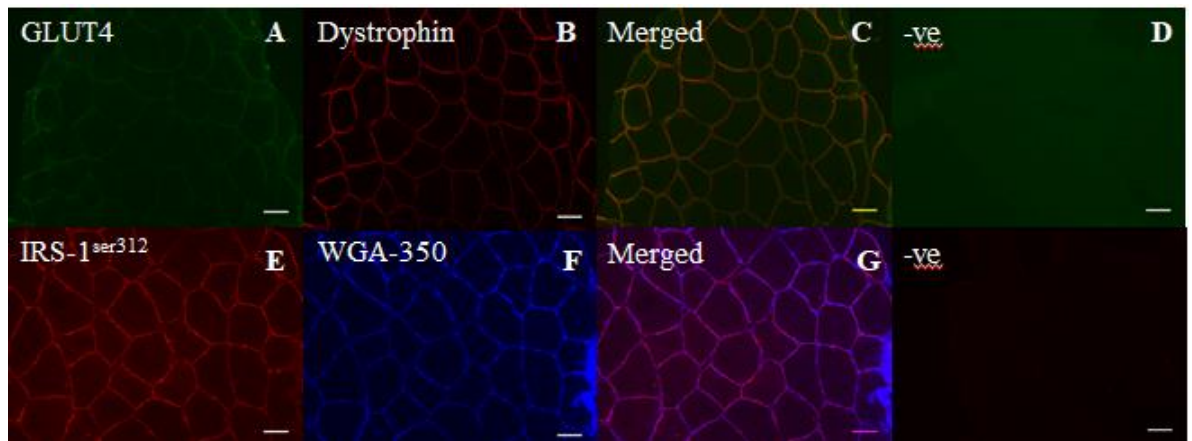
Figure 3.5 – Representative widefield images of skeletal muscle GLUT4 and IRS-1^{ser312} stained in combination with dystrophin (red) or wheat germ agglutinin (blue) to mark the plasma membrane. (A-D) GLUT4 (green), plasma membrane (red), merged to demonstrate translocation of GLUT4 with the plasma membrane and a negative control for GLUT4, and (E-H) IRS-1^{ser312}(red), plasma membrane (blue) and merged images to demonstrate colocalization of IRS-1^{ser312} with skeletal muscle plasma membrane, but not endothelial cells and a negative control. Scale bars are 50µm

3.9.2. Skeletal muscle microvascular staining (eNOS, eNOS^{ser1177}, NOX2)

After blocking for 30 min in 1% BSA and a mild wash, samples were incubated overnight at 4ºC in 1:100 rabbit polyclonal eNOS (Abcam, UK, Ab5589), 1:100 rabbit polyclonal eNOS^{ser1177} (Abcam, UK, Ab184154) or 1:100 rabbit polyclonal NOX2 (Abcam, UK, Ab80508) diluted 1x TBS containing 1% BSA, 0.01% Triton-X and 0.02% Tween-20. Then, samples were washed thoroughly. Samples were targeted with a secondary antibody 1:200 goat anti-rabbit IiG594 (Alexafluor594, Invitrogen, A-11005) and incubated for two hours at room temperature. Samples were washed thoroughly and incubated for one hour at room temperature with 10 µg/mL UEA-I-FITC (Sigma-Aldrich, L9006) and 50 µg/mL WGA-350 (Invitrogen, W11263) in 1x TBS to visualize endothelial cells and skeletal muscle plasma membrane respectively.

After samples were washed thoroughly and incubated for 3 min in 1:200 TrueBlack

(Lab Supply, NZ, 23007) dissolved in 70% ethanol , slides were mounted with 30/70

TBS/glycerol and a coverslip was placed on top of the sample. Representative staining

was displayed in **Figure 3.6**. For proof of principle, the contrast of the images was

enhanced as treatment with TrueBlack dimmed the fluorescent intensity of the

fluorescent label.

3.9.3. Antibody controls

For every round of staining, a standard negative control was included in order to

confirm non-specific secondary antibody binding. The primary antibody was replaced

by a solution of 1X TBS containing 1% BSA. Antibody specificity for IRS-1^{ser312} was

confirmed using paraffin-embedded sections of a T47D cell line to show positive

staining in nuclei positive areas. To confirm primary antibody specificity for eNOS and

eNOS^{ser1177} a positive control was performed. As eNOS and eNOS^{ser1177} is expected to

be present in endothelial cells, the staining protocol was executed on freshly frozen

cryosections of the *a. Aorta descendens* of a 6-months old C57BL/6J mouse.
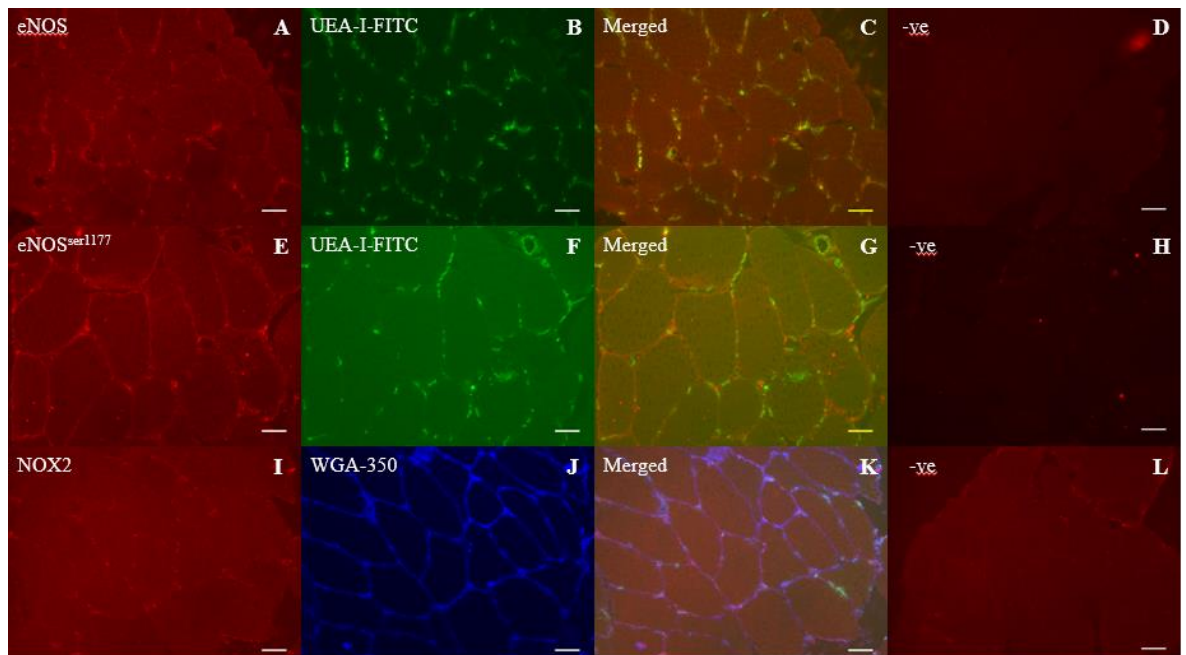
Figure 3.6 – Contrast-enhanced representative widefield images of skeletal muscle eNOS, eNOSser1177 and NOX2 stained in combination with Ulex-Europaeus-FITC to mark endothelial capillaries or Wheat Germ Agglutinin to mark the plasma membrane. (A-D) eNOS (red), endothelial capillaries (green), merged to demonstrate colocalization of eNOS with the microvascular endothelium and a negative control for eNOS, (E-H) phosphorylated eNOS^{ser1177} (red), F) microvascular endothelium (green) merged to demonstrate colocalization of eNOS^{ser1177} with the microvascular endothelium and a negative control for eNOS^{ser1177}(I-L) NOX2 (red), plasma membrane (blue) and merged images to demonstrate colocalization of NOX2 with skeletal muscle plasma membrane, and not endothelial cells. Scale bars are 50μm

3.9.4. Image capturing

Images of cross-sectional muscle fibres were captured using a compound widefield fluorescence microscope (Olympus BX-50, Olympus Corporation, Tokyo, Japan). Images were captured with a Spot-RT slider (SPOT Imaging Solutions: Diagnostic Instruments, Inc., Sterling Heights, MI) cooled digital microscope camera. Illumination

and exposure times were optimized for every single antibody and kept consistent throughout the analysis. The Alexafluor 594 was excited using Olympus U-MWIY (540-580 nm) excitation filter, the Alexafluor 488 and UEA-I-FITC were excited using Olympus U-MWIG (465-495 nm) excitation filter and the WGA-350 was excited using narrow band UV filter (Olympus U-MNU-BP, 330-385 nm). All images were taken using an oil immersed 20x magnification objective.

3.9.5. Image analysis

3.9.5.1. Skeletal muscle fibre analysis

Three images per sample were taken. Images were taken randomly with the only requirement having at least 10 transverse cells in the image. Image J (National Institutes of Health, Bethesda, MD, USA) was used for image analysis. For co-localization of GLUT4 with dystrophin, the Coloc 2 tool was used. Pearson's correlation coefficient (*r)* was used to measure colocalization. Additionally, using the dystrophin image, a mask was created by thresholding the image to only select the plasma membrane region. Next, mean fluorescent intensity was measured within the masked plasma membrane region as a semi-quantitative measure for GLUT4 translocation (**Figure 3.7 Panel A-D)**.
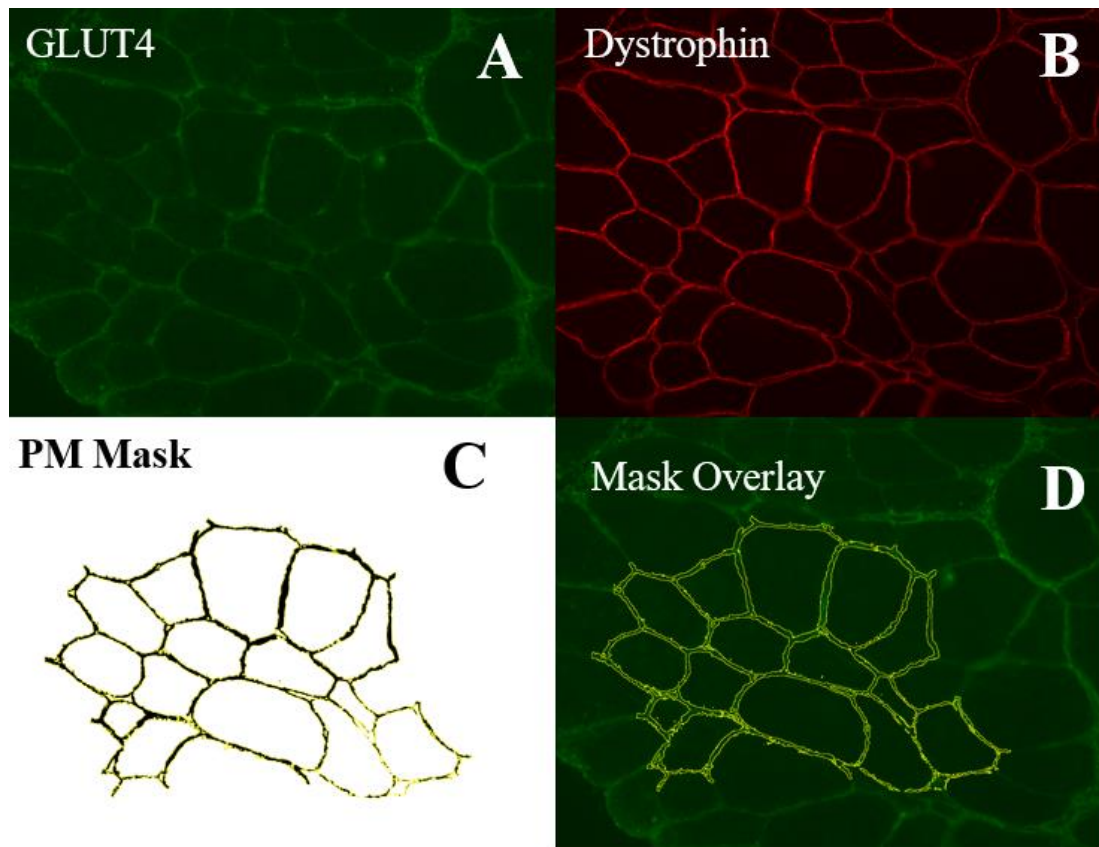
Figure 3.7 – Representative example of the visualization for semi-quantitative measurement of GLUT4 translocation. From the dystrophin image, a mask of the plasma membrane (PM) of at least 10 muscle fibres in the plane of focus was created (C) and overlapped on the GLUT4 (D). Mean fluorescent intensity was measured within the mask.

IRS-1[ser312] was quantified in an equal manner as GLUT4 with the exception that the plasma membrane outline was obtained from a WGA-350 stained image. The WGA-350 image was thresholded to create a mask. The mask was used to measure mean fluorescent intensity of IRS-1[ser312] as a semi-quantitative measure of phosphorylation activity.

3.9.5.2. Microvascular analysis

For analysing microvascular plasticity in the skeletal muscle fibres, capillaries stained with an endothelial marker (UEA-I-FITC) were counted manually. Analysis of capillary contacts (CC), capillaries per fibre on individual basis (C/F$_i$), sharing factor (SF) and capillary-to-fibre-perimeter exchange (CFPE) was performed on 50 fibres per sample as has been used and validated before (Hepple et al., 1997). For measurements of microvascular eNOS, eNOS[ser1177] and NOX2, UEA-FITC images a threshold was applied to extract all capillaries. Next, a mask was created for the capillaries and overlapped on the corresponding NOX2, eNOS/eNOS[ser1177] image. Then, NOX2, eNOS[ser1177]/ eNOS was quantified by measuring mean fluorescent intensity in the regions that corresponded with the capillaries (Cocks et al., 2012) (**Figure 3.8, Panel A-D**). NOX2 in the plasma membrane of skeletal muscle fibres was quantified by creating a mask from the WGA-350 image and overlapped on the corresponding NOX2 image.
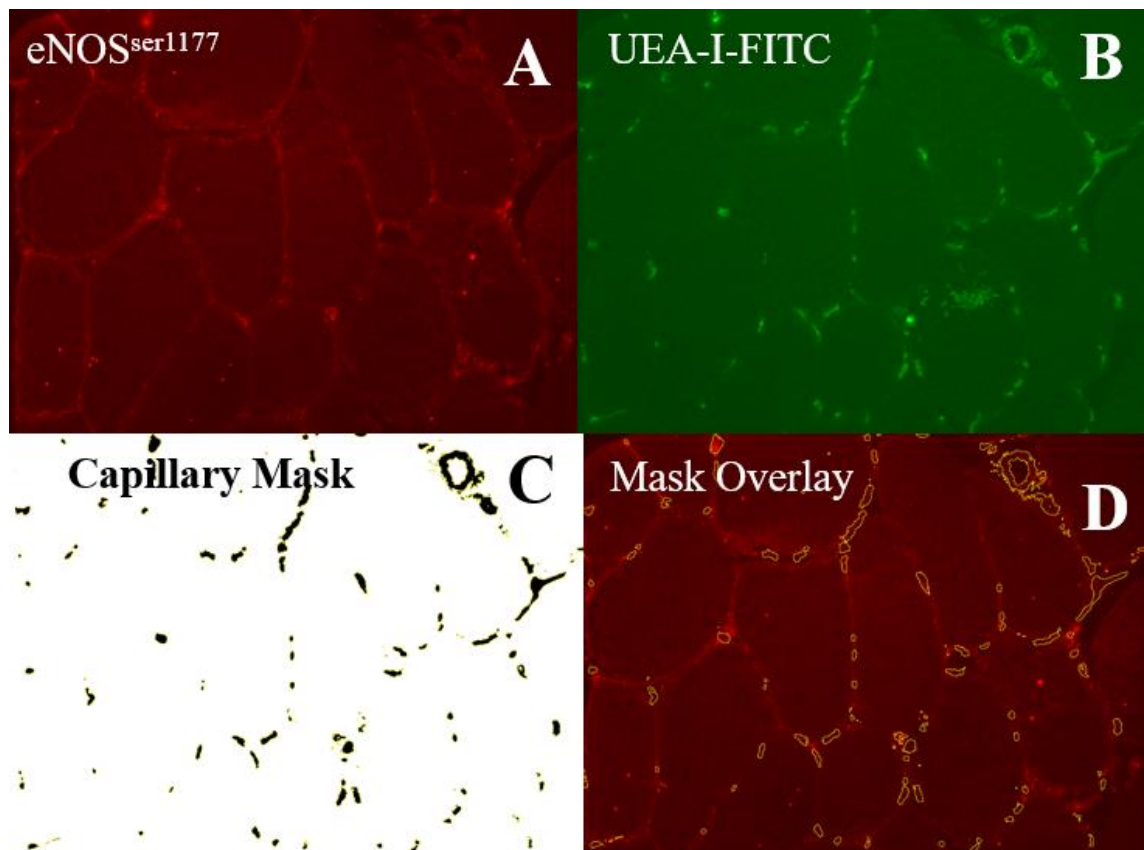
Figure 3.8 – Representative example of the visualization for the semi-quantitative measurement of eNOS, eNOSser1177, and NOX2. From the Ulex europaeus-FITC conjugate (UEA-I-FITC) *image, a mask of the endothelial cells of microvascular capillaries was created (C) and overlapped on the protein of interest image (D). Mean fluorescent intensity was measured within the mask.*

3.10. Immunogold protocol

The staining protocols were optimized by testing different antibody dilutions, dilution compositions and incubation times in order to get optimal images. Samples from the 50/50 PBS/glycerol were washed for 5 minutes in PBS. To avoid non-specific binding, samples were blocked in blocking buffer containing 0.01 M PBS, 50 mM glycine, 0.25% BSA and 0.06% saponin for 30 minutes. Next, samples were incubated with primary antibody against GLUT4 (1:200 polyclonal rabbit GLUT4, Abcam, UK, Ab654) for four hours at room temperature. After three washes of five minutes in blocking buffer, samples were incubated with 1:300 secondary goat anti-rabbit fab fragments conjugated with 1.4nm gold particles (Nanoprobes, Inc., NY) for two hours at room temperature. Next, samples were washed two times one minute in PBS and fixed in PBS containing 2.2% glutaraldehyde for one hour. After a rinse with distilled water, samples underwent silver enhancement for seven minutes, using HQ Silver (Nanoprobes Inc., NY) and rinsed again with distilled water. Next, samples were treated with osmium tetroxide for ten minutes and then dehydrated in graded series of ethanol (15%, 30%, 50%, 70%, 80%, 90%, 100%) before embedding in epoxy resin. Samples were embedded horizontally in fresh epoxy resin moulds and cured overnight at 60ºC. Next, sections of ~90 nm were cut using an ultramicrotome (Riechert-Jung Ultracut E Microtome) and air-dried. Sections were placed on a 300 mesh gold grid and viewed under a Transmission Electron Microscope (Philips CM100 TEM, Eindhoven, The

Netherlands). Images were obtained under light-standardized conditions using a film picture camera (Kodak 4489, Rochester, NY).

3.11. Western blot protocol

Frozen muscle samples (~30 mg) were homogenized in ice cold homogenization buffer (RIPA buffer; 15mM Tris, 167mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100), containing protease (Complete-mini) and phosphatase inhibitor tabs (Complete-mini), using an automatic homogenization blender (IKA) for one minute. Sample lysates were placed in an orbital shaker for 1 hour at 4ºC before centrifuging at 600 rcf for 15 minutes at 4ºC. Next, the supernatant was used for protein concentration determination in triplicate using a commercially available bicinchoninic acid procedure (Pierce BCA Protein Assay Kit, Invitrogen, catalogue # 23227). Under reducing conditions, 40μg of protein extract was boiled for 5 min at 95ºC, before loaded onto Tris-Glycine SDS-PAGE for gel electrophoresis. Proteins were transferred onto PVDF membrane using 18 h wet-transfer procedure. Membranes were blocked for 1 h in 3% BSA, incubated in 1:1000 rabbit polyclonal AS160^{Thr642} (Cell Signalling, catalogue # 4288), 1:1000 rabbit polyclonal PAK1^{Thr423}/PAK2^{Thr402} (Cell Signalling, catalogue # 2601) or 1:1000 mouse monoclonal α-tubulin HRP-conjugated loading control (Abcam, catalogue # ab40742) overnight at 4ºC and for 2 h in 1:5000 anti-rabbit HRP-conjugated secondary antibody at room temperature, followed by chemiluminesence (Thermoscientific, catalogue # 34580) and imaging (iBright^{TM} FL1500, Invitrogen, A44241). Between every step, membranes were washed 3 x 5 min with T-TBS. After imaging, membranes were stripped and incubation steps were repeated for detection of other proteins. Protein quantification analysis of band intensities was performed in Image J software. Protein quantity was expressed relative to loading control protein.

3.12. Statistical analysis

Sample size estimation was based upon the primary outcome glucose disposal rate (GDR) using the available test-retest measures for GDR values reported by Defronzo et al (1979) in a healthy adult population and sample size estimations for magnitude based clinical inference (Hopkins et al., 2016; Hopkins et al., 2009). The typical error of measurement was doubled to allow for uncertainty in variability in a T2DM population and n was increased by 10% to allow for potential dropouts, which brought the required sample to 12 in each group.

The effect of treatment and time on all dependent variables was estimated from mixed models (Proc Mixed, SAS Version 9.1; SAS Institute, Cary, NC). All mechanistic continuous data not including negative values or ratio analysis were log transformed prior to analysis to manage heteroscedasticity and back-transformed to acquire percent change scores. Insulin-stimulated outcomes were defined as the change in number from fasting to one hour of insulin stimulation for protein analysis or 2 hours after insulin stimulation for NIRS measurements. All outcomes were adjusted for the baseline values. A random effects model included additional subject random error for the WDP and WHEY conditions. Uncertainty was presented as 90% confidence limits. Magnitude-based inference, a Bayesian approach with an uniformally-dispersed flat prior, was employed to infer to clinical and mechanistic outcome effects (Batterham et al., 2006).

The threshold for smallest worthwhile clinical change in the primary clinical outcomes were: GDR was 5.4% based upon the effect of 3 months of hypoglycaemic therapy (Metformin) on naïve Type-2 diabetics (Derosa et al., 2009) and HbA1c, 5.5 mmol/mol based on general clinical consensus (Lenters-Westra et al., 2014). The smallest noteworthy magnitude of change for mechanistic parameters was the default smallest

Cohen's *d* standardised difference (0.2 × cohort baseline SD) (Hopkins et al., 2009). The probability that a contrast was at least greater than the smallest Cohen's *d* standardized difference was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain (Hopkins et al., 2009). In the case where the majority (>50%) of the CI lay between the thresholds for positive and negative substantiveness, the effect was qualified trivial (negligible) with the respective probabilities as above (Hopkins et al., 2009). The terms *benefit/increase, trivial (negligible),* and *harm/decrease* refer to the most likely directional outcome, relative to the smallest effect threshold. The terms *unclear* refers to outcomes where the likelihood of both benefit and harm exceeded 5%. Magnitude-based inference was selected as statistical inference model instead of traditional null-hypothesis testing, where a significant effect is detected when the *p*-value < 0.05. Pre- and post-intervention scores are presented in figures as raw means and standard deviations.

Recently, researchers are starting to call for a ban on the use of *p*-values to claim the effectiveness of an intervention as the dichotomous way of inferring might lead to type-I errors (false positive, where researchers wrongfully conclude there is an effect or this effect is not clinically meaningful) and type-II errors (false negative, where researchers wrongfully conclude no effect, such as when *p* = 0.06) (Amrhein et al., 2019; Colquhoun, 2017). Magnitude-based inference has been developed as an alternative to null-hypothesis testing, where inferences are made based upon the likelihood that an intervention is beneficial, trivial or harmful. Despite the fact that MBI is still in its infancy and scrutinized by peer-statisticians (Curran-Everett, 2018; Sainani et al., 2019), the current research acknowledges the strengths and limitations of both statistical methods, but decided to use MBI over null-hypothesis testing.

3.12. Results on immunofluorescence

3.12.1. Antibody validation

The immunogen sequences of all antibodies used, were analysed with Basic Local Alignment Search Tool (BLAST) (www.uniprot.org) to confirm that the amino acid sequence of the proteins was unique and was not present in any other protein. This confirms that the raised antibody only binds to the protein of interest. All the immunogen sequences of all antibodies showed 100% alignment with the protein of interest and were not present in any other protein.

Antibodies for GLUT4, dystrophin (Bradley et al., 2014), eNOS, eNOS[ser1177] and NOX2 (Cocks et al., 2012) have previously been demonstrated for positive staining in skeletal muscle using immunofluorescence. IRS-1[ser312] positive binding was confirmed by staining paraffin-fixed T47D cells (**Figure 3.9**). Pearson correlation coefficient to demonstrate colocalization of IRS-1[ser312] within T47D cell nuclei, stained with 4′,6-diamidino-2-phenylindole (DAPI) showed a correlation of $r = 0.69$.

Lastly, non-specific binding of the secondary antibody was confirmed. During every run, a negative control was included, where incubation of the primary antibody was replaced by the immune diluent solution. No signal was detect, indicating that there was no non-specific binding of secondary antibody.
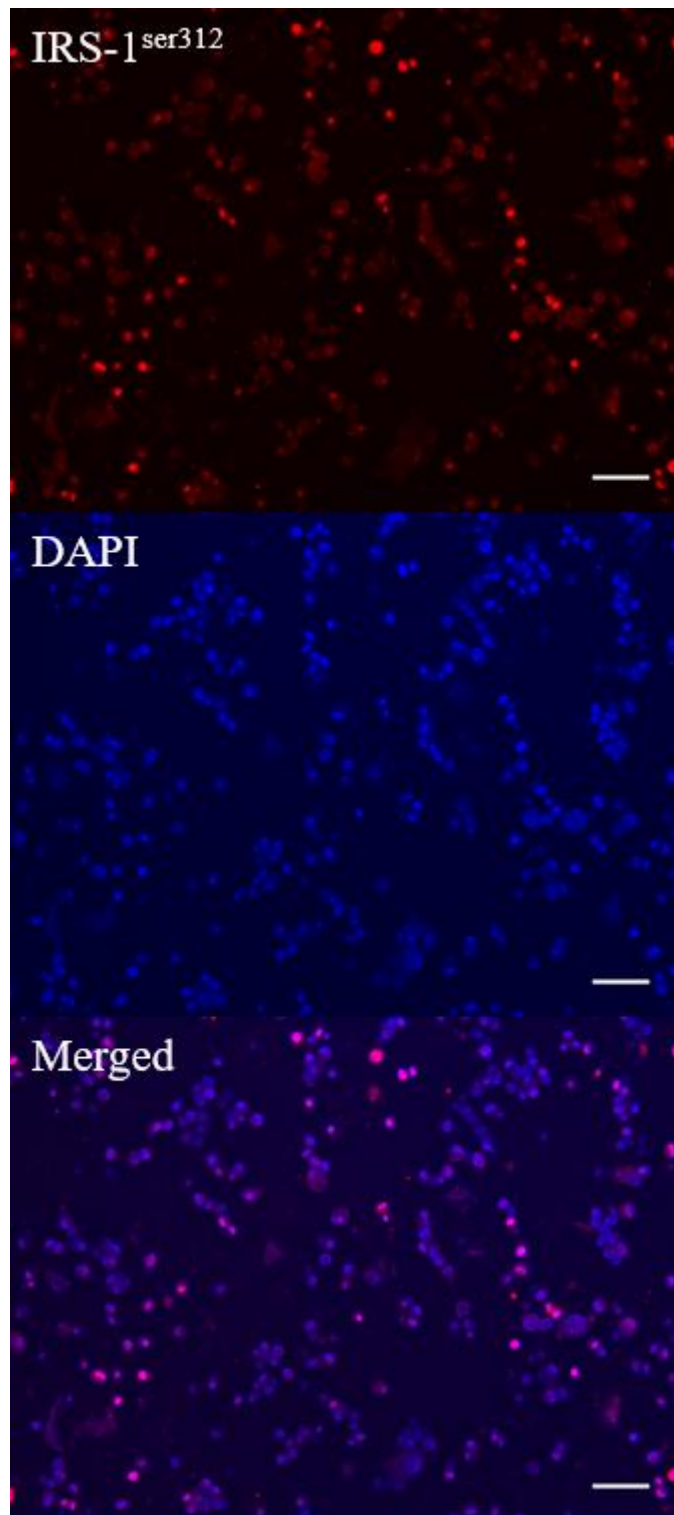
Figure 3.9 – IRS-1^{ser312} antibody validation. The primary antibody specificity was confirmed in T47D cells. IRS-1^{312} was co-stained with DAPI. Scale bars represent 50µm.

CHAPTER 4

KERATIN-BASED PROTEIN ISOLATE MODULATES GLUCOSE METABOLISM, MICROVASCULAR HAEMODYNAMICS AND GLUT4 TRANSLOCATION IN SKELETAL MUSCLE OF ADULTS WITH TYPE 2 DIABETES MELLITUS

An edited version of Chapter 4 was submitted to the American Journal of Clinical Nutrition

4.1. Abstract:

BACKGROUND: Keratin protein is high in amino acids glycine, cysteine and arginine of which isolated supplementation could improve insulin sensitivity in type 2 diabetes mellitus (T2DM). Adaptations in skeletal muscle glucose handling is unclear.

OBJECTIVE: To explore changes in whole-body glucose uptake and skeletal muscle insulin sensitivity following chronic supplementation with novel wool-derived keratin protein, compared to whey or low-protein control added to exercise training.

DESIGN: In a randomized, double-blind, clinical trial, 35 adults with T2DM (age: 56.3 SD 8.3 y, HbA$_1$c: 62, SD 13 mmol/mol) ingested a daily dose of 17 g wool-derived protein and 23 whey protein (WDP), 40 g whey protein (WHEY) or isocaloric amount of maltodextrin (CON) during 14 weeks of a mixed-modality exercise training. At weeks 0 and 15, a 2-hour, 80 mU·kg^{-1}·min^{-1} hyperinsulinaemic isoglycaemic clamp was performed to assess changes in whole-body glucose uptake (GCR). Moreover, *m.vastus lateralis* blood flow and volume were measured using near-infrared spectroscopy. Skeletal muscle biopsies were collected in a fasted and insulin-stimulated state to assess microvascular and muscle fibre insulin signalling proteins eNOS/eNOS^{ser1177}, NOX2, IRS-1^{ser312} and GLUT4 translocation at weeks 0 and 15.

RESULTS: GCR increased (mean change (SD), WDP: 1.8 (1.9), WHEY: 0.6 (1.0), CON: 0.7 (1.7) mL/kg/min) substantially in WDP compared to WHEY (29.4% 90%CI 0.5, 66.4) and CON (26.9% 90%CI -3.0, 64.1). Insulin-stimulated mBF increased (mean (SD) mL/cm^3/min; WDP: 0.29 (0.20) WHEY: 0.08 (0.38), CON: 0.20 (0.20)) substantially in WDP compared to WHEY (Δ 45.7% 90%CI: 9.0, 94.7) and CON (Δ 51.1% 90%CI: 5.4, 116.6), positively associated with changes in GCR (WDP-WHEY: β = +0.60% (90%CI: 0.22, 0.98) mBF·% GCR^{-1}). Insulin-stimulated GLUT4 translocation

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weakly increased with WDP compared to WHEY (Δ 14.9% 90%CI: -1.2, 33.6) and

CON (Δ 17.8% 90%CI: -0.5, 39.4), positively associated with GCR (WDP-CON: $\beta =$

+2.1% (90%C: 0.5, 3.7) GLUT4·%GCR^{-1}).

CONCLUSION: Dietary supplementation with WDP may promote clinically-

favourable insulin-sensitizing effects within skeletal muscle tissue in T2DM. These

findings suggest a potential for digestible wool-derived keratin protein in clinical

nutrition.

Introduction

Keratin protein sources, such as hair, nails and wool, are characterized by tight networks of disulphide bonds, which makes the protein indigestible for human consumption. However, Dias et al. (G. J. Dias et al., 2017) successfully developed a protocol to process sheep wool or feathers into edible dietary keratin protein. Amino acid analysis on the protein revealed high concentrations of cysteine, glycine and arginine. Supplementation of these amino acids have been associated with improvements in glucose homeostasis and insulin sensitivity in type 2 diabetes mellitus (T2DM) via alterations in plasma glutathione (GSH) (Sekhar et al., 2011) and nitric oxide (NO)-associated processes (Piatti et al., 2001), controlling antioxidant and endothelial blood flow pathways respectively. However, how these pathways affect insulin-sensitive peripheral organs remains unclear.

Skeletal muscle is considered the largest site for insulin-stimulated glucose disposal, and therefore, has an important role in controlling glucose homeostasis (Katz et al., 1983). Two primary mechanisms involved in insulin-stimulated glucose uptake in skeletal muscle are the microvascular response to insulin, dependent on NO bioavailability (Vincent et al., 2003) and the translocation of the sarcoplasmic glucose transporter (GLUT4) to the plasma membrane of the muscle fibre (Wasserman et al., 2011). These two mechanisms are both impaired in T2DM (Baron et al., 1990; Keske et al., 2009; Laakso et al., 1992; Vind et al., 2011; Zierath et al., 1996) as a result of skeletal muscle insulin resistance and contribute to impaired whole-body glucose uptake observed in T2DM (Bonadonna et al., 1998) but are both in part remediated by chronic exercise (Cocks et al., 2013; Cocks et al., 2016b; Dela et al., 1994; Hoier et al., 2014; Holten et al., 2004; Little et al., 2011; Wagenmakers et al., 2016).

Additionally, specific dietary proteins may have differential effects on muscle insulin sensitivity and endothelial function. Chronic dietary protein intake positively affects metabolic health and insulin sensitivity (Ouellet et al., 2007; Ouellet et al., 2008), potentially via alterations in GLUT4 translocation (Lavigne et al., 2001; Tremblay et al., 2003) and endothelial function (Fekete et al., 2016; Monti et al., 2013; Piatti et al., 2001). Moreover, in young, healthy people, acute post-exercise protein ingestion further accelerates adaptations in skeletal muscle plasticity via increased muscle protein synthesis (Biolo et al., 1997; Tang et al., 2009; Witard et al., 2014), and increased transcriptome signalling involved in muscle development, mitochondrial biogenesis and energy metabolism (Rowlands et al., 2011), inflammation and muscle growth (Rowlands et al., 2016), leading to improved muscle plasticity when applied chronically. However, the effect of chronic post-exercise protein ingestion on skeletal muscle insulin signalling is currently unknown. As exercise training and dietary protein interventions independently seem be able to improve insulin sensitivity, exploring potential synergetic effects are of relevance to optimize treatment in T2DM.

To date, only a few trials have investigated the clinical effect of combined exercise and protein ingestion in T2DM. Using milk-derived protein sources, with high concentrations of essential amino acids, including leucine, which is identified as a nutritional activator of muscle protein synthesis (Anthony et al., 2001), researchers have been unable to identify a synergistic effect of combined exercise training and protein ingestion on insulin sensitivity (Francois et al., 2017; Gaffney et al., 2017; Wycherley et al., 2010a). However, keratin, high in cysteine, glycine and arginine might be a better alternative protein source to overcome impairments in GSH and microvascular blood flow in T2DM and improve insulin-stimulated glucose uptake within skeletal muscle.

Therefore, we explored the efficacy of 14 weeks of exercise training and twice-daily consumption of keratin-derived protein-whey protein blend, whey protein or a placebo on skeletal muscle insulin signalling involving the microvascular and muscle fibre response to insulin stimulation in skeletal muscle of T2DM. We hypothesized that the addition of keratin-derived protein, with amino acid precursors for glutathione and nitric oxide, to exercise training would result in improved whole-body glucose uptake, accompanied by improved insulin signalling leading to insulin-stimulated GLUT4 translocation to the plasma membrane of skeletal muscle.

4.3. Methods

Refer to Chapter 3 for General methods.

Study design: paragraph 3.1, 3.2, 3.3, 3.4

Analytical methods: paragraph 3.5, 3.6, 3.7, 3.8, 3.9

Statistical methods: paragraph 3.11

4.4. Results

4.4.1. Participant characteristics

After recruitment, 43 participants were randomized into an intervention group. Eight participants were lost in the follow-up, which resulted in a total of 35 participants who finished the intervention and were included in the per protocol analysis. Baseline characteristics are displayed in **Table 4.1**. An adipose thickness of > 2 cm was detected in 4 participants. Accordingly, mBF and mBV was performed on CON (n = 12), WHEY (n = 10) and WDP (n = 9) respectively. Due to participants' rejection for muscle biopsy harvesting (n = 3) and poor sample quality for immunofluorescence at minimally one time point (n = 4), analysis of eNOS/eNOS^{ser1177}, NOX2, GLUT4 and IRS-1^{ser312} was performed on CON (n = 10), WHEY (n = 9) and WDP (n = 9). Sampling for western

blots resulted in analysis of CON (n = 12), WHEY (n = 8) and WDP (n = 10).

Table 4.1 – Baseline characteristics of the participants who completed the study (per protocol dataset).

	Treatment Group		
	CON (n = 12)	WHEY (n = 12)	WDP (n = 11)
Age, yr	58.3 (8.4)	54.8 (8.0)	56.9 (8.1)
Sex, n (female %)	3 (25)	4 (33)	3 (27)
Peak Power, W	167 (48)	169 (54)	164 (38)
Leg press 1RM, kg	191 (72)	184 (81)	205 (49)
GCR, mL·min^{-1}·kg^{-1}	4.0 (2.1)	4.2 (1.6)	4.5 (4.3)
Weight, kg	96.6 (13.9)	94.2 (21.3)	93.2 (25.3)
Height, cm	174 (10)	172 (10)	172 (7)
BMI, kg·m^{-2}	32.0 (4.7)	31.9 (6.4)	32.5 (7.5)
Waist, cm	108 (11)	108 (15)	111 (18)
Hip, cm	111 (10)	114 (12)	111 (13)
Diabetes duration, yr	6.0 (3.5)	8.3 (5.5)	6.5 (6.2)
Blood glucose, mmol	7.8 (2.5)	9.3 (2.2)	8.3 (2.4)
HbA1c, mmol·mol^{-1}	64.0 (15.6)	64.5 (13.0)	56.5 (8.8)

All values are mean (SD). Participant randomization was based on minimization. The randomization variables were age, sex, peak power, leg press one repetition maximum (1RM) and glucose clearance rate (GCR).

4.4.2. Hyperinsulinaemic isoglycaemic clamp and HbA$_1$c

During the clamps in week 0 and week 15, blood glucose concentrations throughout the

2 hours remained stable (**Figure 4.1A**) within all treatments and were clamped during

the last 20 minutes at similar concentrations in week 0 and week 15. As expected, after

the start of insulin infusion, serum insulin concentrations increased substantially and

remained elevated throughout the clamp in all treatments (**Figure 4.1B**). No differences

were observed within treatments between week 0 and week 15 insulin concentrations.

Statistical analysis and inferences on changes in glycaemic control outcomes are

presented in **Table 4.2**. Glucose clearance rate (GCR) improved substantially relative to

the threshold for smallest worthwhile clinical change (5.4%) in all intervention groups

(**Figure 4.1C-D**). The change in GCR for WDP was likely higher, relative to CON and

WHEY, and the clinical benefit:harm odds ratio was above the threshold for adoption

(>66:1). HbA$_1$c substantially decreased for WDP and CON relative to the threshold for

smallest worthwhile clinical change (-5.5 mmol/mol), but was trivial for WHEY

(**Figure 4.1E-F**). Between-group contrast was likely different between CON and

WHEY and possibly different between WDP-CON and WDP-WHEY and the clinical

benefit:harm odds ratio was above the threshold for adoption between WDP and WHEY

(>66:1). Simple linear regressions were used for associations between post-pre changes

in GCR with unpublished skeletal muscle oxidative stress outcomes (**Appendix F**). For

WDP, change in GCR was very likely, negatively associated with change in fasting

glutathione (GSH; β = -0.26 (90%CI -0.43, -0.08)) compared to CON (β = -0.42 (-0.77,

-0.07)) and WHEY (β = -0.30 (-0.54, -0.05)). For WDP, change in GCR was likely,

positively associated with change in fasting oxidised peroxiredoxin 2 (PRX2; β = 0.66

(-0.07, 1.41)) compared to WHEY (β = 1.11 (-0.16, 2.40)).
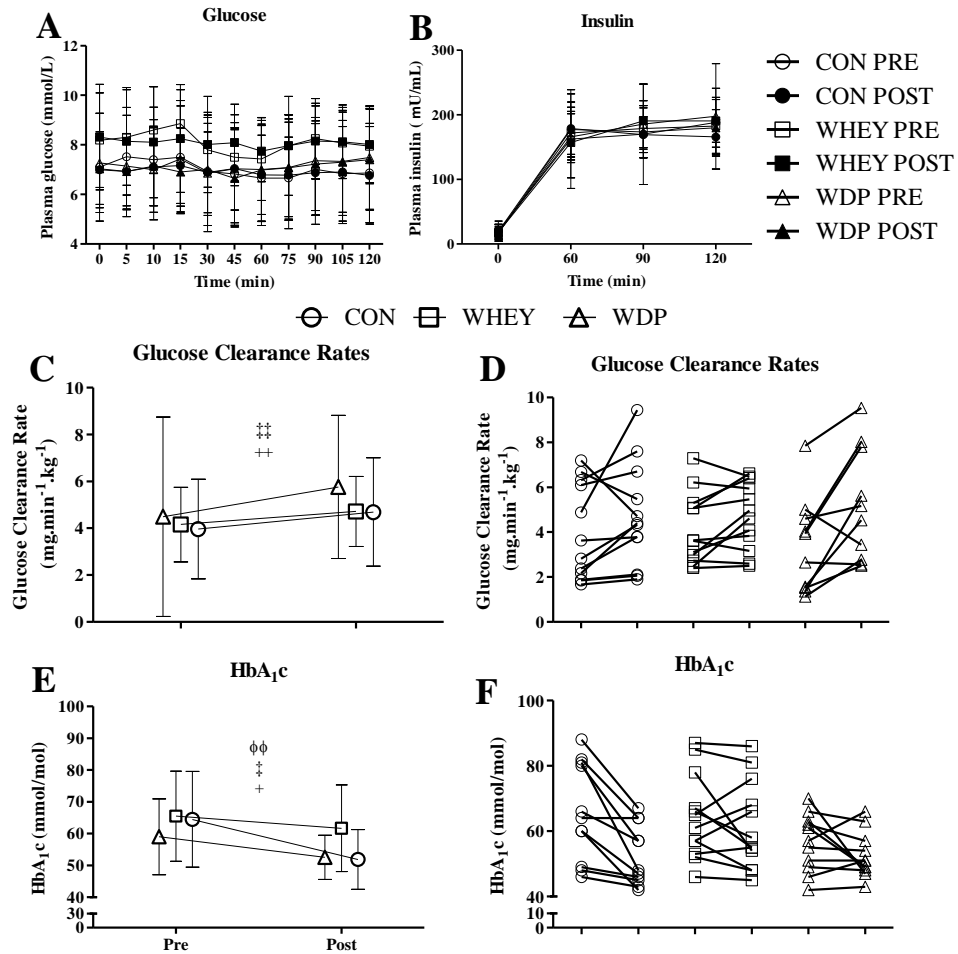
*Figure 4.1 – Group and individual effect of CON, WHEY and WDP in response to 14 weeks of protein and exercise training on glycaemic control outcomes in adult men and women with type-2 diabetes. Mean (SD) of plasma glucose (A) and insulin (B) concentrations throughout the 2 hour hyperinsulinaemic isoglycaemic clamp. (C-D) Glucose clearance rates were calculated over the last stable 20 minutes of the clamp. (E-F) HbA$_1$c was measured from fasted serum samples. Statistical symbols above the data represent magnitude-based inferences (for detail see Table 4.2), with a clinical inferential decision based on the magnitude-based benefit:harm ratio of >66:1. Abbreviations: ϕ = WHEY vs CON; + = WDP vs CON; ‡ = WDP vs WHEY. The number of letters or symbols denotes likelihood of change where * = possible; ** = likely; *** = very likely; **** = almost certain.*

Table 4.2 – The effect of 14-weeks of protein supplementation with exercise on clinical measures of glycaemic control

	Unadjusted Raw Unit Mean Change (SD)			Outcome Statistics[a] Estimate (90%CI L, U) Likelihood Increase/Decrease (%); Inference[b]; Benefit odds[b]		
Treatment, Contrast[a]	CON	WHEY	WDP	WHEY – CON[c]	WDP – CON[c]	WDP – WHEY[c]
Glucose Clearance Rate $(ml \cdot kg^{-1} \cdot min^{-1})$[b]	0.7 (1.7)	0.6 (1.0)	1.8 (1.9)	-1.9 (-19.5, 19.5; 0.87)[UC] 0.6:1.7; inconclusive[c]	26.9 (-3.0, 64.1; 0.14)** 241:<1; use[c]	29.4 (0.5, 66.4; 0.09)** N/A
HbA$_1$c $(mmol \cdot mol^{-1})$[b]	-8.5 (18.8)	-2.8 (9.8)	-4.0 (9.1)	9.2 (3.3, 15.1; 0.015)** <1:56940; don't use[c]	2.9 (-2.8, 8.6; 0.384)[++] <1:33; inconclusive[c]	-6.3 (-13.0, 0.4)* N/A

[a]Least-squares (LS) estimate mean post-pre intervention change score, adjusted for baseline. The likelihoods of change relative to smallest threshold was: 25-75% possible*, 75-95% likely**, 95-99.5% very likely***, >99.5% almost certain****. Likelihood trivial effect size (100-(harm+benefit)) was denoted with [+] symbols. Unclear ([UC]) refers to outcomes where the likelihood of both benefit and harm exceeded 5%. Output estimates and confidence limits are geometrically scaled within the analysis of log-transformed data. [b]The smallest clinical effect thresholds in the two primary clinical outcomes were: glucose clearance rate, 5.4% based upon the effect size of 3 months of hypoglycaemic therapy (Metformin) on naïve Type-2 diabetics (Derosa et al., 2009) and HbA1c, 5.5 mmol/mol based on general clinical consensus (Lenters-Westra et al., 2014). For other secondary clinical (fasting glucose), between-subject criteria (Cohen's *d* standardized difference) was used; where, the effect size for small was 0.2 (Hopkins et al., 2009). Glucose concentration estimates are percent change. [c]Clinical inferences were referenced for comparisons with control (CON) and non-clinical for comparisons of the two active treatments. The clinical decision threshold was a benefit harm odds ratio >66:1 (Hopkins et al., 2009); N/A denotes clinical contrasts not applicable.

4.4.3. Insulin-stimulated mBF and mBV

Statistical outcomes and inferences were presented in **Table 4.3**. In the current study, improvements in insulin-stimulated blood flow for CON, WHEY and WDP were very likely, likely and almost certain respectively (**Figure 4.2**). Improvements in insulin-stimulated blood flow (mBF) were substantially larger in WDP compared to WHEY and CON (**Figure 4.2B**). Insulin-stimulated blood perfusion (mBV) substantially increased in CON with unclear effects for WDP and WHEY. Comparing slopes using simple linear regression revealed a positive association between change in mBF and GCR for WDP. For every % increase in GCR, there was a 0.53% increase in mBF ($\beta = 0.53\%$ 90%CI 0.25, 0.82), with the WDP-WHEY difference ($\beta = 0.60\%$ (90%CI 0.22, 0.98) (**Figure 4.2C**). No clear associations were found for CON ($\beta = 0.23\%$ 90%CI: -0.27, 0.74) and WHEY ($\beta = -0.06\%$ 90%CI: -0.31, 0.19).
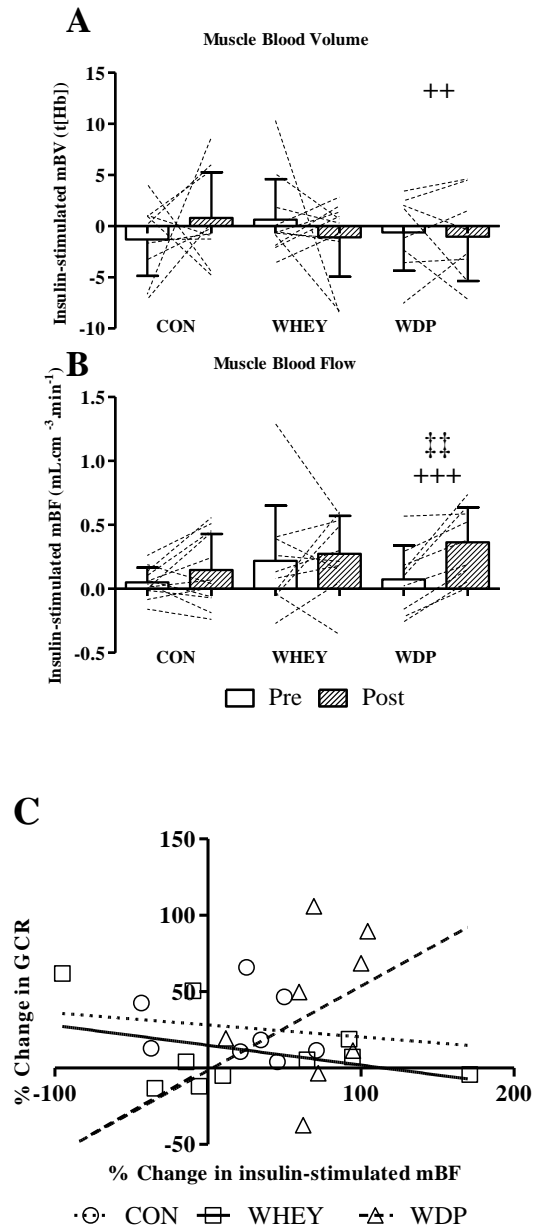
*Figure 4.2 – Effect of CON, WHEY and WDP on A) mBV, B) mBF and C) associations between changes in insulin-stimulated mBF and GCR in adult men and women with type-2 diabetes. Data are raw means and SDs. Statistical symbols represent magnitude-based mechanistic inferences based on log-transformed mixed models. Abbreviations: + = WDP vs CON; ‡ = WDP vs WHEY. The number of letters or symbols denotes likelihood of change where * = possible; ** = likely; *** = very likely; **** = almost certain*

Table 4.3 – The effect of 14-weeks of WDP, whey-protein supplementation with exercise on skeletal muscle microvascular hemodynamics

	Unadjusted Raw Unit Mean Change (SD)			Outcome Statistics[a]; Estimate (90%CI); Likelihood Increase/Decrease (%); Inference[b];		
Treatment, Contrast[a]	CON	WHEY	WDP	WHEY – CON	WDP – CON	WDP – WHEY
Insulin-stimulated muscle blood flow (mL/cm³/min)	0.20 (0.20)	0.08 (0.38)	0.29 (0.20)	-3.6 (-33.1, 38.9) 22.3/33.9; UC	51.1 (5.4, 116.6) 93.5/0.7; **	45.7 (9.0, 94.7) 95.4/0.2; ***
Insulin-stimulated muscle blood volume (t[Hb])	3.3 (5.9)	-2.2 (6.7)	-0.4 (3.9)	-4.7 (-13.4, 4.9) 10.3/66.4; UC	-12.0 (-24.8, 3.1) 4.4/87.5; **	-7.6 (-20.6, 7.5) 10.8/74.7; UC

[a] Data for each contrast are post-pre covariate adjusted for the baseline value. [b] Determination for the threshold for smallest worthwhile change in mechanistic outcomes, between-subject criteria (Cohen's *d* standardized difference) was used; where, the effect size for small was 0.2*SD at baseline (Hopkins et al., 2009). The likelihood that a contrast was at least greater than Cohen's *d* threshold was: 25-75% possible *, 75-95% likely **, 95-99.5% very likely ***, >99.5% almost certain ****; likelihood trivial effect size calculable from 100-(increase + decrease). Unclear (UC) refers to outcomes where the likelihood of both increase and decrease exceeded 5%.

4.4.4. Skeletal muscle capillarization

Statistical outcomes were presented in **Table 4.4**. Skeletal muscle capillarization, measured as CC, C/F_i, CFPE and SF, increased substantially in all groups, without clear treatment effects (**Figure 4.3**).
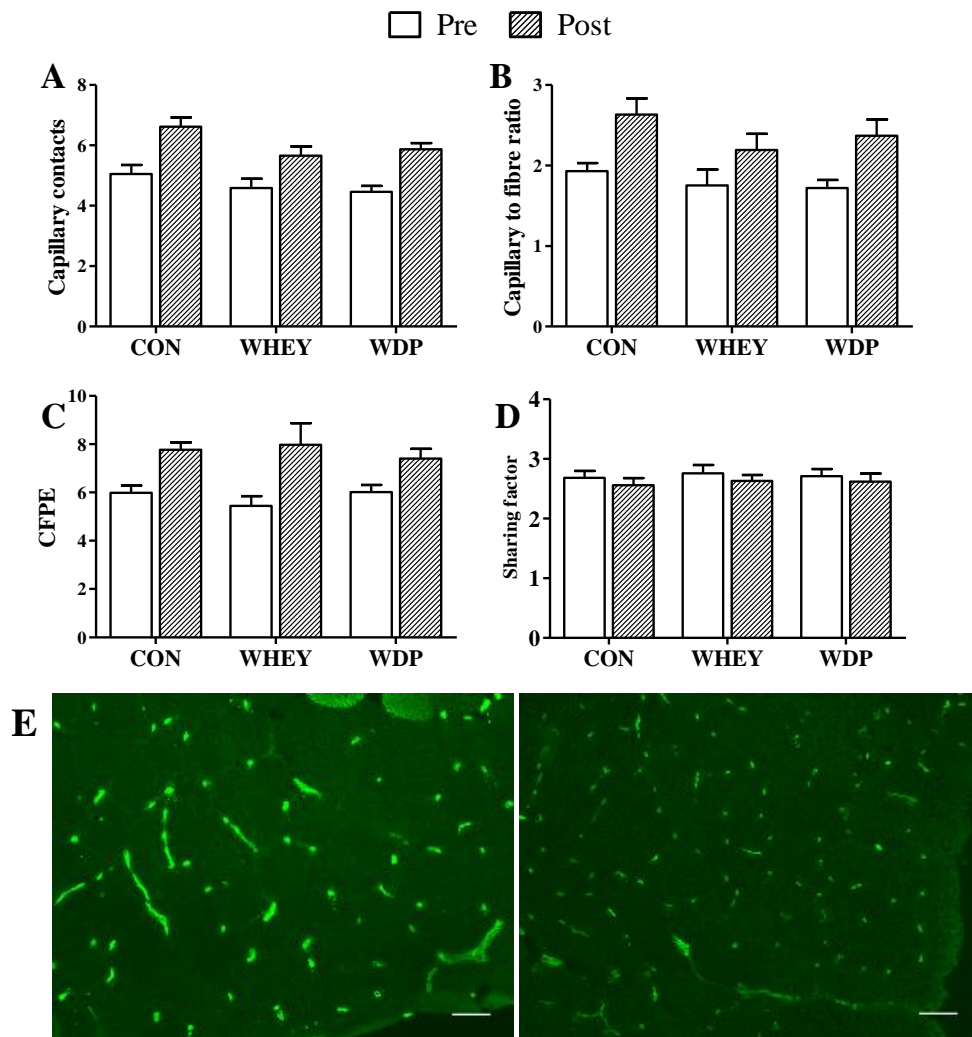
Figure 4.3 – Changes in skeletal muscle capillarization parameters pre and post-intervention. Values are Mean (SD). CFPE, capillary-per-fibre perimeter exchange.

Table 4.4 – The effect of 14-weeks of WDP, whey-protein supplementation with exercise on skeletal muscle capillarization

Treatment, Contrast[a]	Unadjusted Raw Unit Mean Change (SD)			Outcome Statistics[a] ; Estimate (90%CI); Likelihood Increase/Decrease (%); Inference[b]		
	CON (n = 11)	WHEY (n = 10)	WDP (n = 10)	WHEY – CON	WDP – CON	WDP – WHEY
Sharing Factor (#)	-0.11 (0.15)	-0.15 (0.12)	-0.09 (0.12)	0.3 (-3.1, 3.8) 37.7/26.4; [UC]	1.4 (-2.2, 5.2) 60.2/12.5; [UC]	1.4 (-2.2, 5.2) 60.2/12.5; [UC]
Capillary Counts (#)	1.6 (1.0)	1.3 (0.9)	1.4 (1.1)	-6.2 (-16.7, 5.4) 5.2/64.6; [UC]	-6.5 (-20.1, 9.4) 10.6/62.0; [UC]	-0.2 (-14.4, 16.4) 31/32.4; [UC]
C/F$_i$ (#)	0.7 (0.5)	0.6 (0.5)	0.6 (0.5)	-5.9 (-18.3, 8.3) 7.9/56.5; [UC]	-4.9 (-20.0, 12.9) 14.7/50.9; [UC]	1.1 (-14.6, 19.6) 34.1/26.1; [UC]
CFPE (#)	1.8 (1.4)	2.7 (2.6)	1.4 (1.2)	7.1 (-10.3, 27.9) 63.8/14.5; [UC]	-13.0 (-28.7, 6.1) 5.5/83.3; [UC]	-6.8 (-18.3, 6.3) 7.3/69.3; [UC]

[a] Data for each contrast are post-pre covariate adjusted for the baseline value. [b] Determination for the threshold for smallest worthwhile change in mechanistic outcomes, between-subject criteria (Cohen's *d* standardized difference) was used; where, the effect size for small was 0.2*SD at baseline (Hopkins et al., 2009). The likelihood that a contrast was at least greater than Cohen's *d* threshold was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain; likelihood trivial effect size calculable from 100-(increase + decrease). Unclear ([UC]) refers to outcomes where the likelihood of both increase and decrease exceeded 5%.

4.4.5. eNOS, eNOS^{ser1177} and NOX2 expression

NOX2, eNOS and eNOS^{ser1177} were detected in endothelial cells of capillaries and terminal arterioles (**Figure 3.6**) as observed previously (Cocks et al., 2012). **Figure 4.4** visualizes pre and post-intervention protein expression under basal conditions and after 1 hour of insulin infusion. The average CV for eNOS, eNOS^{ser1177} and NOX2 were 9.1%, 10.3% and 6.8% respectively. All between-group effects for insulin-stimulated microvascular protein expression were unclear (**Figure 4.5**). When expressed as a ratio of eNOS^{ser1177}/eNOS, protein content for WHEY and WDP showed possible increases compared to CON in the fasted state (**Table 4.5**). There was an increase of eNOS^{ser1177} in WDP in the fasted state with no clear group changes. Increases for insulin-stimulated eNOS^{ser1177}, eNOS^{ser1177}/eNOS and eNOS^{ser1177}/NOX2 ratio were observed in WHEY but with unclear between-group effects. Insulin-stimulated NOX2 in the skeletal muscle plasma membrane likely increased in WDP compared to WHEY and insulin-stimulated NOX2 in the plasma membrane decreased in WHEY but differences between groups were unclear.
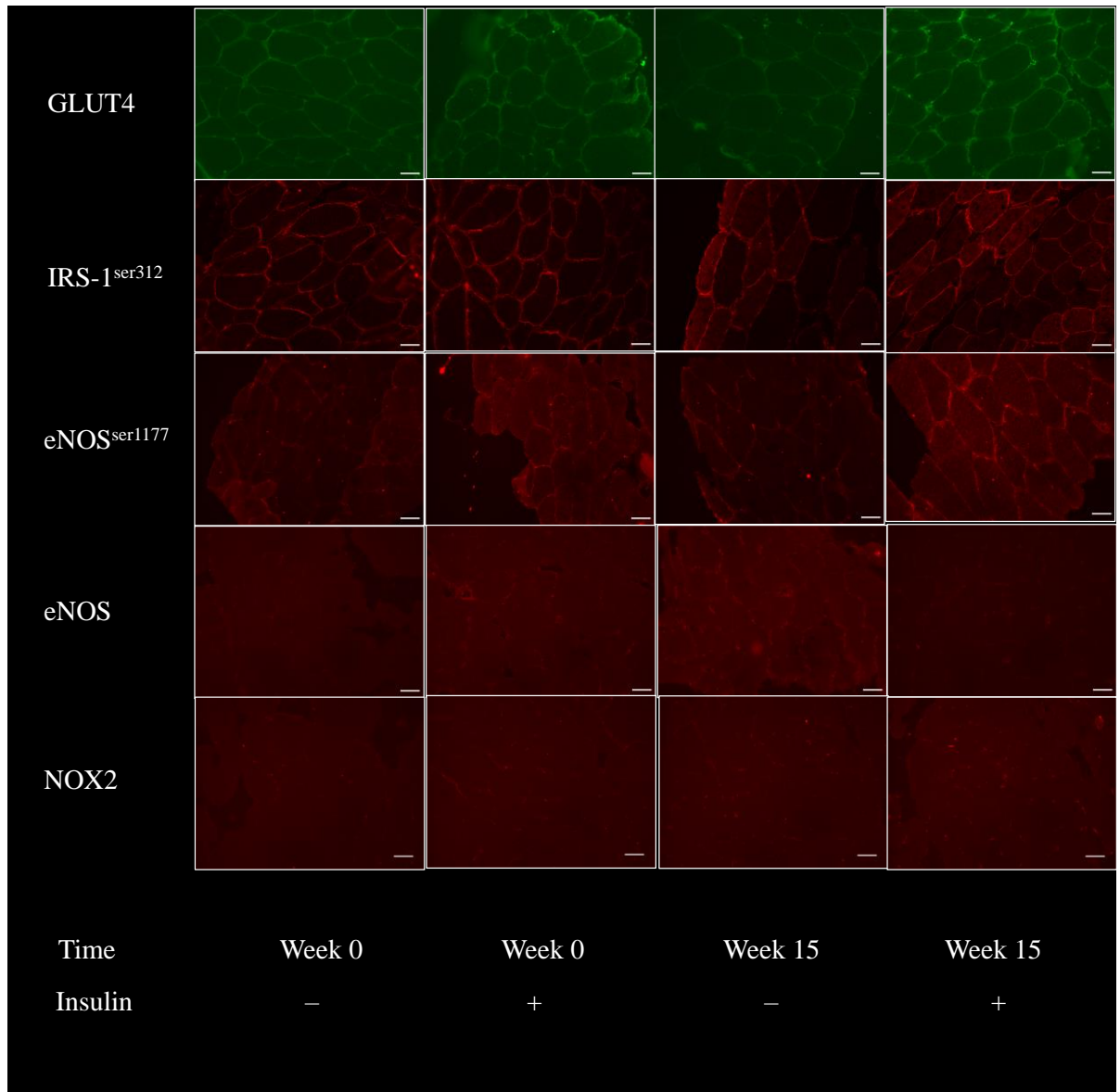
Figure 4.4 – Representative widefield images of GLUT4 and p-IRS-1ser312, eNOS, p-eNOSser1177 and NOX2 at week 0 and week 15 in under basal conditions and 1 hour after insulin infusion. Brightness for eNOSser1177, eNOS and NOX2 was enhanced by 50% for demonstration, but analysis was on performed on raw images. Bars are 50 µm.
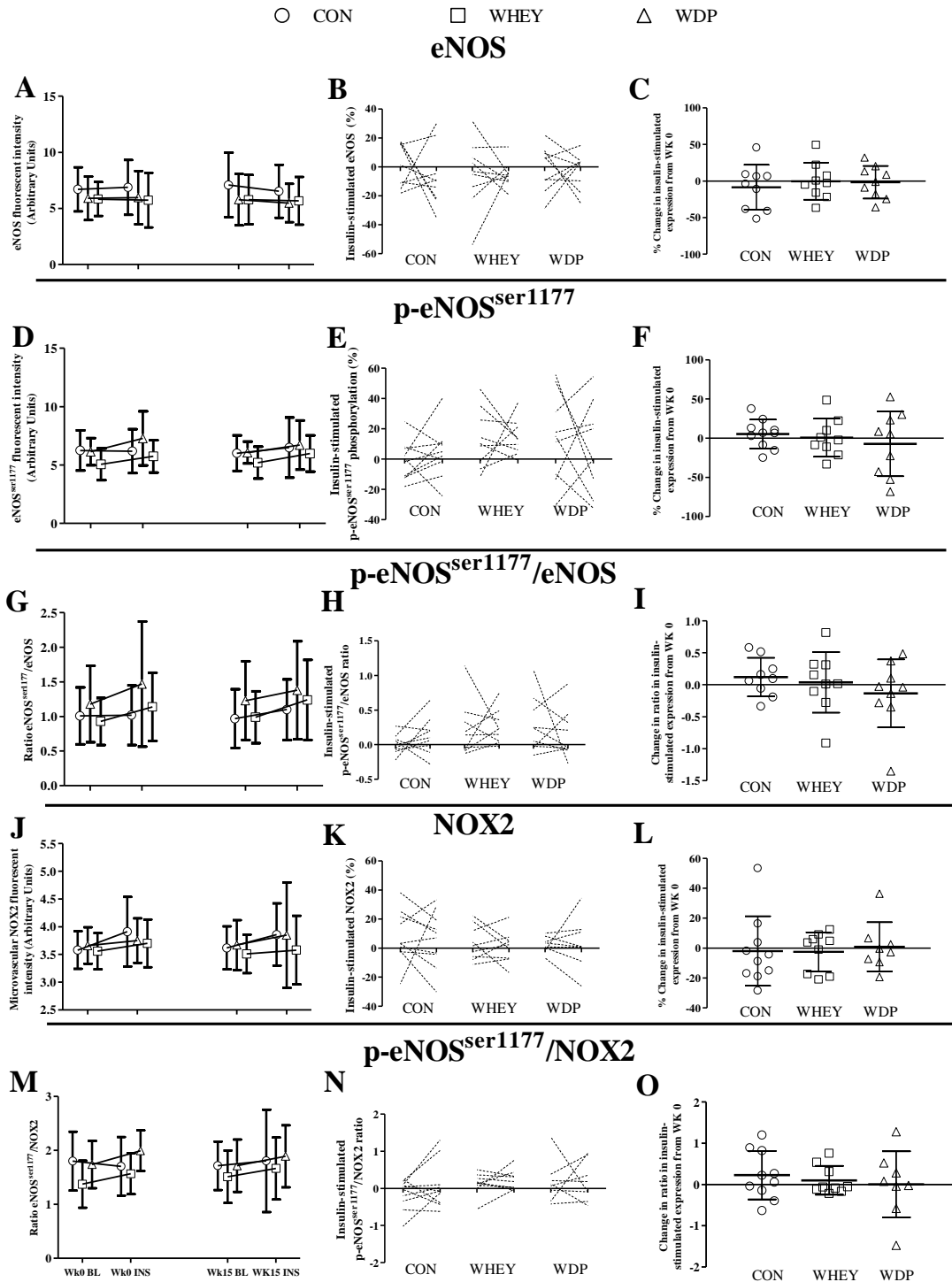
Figure 4.5 – Effect of WDP and whey protein supplementation in response to 14 weeks of exercise training on enzymatic expression of proteins controlling skeletal muscle NO bioavailability in adult men and women with type-2 diabetes. The left panels are raw mean fluorescent intensity of protein expression obtained from muscle

biopsies during the week 0 and week 15 hyperinsulinaemic isoglycaemic clamp. The middle panel represents individual responses in insulin-stimulated (change from BL to INS) conditions before and after the intervention, presented as % change scores. The right panel represents the mean % change (SD) in insulin-stimulated protein expression from week 0 to week 15.

Table 4.5 – The effect of 14-weeks of WDP, whey-protein supplementation with exercise on skeletal muscle microvascular enzyme activity

Treatment, Contrast[a]	Condition	Week 15-0 Mean Change (SD)[a]			Estimate (90%CI)[b] Likelihood Increase/Decrease (%); Inference[c]		
		CON	WHEY	WDP	WHEY – CON[c]	WDP – CON[c]	WDP – WHEY[c]
eNOS protein expression (%)	*Basal*	4.2 (24.3)	-3.4 (26.0)	-3.9 (19.6)	-7.0 (-23.9, 13.7) 10.7/53.5; [UC]	-7.8 (-23.0, 10.6) 7.3/57.2; [UC]	-0.8 (-18.0, 19.9) 24.5/29.8; [UC]
	Insulin-stimulated	-8.1 (35.9)	-0.2 (28.7)	-1.5 (24.9)	0.7 (-12.9, 16.4) 35.6/29.2; [UC]	3.4 (-11.5, 20.7) 48.9/20.3; [UC]	2.7 (-7.3, 13.7) 43.4/13.1; [UC]
eNOS^{ser1177} protein expression (%)	*Basal*	-3.2 (20.2)	2.9 (10.4)	-0.4 (26.8)	3.0 (-9.4, 17.1) 37.5/12.0; [UC]	7.8 (-6.9, 24.8) 61.1/5.6; [UC]	4.6 (-7.6, 18.4) 45.7/7.6; [UC]
	Insulin-stimulated	5.5 (20.2)	0.7 (27.5)	-7.0 (51.1)	7.8 (-7.4, 25.4) 65.7/7.7; [UC]	-0.2 (-21.4, 26.7) 36.5/37.9; [UC]	-7.5 (-25.4, 14.8) 15.2/62.0; [UC]
eNOS^{ser1177}/eNOS (Ratio)	*Basal*	-0.08 (0.19)	0.06 (0.23)	0.04 (0.21)	0.12 (-0.08, 0.32) 61.1/2.5; *	0.13 (-0.05, 0.30) 64.3/1.2; *	0.0 (-0.22, 0.23) 23.6/21.4; [UC]
	Insulin-stimulated	0.12 (0.30)	0.04 (0.47)	-0.13 (0.53)	0.12 (-0.21, 0.45) 61.5/14.5; [UC]	0.02 (-0.36, 0.4) 40.5/33.5; [UC]	-0.1(-0.4, 0.2) 14.9/58.1; [UC]
eNOS^{ser1177}/NOX2 (Ratio)	*Basal*	-0.08 (0.32)	0.06 (0.28)	-0.02 (0.53)	0.08 (-0.18, 0.35) 45.7/11.1; [UC]	0.09 (-0.23, 0.41) 49.0/13.9; [UC]	0.01 (-0.32, 0.35) 32.0/27.7; [UC]
	Insulin-stimulated	0.23 (0.59)	0.10 (0.34)	0.0 (0.81)	0.01 (-0.42, 0.45) 38.1/33.8; [UC]	0.0 (-0.55, 0.55) 38.4/39.4; [UC]	-0.02 (-0.44, 0.41) 33.0/38.6; [UC]
NOX2 protein expression (%)	*Basal*	1.0 (12.6)	-1.5 (11.8)	-0.1 (10.3)	-2.8 (-11.5, 6.8) 17.6/58.9; [UC]	-0.4 (-9.8, 9.9) 33.9/39.9; [UC]	2.4 (-7.2, 13.0) 55.3/21.0; [UC]
	Insulin-stimulated	-1.9 (26.2)	-2.4 (13.9)	0.9 (17.8)	-3.8 (-16.2, 10.5) 19.7/57.0; [UC]	0.7 (-15.6, 20.1) 42.5/36.7; [UC]	4.6 (-9.5, 21.1) 60.2/18.5; [UC]

[a]Data are raw mean (SD). [b]Data are the mean estimate of the baseline-adjusted post-pre difference expressed as a percent derived from the analysis of log-transformed data. [c]The threshold for smallest substantial change for mechanisms was 0.2*SD at baseline derived from the Cohen's *d* threshold (Hopkins et al., 2009), where the likelihood for substantial change was 25-75% possible *, 75-95% likely **, 95-99.5% very likely ***, >99.5% almost certain ****. Unclear ([UC]) refers to outcomes where the likelihood of both increase and decrease exceeded 5%. C/F$_i$, capillaries per fibre; CFPE, capillary to fibre perimeter exchange; PM, plasma membrane.

4.4.6. Insulin signalling protein phosphorylation activity in skeletal muscle fibre

The average CV within three images per sample was 10.7%, 9.1% and 14.8% for GLUT4 mean fluorescent intensity (MFI), Pearson's correlation coefficient (PCC) and IRS-1^{ser312} mean fluorescent intensity respectively. Representative widefield images for the immunofluorescence analysis at four timepoints are displayed in **Figure 4.4**. Representative pairs of Western blot analysis at four timepoints are displayed in **Figure 4.6**. Fasted IRS-1^{ser312} phosphorylation activity was not clearly different for all within-group and between-group contrast, whereas insulin-stimulated IRS-1^{ser312} weakly and possibly decreased in WHEY relative to WDP and CON respectively (**Figure 4.7A; Table 4.6**). Fasted AS160^{Thr642} phosphorylation decreased after the intervention for CON and WHEY, however all between-treatment effects were unclear. Insulin-stimulated AS160^{Thr642} substantially increased in all groups after the intervention but all between-group contrasts were unclear (**Figure 4.7B**). There was an almost certain and very likely decrease in fasted PAK1^{Thr423}/PAK2^{Thr402} in WHEY compared to WDP and CON respectively. Insulin-stimulated PAK1^{Thr423}/PAK2^{Thr402} substantially increased within all groups, but the treatment effects were unclear (**Figure 4.7C**).
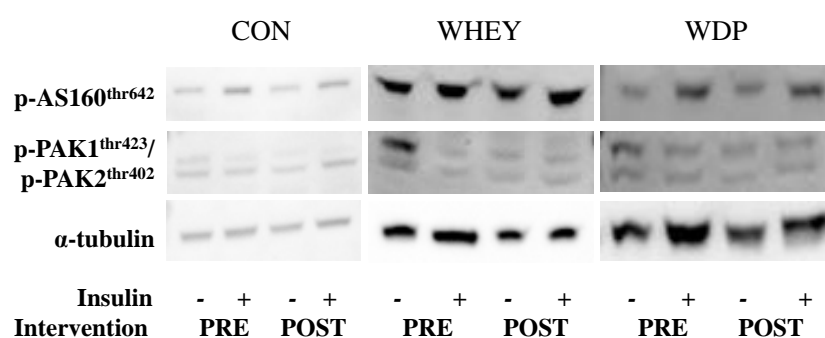
Figure 4.6 – Representative Western blots for p-AS160thr642, p-PAK1thr423/p-PAK2thr402 and α-tubulin as loading control at weeks 0 and 15 in basal conditions and 1 hour after insulin infusion.

4.4.7. GLUT4 translocation in the skeletal muscle plasma membrane

Fasting GLUT4 density in the skeletal muscle plasma membrane region as a proxy for GLUT4 translocation decreased within WDP after the intervention, but differences between groups were inconclusive (**Table 4.6**). Insulin-stimulated GLUT4 translocation increased within all groups and WDP treatment resulted in likely increased GLUT4 translocation compared to CON and WHEY (**Figure 4.7D**). When using PCC (*r*) as a measure of GLUT4 translocation as described previously (Bradley et al., 2015), fasted GLUT4 in the plasma membrane decreased in CON and WHEY, but comparison between groups showed unclear effects. Insulin-stimulated GLUT4 translocation, using PCC, increased very likely for WDP, but differences between groups were unclear (**Appendix G, Figure D-F**). Comparing slopes using simple linear regression revealed a positive association between change in insulin-stimulated GLUT4 translocation and GCR for WDP. For every % increase in insulin-stimulated GLUT4 translocation, there was a 2.1% increase in GCR in WDP ($\beta = 2.1\%$ 90%CI 0.9, 3.2), but the associations within CON ($\beta = 0.0\%$ 90%CI -1.1, 1.0) and WHEY ($\beta = 0.4\%$ 90%CI -1.1, 1.9) were unclear (**Appendix G, Figure J**). The regression slope was substantially different for WDP-CON ($\beta = 2.1\%$ 90%CI 0.5, 3.7) and WDP-WHEY ($\beta = 1.6\%$ 90%CI -0.3, 3.6), but unclear for WHEY-CON ($\beta = 0.4\%$ 90%CI: -1.4, 2.3)). Changes in chronic skeletal muscle [GSH], as reported elsewhere (**Appendix F**), tended to be negatively associated with insulin-stimulated GLUT4 translocation in WDP. For every % decrease in [GSH], there was a 0.10% increase in GLUT4 translocation for WDP ($\beta = -0.1\%$ 90%CI -0.18, -0.01), however the association between WDP-CON ($\beta = -0.1\%$ 90%CI -0.28, 0.09), WDP-WHEY ($\beta = -0.04\%$ 90%CI -0.17, 0.09) and WHEY-CON ($\beta = -0.05\%$ 90%CI -0.24, 0.14) were unclear (data not shown). No associations were observed between GLUT4 and HbA1c (CON: $\beta = 0.16$ 90%CI -0.06, 0.39, WHEY: $\beta = -0.13$ 90%CI -

0.54, 0.28, WDP: $\beta = 0.13$ 90%CI –0.09, 0.35) (**Appendix G, Figure K**). Associations between pre-post changes in insulin-stimulated GLUT4 translocation with unpublished changes in fasting skeletal muscle GSH (**Appendix F**) showed a negative association within WHEY ($\beta = -7.0$ 90%CI -12.2, -1.7), but unclear associations within WDP ($\beta = -2.5$ 90%CI -6.6, 1.6) and CON ($\beta = -0.6$ 90%%CI –3.5, 2.3) Simple linear regression did not demonstrate an association between insulin-stimulated IRS-1[ser312] and GLUT4 within the three treatments (CON: $\beta = 0.1$ 90%CI -1.3, 1.4, WHEY: $\beta = -0.9$ 90%CI -3.3, 1.5, WDP: $\beta = 1.3$ 90%CI -0.6, 3.1) (**Appendix G, Figure L**).
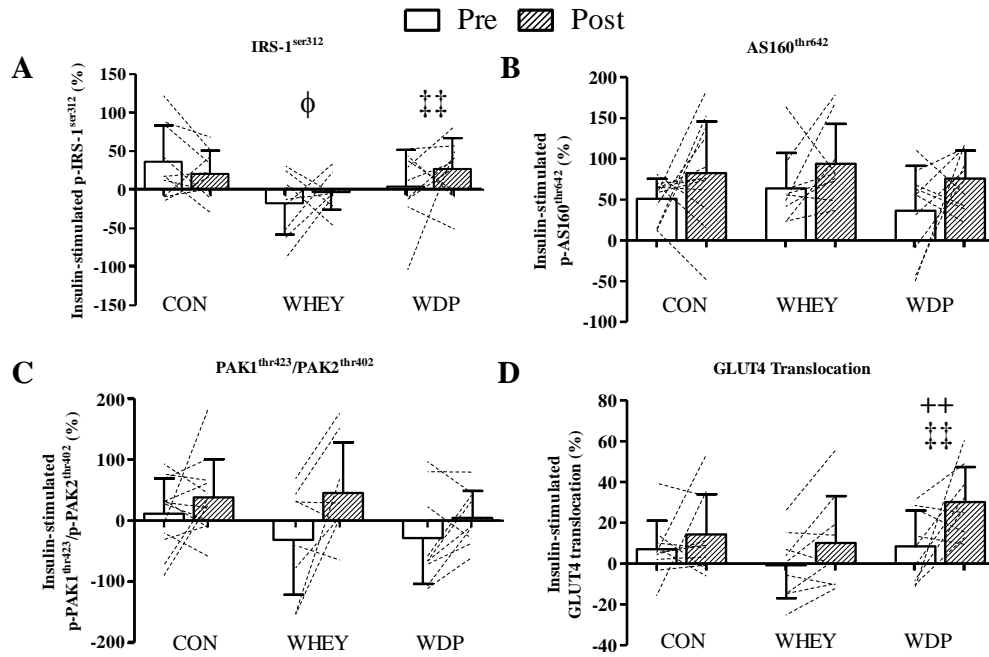
Figure 4.7 – Mean (SD) and individual % change in insulin-stimulated phosphorylation of IRS-1ser312 (A), AS160thr642 (B), PAK1thr423/PAK2thr402 (C) and GLUT4 translocation (D) in adult men and women with T2DM before and after the intervention. Data were analyzed using linear mixed models and statistical symbols represent magnitude-based decisions (for detail see Table 4.6). Symbols: ϕ = WHEY vs CON; + = WDP vs CON; ‡ = WDP vs WHEY. The number of letters or symbols denotes probability of substantial change where * possible (50-75%); ** likely (75-95%); *** very likely (95-99.5%); **** almost certain (>99.5%).

Table 4.6 – Effects of CON, WHEY and WDP on insulin signaling phosphorylation activity and GLUT4 fluorescent intensity in skeletal muscle plasma membrane as a proxy for GLUT4 translocation.

		Week 15-0 Mean Change			Outcome Statistics[a]		
		(SD)			Estimate (90%CI)		
					Likelihood Increase/Decrease (%); Inference[b]		
Treatment, Contrast[a]		CON	WHEY	WDP	WHEY – CON[c]	WDP – CON[c]	WDP – WHEY[c]
IRS^{ser312} protein expression (%)	*Fasting*	-10.6 (46.3)	-11.7 (49.3)	0.9 (33.1)	0.4 (-26.1, 36.3) 34.2/32.6; [UC]	10.2 (-12.8, 39.4) 58.2/9.3;[UC]	9.8 (-16.7, 44.8) 56.1/13.6; [UC]
	Insulin-stimulated	-14.6 (50.2)	15.8 (69.4)	25.8 (88.0)	-17.0 (-35.8, 7.3) 2.2/74.0; *	17.2 (-15.6, 62.7) 63.7/7.6;[UC]	41.2 (2.5, 94.6) 92.3/0.6; **
AS160^{Thr642} protein expression (%)	*Fasting*	-22.7 (109.1)	-9.2 (97.8)	-5.1 (41.7)	5.1 (-34.1, 67.5) 34.9/22.0; [UC]	21.5 (-21.5, 88.1) 57.2/7.8;[UC]	15.6 (-13.9, 55.3) 48.7/3.8; [UC]
	Insulin-stimulated	37.0 (104.4)	35.4 (79.4)	47.9 (113.5)	8.6 (-32.4, 74.4) 50.1/26.6; [UC]	-17.3 (-43.9, 22.0) 10.0/69.2; [UC]	10.0 (-39.4, 33.7) 19.7/54.3; [UC]
PAK1^{Thr423}/PAK2^{Thr402} protein expression (%)	*Fasting*	-0.6 (95.5)	-47.0 (69.7)	10.6 (127.6)	-48.3 (-67.7, -17.2) 0.1/97.3; ***	25.8 (-20.1, 98.2) 61.7/6.4; [UC]	143.3 (66.0, 256.6) 99.9/0.0; ****
	Insulin-stimulated	12.2 (78.1)	-27.0 (144.6)	-28.9 (93.1)	22.4 (-36.4, 140.0) 57.2/16.9; [UC]	-15.2 (-45.5, 32.0) 10.4/54.6; [UC]	-30.8 (-61.4, 24.3) 5.5/76.6; [UC]
GLUT4 fluorescent intensity (%)	*Fasting*	-3.1 (20.7)	-4.5 (28.9)	-9.0 (11.9)	-0.9 (-17.2, 18.5) 30.0/37.0; [UC]	-5.3 (-17.5, 8.8) 10.2/56.6; [UC]	-4.4 (-18.1, 11.7) 15.3/51.5; [UC]
	Insulin-stimulated	7.6 (26.5)	11.6 (14.9)	24.2 (29.8)	2.5 (-12.4, 20.1) 47.4/25.6; [UC]	17.8 (-0.5, 39.4) 92.8/1.6; **	14.9 (-1.2, 33.6) 90.7/1.9; **

[a] Data for each contrast are presented as % scores for post-pre estimates covariate adjusted for the pre-intervention value. [b] The threshold for smallest worthwhile change for mechanistic changes, between-subject criteria (Cohen's d standardized difference) was used; where the effect size for small was 0.2*SD at baseline (Hopkins et al., 2009). The likelihood that a contrast was at least greater than Cohen's *d* threshold was: 25-75% possible *, 75-95% likely **, 95-99.5% very likely ***, >99.5% almost certain ****; likelihood trivial effect size calculable from 100-(increase + decrease). Unclear ([UC]) refers to outcomes where the likelihood of both increase and decrease exceeded 5%.

4.5. Discussion

The current study was the first to explore the effects of different dietary protein types combined with exercise training on changes in whole-body glucose uptake, skeletal muscle microvascular haemodynamics, endothelial eNOS activity and GLUT4 translocation *in vivo* in humans with T2DM. Insulin-stimulated skeletal muscle mBF increased twofold after 14 weeks of twice-daily consumption of a novel uniquely-processed keratin protein isolate and whey protein blend (WDP) in conjunction with mixed-modality, interval-based. Moreover, the probability that the increase was larger compared to WHEY and CON was very likely and likely respectively and was positively associated with GCR. However, it was unclear whether changes in protein expression of enzymes controlling NO bioavailability contributed to improvements in mBF. Furthermore, insulin-stimulated GLUT4 translocation was likely enhanced in WDP, compared to WHEY and CON.

The GCR and GLUT4 translocation out comes suggest WDP protein may support exercise-mediated skeletal muscle adaptive plasticity compared to whey protein and a placebo, which was hypothesized to be attributed to the higher concentrations of cysteine, glycine and arginine (**Table 3.1**). Both chronic cysteine, glycine, when supplemented as n-acetylcysteine (Sekhar et al., 2011) and arginine (Piatti et al., 2001) supplementation has been associated with improved insulin sensitivity in adults with T2DM. Cysteine treatment increased erythrocyte glutathione concentrations as a marker of reduced oxidative stress in both older adults and in adults with uncontrolled T2DM (Sekhar et al., 2011). In contrast, in the current cohort, erythrocyte GSH was unchanged with WDP, but decreased skeletal muscle glutathione concentrations and increased oxidised skeletal muscle peroxiredoxin 2 (**Appendix F**) in the same study cohort and in

rat *m. gastrocnemius*, although skeletal muscle glutathione was highly variable in WDP at baseline. These findings suggests that improvements in insulin sensitivity occurred under increased pro-oxidative cellular conditions, which contradicts current understanding of cellular redox status on insulin resistance, since increased oxidative stress is a hallmark in the development of insulin resistance and T2DM (Evans et al., 2002; Nourooz-Zadeh et al., 1997). However, in line with the current study, previously high intake of n-acetylcysteine (2.4 g/d) has been reported to reduce GSH in erythrocyte (Kleinveld et al., 1992). However, the current study found no association between changes in GCR and changes in fasted skeletal muscle glutathione and oxidised PRX2 within WDP or between groups which indicates that changes in glutathione and oxidised PRX2 might not affect changes in insulin sensitivity. However, further research should explore the relationship between keratin protein mediated changes in the cellular redox environment within insulin sensitive tissues of interest to systemic glucose metabolism.

The current *a priori* hypothesis was that, amongst cysteine and glycine, chronic enrichment of dietary arginine content from the WDP condition (1.9 g/d; 2-fold higher than WHEY and several-fold higher than CON) would assist increasing fasting NO bioavailability. NO could be utilized during insulin stimulation (Muniyappa et al., 2007) to improve nutrient and insulin delivery to the skeletal muscle fibre via increased blood flow, vasodilation in terminal arterioles and trans-endothelial insulin transport. (Barrett et al., 2011; Steinberg et al., 1994; Vincent et al., 2003). Indeed, insulin-stimulated blood flow was improved in WDP to a greater magnitude compared to CON and WHEY and was positively associated with changes in GCR. Existing evidence that arginine ingestion improves insulin sensitivity via increased NO bioavailability, which

was measured by an increase in fasting plasma L-arginine (precursor for NO synthesis) and cGMP (a second messenger of NO) (Lucotti et al., 2006; Monti et al., 2013; Piatti et al., 2001) does support our hypothesis, although these studies used considerably higher supplemental doses (>3g per day) for shorter durations ($\leq$ 2 months) compared to the current study. In the current study, no direct assessment of NO bioavailability was made, however indirect investigation via measurement of enzyme activity controlling skeletal muscle NO bioavailability (eNOS[ser1177] phosphorylation and NOX2) yielded no clear evidence on changes within or between groups. The probability that fasting eNOS[ser1177]/eNOS ratio was increased in WDP and WHEY relative to CON was only possibly and other between-group contrasts remained unclear. The discrepancy between the phenotype observations of group contrasts for insulin-stimulated skeletal muscle blood flow and the unclear mechanistic effects on endothelial enzyme activity could be for a few reasons. First, no distinction was made between changes in enzyme activity in terminal arterioles and capillaries. Differential effects of exercise training in terminal arterioles and capillary eNOS[ser1177] have been reported previously as Cocks et al. (2016a) did find an increase in eNOS[ser1177] activity after exercise training in terminal arterioles but not capillaries when normalised for total eNOS, suggesting that enzyme activity could differ at multiple levels of the microvasculature. Alternatively, insulin is also known to activate endothelin-1 (ET-1) that has been shown to act as a vasoconstrictor (Eringa et al., 2006). Skeletal muscle ET-1 protein expression is higher in T2DM compared to healthy individuals, contributing to an impaired insulin-mediated blood flow and glucose disposal (Reynolds et al., 2017). Moreover, supplementation of L-arginine during exercise training under hypocaloric conditions in T2DM lowered plasma ET-1 and improved daily blood glucose profiles compared to placebo,

highlighting the role of ET-1 on insulin sensitivity and endothelial function (Lucotti et al., 2006). Thus, further explorations in the underlying mechanisms are required to provide a more detailed answer to the beneficial effects of WDP on insulin-stimulated blood flow.

The current study found that the probability of an increase in insulin-stimulated GLUT4 translocation in WDP compared to CON and WHEY was likely, indicating that combined protein and exercise intervention might modulate skeletal muscle insulin signalling. Using Western blot methods, insulin-stimulated GLUT4 translocation, a rate limiting step in glucose transport, has previously been shown to increase 27% in skeletal muscle plasma membrane GLUT4 after glucose ingestion (Goodyear et al., 1996) and a 61% increase after insulin infusion (Zierath et al., 1996) in healthy humans. Moreover, insulin-stimulated GLUT4 translocation was absent in humans with T2DM (Garvey et al., 1998; Stuart et al., 2010; Zierath et al., 1996). No *in vivo* human studies to date have investigated the change in insulin-stimulated GLUT4 translocation in response to a clinical intervention, but have measured change in total GLUT4 protein expression (Dela et al., 1994; Guadalupe-Grau et al., 2017; Holten et al., 2004). However, change in total GLUT4 protein expression might not be an accurate measure for skeletal muscle insulin sensitivity, as evidence suggests that changes in GLUT4 protein expression is dependent on the duration of T2DM, with no changes in GLUT4 in early T2DM compared to healthy control (Handberg et al. (1990); Pedersen et al. (1990). Therefore, measuring changes in insulin-stimulated GLUT4 translocation would be a better reflection of changes in skeletal muscle insulin sensitivity. The current study found that the probability of an increase in insulin-stimulated GLUT4 translocation in WDP compared to CON and WHEY was weakly, indicating that combined protein and

exercise intervention might modulate skeletal muscle insulin signalling. The *a priori* hypothesis was that cysteine and glycine-rich WDP would increase cellular GSH and alleviate oxidative stress-induced insulin resistance. However, a substantial decrease in skeletal muscle GSH was observed in WDP compared to WHEY and CON (**Appendix F**), not supporting the hypothesis. Moreover, there was no clear association between change in basal skeletal muscle GSH and changes in insulin-stimulated GLUT4 translocation, suggesting no clear role for GSH concentrations in skeletal muscle glucose uptake. Some limited evidence found that L-cysteine supplementation to adipocytes exposed to high glucose concentrations increased GLUT4 expression (Achari et al., 2016) and cod protein supplementation, high in arginine restored GLUT4 translocation in skeletal muscle of high-fed rats via restored Akt signalling compared to soy and casein (Lavigne et al., 2001; Tremblay et al., 2003) and improved insulin sensitivity in insulin-resistant women (Ouellet et al., 2007). Therefore, WDP high in arginine and cysteine was expected to increase insulin-stimulated AS160 phosphorylation downstream of Akt. However, no clear treatment effects were found for insulin-stimulated AS160^{thr642} phosphorylation, which suggests that phosphorylation at the threonine residue might not explain observations of favourable effects of WDP on GLUT4 translocation. Insulin induces AS160 phosphorylation at several sites (Consitt et al., 2013) and assessing several markers of AS160 phosphorylation would have provided better insight in insulin-stimulated mechanisms leading to likely improved GLUT4 translocation in WDP compared to WHEY and CON.

4.5.1. Limitations

A few limitations present in the current study need to be further addressed in future studies. Firstly, the fate of the partically-hydrolysed dietary keratin isolate after

ingestion remains unknown. Previously, Sekhar et al. (Sekhar et al., 2011) used free glycine and n-acetylcysteine as supplement to restore GSH synthesis and improve insulin sensitivity in T2DM and free L-arginine was administered in T2DM to improve NO bioavailability (Lucotti et al., 2006; Piatti et al., 2001). The digestion and absorption kinetics of a whole-protein keratin could be assumed to differ from these sources, resulting in different postprandial protein handling. Although some preliminary evidence has confirmed the tolerance and digestibility of keratin in healthy humans (Houltham et al., 2014), future studies could develop intrinsically labelled stable isotope techniques to label glycine, cysteine or arginine residues in keratin to trace the fate of these amino acids after consumption as has been applied for other dietary proteins sources previously (Pennings et al., 2011b). Secondly, one major concern in interpreting the data is the limited sample size. The study was appropriately powered for detectable improvements in the primary outcome GCR, but may not have been adequate for all the mechanistic outcomes including mBF or GLUT4 translocation. Moreover, skeletal muscle analysis was performed in a reduced number of participants due to rejection of muscle biopsies and loss in tissue during analysis. This reduced the statistical power in the analysis and therefore warrants larger study populations in follow-up studies. However, sample size given the scale of the intervention was enough for goals of the current exploratory investigation of the novel protein; initial findings seem promising.

4.5.2. Conclusion

In the current explorative study, we provide some novel evidence that a keratin-derived wool protein in addition to an exercise intervention could modulate whole-body insulin sensitivity, skeletal muscle microvascular haemodynamics and skeletal muscle insulin signalling mechanisms. The exact underlying mechanisms that could contribute to these

observed changes remain to be elucidated, however some data suggest redox-mediated alterations as explanation.

Keratin-derived protein seems to be able to modulate insulin sensitivity and might be an effective treatment to improve glycaemic control in T2DM. However, follow-up studies are warranted to further confirm the probability that dietary keratin protein can modulate insulin sensitivity in T2DM.

CHAPTER 5

ATTEMPTS TO QUANTIFY GLUT4 TRANSLOCATION WITH IMMUNOGOLD

5.1. Abstract

Introduction: Insulin-stimulated glucose transport across the membrane of skeletal muscle is facilitated by the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Many methods have been utilized to measure the amount of GLUT4 in the plasma membrane, including immunofluorescence used in this thesis; however, these methods have localization and quantification limitations. The aim of this study was to develop and validate immunogold electron microscopy (IEM) as a technique to quantify GLUT4 translocation in human skeletal muscle tissue.

Methods: Skeletal muscle tissue of both a healthy person (M, 25.3 yr) and a person diagnosed with T2D (F, 56.3 yrs) was obtained after ingestion of a mixed meal or one hour into a hyperinsulinaemic isoglycaemic clamp respectively. Various protocols were tested to establish reliable GLUT4 signal detection using electron microscopy for validation.

Results: Testing a series of protocols did not result in the establishment of a reliable protocol. Negative control procedures showed a high quantity of non-specific binding, causing high background noise and making it unsure to distinguish between true signal detection and non-specific binding detection

Discussion: This study was not able to develop and validate the measurement of human skeletal muscle GLUT4 translocation with the plasma membrane. Until protocols are optimized and a reliable protocol is established for IEM, other methods with compromised spatial resolution have to be used to investigate GLUT4 translocation.

5.2. Introduction

Glucose transport across the plasma membrane is, together with delivery of glucose to the muscle and phosphorylation of glucose by hexokinase, one of the three rate-limiting steps in the process of glucose uptake in skeletal muscle (Wasserman et al., 2011). The translocation and incorporation of glucose transporter 4 (GLUT4) into the plasma membrane of the skeletal muscle fibre facilitates skeletal muscle glucose uptake. Over the past decades, understanding of the molecular signalling events that regulate the translocation of GLUT4 to the skeletal muscle plasma membrane, in particular in response to insulin, has evolved (Foley et al., 2011; Klip et al., 2014). This has led to important discoveries in the process of impaired glucose uptake due to insulin resistance as observed in type-2 diabetes mellitus (Zierath et al., 1996). Even though much is known about the molecular signalling events that initiate and regulate the translocation of GLUT4, there is very limited amount of reliable and validated techniques to quantify GLUT4 translocation in humans *in vivo* (Li et al., 1997).

Methods that have been applied to measure GLUT4 translocation, including in chapter 4, involve the use of widefield microscopy (Bradley et al., 2015; Bradley et al., 2014). However, widefield microscopy has limitations when it comes to analysing GLUT4 translocation. The most important limitation is the spatial resolution. When fluorescent labelled specimens are viewed under the widefield microscope, the light emitted, penetrating the full depth of the specimen, creates an out-of-focus haze equivalent to the wavelength of the light (>100nm). This results in lower spatial accuracy and overlap between structures, where there is no overlap (Harvath, 1999; Shaw, 1994). In both the

post-absorptive and postprandial state, GLUT4 resides intracellularly in perinuclear regions and is continuously moving between intracellular compartments and the plasma membrane at relatively slow rates in the post-absorptive rate and rapidly redistributed to the plasma membrane in the presence of insulin (Lauritzen et al., 2006; Ploug et al., 1998). Intracellular compartments that have been identified as GLUT4 carriers are early endosomes (EE), endosome recycling compartments (ERC), trans-golgi networks (TGN) and GLUT4 storage vesicles (GSV) (Foley et al., 2011). Ploug et al. (1998) showed that in rat skeletal muscle clusters of GSV's are observed within 0.5 µm of the plasma membrane. However, GLUT4 is only effective for glucose transport when fully incorporated in the plasma membrane. Given the shortcomings of widefield microscopy it is unclear whether reported GLUT4 translocation reflects actual insertion into the plasma membrane or the number of cellular structures in close proximity to the plasma membrane that contain the transporter.

The transmission electron microscope (TEM) has higher spatial resolution (as high as 0.3nm) compared to the widefield microscope and could therefore be introduced to overcome the limitations that are accompanied with widefield microscopy (Massover, 2011). Studies in animals have used TEM to assess GLUT4 translocation in adipose tissue (Malide et al., 2000; Slot et al., 1991b) and skeletal muscle of rats (Ploug et al., 1998) or transgenic mice (W. Wang et al., 1996) using antibody-binding with gold particles (immunogold), detectable by the electron microscope. Findings showed a sevenfold increase in GLUT4 translocation in the plasma membrane of rat skeletal muscle upon insulin stimulation (Ploug et al., 1998). However, these results were obtained under extremely high concentrations of insulin (20U), which do not correspond to physiological conditions *in vivo*. Infusion of lower insulin concentrations

(20 mU/kg/min) resulted in a threefold increase in plasma membrane GLUT4 in skeletal muscle of transgenic mice (W. Wang et al., 1996). Thus far, only one study has used immunogold TEM to measure GLUT4 translocation in humans (Friedman et al., 1991). Interestingly, GLUT4 was not detected in the plasma membrane after insulin stimulation, but was observed in the transverse tubules and terminal cisternae (Friedman et al., 1991).

In order to gain further understanding on the magnitude of GLUT4 translocation by overcoming spatial accuracy limitations in human skeletal muscle *in vivo*, during this Ph.D., attempts were made to develop and validate TEM with immunogold staining as a methods to quantify GLUT4 translocation in the plasma membrane. Here, the process of the trialling will be evaluated.

5.3. Methods

The general methods can be found in Chapter 3. Corresponding paragraphs are **3.7, 3.9.**

5.3.1. Human skeletal muscle collection

Briefly, after applying local anaesthesia (1% Xylocaine), skeletal muscle samples were obtained from two specimens from the *m. Vastus Lateralis* using a Bergstrom biopsy needle as described in chapter 3. One samples was harvested from T2DM human skeletal muscle (56.3 yr) and one from a young healthy adult (25.3 yr). To increase the likelihood to detect GLUT4 in the plasma membrane, the skeletal muscle from the T2DM was obtained after one hour into a 80 mU.kg^{-1}.min^{-1} hyperinsulinaemic isoglycaemic clamp. Skeletal muscle in the young healthy adult was obtained one hour after ingestion of a mixed meal (283 kcal; 73%/11%/12%; Carbohydrate/Fat/Protein).

5.3.2 Immunogold protocol

Samples were handled for two processing protocols. For the first protocol, samples were put in 0.1M cacodylate buffer containing 3% paraformaldehyde and were left at room temperature for two hours until samples were transferred into 0.1 M sodium cacodylate buffer (CB) and stored at 4^0C until further analysis. For the second processing protocol, samples were fixed in in Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, gasified with 5% CO_2/95% O_2 for 30 minutes) containing 24 mg procaine (Sigma-Aldrich, P9879) for five minutes (Ploug et al., 1998), transferred to Zamboni solution (PBS containing 22 g/L paraformaldehyde, 15% saturated picrid acid) for 30 minutes at room temperature, and put in fresh Zamboni for an additional 3.5 h at 4°C (Stefanini et al., 1967). Next, muscle samples were dissected in bundles of single muscle fibres in 0.1M PBS, transferred to 50/50 PBS/glycerol and placed in the fridge for 24 h before storing it at -20°C until further analysis.

5.3.2.1. Pre-embedding protocol

Samples from the 50/50 PBS/glycerol were washed for 5 minutes in PBS. To avoid non-specific binding, samples were blocked in blocking buffer containing 0.01 M PBS, 50 mM glycine, 0.25% BSA and 0.06% saponin for 30 minutes. Next, samples were incubated with primary antibody against GLUT4 (1:200 polyclonal rabbit GLUT4, Abcam, UK, Ab654) for four hours at room temperature. After three washes of five minutes in blocking buffer, samples were incubated with 1:300 secondary goat anti-rabbit fab fragments conjugated with 1.4nm gold particles (Nanoprobes, Inc., NY) for two hours at room temperature. Next, samples were washed twice for one minute in PBS and fixed in PBS containing 2.2% glutaraldehyde for one hour. After a rinse with

distilled water, samples underwent silver enhancement for seven minutes, using HQ

Silver (Nanoprobes Inc., NY) and rinsed again with distilled water. Next, samples were

treated with osmium tetroxide for ten minutes and then dehydrated in graded series of

ethanol (15%, 30%, 50%, 70%, 80%, 90%, 100%) before embedding in epoxy resin.

Samples were embedded horizontally in fresh epoxy resin moulds and cured overnight

at 60℃. Next, sections of ~90 nm were cut using an ultramicrotome (details) and air-

dried.

5.3.2.2. Post-embedding protocol

Samples, stored in 0.1 M sodium cacodylate, were dehydrated in graded series of

alcohol as per pre-embedding protocol, embedded in resin and cured overnight at 60^0C.

80-90nm sections were cut using an ultramicrotome. Next, samples were blocked by

floating the section on 0.01 M PBS, containing 1% BSA for 5 minutes, followed by a

wash in PBS, containing 1% gelatin for 10 minutes, followed by a wash in PBS,

containing 0.02 M glycine for 10 minutes. Next, samples were incubated in primary

antibody (1:200 GLUT4 polyclonal rabbit GLUT4, Abcam, UK, Ab654) for 1 hour,

washed 5 x 1 min in PBS, and incubated in secondary antibody (Protein A, conjugated

with 10nm gold particles) for 1 hour. After washing in PBS, samples were fixed in 1%

glutaraldehyde for 3 min, rinsed in distilled water 5 x 1 min and subsequently stained

with uranyl acetate and lead citrate for 10 min and airdried.

5.3.3. Image Capturing

Sections were placed on a 300 mesh gold grid and viewed under a Transmission

Electron Microscope (Philips CM100 TEM, Eindhoven, The Netherlands). Images were

obtained under light-standardized conditions using a film picture camera (Kodak 4489, Rochester, NY).

5.4. Results

5.4.1. Post-embedding versus pre-embedding protocol

Both post-embedding and pre-embedding variant for immunogold staining were trialled. Post-embedding refers to the situation where antibody incubation is performed after samples are embedded and cured in a resin and sectioned with an ultramicrotome. The advantage of the post-embedding method is that intracellular protein epitopes can be targeted and intracellular localization of GLUT4 could be visualized. The pre-embedding protocol refers to the situation where antibody incubation is performed before samples are being embedded and cured into the resin. This has the advantage that immunolabelling sensitivity is not reduced by potential degradation of the antigen exposed to chemicals used in the post-embedding technique. Moreover, pre-embedding allows for detection of all present antigens in the tissue, whereas in the post-embedding technique only antigens present at the surface of the ultrathin section are available for immunolabelling.

The post-embedding protocol, which was performed on samples fixed in CB, did not show clear staining patterns in intracellular regions of skeletal muscle (**Figure 5.1**), but rather a non-specific pattern. Moreover, the total amount of gold particles was very poor using the post-embedding protocol.
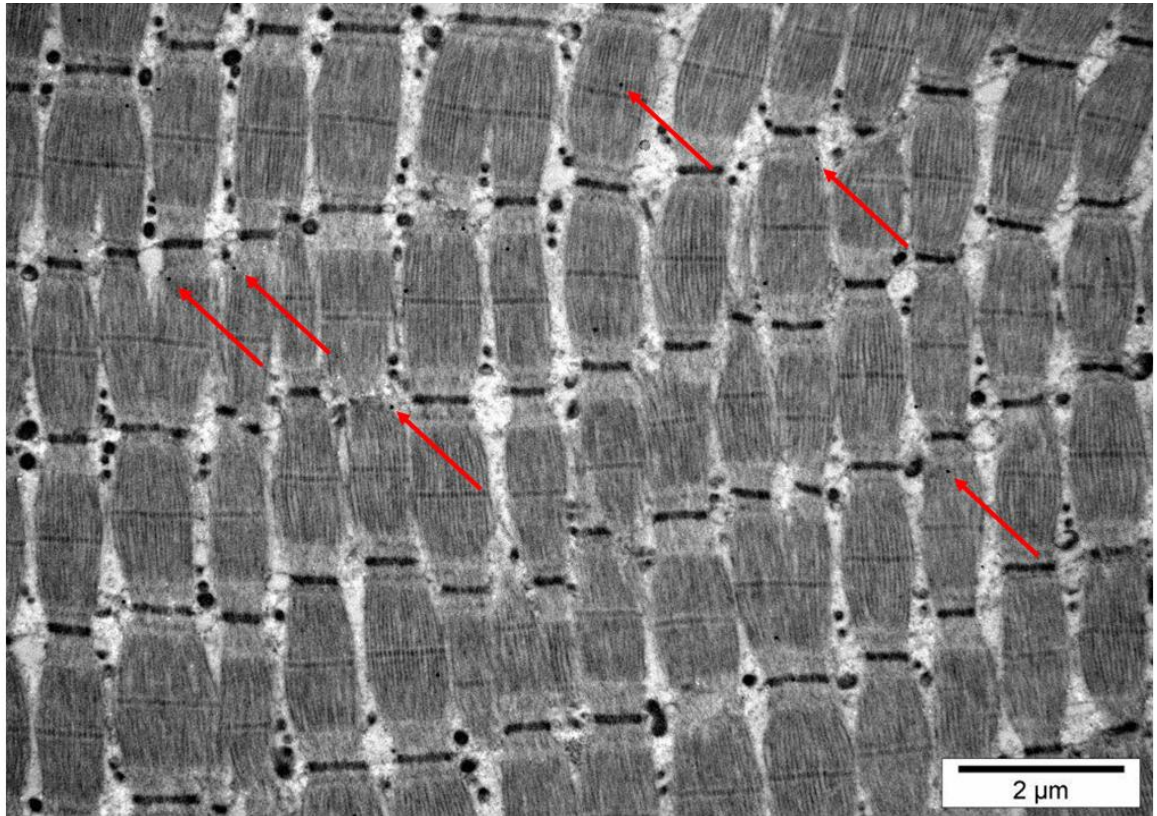
Figure 5.1 - Representative example of GLUT4 antibody binding with post-embedding protocol. Red arrows indicate gold particles.

Therefore, next we tried to develop a pre-embedding protocol. We used bundles of whole single muscle fibres derived from the Zamboni protocol, stored in 50/50 PBS/Glycerol. As these cells are intact, antibodies need to penetrate the plasma membrane to bind to GLUT4, as the GLUT4 antibody (ab654) binds to the amino acid sequence that is located at the C-terminus of the cytoplasmic domain (https://www.uniprot.org/uniprot/P19357). Penetration of the antibody across the plasma membrane is made possible by adding a detergent to the antibody dilution

medium. Saponin is a detergent that only washes cholesterol molecules out of the

plasma membrane. Primary and 1.4 nm conjugated gold secondary antibodies would be
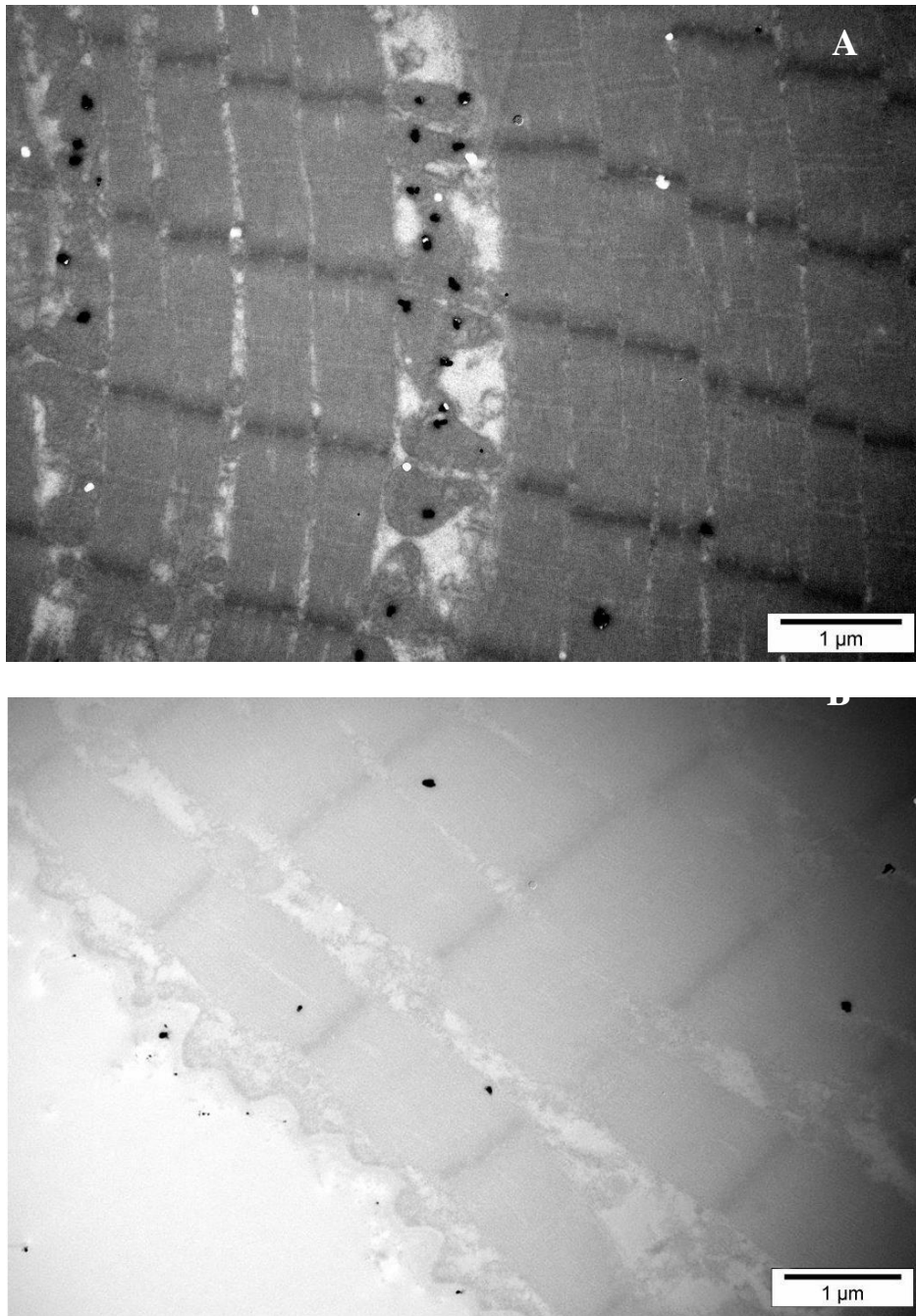
Figure 5.2 - Representative example of the GLUT4 antibody binding with pre-

embedding protocol. 1.4 nm Gold particles underwent silver enhancement to increase

detectability on the electron microscope. A) gold particles were detected at

intermyofibrillar mitochondria. B) No clear gold particles were observed in plasma membrane regions.

able to penetrate through the pores and bind to the GLUT4 epitope expressed on the cytoplasmic domain. After antibody incubation, samples were treated with a silver enhancement kit that nucleates the gold particles which increases the size and hence detectability of the gold particles on the electron microscope. Results derived from the pre-embedding protocol showed a more consistent staining pattern (**Figure 5.2A**) with gold particles associated with mitochondrial compartments, but no staining was observed in the plasma membrane (**Figure 5.2B**).

5.4.2. Negative control samples

A negative control procedure is a standard protocol to evaluate non-specific binding, i.e. whether the secondary antibody only binds to the primary antibody or binds to non-specific proteins. In such protocol, the primary antibody is replaced by the antibody dilution solution, mostly blocking buffer with BSA. As secondary antibodies should only detect and bind to the primary antibody, any gold particles detected under the microscope in the negative control procedure should be the result of non-specific binding of the secondary antibody to an unknown molecule in the cell. If there is non-specific binding present in the cell during the negative control procedure, uncertainty about GLUT4 binding or non-specific binding will compromise obtained results from any sample. After detecting gold particles in skeletal muscle tissue of a healthy subject (**Figure 5.2**), suggesting GLUT4 binding in intracellular regions between myofibrils and in the region of the plasma membrane, a negative control trial was executed, which revealed high non-specific binding in the tissue (**Figure 5.3**).

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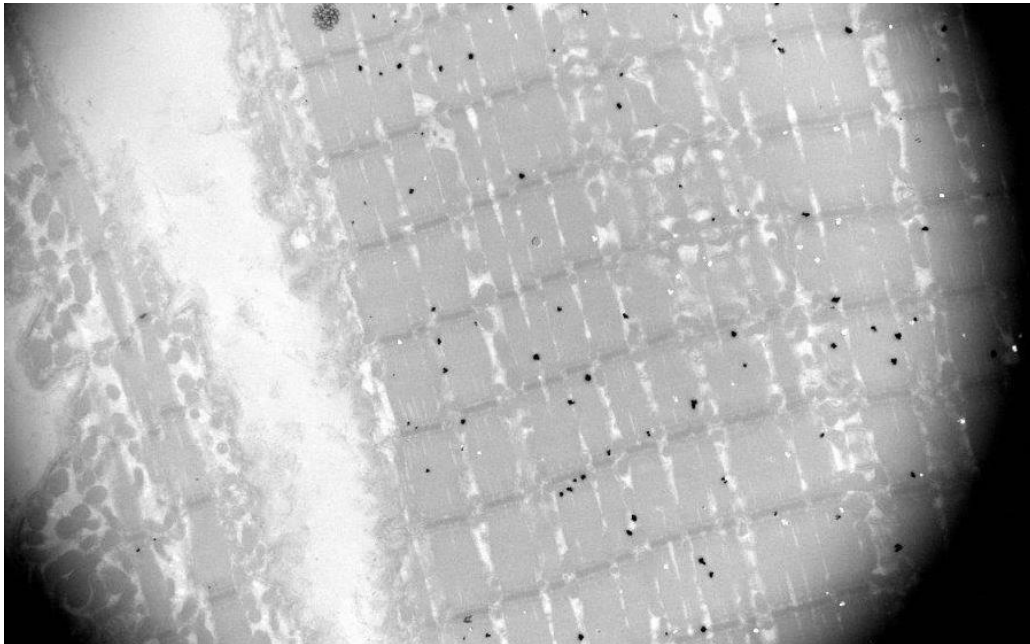
Figure 5.3 – Representative example of a negative control protocol, where primary antibody was removed from the antibody dilution and replaced by 5% BSA.

5.5. Discussion

In the current study, attempts were made to develop and validate a protocol to quantify GLUT4 translocation with the plasma membrane, using TEM to overcome spatial resolution limitations associated with the use of widefield light microscopy. Despite trialling various protocols, we could not establish a reliable protocol with low non-specific binding and therefore aborted the attempts to develop and validate the use of TEM for GLUT4 translocation.

The first protocol was the post-embedding protocol, where samples were cured in resin and cut in ultrathin sections before antibody incubation was performed. The post-embedding protocol did not result in a clear staining pattern and only a very low

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quantity of gold particles was observed throughout the ultrathin section. These results were in line with previous observations from Ploug et al. (1998) who compared GLUT4 quantification between a post-embedding or pre-embedding protocol in rat skeletal muscle and suggested that the pre-embedding protocol was better for antibody detection of GLUT4 in skeletal muscle. Despite post-embedding protocols being used previously in adipose (Slot et al., 1991b) and cardiac tissue (Slot et al., 1991a) in rat, a disadvantage of post-embedding technique is that only antigens present at the surface of the ultrathin section could be detected for immunolabelling, as antibodies will not be able to penetrate through resin and detect antigens that are not exposed to the section surface.

The pre-embedding protocol revealed a more consistent pattern of gold particles binding, with clusters of particles associated with mitochondrial structures residing in between myofibrils (**Figure 5.2A**), but without gold particles associated with the plasma membrane (**Figure 5.2B**). The first observation was somewhat surprising, as literature does not report on GLUT4 localized in mitochondrial structures. Instead, GLUT4 has been reported to be distributed at perinuclear regions, the golgi-apparatus and in vesicles travelling between the intracellular domain and the plasma membrane (Foley et al., 2011; Ploug et al., 1998; Slot et al., 1991a; Slot et al., 1991b). It was therefore unclear whether the staining at the mitochondrial structures was specific or non-specific staining, which needed to be confirmed by running a negative control protocol. Another observation in the pre-embedding protocol was the absence of gold particles at the plasma membrane (**Figure 5.2B**). The skeletal muscle sample for the pre-embedding protocol was harvested 1 hour after ingestion of a mixed meal, containing ~50 grams of carbohydrates. Plasma insulin concentrations have been reported to peak between 30

minutes and 1 hour after ingestion of a mixed meal (Capaldo et al., 1999) or after oral glucose ingestion (Anderwald et al., 2011). Therefore, we expected GLUT4 translocation at the plasma membrane to be present however, this was not observed. Previous findings, using TEM, did not show labelling of GLUT4 in the plasma membrane in the fasted state, but a rapid redistribution towards the plasma membrane occurred after insulin stimulation in rat adipocytes (Slot et al., 1991b), cardiac myocytes (Slot et al., 1991a) and skeletal muscle (Ploug et al., 1998). However, in one study using human skeletal muscle, Friedman et al. (1991) did not observe GLUT4 labelling at the plasma membrane under both basal and insulin-stimulated conditions, but found labelling around the T-tubules and their terminal cisternae. We did not look for GLUT4 labelling in these cellular compartments, as the study design was to validate TEM observations at the plasma membrane with widefield microscopy utilized in this thesis. Thereby, it could be argued that the current study might have missed signal detection at different cellular structures.

A negative control protocol, a common procedure in immunohistochemistry methods, was executed to assess the degree of non-specific binding and to analyse whether the gold particles associated with the mitochondria was a true signal or non-specific binding. Results showed an extremely high degree of non-specific binding in myofibrillar regions (**Figure 5.3**). As the high background noise would interfere with interpretation of the results, we concluded that the current protocol was not reliable and therefore the attempts to validate and quantify GLUT4 translocation using TEM were aborted.

5.5.1. Future recommendations to establish GLUT4 translocation using TEM in human skeletal muscle

Time constraints prevented us from making further attempts to establish a reliable protocol for GLUT4 translocation using TEM. However, based on the observations from the current protocols, we could provide recommendations for any future attempts. Firstly, based on experiments conducted by Ploug et al. (1998) who compared a post and pre-embedding protocol, it would be recommended to use the pre-embedding protocol. To maintain cell integrity, it would be recommended to not use a strong detergent to access the cell interior. Saponin could be considered as added detergent to keep cell membranes intact, as saponin exclusively removes cholesterol groups in the plasma membrane. Consequently, openings in the cell membrane are small, which prevents larger molecules to penetrate into the cell interior. However, based on our observations and others (Ploug et al., 1998), primary antibodies and 1.4 nm gold-conjugated secondary antibodies are able to pass the cell membrane, suggesting that these molecules are able to penetrate through the plasma membrane.

Antibody incubation times could be extended to provide antibodies with more time to detect and bind to the protein of interest. Here we incubated primary antibodies for 4 hours at room temperature and secondary antibodies for 2 hours at room temperature, which are standard incubation times for immunohistochemistry. However, others have performed incubations of primary antibodies overnight at 4^0C (Friedman et al., 1991; Ploug et al., 1998; Ralston et al., 1999), which could be tested in future trials.

Another point of attention could be washing times that are performed in between antibody incubations. In the pre-embedding protocol, we washed samples for 3 x 5

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minutes after primary antibody incubation and 2 x 1 minute after secondary incubation. It could be argued that these washing times did not provide sufficient time for the unbound antibodies to escape the cell interior through the holes in the plasma membrane, with the consequences of being detected under the TEM, whilst not bound to the target protein and therefore causing high background noise. This could explain why we observed the presence of gold particles in the negative control protocol, as secondary antibodies would not have been able to be washed out of the cell. Therefore, longer washing times could be recommended for future trials to establish whether this would increase signal specificity.

5.5.2. Conclusion

In conclusion, we were not able to develop and validate a reliable protocol for the detection of GLUT4 translocation within the plasma membrane using TEM. Until a reliable protocol is established for GLUT4 translocation using TEM, methods with lower spatial resolution have to be utilized to investigate the trafficking of GLUT4 in response to insulin in populations where GLUT4 translocation is impaired, such as type-2 diabetes mellitus.

CHAPTER 6

GENERAL DISCUSSION

6.1. Introduction

Over the last decade, data indicate that there is a concerning increase in the global prevalence of T2DM (WHO, 2016). Decreased physical activity and excess caloric intake are proposed to be at the core of the development of insulin resistance leading to tissue lipid accumulation and multiple biochemical and cellular changes linked to impaired disposal of glucose into insulin-sensitive tissues (**Figure 2.3**). Skeletal muscle has the greatest contribution in the postprandial uptake of glucose with evidence showing approximately half of all glucose is disposed towards skeletal muscle after ingestion of a glucose load or a mixed meal in healthy adults (Capaldo et al., 1999; Katz et al., 1983). In T2DM, glucose uptake into skeletal muscle is impaired due to insulin resistance (DeFronzo et al., 1985). Therefore, intervening upon the skeletal muscle tissue to attenuate insulin resistance is likely to have meaningful impact effect on capacity for glucose uptake.

Exercise training is an effective strategy to improve skeletal muscle insulin sensitivity as it reverses the maladaptations in skeletal muscle associated with decreased physical activity in T2DM (**Figure 2.3**) via increasing muscle mass (Egger et al., 2013), increasing mitochondrial content (Meex et al., 2010), altering intramyocellular lipid composition (Meex et al., 2010; Schenk et al., 2007) and increasing microvascular function (Mortensen et al., 2018; Prior et al., 2014). These adaptations to exercise training improve the delivery of insulin and glucose to the muscle fibre and improve the insulin signalling cascade which leads to improved expression of glucose transporter 4 (GLUT4) which facilitates glucose transport (Dela et al., 1994; Holten et al., 2004).

Post-exercise protein consumption has been shown to accelerate processes involved in adaptive remodelling of skeletal muscle to an exercise stimuli via increased muscle protein synthesis rates in young and older people (Cermak et al., 2012), after both endurance (Rowlands et al., 2015) and resistance exercise (Witard et al., 2014). Additionally, protein ingestion after exercise increases upregulation of the transcriptome involved in energy metabolism (Rowlands et al., 2011) and inflammation-mediated muscle growth processes (Rowlands et al., 2016). Therefore, consuming protein after exercise could be proposed as an adjunct therapy to exercise to further improve skeletal muscle function, which may include insulin sensitivity and glucose uptake. However, to date, there is limited evidence to support additional benefits of consuming supplemental dietary protein after exercise in T2DM to further improve insulin sensitivity as reviewed in **Chapter 2.9**.

One possible explanation for the observation in Chapter 2 could be that thus far, only milk-derived proteins, including whey protein (Gaffney et al., 2017), have been investigated as an adjunct therapy to exercise. However, from feeding studies, there is emerging evidence that improvements in insulin sensitivity might be dependent on the type of protein i.e. the amino acid composition of the protein (Ouellet et al., 2007). Ouellet et al. (2007) demonstrated improved insulin sensitivity in insulin-resistant humans after a 4-week diet containing cod protein compared to a diet containing beef, pork, eggs and milk protein, which the authors attributed to the higher arginine and lower branched-chain amino acid content in the cod diet. Therefore, the overall aim of the research described in the thesis was to investigate the effect of ingestion of proteins with different amino acid composition additional to a fourteen-week exercise training intervention on skeletal muscle plasticity and insulin sensitivity. Specifically, the thesis

focussed on rate-limiting mechanistic changes in skeletal muscle glucose uptake (GLUT4 translocation and microvascular function in response to insulin) of humans with T2DM. A novel keratin-derived protein was blended with whey and compared against whey protein and a control group ingesting an isoenergetic whilst participants followed a similar exercise protocol for fourteen weeks.

6.2. Novel findings and relevance to existing literature

Throughout the thesis, several novel findings were found, which could potentially have an impact for future treatment therapies in T2DM.

6.2.1. Glycaemic control after fourteen weeks of post-exercise protein ingestion

For the first time, it was shown that fourteen weeks of twice-daily consumption of a 20 grams WDP-whey blend protein (40 g/d, ratio WDP-Whey: 17/23 g), in addition to exercise training resulted in a substantial improvement in whole-body glucose disposal, (GCR) as a measure of acute glycaemic control and insulin sensitivity, compared to consumption of whey protein or an isoenergetic control supplement. Previous researchers have failed to find clear improvements in insulin sensitivity when protein consumption was added to exercise compared to exercise alone combined with a placebo supplement (Francois et al., 2017; Gaffney et al., 2017; Wycherley et al., 2010b), so the results of the current study could have a meaningful impact for future research and treatments in T2DM.

The study in the thesis was designed to manipulate dietary protein intake after exercise. As the exercise-training program in the current study was similar for each participant with intensities progressively increasing at fixed rates relative to participant's baseline, the *a priori* hypothesis was that the difference in amino acid composition, with relative

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high concentrations of amino acids cysteine, glycine and arginine in the WDP arm compared to WHEY and CON, was the factor that would lead to between-group effects in outcome parameters via cellular redox-mediated (i.e. glutathione synthesis) adaptations. In line with this hypothesis, the probability of a beneficial effect of WDP on glucose clearance rate (GCR) compared to WHEY and CON was likely. However, additional data produced by Gram et al. (Appendix F) contradicts the hypothesis by showing a substantial reduction in fasting skeletal muscle glutathione concentrations and increased oxidised peroxiredoxin 2 in the WDP compared to WHEY and CON in both humans and rats. Of note is the large range for fasting skeletal muscle glutathione concentrations at baseline in WDP, which could be an error of the measurement and therefore does not reflect the true effect of WDP on glutathione. However, since baseline measures were used as a statistical covariate and a very likely increase was observed in oxidised PRX2 in WDP compared to WHEY and CON, indicates a modulation towards a pro-oxidative environment. Currently, it is unclear how an increased cellular oxidative environment would improve insulin sensitivity and this warrants further investigations.

Within-group changes for WHEY and CON showed improvements in glucose disposal relative to the smallest worthwhile clinical change of 5.4% (Derosa et al., 2009), however the between-group contrast was unclear. This aligns with previous findings, suggesting no clear benefit of whey protein ingestion compared to exercise alone (Gaffney et al., 2017). The current study used a fixed rate of progressive exercise intensity (1%-2% per week based of individuals baseline W_{max}), whereas Gaffney et al. (2017) increased the exercise intensity based on individuals fortnightly performance, which could have interfered with the effects of whey due to variability in individuals

exercise responses. Using a fixed rate would have increased the sensitivity of the action of whey on glucose disposal. However, the different approach in the current study found similar, unclear changes in glucose disposal between WHEY and CON. Acute responses to insulin in T2DM, such as glucose disposal rates have previously been demonstrated to be inversely associated with HbA_1c as a measure of systemic glucose homeostasis (Lindmark et al., 2006). Therefore it would be expected that improvements in glucose disposal would be accompanied by improvements in HbA_1c.

In the current thesis, HbA_1c substantially improved in both CON (-12.3 mmol/mol) and WDP (-8.7 mmol/mol) relative to the smallest clinical important effect of -5.5 mmol/mol (Lenters-Westra et al., 2014). Meanwhile, changes within WHEY (-2.5 mmol/mol) where inconclusive. Additionally, in the between-group analysis HbA_1c in WHEY was likely and possibly different compared to CON and WDP and a likely trivial difference was found between CON and WDP, suggesting an inhibition in exercise-induced improvements in systemic glycaemic control when exercise is combined with protein ingestion. These observations do not align with previous research, which suggest a positive or neutral effect of whey protein on HbA_1c. Francois et al. (2017) found unclear effects of high-intensity interval exercise for 12 weeks combined with ingestion of milk protein or a carbohydrate placebo on HbA_1c in T2DM. Additionally, multiple lines of evidence have reported that whey protein has positive effects on HbA_1c in T2DM when ingested prior to a meal (Jakubowicz et al., 2017; Ma et al., 2009; Watson et al., 2018) because of lower gastric emptying rates and thereby reducing postprandial blood glucose levels (Bjornshave et al., 2018), however these studies did not combine whey protein intake with exercise.

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One suggestion which might explain the likely and possible difference for WHEY and WDP compared to exercise-induced (Control) improvements in HbA_1c is the positive association between increased levels of plasma branched-chain amino acids (BCAA) and the development of T2DM (Flores-Guerrero et al., 2018; Giesbertz et al., 2016; Saleem et al., 2019). Moreover, in a meta-analysis Okekunle et al. (2019) reported that higher dietary intake of BCAA was associated with an increased risk in developing T2DM. Whey protein contains relatively high concentrations of BCAA and leucine (**Table 3.1**) compared to soy and casein protein (Phillips et al., 2009). Leucine is a BCAA and has been identified as a nutritional activator of muscle protein synthesis via mTORC1 activation (Anthony et al., 2000). However, mTORC1 has also been demonstrated to act as a negative feedback loop on insulin signalling via serine phosphorylation of insulin receptor substrate 1 (IRS-1) in skeletal muscle, thereby inducing insulin resistance (Newgard et al., 2009). It could be argued that chronic ingestion of leucine in both the WDP and WHEY, with higher concentrations of leucine in WHEY, resulted in repeated increases in plasma BCAA concentrations and thereby overactivation of the skeletal muscle mTORC1/S6K1 pathway (Tremblay et al., 2005), thereby suppressing exercise-induced improvements in HbA_1c (CON) however, no measures of mTORC1 or plasma BCAA concentrations were taken in the current thesis to confirm this argument. Analysing mTORC1 with Western blot analysis or measuring plasma BCAA concentrations using mass spectroscopy would have provided some further insights in a potential role of dietary protein on HbA_1c.

6.2.2. Insulin-stimulated GLUT4 translocation after fourteen weeks of post-exercise

protein ingestion

In the current study, insulin-stimulated GLUT4 translocation in skeletal muscle was

measured in response to combined exercise and dietary interventions in humans with

T2DM. Chapter 4 provided evidence that insulin-stimulated GLUT4 translocation was

substantially improved in all three groups after the 14-week intervention and that the

improvement was likely higher in the WDP compared to WHEY or CON. Skeletal

muscle insulin-stimulated glucose transport, which is facilitated by GLUT4

translocation, is positively correlated to whole-body glucose uptake (Zierath et al.,

1996). Therefore, improvements of GLUT4 translocation would be expected to be

associated with improvements in whole-body glucose disposal and systemic glucose

homeostasis. However, using simple linear regression to compare regression slopes,

associations between improvements in GLUT4 translocation and GCR or HbA$_1$c were

unclear both within and between groups. The absence of an association might be

explained due to limited sample size (n = 9 per group). Alternatively, other

mechanisms, such as improvements in liver glucose uptake (Honka et al., 2018) or

skeletal muscle microvascular function (Barrett et al., 2009)(discussed below in **section

6.2.3**), may play a more dominant role in improvements in glucose uptake and systemic

glucose homeostasis.

Previous studies have recorded GLUT4 translocation after insulin stimulation (Zierath

et al., 1996) or glucose ingestion (Bradley et al., 2015) on a cross-sectional cohort, or

have measured changes in total fasting GLUT4 protein expression in response to both

strength (Holten et al., 2004) and endurance exercise training (Dela et al., 1994;

O'Gorman et al., 2006). However, fasting GLUT4 protein expression might not be an

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accurate measure for skeletal muscle insulin sensitivity, as Pedersen et al. (1990) and Handberg et al. (1990) found no differences in fasting GLUT4 expression *in vivo* between healthy and early T2DM. In contrast, GLUT4 translocation in response to insulin is a key step in glucose transport and is impaired with insulin resistance in T2DM compared to healthy humans (Zierath et al., 1996). Future studies investigating changes in GLUT4 in response to an intervention, should therefore consider the assessment of change in GLUT4 translocation upon insulin stimulation. This can be done, as executed in the current thesis, by taking muscle biopsies before and after an intervention in both the fasted and insulin-stimulated state, measure plasma membrane or T-Tubules GLUT4 content and compare the change in GLUT4 content from fasted to insulin-stimulated state as a proxy for GLUT4 translocation before and after the intervention to assess changes in insulin-stimulated GLUT4 translocation.

In summary, the current findings give indications that exercise and co-ingestion of WDP protein may restore insulin-stimulated GLUT4 translocation in skeletal muscle of T2DM compared to WHEY and CON, which might partially contribute towards increased whole-body glucose disposal with WDP treatment relative to CON and WHEY.

6.2.3. Insulin-stimulated skeletal muscle microvascular function after fourteen weeks of post-exercise protein ingestion

For the first time, the effects of twice-daily consumption of a 20 grams dietary protein (40 grams per day), in addition to exercise training on microvascular function were investigated. Chapter 4 provided evidence that insulin-stimulated muscle blood flow, but not blood volume, was very likely and likely improved with WDP supplementation

compared to WHEY and CON respectively (**Table 4.3**). Moreover, insulin-stimulated blood flow in WDP was positively associated with improvements in whole-body GCR. Previously a strong correlation was found between changes in blood flow and whole-body glucose uptake (Baron et al., 1990). This suggests that improvements in whole-body insulin sensitivity are partially explained by improvements in microvascular function with WDP. Capillarization of the skeletal muscle microvasculature as expressed as sharing factor, capillary counts, capillaries-per-fibre and capillary-to-fibre perimeter exchange improved within all groups, with unclear between-group differences. It would be expected that an increase in capillarization would also increase insulin-stimulated blood volume, however only in CON did insulin-stimulated muscle blood volume increase relative to baseline, an increase which was likely higher compared to WDP. Insulin-stimulated blood volume measures were obtained as the difference between fasting blood volume and 2 h after insulin stimulation. However, evidence derived from rat studies have shown that insulin produces a response in blood volume via capillary recruitment after 5-10 minutes (Vincent et al., 2004). Sampling of blood volume at only two time points might have missed a potential early response in muscle blood volume changes, although Coggins et al. (2001) showed that microvascular blood volume in skeletal muscle of healthy humans was elevated 4 h after insulin infusion. Lastly, enzymes involved in controlling nitric oxide (NO) bioavailability provide some limited evidence that fasting endothelial nitric oxide synthase (eNOS) phosphorylation at serine 1177 residue was possibly increased in WDP and WHEY compared to CON. Additionally, WHEY produced possible improvements on insulin-stimulated eNOS^{ser1177} phosphorylation and likely improvements when normalized to NOX2 but with unclear difference compared to

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CON and WDP. Collectively, these findings suggest that WDP ingestion in conjunction with exercise therapy might have beneficial effects on microvascular function compared to CON and WHEY, which overall would have contributed to improved whole-body glucose uptake. However, enzymatic activity of proteins controlling NO bioavailability did not provide clear a clear explanation for the observed improvements in muscle blood flow.

The findings in the current thesis extend on currently limited evidence that protein ingestion, by providing arginine as a precursor for NO synthesis, might improve endothelial function (Fekete et al., 2016; Lucotti et al., 2006; Monti et al., 2013; Piatti et al., 2001), however, no measurement was taken in the current study to confirm increased levels of NO bioavailability. The addition of measurements including plasma NO_x, cGMP and L-arginine as described previously (Lucotti et al., 2006; Monti et al., 2013; Piatti et al., 2001) would have provided further insights in the mechanisms of WDP-related improvements in microvascular function.

6.3. Comments and limitations on methodology in relation to the published literature

6.3.1. Study design

One limitation in the current thesis was the relative low number of study participants. Although, the study was powered to detect clinically meaningful effects on whole-body glucose disposal, which required n = 12 participants per group, larger follow up studies are needed to increase the confidence and statistical power on the primary outcomes and test for reproducibility. Post-hoc sample size calculations, using published spreadsheet (http://www.sportsci.org/2006/wghss.htm) produced n = 16 per group to detect a change

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in GCR according to the current study (WDP-CON: 25.8% (-3.6, 66.3)) with within-subject variability of 35.2% and a between-subject variability of 46.9%. Moreover, for the skeletal muscle immunofluorescence analysis, participant numbers were lowered (n = 9-10 per group) due to several reasons including participant's rejection to a biopsy or loss of tissue in the follow up to the analysis. Including more participants and powering for the within-subject error involved in the mechanistic parameters in follow up studies would increase the statistical power on the molecular mechanisms that might explain the benefits of ingesting the WDP.

Another limitation in the current study is the lack of a non-exercising, non-dietary intervention control group. We combined the use of keratin-derived protein and whey with exercise training and compared it against whey and exercise or an exercise control group. A few outcomes only demonstrated within-group changes, but with unclear effects between groups, including capillarization and eNOS[ser177]/NOX2. However, within-group changes are largely meaningless, because they are not compared against a fully controlled contrast group. Presentation of significant within-group changes has been reported to lead to misinterpretation of results and misleading communication (Bland et al., 2011a, 2011b). Implementing a non-exercising, non-dietary intervention group would have enabled the study to compare these within-group changes against a group where no change would have been hypothesized to occur. However, the approach in the current study was applied in order to expose all participants to an intervention (i.e. exercise) that is established to improve insulin sensitivity. Previously, it has been reported that particularly Maori and New Zealand Polynesian participants would find it unethical to be randomized into a group, which would unlikely improve health outcomes (McAuley et al., 2003; Sukala et al., 2012). Since the study as designed to

include multiple ethnicities, including Maori and Polynesian participants, considerations had to be made to overcome these ethical questions. One option would have been to implement a crossover design where, additional to the intervention, participants would be followed for fourteen weeks without a nutritional or exercise intervention. However, due to time and budget constraints, a crossover design was not feasible for the current study. Therefore, the study design in the current trial was therefore not able to investigate the effects of chronic isolated WDP intake on insulin sensitivity and compare it with a non-exercising, non-dietary intervention group.

6.3.2. Immunofluorescence analysis

The current thesis mainly used immunofluorescence technique and imaging with a widefield microscope whilst analysing skeletal muscle sections in the transverse, cross-sectional orientation. In paragraph 2.10, a brief commentary was provided on various methods to measure GLUT4 translocation with the plasma membrane in skeletal muscle. Recently, Bradley et al. (2015) developed an immunofluorescence method for GLUT4 translocation to the plasma membrane where GLUT4 was colocalized with the plasma membrane marker dystrophin using a Pearson's correlation coefficient (PCC) analysis. With this method, the authors demonstrated an increase in GLUT4 co-localization with dystrophin after 30 minutes of exercise and 20 minutes after glucose ingestion. The current thesis adopted this similar method, demonstrating a very likely improvement in GLUT4 translocation in the WDP, but no effects in the other groups or between treatments (**Table 4.6**). Although PCC is used frequently in immunofluorescence image analysis (Dunn et al., 2011), caution is warranted with the use of this analysis model. PCC measures the pixel intensity in two colour channels (i.e.

green GLUT4 and red dystrophin) and compares the intensity of one pixel to the average intensity of the whole image (equation 1),

Equation 1: $$PCC = \frac{\sum i (\text{Ri}-\bar{R}) \times (Gi-\bar{G})}{\sqrt{\sum i\ (Ri-\bar{R})^2 \times \sum i\ (Gi-\bar{G})^2}}$$

Where Ri and Gi refer to the intensity values of the red and green channels respectively of pixel i, and R̄ and Ḡ refer to the mean intensities of the red and green channels across the entire image (Dunn et al., 2011). From this equation, the PCC exists between -1 which means anti correlation and +1 which means a perfect correlation. Herein lies a first limitation of the PCC method: the method is qualitative and has limited detection in the magnitude of increases in colocalization due to the -1 to +1 range. This is in contrast with studies using semi-quantitative measures, including density-derived numbers from western blots or immunofluorescence measuring mean fluorescent intensity as these are able to investigate the magnitude of change.

Secondly, using PCC for cross-sectional analysis of skeletal muscle must be taken with caution. The largest area covered in an image with cross-sectional orientation is the intramyocellular area and will therefore largely determine the mean intensity across the entire image. Since both dystrophin and GLUT4 reside close to the plasma membrane, it is likely that individual pixels in the intramyocellular space have lower intensities to the mean image intensity. However, according to equation 1, when a pixel in the red and green channel are both lower than the mean intensity, there is a positive correlation. So, as the intramyocellular area covers the largest area of an image, there may be a positive correlation already, leaving only small, possibly undetectable margins for changes in colocalization in areas where the pixel intensity in both channels are higher than the

mean i.e. the plasma membrane. This is even more important when measuring co-localization in skeletal muscle in response to an exercise training intervention, as hypertrophy, observed during resistance exercise training, further increases the intramyocellular area. One option to eliminate the interference of intramyocellular space in correlation is to only measure PCC above a defined intensity threshold where only pixels are included that at least in one channel are higher than the threshold. ImageJ has an algorithm for this option (Coloc2), which has been used in this thesis to analyse GLUT4 translocation (unpublished analysis), however, the algorithm selected highly inconsistent thresholds within the three images per sample and was considered unreliable. Therefore, in Chapter 4 we opted to measure mean fluorescent intensity of GLUT4 in regions that were positive for dystrophin staining as a semi-quantitative method to measure insulin-stimulated GLUT4 translocation with the plasma membrane. This method provided evidence that all three treatments improved GLUT4 translocation after the intervention with substantially larger improvements in the WDP compared to exercise and whey protein.

However, as described in paragraph 2.10 of this thesis, using widefield microscopy with immunofluorescence has spatial accuracy limitations. Studies have shown that GLUT4 storage vesicles reside within 100 nm of the plasma membrane, which is beyond the spatial accuracy that widefield microscopy can produce. Therefore, in this thesis attempts were made to use electron microscopy combined with immunogold. This method has been utilized to measure GLUT4 translocation to the plasma membrane of skeletal muscle in rodents where it showed a several-fold increase in GLUT4 translocation upon insulin stimulation and contraction-stimulated GLUT4 translocation. In Chapter 5, various experiments were conducted to develop a reliable protocol for *in*

vivo skeletal muscle GLUT4 translocation using immunogold electron microscopy with the aim to validate and compare this method against the immunofluorescence method utilized in Chapter 4. However, extensive trialling did not result in the validation of a reliable protocol. Future research could further trial the immunogold electron microscopy method based on recommendation provided in Chapter 5, as this technique might produce more detailed information on the process of GLUT4 translocation to the plasma membrane in skeletal muscle.

6.4. Practical implications of the current findings

The current study has provided several strong implications, which could be of relevance to society. Primarily, the current thesis has provided novel evidence that WDP has the potential to be introduced as an adjunct therapy to exercise for the treatment in T2DM and could therefore be considered as a nutritional replacement to drug therapies, including standard metformin treatment.

WDP is produced from sheep wool, which means it is a sustainable source of protein via shearing. Particularly in New Zealand, the wool industry plays a major part in the economy and the introduction of the production of WDP could further enhance the revenue derived from sheep wool. Additionally, the costs for production of WDP (<$10/kg protein) has been estimated to be half compared to whey protein (~$25/kg). Lastly, the environmental impact for WDP production from sheep, while still a ruminant producing methane, is suggested to be lower (Dougherty et al., 2019) compared to the dairy industry, which produces whey protein that is derived from cow milk that is known for its large production of greenhouse gasses and carbon dioxide emission (Hagemann et al., 2012).

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In contrast to WDP, whey protein in conjunction with exercise did not demonstrate a further improvement in exercise-induced insulin sensitivity, apart from possible improvements in enzymatic activity of proteins controlling NO bioavailability. Moreover, the whey group in the current study demonstrated an inhibition of exercise-induced improvements in chronic glycaemic control i.e. HbA$_1$c. Although the mechanisms by which whey protein might suppress these improvements are currently unknown and therefore warrant further investigations, the practical implications for the use of whey protein in conjunction with exercise in T2DM would currently be unsubstantiated.

6.5. Suggestions for future research

Until now, previous studies combining exercise training and post-exercise protein ingestion in T2DM found no benefit of post-exercise protein ingestion on insulin sensitivity compared to exercise alone. The current thesis provides novel explorative evidence that post-exercise ingestion of proteins with a different amino acid composition may improve insulin sensitivity in T2DM. These findings could open up avenues for future research to elaborate on the post-exercise protein ingestion paradigm.

Firstly, future research should focus on the molecular mechanisms that might explain superior adaptations in insulin sensitivity with consumption of a WDP. In the current thesis, we demonstrated that there was a likely probability that WDP was beneficial for increasing whole-body glucose disposal via increased insulin-stimulated blood flow and GLUT4 translocation compared to WHEY and CON. However, it remains unclear what mechanism underlies these improvements. The *a priori* hypothesis was that the higher concentrations of the amino acids glycine, cysteine and arginine in the WDP protein

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would reduce cellular oxidative stress via increased synthesis of glutathione and improved nitric oxide bioavailability. Oxidative stress has been suggested to induce inflammation that activates cellular kinase activity, inhibiting signalling events in the insulin-signalling cascade (Wei et al., 2008). Indeed, previous research has found that glycine and cysteine supplementation increased glutathione and improves insulin sensitivity in older and T2DM humans (Sekhar et al., 2011). However, results derived from the current study, as part of Dr. Gram's project (Unpublished observations, Appendix F), showed a substantial decrease in skeletal muscle glutathione and an increase in oxidised peroxiredoxin 2 (PRX2) in WDP compared to CON and WHEY, suggesting that improved insulin sensitivity was accompanied by an increase in the oxidative cellular environment. This is a surprising finding, which warrants further investigation for reproducibility purposes and on how a pro-oxidative environment results in favourable cellular adaptations.

Future studies should investigate the quality and acute effects of isolated WDP intake on digestibility and absorption, referred to as the protein digestibility-corrected amino acid score (PDCAAS) (Schaafsma, 2012) or digestible indispensable amino acid score (DIAAS) (Wolfe et al., 2016). These scores quantify the quality of a protein based on the amino acid profile and relative amounts of indispensable amino acids expressed relative to a profile of amino acid requirements (FAO, 1991). Whey protein, used in the thesis, has a PDCAAS score of 1 (Hoffman et al., 2004), meaning it is a high quality protein score meeting amino acid requirements of all indispensable amino acids. Moreover, whey protein is quickly digested and absorbed, resulting in a rapid and potent rise of plasma amino acids (hyperaminoacidemia), which stimulates muscle protein synthesis (Pennings et al., 2011a). The PDCAAS of raw WDP was calculated as

0.24 and in combination with the whey blend 0.58. However, digestion and absorption characteristics of WDP are currently unknown, but warrant further investigation given the possible benefits demonstrated in the current thesis. Digestion and absorption kinetics for WDP would be required to investigate the acute effects of WDP on several metabolic responses, including postprandial peak plasma glucose and area under the curve, which previously has been shown to be reduced after whey ingestion (Jakubowicz et al., 2014; Ma et al., 2009), but also acute changes in redox-related measures, including glutathione synthesis. Studies could be designed where participants ingest a single bolus of WDP after which the fate of WDP could be determined via measuring kinetics as rate of appearance and disappearance of specific amino acids in plasma. Furthermore, WDP could be co-ingested with a glucose load to establish acute effects of WDP on postprandial glucose handling as has been done previously by Jakubowicz et al. (2014) and Ma et al. (2009). Additionally, chronic trials should investigate the effects of isolated WDP intake on insulin sensitivity, as the current study only investigated the combined effect of WDP and exercise, but not WDP supplementation alone.

In line with investigating the effects of WDP ingestion, different types of protein could be compared against each other to further elucidate the hypothesis that the effectiveness of post-exercise protein ingestion on insulin sensitivity in T2DM is dependent on the amino acid composition of the protein. Previous research comparing cod protein to a diet containing beef, pork, milk and eggs in T2DM humans, showed improved insulin sensitivity after four weeks of cod protein ingestion compared to the other diet (Ouellet et al., 2007). It would be of interest to combine the dietary intervention with an exercise intervention to see if this further increases insulin sensitivity. In addition, the

effectiveness of plant-based protein, including soy protein or pea protein, could be combined with exercise, as epidemiologic evidence suggests that high intake of plant-based protein decreases the risk of developing T2DM (Sluik et al., 2019) compared to the consumption of high animal-based protein diet (Ye et al., 2019), although the beneficial effects of plant-based protein intake might be attributed to the higher dietary fibre content (Richter et al., 2015).

Future studies could focus on a dose-dependent relationship and timing of protein consumption. Previously, 20 grams of post-exercise whey protein ingestion immediately post-exercise was found to maximally stimulate muscle protein synthesis in young healthy men, with no further increase with doses higher than 20 grams (Witard et al., 2014). In addition, Areta et al. (Areta et al., 2014) showed that ingesting 20 grams of protein every three hours resulted in the highest rates of muscle protein synthesis compared to smaller, but more frequent or larger, but less frequent portions. However, these studies were performed with unilateral leg exercise (Witard et al., 2014) or bilateral leg exercise. When performing whole-body resistance exercise, 40 grams of whey protein increased muscle protein synthesis to a larger extent compared to 20 grams (Macnaughton et al., 2016). The current study adopted evidence from Witard et al. (2014) and Areta et al. (2013) by providing people with T2DM with 20 grams of protein immediately post-exercise and providing 20 grams of protein in the evening, as some evidence suggests that consuming protein in the evening increases muscle protein synthesis overnight (Kouw et al., 2017). The 20 grams of exercise could be sufficient after the cycling exercise in the current study; however, the exercise training protocol included a whole-body resistance exercise one day per week where potentially higher

intake of protein could have made a larger impact on muscle remodelling and therefore insulin sensitivity.

6.6. Final Conclusions

In conclusion, the thesis has provided the first evidence and indications of potentially beneficial effects of a novel keratin-derived protein as an adjunct therapy to exercise for improving insulin sensitivity in T2DM after a 14-week intervention. Beneficial effects of WDP are improved whole-body glucose disposal and skeletal muscle insulin-stimulated blood flow and insulin-stimulated GLUT4 translocation. These findings have opened up new avenues for further research on dietary protein as an adjunct therapy to exercise to improve insulin sensitivity in T2DM.

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Appendices

Appendix A Search Terms used for a systematic search of the effects of exercise and protein supplementation on glycaemic control in T2DM

PubMed: Exercise[Title/Abstract] AND (diabet*[Title/Abstract] OR metabolic syndrome[Title/Abstract] OR obes*[Title/Abstract] OR insulin sensitivity[Title/Abstract] OR insulin resistance[Title/Abstract] OR glycemic control[Title/Abstract]) AND (protein administ*[Title/Abstract] OR protein intake[Title/Abstract] OR protein consum*[Title/Abstract] OR protein supplement*[Title/Abstract] OR protein ingest*[Title/Abstract] OR protein diet[Title/Abstract])

Web of Science: TI=exercise AND TI=(diabet* OR "metabolic syndrome" OR obes* OR "insulin resistance" OR "insulin sensitivity" OR "glycemic control") AND TI=("protein administ*" OR "protein intake" OR "protein consum*" OR "protein supplement*" OR "protein ingest*" OR "protein diet")

Scopus: TITLE-ABS (exercise) AND TITLE-ABS (diabet* OR "metabolic syndrome" OR obes* OR "insulin sensitivity" OR "insulin resistance" OR "glycemic control") AND TITLE-ABS ("protein administ*" OR "protein intake" OR "protein consum*" OR "protein supplement*" OR "protein ingest*" OR "protein diet")

[1] *The systematic search was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).*

Participant Information Sheet

Optional Future Unspecified Use of Human Tissue

Study title:	Can protein and exercise improve glucose tolerance in older adults with type-2 diabetes?		
Locality:	Wellington and Palmerston North	Ethics committee ref.:	14/CEN/194
Lead investigator:	Assoc. Prof. David Rowlands	Contact phone number:	0800 Massey (63167); 04.801.5799 ext 63295

You are invited to take part in a study looking at the effects of protein supplementation on glucose tolerance in Type-2 diabetics. As part of this study we will collect skeletal muscle tissue before and during the glucose clamp to measure your muscle insulin resistance. Besides being used to answer specific study questions, some tissue will be stored for future unspecified research. Whether or not you agree to this future unspecified use is your choice. If you do not want this tissue to be available for future unspecified use you will still be able to participate in the study, you do not have to give a reason, and it will not affect the care you receive.

This *Participant Information Sheet* will help you decide if you'd like to take part. We will go through this information with you and answer any questions you may have. You may also want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Please feel free to do this.

If you agree to future unspecified tissue use, you will be asked to sign the *Consent Form* at the end of this document. You will be given a copy of both the *Participant Information Sheet* and the *Consent Form* to keep.

This document is 4 pages long. Please make sure you have all the pages.

Why are we doing the study?

This project will focus on people with Type 2 diabetes. The purpose is to determine whether dietary protein supplementation with exercise can be used to improve glucose control. As part of this study we will measure the effect of protein treatment on insulin sensitivity and skeletal muscle health. This study may allow us to identify important target mechanisms for improving glucose control. As such, future analysis may contribute to the development of effective rehabilitation and treatment plans for Type 2 diabetes.

The project coordinator is Assoc Prof David Rowlands, an exercise physiologist in the School of Sport and Exercise. Medical co-ordination will be provided by Dr. Jeremy Krebs, the Director of the Endocrine, Diabetes, and Research Centre at Wellington Hospital.

The study has been approved by the Central Health and Disability Ethics Committee.

What would your participation involve?

If you choose to participate in the research you will be asked to commit to an 17-week study period. Before the training/supplementation starts and after it ends we will collect a total of 4 muscle biopsy samples during two visits at the *Clinical Trials Unit, Wellington Hospital*.

A small muscle tissue sample will be taken from the <u>outer thigh</u> to determine changes in muscle cell structure, function and molecular regulation in order to explain the effects of treatment on glucose metabolism. The sample will be taken under local anaesthetic and then sealed with a butterfly strip. The sample is so small it does not require stitches. A detailed explanation of the biopsy procedure and associated risks will also be provided.

What will happen after the study ends?

At the completion of the study remaining tissue samples will be destroyed unless required for future analysis. Stored tissue samples will be kept in a swipecard access locked laboratory at Massey University. These stored samples will be linked to a personal identity code, which only the research team will have access to. Tissue samples will be stored for 10 years, after which they will be destroyed.

Future analysis may take place at research laboratories in New Zealand, at McMaster University in <u>Canada</u>, the Center for Genetic Medicine Research in Washington D.C., <u>USA,</u> or the Panum Institute, University of Copenhagen, <u>Denmark</u>. Stored samples will not be sent to commercial companies, and will not be used for genetic inheritance, stem cell, or other research.

If requested, we can return the unused and processed tissue samples that are being analysed in New Zealand. However, due to logistics and cost we are unable to return samples that are being shipped overseas. Māori participants may request to have overseas laboratories blessed.

All future unspecified research in New Zealand will be subject to ethical review. However, if the tissue sample is sent overseas, this future research may be considered by an overseas ethics committee without New Zealand representation.

In the event of future unspecified research, a summary of findings can be sent at your request.

What are the rights of participants in the study?

Participation in the study is voluntary and you will have the right to withdraw at any time without any disadvantage to yourself.

At any time, you will also have the right to:

- decline to answer any particular question;

- ask any questions about the study at any time during participation;
- access any personal information collected during the study;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be told of any new information about adverse or beneficial effects related to the study that becomes available during the study that may have an impact on your health

The privacy and confidentiality of participants will be maintained during and after the study. Only the study researchers will have access to personal health information or collected data.

5. **Compensation**

No direct compensation will be provided for this study.

Where can you go for more information about the study, or to raise concerns or complaints?

If you have any questions, concerns or complaints about the study at any stage, please contact:

Dr. Martin Gram, Research Officer

Telephone: 04.801 5799

Mobile: 0221692343

Email: M.Gram@massey.ac.nz

Or

Assoc. Prof. David Rowlands*, Coordinating Investigator*

Telephone: 0800 Massey (63167); 04.801.5799 ext 63295

Email: d.s.rowlands@massey.ac.nz

If you wish to seek Māori support please contact:

Prof. Chris Cunningham, Research Center for Māori Health and Development

Telephone: 04.801.4994.5799 ext. 63242

Email: c.w.cunningham@massey.ac.nz

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

Phone: 0800 555 050
Fax: 0800 2 SUPPORT (0800 2787 7678)
Email: advocacy@hdc.org.nz

You can also contact the ethics committee that reviewed and approved this study on:

Phone: 0800 4 ETHICS

Email: hdecs@moh.govt.nz

Participant Consent Form

Optional Future Unspecified Use of Human Tissue

Study title:	Can protein and exercise improve glucose tolerance in older adults with type-2 diabetes?		
Locality:	**Wellington and Palmerston North**	Ethics committee ref.:	**14/CEN/194**
Lead investigator:	**Assoc. Prof. David Rowlands**	Contact phone number:	**0800 Massey (63167); 04.801.5799 ext 63295**

Declaration by participant:

- I have read, or have had read to me in my first language, this Participant Information Sheet. I understand the information in this Participant Information Sheet. I have had the opportunity to ask questions and I am satisfied with the answers I have received.
- I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.
- I have been given a copy of the Participant Information Sheet and Consent Form to keep.
- I agree to the tissue samples, collected from the outer thigh, being accessible for future unspecified use.
- I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.
- I understand taking part in the study is voluntary.
- I agree to my tissue samples being sent overseas.
- I agree for my tissue samples to be stored and used in future research but only on the same subject as the current research project [give name of current research].
- I acknowledge that I am fully aware that I will not own any intellectual property that may arise from any future research.

If applicable please tick the following box(es):

☐ I would like my tissue samples to be de-identified.

☐ Please contact me for consent prior to the use of tissue for future unspecified research.

Participant's name:

_____

Signature: Date:

_____

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant, and have answered the participant's questions about it.

I believe that the participant understands the tissue samples may be made available for future unspecified and has given informed consent to participate.

Researcher's name:

_____

Signature: Date:

_____

Appendix C Recruitment Pamphlets

For enquires:

Dr. Martin Gram, Research Officer

Telephone: 04.801.5799

Mobile: 0221692343

Email: m.gram@massey.ac.nz

Dear Doctor, Nurse

Can protein and exercise improve glucose tolerance in older adults with type-2 diabetes?

The Massey University College of Health is currently investigating the effect of a novel keratin-derived protein (WDP) on type 2 diabetes health. Recent research suggests that protein supplementation increases muscle mass and glucose control. Previous studies have typically used protein from dairy sources; however, protein from dairy sources is relatively expensive and not environmentally sustainable. Alternatively, the WDP extract is uniquely rich in certain amino acids and minerals that are key components in rebuilding glutathione – an important antioxidant. Supplementing with WDP protein may boost the body's antioxidant defence and improve glucose control. If WDP improves the regulation of glucose in the blood of individuals with type 2 diabetes it will lead to better treatment for Type 2 Diabetes.

The study is a collaboration between: Massey University's College of Health; the Centre for Endocrine and Diabetes Research at Wellington Hospital; the Free Radical Research Group at Christchurch Hospital; and the Department of Anatomy at the University of Otago.

We are seeking to recruit 36 (18 male, 18 female) patients who meet the following study inclusion criteria:

- Male or female aged 35-65
- Diagnosed Type-2 diabetic and not requiring insulin therapy
- HbA1c > 48 mmol/mol

- Over the past 6 months have not achieved a minimum of 150 minutes of moderate to vigorous intensity exercise each week. (American College of Sports Medicine and American Diabetes Association guidelines)
- Exclusion criteria are moderate to severe retinopathy, nephropathy and neuropathy, history of cerebrovascular or cardiovascular diseases
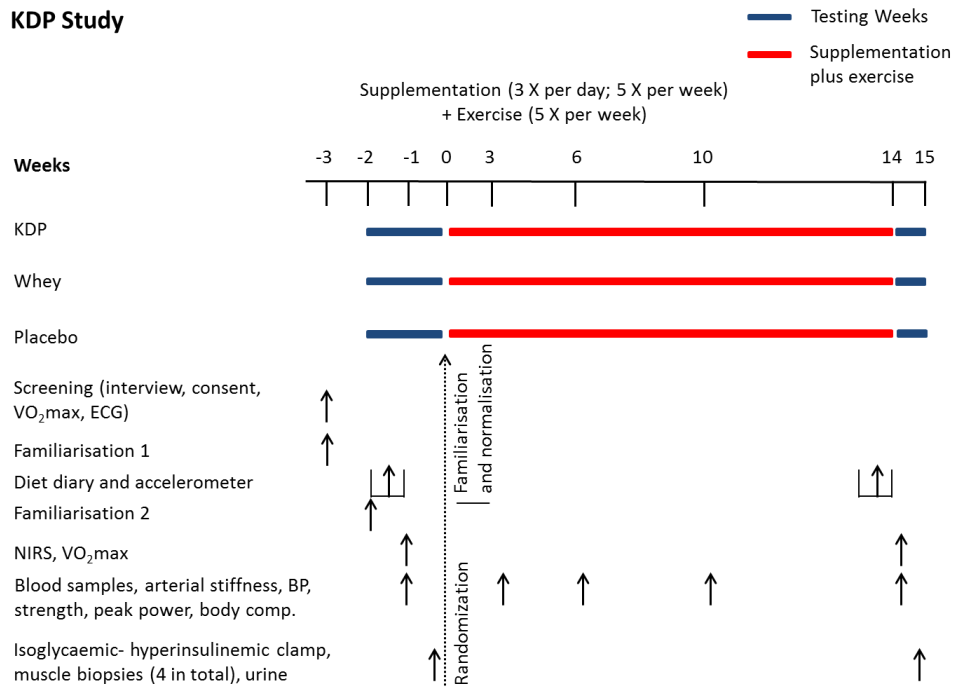
Study Design

The study is a randomised controlled trial. The study duration will be 17 weeks, which includes an initial two testing weeks followed by 14 weeks of supervised intermittent high-intensity exercise (~50-min/day, 5 days/week) and finally the participants will be tested in the last week. Participants will be randomly allocated to 1 of 3 dietary supplementation groups: 1) keratin derived protein 2) whey supplement or 3) placebo (exercise only). See figure below.

What is involved from the practice?

- For practice staff to identify potential participants and invite them to contact us for further information about the study. This can be done in three ways:
  - The best and most efficient way is to identify eligible participants through a database search and send out a personal letter. We can assist with prepaid envelopes; a draft letter and we can even come out and help with the database search.
  - The second best method is for practice staff to mention the study to eligible participants when doing their regular check-up
  - The third most efficient way is for us to hang up flyers and pamphlets in the waiting room
- Once consent has been obtained from potential participants, to share patient health information with the research team.

KDP Study

Legend: Testing Weeks; Supplementation plus exercise

Supplementation (3 X per day; 5 X per week) + Exercise (5 X per week)

Weeks: -3 -2 -1 0 3 6 10 14 15

KDP
Whey
Placebo

Screening (interview, consent, VO$_2$max, ECG)
Familiarisation 1
Diet diary and accelerometer
Familiarisation 2
NIRS, VO$_2$max
Blood samples, arterial stiffness, BP, strength, peak power, body comp.
Isoglycaemic- hyperinsulinemic clamp, muscle biopsies (4 in total), urine

Familiarisation and normalisation
Randomization

Study outline

Benefits of the study to participants?

- 14 weeks of supervised training 5 days per week with clinical exercise physiologists
- Focused exercises targeted at lowering blood glucose levels
- Results from comprehensive health evaluations will be available
- Assistance with travel costs available if required
-

Current findings suggest that each of the protein therapies we will be testing will improve glycaemic control and so far all participant in this study have experienced a decrease in hbA1c (average decrease = 15 mmol/mol, n=8). This decrease is likely caused by improved glucose disposal rate (a recent study from our laboratory (13NTB69) indicates an average 25% improvement). We will provide full support for participants to continue with exercise if they wish following the study. If effective, the study findings may provide a new simple and practical direction for improving diabetic rehabilitation.

Who to contact for more information

The point of contact for this study is Dr Martin Gram, who has a PhD in clinical exercise physiology, or Assoc. Prof David Rowlands, the principle investigator.

I look forward to your involvement in this project.

Yours sincerely

Martin Gram

Dr. Martin Gram

HDEC: 14/CEN/194

Principal investigator Assoc. Prof David Rowlands

Telephone: 04.801.4994.5799 ext. 63295

Mobile: 0272099383

Email: d.s.rowlands@massey.ac.nz

Appendix D Supplement Nutritional Profile

Amino acid composition of raw protein powders

	WDP	Whey (Alacen895, Fonterra, New Zealand)
Protein	78.7	93.5
Cho	0	0.9
Fat	0	0.4
AMINO ACIDS		
Alanine	2.9	4.8
Arginine	7.4	2.0
Aspartic Acid	2.4	9.6
Cysteine*	8.8	2.5
Glutamic Acid	10.3	16.2
Glycine	4.2	1.6
Histidine	0.6	1.6
Isoleucine	3.0	6.1
Leucine	6.2	9.6
Lysine	2.2	8.8
Methionine	0.4	2.0
Phenylalanine	3.4	2.8
Proline	5.7	5.5
Serine	5.8	4.3
Threonine	4.8	6.3
Tryptophan	0.7	1.6
Tyrosine	4.8	2.9
Valine	5.1	5.5
	mg/100 mg powder	mg/100 mg powder

Amino acid composition of the protein powder used for the supplements. In the WDP powder 10.3 g/100g of non-caloric additives are original citric acid remaining after the microwave hydrolysis process of wool. In addition, 7.2 g/100 g of ash and moisture is present. The remaining part of the WDP powder is presumably protein such as selenocysteine and pyrrolysine. The amino acids in the WDP powder was analysed in triplicate in the two fractions (plug 50% and precipitate 50%) with a standard hydrochloric acid hydrolysis followed by RP HPLC separation using AccQ Tag derivatization. Cysteine/methionine were analysed using performic acid oxidation (AOAC 994.12). Whey protein isolate (Alacen895) was bought from Fonterra, New Zealand. *In whey protein the cysteine content was not available, so the value represents cystine content.

Muffin ingredients list

| Ingredients (g)	WDP	Whey	Placebo
Whey	46	74	0
WDP	28	0	0
Maltodextrin	0	0	37
Gluten free flour	0	0	37
Butter	45	45	45
Golden syrup	30	45	30
Egg	25	25	25
Flour	5	20	5
Ginger	4	4	4
Passion fruit flavour	1	1	1
Baking soda	3	3	3
Sucralose	40 mg	20 mg	20 mg
Guar gum	20 mg	20 mg	20 mg
Cinnamon	1	1	1
Dates	30	0	30
Apricots	25	25	25
Coconut thread	20	20	20
Vanilla essence	7	7	7
Clove oil	0.7	0.7	0.7
0.1M NaOH	4	4	4
Rice wine	40	40	40
Water	60	60	60

Above recipe allows for 4 muffins each weighing 70 g.

Preparation

Chop the ingredients into small pieces and mix the ingredients into a smooth dough.
Add 40g of rice wine and 60 mL of water cook for 45 seconds in the microwave. Let the
mix rest for an hour before baking. Cook at 150 degrees for 15 minutes.

Appendix E Optimization of immunofluorescence protocol

Antibody dilution

GLUT4 and IRS-1^{ser312}
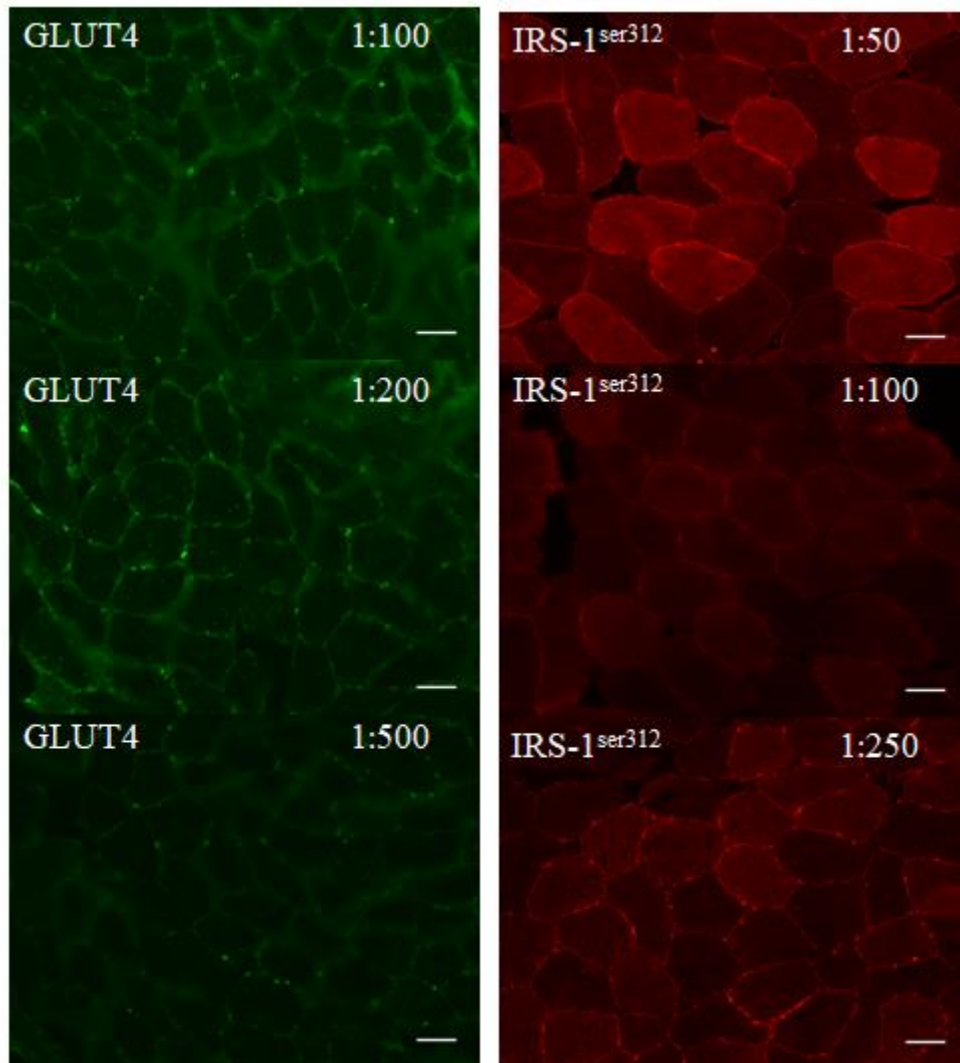
Under identical widefield microscope settings (exposure time: 1500 ms, gain: 4) different antibody dilutions were tested for GLUT4 and IRS-1^{ser312} in order to determine optimal antibody concentration. Ultimately, both GLUT4 and IRS-1^{ser312} were used with a 1:250 dilution to provide a reliable antibody signal to avoid a loss of sensitivity or excess background signal. Scale bars represent 50 µm.
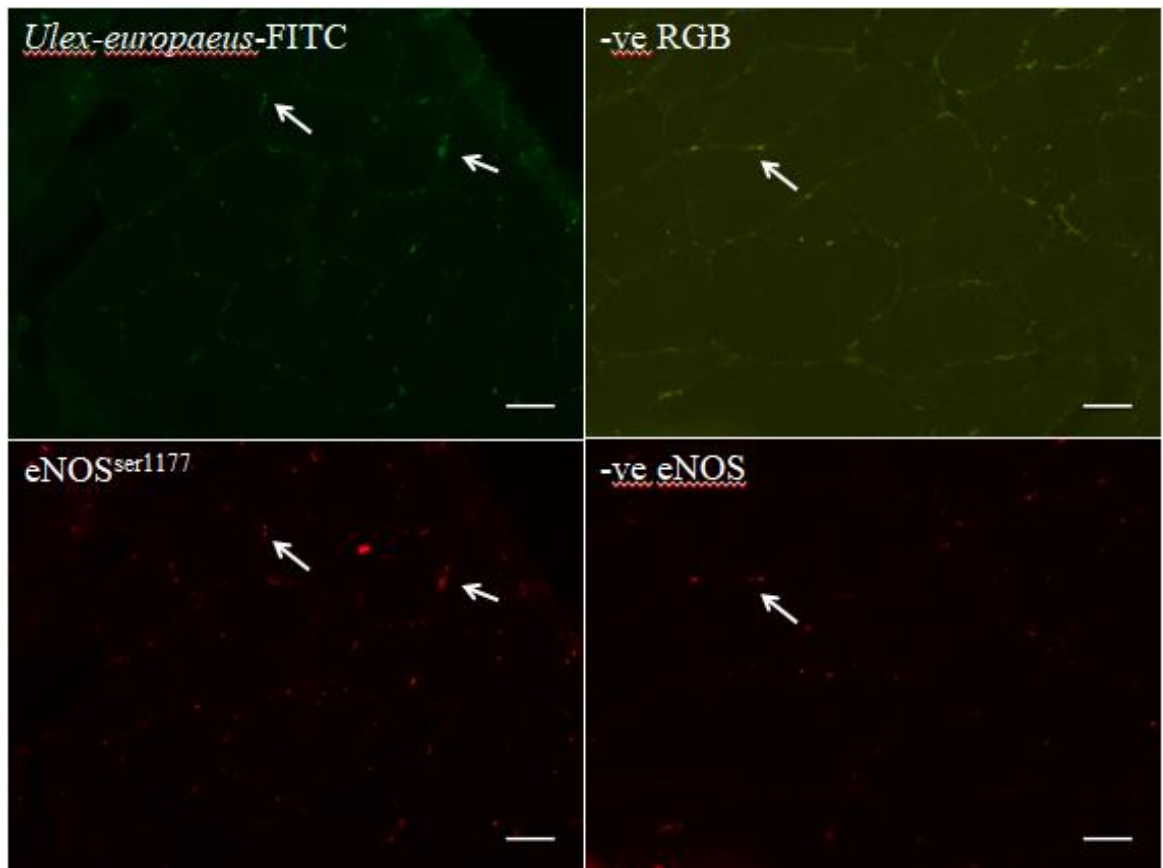
Initially, eNOS and eNOS^{ser1177} appeared to produce a positive signal at a dilution of 1:100. However, after several trials, the signals in the red and green channel for both eNOS and eNOS^{ser1177} appeared to be perfectly matched. This is indicated by the white arrows in *Ulex-europaeus*-FITC and eNOS^{ser1177} image. Moreover, these spots of allegedly positive staining were also detected in negative controls where no antibodies are used. A negative control should produce a dark background image, however as indicated with the white arrows in –ve RGB and –ve eNOS, spots of autofluorescent signal was detected in the negative controls. These spots were identified as lipofuscin granules, molecules that are formed during incomplete lipid oxidation and are associated with aging. TrueBlack was used (Section 3.9) to remove autofluorescent signal and only detect positive antibody-binding.

Appendix F Outcomes Glutathione – oxidised peroxiredoxin 2
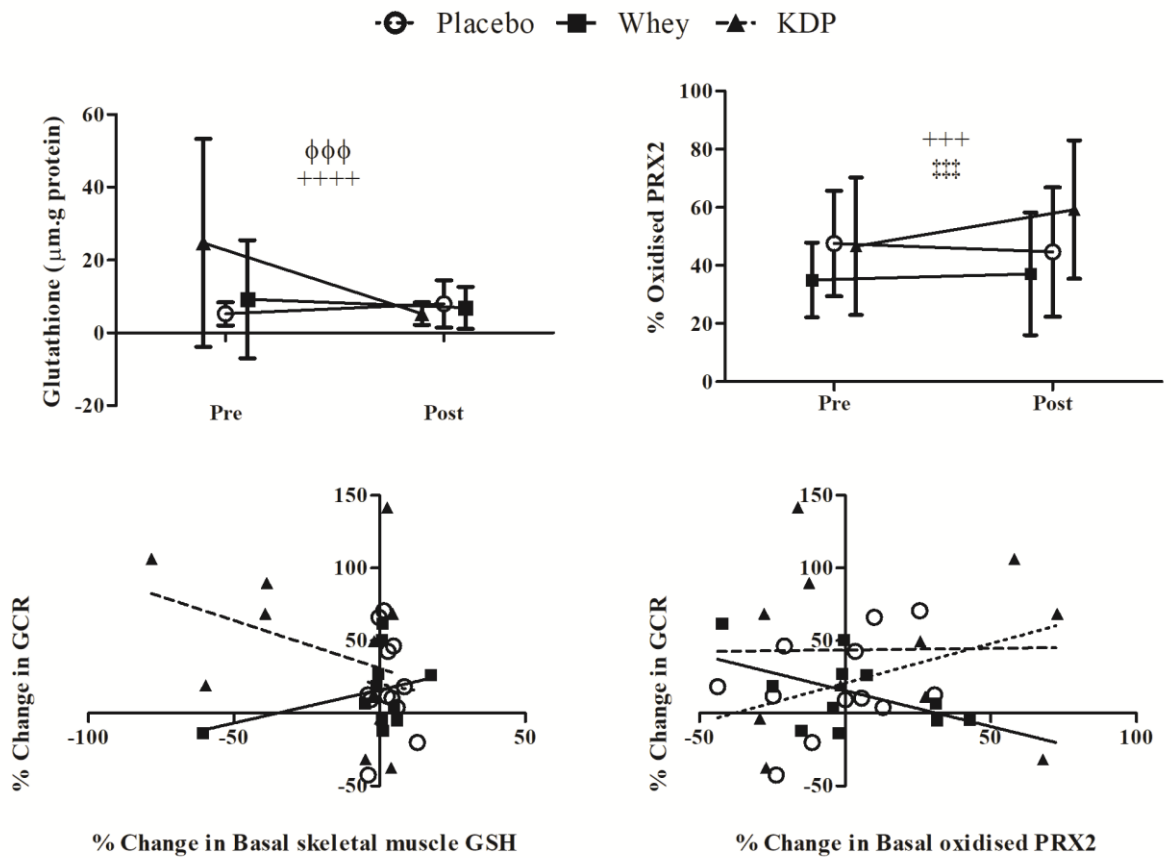
*Effect of WDP and whey protein supplementation in response to 14 weeks of exercise training on skeletal muscle cellular redox environment and associations with glucose disposal rates in adult men and women with type-2 diabetes. Data are raw means and SDs. Statistical symbols to the right of data points represent magnitude-based clinical. Abbreviations: φ = whey vs control; + = WDP vs control; ‡ = WDP vs whey. The number of letters or symbols denotes likelihood of change where * = possible; ** = likely; *** = very likely; **** = almost certain. Data is drawn from unpublished observations from Gram et al.*

Appendix G Supporting figures GLUT4 Pearson's correlation coefficient, linear regressions
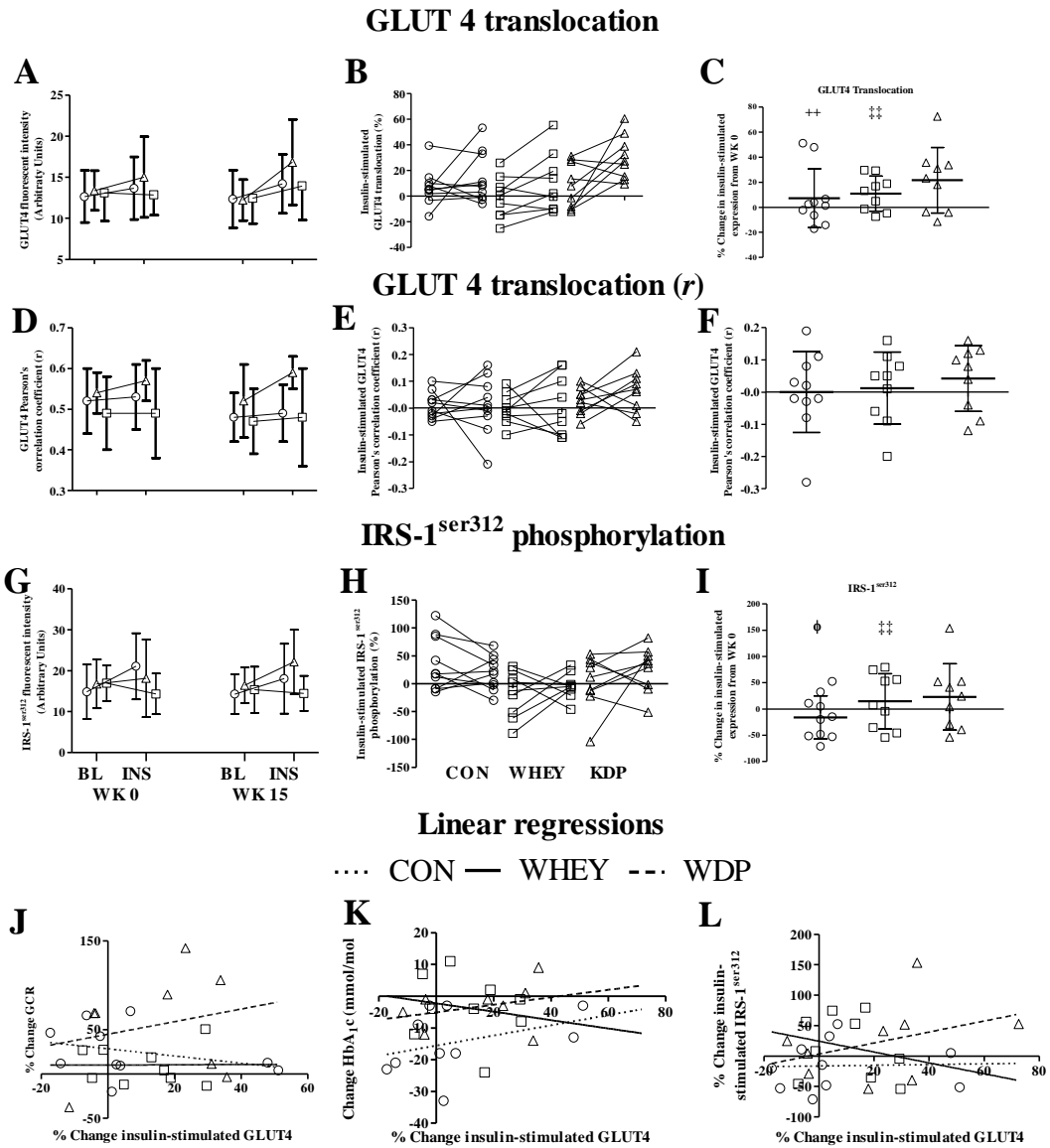
Effect of WDP and whey protein supplementation in response to 14 weeks of exercise training on GLUT4, IRS-1ser312 and associations between GLUT4 and GCR, HbA1c and IRS-1ser312 in adult men and women with type-2 diabetes. Data are means and SDs. The left panels are raw mean fluorescent intensity of protein expression obtained from muscle biopsies during the week 0 and week 15 hyperinsulinaemic isoglycaemic clamp. The middle panel represents individual responses in insulin-stimulated (change

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*from BL to INS) conditions before and after the intervention, presented as % change scores. The right panel represents the mean change (%) in insulin-stimulated protein expression from week 0 to week 15. Statistical symbols to the right of data points represent magnitude-based mechanistic inferences (for detail see Table 4.4). Abbreviations: ϕ = WHEY vs CON; + = WDP vs CON; ‡ = WDP vs WHEY. The number of letters or symbols denotes likelihood of change where * = possible; ** = likely; *** = very likely; **** = almost certain.*