The Effect of 10 Weeks of Peri-Training Whey Protein Supplementation on Systemic, Metabolic, and Skeletal Muscle Molecular Responses in Type-2 Diabetes

A Thesis

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

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ABSTRACT

Introduction: Type-2 diabetes (T2D) is a modern global epidemic associated with multiple health complications and economic burden. Exercise improves glycaemic control in populations with T2D with greater insulin sensitivity, muscle hypertrophy, and reduced emotional distress as possible mediators. Milk protein supplementation has been shown to produce similar benefits, raising the potential of an adjunct therapy. Therefore, the primary purpose of the thesis was to determine if whey-protein supplementation can promote skeletal muscle plasticity associated with improved glycaemic control in exercising men with T2D. Secondary aims were to determine if improvements in functional capacity and glycaemic control led to better mood and quality of life.

Methods: In a randomized, double blind clinical trial, 24 non-insulin dependent middle-aged men with T2D were allocated to a pre- and post-training whey-carbohydrate (20 grams-10 grams) supplement or isocaloric carbohydrate-only control. Participants completed 45 high-intensity endurance and resistance exercise sessions over 10 weeks. Insulin sensitivity was determined from glucose disposal rates (GDR) during a euglycaemic insulin clamp, with fasting blood glucose concentration (FBG) and the homeostatic model of assessment of insulin resistance (HOMA-IR) providing secondary measures of glycaemic control. Insulin-mediated haemodynamics; microvascular blood flow (mBF) and microvascular blood volume (mBV) were assessed at the vastus lateralis (VL) muscle via near-infrared spectroscopy. VL muscle biopsies were used to determine capillarity, intramyofibrillar mitochondrial and lipid density, citrate synthase (CS) and cytochrome c oxidase (COX) activity, and mRNA content of angiogenic and mitochondrial markers: eNOS, VEGFA, VEGFR2, PGC1-α, CS, NRF1. Aerobic capacity (VO_2peak), strength (1-repetition maximum), VL muscle and subcutaneous

adipose thickness, and survey-rated mood and quality of life (DASS42; SF-36) were also assessed.

Results: There were substantial increases in GDR (27.5%; 90%CI 1.2%, 60.7% and 24.8%; -5.4%, 64.8%), capillarisation (24.5%; -0.1%, 55.0 and 26.3%; 1.9%, 56.6%), and mitochondrial density (24.3%; 13.8%, 35.8% and 26.7%; 16.8%, 37.5%) in the control and whey groups respectively, with no group differences. Lipid density, COX enzyme activity, VL muscle thickness, VO_2peak, 1RM strength, mood, and quality of life were also substantially increased with no group differences. Exercise training had no effect on microvascular haemodynamics; however, whey supplementation produced likely and possible improvements in mBV (16.8%; -4.3%, 42.6%) and mBF (5.9%; -3.7%, 16.3%) respectively at rest and likely improvements in both mBV (17.5%; -3.7%, 43.5%) and mBF (10.2%; 0.3%, 21.1%) under insulin-stimulated conditions. Regression analysis of the pooled 10-week change outcomes showed a positive relationship between the change in lipid density and the change in GDR (r = 0.29); negative associations between basal mBV and FBG (r=.27) and HOMA-IR (r=.30); a negative association between basal mBF and HOMA-IR (r=.48); and a positive association (r = 0.39) between the total DASS score and the change in FBG.

Conclusion: Peri-training whey protein supplementation elevated microvascular blood kinetics in middle-aged men with T2D; but did not accentuate the substantial improvements produced by the intense mixed-mode exercise training on tissue and cellular remodelling, insulin sensitivity, glycaemia, exercise capacity, mood or quality of life. The findings support the use of adjunct whey protein supplementation for elevating microvascular blood kinetics in populations with T2D, an outcome that could potentially improve the treatment of vascular diseases where microcirculation contributes to disease pathology and therefore warrants further exploration. The observation that myocellular lipid density was increased by

intense exercise training and not detrimental to insulin sensitivity supports recent evidence that lipid accrual may be a favourable adaptation to exercise in populations with T2D.

ACKNOWLEDGEMENTS

Having finally completed this study, I am extremely thankful to quite a few people who participated. I am very much obliged for the commitment of a number of research institutions who through physical, intellectual and financial investment made this project possible: Massey University, CCDHB Endocrine and Research Centre, Otago Medical School, Environmental Science Research (ESR), and the Wellington Hospital Clinical Trials Unit. I am grateful to the study volunteers who let the research team prod and poke them quite a few times and the staff at the medical centres who sent them: Island Bay; Port Nicholson; Newtown; Kelburn; Thorndon; Miramar; The Terrace; Brooklyn; City; Courtney; Kilbirnie; Capital; and Te Aro Health.

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STATEMENT OF CONTRIBUTION

Study conception and design was by Kim Gaffney, Dr David Rowlands and Dr Lee Stoner. Ethics proposal was written by Kim Gaffney and Dr David Rowlands. Participants were recruited and the study co-ordinated by Kim Gaffney. Supplements were designed by Kim Gaffney and Dr David Rowlands. Exercise training sessions were supervised by Kim Gaffney and PhD candidate Adam Lucero. Research assistants helped with exercise sessions, supplement production, data collection and analysis of microscopy imaging throughout the project.

CHAPTER 4: NIL WHEY PROTEIN EFFECT ON GLYCAEMIC CONTROL AFTER INTENSE MIXED-MODE TRAINING IN TYPE-2 DIABETICS.

Maximal cycling workload and breath-gas collection and ECG testing was by Kim Gaffney, Adam Lucero, and Dr James Faulkner. Insulin-clamps were supervised by Kim Gaffney, Adam Lucero, and one of three general practitioners Patricia Whitfield, Brian Corley and Nick Oscroft, with assistance from Dr David Rowlands and Dr Barry Clarke. NIRS measurement was completed by Adam Lucero or Kim Gaffney. General blood analysis was conducted at Wellington Hospital and insulin concentration the Nutrition Laboratory, Massey University, Palmerston North. Statistical analyses were performed by Dr David Rowlands and Kim Gaffney. The manuscript was written and prepared by Kim Gaffney with guidance from Dr David Rowlands and feedback from Dr Lee Stoner, Dr James Faulkner, Dr Jeremy Krebs, and Dr Patricia Whitfield.

CHAPTER 5: WHEY SUPPLEMENTATION IMPROVES MICROCIRCULATION AFTER 10 WEEKS IN EXERCISING MEN WITH T2D.

NIRS data collection methods were developed by Adam Lucero, Kim Gaffney, Dr Lee Stoner, and Dr David Rowlands. Data collection was performed by Adam Lucero and Kim Gaffney. Capillary density methods were developed by Kim Gaffney with assistance from Jane Anderson and St John Wakefield at the Otago Medical School EM Laboratory; images were analysed by Kim Gaffney with help from research assistants; PCR analysis of mRNA was performed by Jane Clapham and Donia McCartney at Environmental Science Research. Data analysis was conducted by Kim Gaffney, Adam Lucero, and Dr David Rowlands.

CHAPTER 6: THE EFFECT OF 10 WEEKS OF PERI-EXERCISE WHEY PROTEIN SUPPLEMENTATION ON MITOCHONDRIAL CONTENT IN MEN WITH TYPE-2 DIABETES.

Muscle biopsies were conducted by a research general practitioner with assistance from Kim Gaffney. Tissue was processed for analysis by Kim Gaffney. Electron imaging methods were designed by Kim Gaffney, Dr David Rowlands, and Dr St John Wakefield. Electron microscopy preparation and imaging was performed by the Electron Microscopy Lab at Otago Medical School, Wellington NZ. Imaging analysis protocols were designed by Kim Gaffney and Adam Lucero and analysis was conducted by research assistants. Mitochondrial enzyme analysis was conducted by Adam Lucero. Statistical analysis was conducted by Dr David Rowlands, Kim Gaffney and Adam Lucero.

CHAPTER 7: NIL WHEY PROTEIN EFFECT ON MOOD AFTER 10 WEEKS OF EXERCISE IN TYPE-2 DIABETES.

Psychometic analysis methods were developed by Kim Gaffney. Data was collected by Kim Gaffney and Adam Lucero. Data was prepared by research assistants and analysed by Kim Gaffney and Dr David Rowlands.

RESEARCH ETHICS

Ethics approval was obtained from the Northern B Health and Disability Ethics Committee, Wellington, NZ for the study conducted. The potential risks, and management of the risks involved are detailed below:

The main risks in this study were 1) tissue sampling, which were minimized through strict adherence to Hospital and University safety protocols, and; 2) exercise risks, which were minimized through baseline health screening and ECG testing during maximal exercise, and by having exercise physiologists supervise each exercise session.

Social and psychological risks were minimised by ensuring privacy and confidentiality of participants throughout data collection and data storage periods. Initially we obtained informed consent and communicated to participants their right to discontinue or withdraw.

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LIST OF ABBREVIATIONS

1RM	1-repetition maximum
Akt	protein kinase B
AS160	Akt substrate of 160 kDa
COX	citochrome c oxidase
CS	citrate synthase
DASS	Depression, Anxiety and Stress Scale
eNOS	endothelial nitric oxide synthase
FBG	fasting blood glucose
FMD	flow-mediated dilation
GDR	glucose disposal rate
GLUT4	glucose transporter 4
HOMA-IR	homeostatic model assessment of insulin resistance
IRS	insulin receptor substrate
mBF	microvascular blood flow
mBV	microvascular blood volume
MPS	muscle protein synthesis
NRF1	nuclear respiratory factor 1
NIRS	near-infrared spectroscopy
NO	nitric oxide
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositide 3-kinase
ROS	reactive oxygen species
SF-36	Short-form (36) health survey
T2D	type-2 diabetes
VEGFA	vascular endothelial growth factor A
VEGFR2	vascular endothelial growth receptor 2
VO_2peak	peak oxygen consumption

CHAPTER 1

INTRODUCTION

Type-2 diabetes is a modern global epidemic with prevalence increasing four-fold between 1980 and 2014 and currently affecting ~400 million people worldwide (Taylor 2016, WHO 2016) and ~200, 000 adults in New Zealand (NZMOH 2016). In the US the average lifetime cost of treating diabetes and associated disease complications has been estimated at $85,000, which is substantially increased with early age (age <44 y) diagnosis (Zhuo 2013). Health complications associated with the disease include: muscle wasting (Kim 2010, Lee 2011), kidney failure, leg amputation, vision loss and nerve damage (Pantalone 2018); increased risk of cardio- and cerebrovascular complications (stroke and myocardial infarction) (Molinaro 2017); and increased risk of experiencing mood disorders (Bener 2011, Papelbaum 2011). While hypoglycaemic agents are the primary intervention in disease management and readily available, they do not restore the constellation of physiological irregularities that have been associated with the disease such as insulin resistance, vascular dysfunction, and accelerated age-related muscle wasting which have been well-reviewed (Barrett 2011, Bjornholm 2005, Khamseh 2011, Kolluru 2012, Rattigan 2013). Therapies that restore normal physiological function are needed to lower disease prevalence, improve disease management, and reduce the increasing economic burden of treating the condition and its associated complications.

The central pathology of T2D is hyperglycaemia, a state where blood glucose concentrations remain chronically elevated. Insulin resistance is a primary mediator of hyperglycaemia, characterised by a decrease in the reactivity of glucose regulating tissues to insulin, the main hormone of glucose disposal. In normal health, insulin lowers blood glucose concentration after eating by suppressing glucose production in hepatic tissue (Kahn 2014),

increasing glucose transport through the vascular system (Fugmann 2003), and upregulating intracellular uptake at disposal tissues, of which skeletal muscle is chief (Bogan 2012). In individuals with T2D the normal concerted response to insulin is diminished, glucose disposal rates are lowered, and blood glucose levels remain chronically elevated.

Exercise is a well-documented therapy for improving glycaemia in populations with T2D and well-reviewed (Bassuk 2005, Colberg 2010, Hansen 2013). Aerobic and resistance exercise training have both been shown to significantly improve skeletal muscle insulin sensitivity and glycaemic control (Abd El-Kader 2011, Bacchi 2012, Castaneda 2002) and there is substantial evidence that a mixed-mode regimen which includes both modalities produces larger improvements in glycaemia than single-mode therapies (Church 2010, Sigal 2007, Zanuso 2010). Intense interval training has emerged in recent years an effective and time-saving alternative to the endurance modalities such as walking and cycling that have been traditionally used to improve glycaemic control. High intensity treadmill and cycle training has been shown to significantly improve insulin sensitivity and glycaemic control (Bird 2012); however, it is unknown if utilising a mixed-mode regimen would increase improvements to a greater extent and further investigation is warranted.

Milk protein supplementation has shown promise as a T2D therapy that could be complementary to intense interval training. As an individual therapy, whey protein supplementation has been shown to significantly improve glucose tolerance and FBG after 8 weeks in insulin resistant rats (Belobrajdic 2004, Tong 2014) and HOMA-IR after 12 weeks in overweight and obese adults (Pal 2010). Utilised together, milk protein supplementation has been shown to enhance gains in muscle mass (Esmarck 2001, Taylor 2016) and aerobic capacity (Robinson 2011) during exercise training, both of which have been positively associated with better glycaemic control (Kang 2009, Larose 2011, Lee 2011). As the

primary tissue of postprandial glucose disposal (DeFronzo 1985, Thiebaud 1982) and responsive to both exercise and nutritional stimulus, skeletal muscle is a central target for therapy of this kind. It is a well-established phenomenon that consuming a rich source of amino acids proximal to exercise increases the synthesis of skeletal muscle proteins during exercise recovery (Beelen 2008, Dideriksen 2011, Howarth 2009, Moore 2011). As there are several proteins important to glucose metabolism and disposal that have been shown to be expressed at diminished levels in T2D muscle tissue (Stentz 2007), there is potential for combined therapies to help restore these irregularities.

Endothelial nitric oxide synthase (eNOS) is an insulin-sensitive enzyme that modulates glucose disposal by altering haemodynamic rates through the microvascular system (Barrett 2011, Vincent 2004, Zhang 2004). eNOS drives the formation of nitric oxide, a vasodilator that increases blood flow rates and capillary recruitment; both of which are diminished in populations with T2D (Clerk 2007, Padilla 2006, St-Pierre 2010, Wallis 2002). It has also been speculated that eNOS may mediate the movement of insulin through the interstitium to increase its accessibility to myocellular membranes where glucose uptake occurs (Barrett 2011). Individually, exercise training and milk protein supplementation have both been shown to increase the synthesis of eNOS (Cocks 2012, Sanchez 2011) and vasodilatory responses to insulin (Fekete 2016). As an adjunct to exercise, milk protein supplementation has been shown to increase arterial vasodilation in older women (Yoshizawa 2010) suggesting that insulin-mediated haemodynamic responses could also be enhanced.

Impaired formation of mitochondrial proteins is another characteristic of T2D skeletal muscle that may be improved by combined therapies. Mitochondrial rarefaction is a well-established irregularity of T2D skeletal muscle that has been associated with insulin resistance and disease severity (Chomentowski 2011, Hsieh 2011, Kelley 2002, Meex 2010, Mogensen 2007). It has been speculated that mitochondrial rarefaction impairs normal lipid

oxidation producing a state of cellular lipotoxicity that is detrimental to insulin-mediated molecular signalling (Coen 2012, Furler 2001, Goodpaster 2001, Peterson 2004). High-intensity cycling has been shown to significantly increase the expression of skeletal muscle mitochondrial enzymes after only 6 sessions (Little 2011) and chronic amino acid supplementation has been observed to substantially increase mitochondrial volume after 90 days in aging mice (Corsetti 2008). Investigations of combined therapies has produced mixed results; whey protein supplementation for 2 weeks significantly increased molecular signalling (PGC-1α mRNA) for mitochondrial biogenesis during endurance cycle training (Hill 2013) and consumption of branch chain amino acids in drinking water significantly increased mitochondrial volume after 30 days in treadmill trained rats (D'Antona 2010); however, consumption of a whey protein-carbohydrate supplement after a bout of endurance cycling did not enhance mitochondrial synthetic rates compared to carbohydrate alone (Breen 2011) and milk protein supplementation for 6 weeks did not increase mitochondrial DNA in treadmill trained men (Robinson 2011). These mixed findings show that it is unclear whether mitochondrial density can be increased by combined therapies in human populations and further investigation of this phenomenon is warranted.

Increasing skeletal muscle mass is a likely mechanism by which combined milk protein supplementation and exercise training could improve glycaemic control. It has been shown that populations with T2D lose muscle mass with aging at a 2- to 3-fold greater rate than non-diabetic populations (Lee 2010, Park 2009) and that loss of lean mass has been negatively associated with glycaemic control (Lee 2011). Milk protein supplementation has been investigated extensively as an adjunct to resistance training for promoting skeletal muscle hypertrophy; however, while there have been numerous observations of a positive effect, overall findings have been mixed. A recent review reported that milk protein supplementation produced trivial benefits on lean mass (Reidy 2016); however, a meta-

analysis of studies that used whey protein supplements found that lean mass was significantly increased after resistance training (Miller 2014) suggesting that the protein source may be important to the magnitude of the hypertrophic response.

Mood is another clinical outcome that could potentially be enhanced by combined therapies. The prevalence of mood disorders including depression and anxiety is substantially increased in populations with T2D (Kaur 2013, Khuwaja 2010); in some countries ~40-60% of the diabetic population suffer from some form of mood disorder (Khuwaja 2010, Tovilla-Zarate 2012). Emotional distress has been linked to hyperglycaemia via increased adrenal secretion of the glucose-releasing hormone cortisol (Adam 2010, Evans 2008), indicating that therapies which improve mood may also lower glycaemia.

In older adults with T2D exercise training has been shown to improve functional capacity and mood together (Baptista 2017) suggesting that physical function may be an important target for therapeutic intervention. Milk protein supplementation has been shown to significantly increase several markers of physical function during exercise training, including: muscle mass and strength (Esmarck 2001, Taylor 2016) and aerobic fitness (Robinson 2011). Furthermore, in patients with chronic obstructive pulmonary disorder, twice daily supplementation with a whey-based nutritional drink was shown to enhance both mobility and survey rated functional capacity compared to exercise alone (Sugawara 2012). If combined therapies does enhance physical function, then both mood and glycaemia could be improved together.

In summary, there are several plausible mechanisms, by which milk-protein supplementation could improve glycaemia in individuals with T2D during intense exercise training, including:

1. Enhanced eNOS regulated delivery of glucose and insulin through the microvascular system;

2. Enhanced myocellular insulin signal transduction due to increased mitochondrial oxidative capacity and reduced formation of metabolites that interfere with insulin-mediated molecular signalling;
3. Enhanced muscle growth increasing tissue mass for glucose disposal;
4. Reduced cortisol-driven elevation of blood glucose concentration.

If effective, milk-protein supplementation could be a simple adjunct therapy to exercise training for improving rehabilitation outcomes in populations with T2D, which may be particularly beneficial to populations where exercise capacity is limited by age, mobility, or other physical impairments.

PURPOSE OF THE THESIS

The clinical trial described in this thesis was designed to test whether milk-protein supplementation could be used as an adjunct therapy to exercise training to improve clinical outcomes relevant to glycaemic control.

The aims of the study were to explore the possible benefit of consuming whey protein before and after exercise during a 10-week high-intensity mixed-mode training program to:

1. vascular and whole-body insulin sensitivity, and glycaemic control;
2. mitochondrial content;
3. body composition and functional capacity (exercise performance); and
4. mood status.

It was hypothesised that whey protein supplementation would upregulate the expression of proteins that have been previously reported to be expressed at low levels in T2D in both vascular and skeletal muscle tissue during exercise recovery and that the improvements would lead to better insulin sensitivity and glycaemic control. More specifically it was hypothesised that supplementation would improve:

1. glucose disposal rate during a euglycaemic insulin clamp, fasting blood glucose, and HOMA-IR;
2. insulin-mediated microvascular blood flow and perfusion;
3. mitochondrial volume and enzyme concentration;
4. skeletal muscle thickness, VO_2peak, and 1-repetition maximum strength;

   survey-rated mood.

CHAPTER 2

LITERATURE REVIEW

MILK PROTEIN SUPPLEMENTATION AS AN ADJUNCT THERAPY TO EXERCISE FOR IMPROVING GLYCAEMIC CONTROL IN TYPE-2 DIABETICS

2.1 Introduction

Consumption of milk-proteins proximal to exercise has been repeatedly shown to upregulate protein synthetic rates in skeletal muscle during exercise recovery (Beelen 2008, Dideriksen 2011, Howarth 2009, West 2011). While the benefit of this phenomenon has largely been explored to enhance muscle growth and athletic performance; see reviews (Miller 2014, Reidy 2016), there has been an increasing number of studies showing heightened adaptations beneficial to the treatment of hypertension (Yoshizawa 2010), obesity (Arciero 2014) and sarcopenia (Cribb 2006). Type-2 diabetes (T2D) is a disease characterised by impaired expression of a constellation of proteins in vascular and skeletal muscle tissue that are important to normal glycaemic control, including proteins that regulate vascular relaxation (Khoo 2005), mitochondrial function (Chomentowski 2011) and contractile tissue mass (Lee 2010). Both exercise and milk-protein supplementation have been shown to independently increase the expression of these proteins (Bacchi 2012, Björkman 2012, Cocks 2012, Hill 2013, Little 2011, Sanchez 2011), suggesting that combined therapies could enhance glycaemic control in populations with T2D.

2.2 Pathology of T2D

The pathology of T2D is chronically elevated blood glucose concentration, i.e. hyperglycaemia. Treating the disease effectively is complex as hyperglycaemia can be mediated by physiological irregularities such as insulin resistance (Bjornholm 2005) and sarcopenia (Lee 2011, Park 2009); and/or heightened emotional status such as depression, anxiety and stress (Adam 2010, Evans 2008, Stetler 2011). Exercise and milk-protein supplementation are particularly effective modes of T2D therapy because they have been

shown to produce improvements in multiple mediators of hyperglycaemia including insulin resistance (Belobrajdic 2004, Krisan 2004, Pal 2010, Tong 2014, Yaspelkis 2006), muscle mass (Gillen 2016, Kang 2009), and mood (Archer 2014, Heinzel 2015, Lincoln 2011).

2.3 Insulin Resistance

Insulin resistance is the central mechanism of dysfunction in T2D. In populations with T2D glycaemic regulating tissues have become less responsive to insulin, the primary hormone of postprandial glucose disposal. In normal health, insulin lowers blood glucose concentration by suppressing glucose production in hepatic tissue (Kahn 2014), increasing glucose transport through the vascular system (Fugmann 2003), and upregulating intracellular uptake at disposal tissues (Bogan 2012). In individuals with T2D the normal concerted response to insulin has diminished, glucose disposal rates are lowered, and elevated blood glucose levels have become chronic (Barrett 2011, Bjornholm 2005, Melvin 2017).

Exercise is a well-documented therapeutic mode for improving insulin resistance in both vascular and skeletal muscle tissue (Cocks 2012, Yaspelkis 2006) and well-reviewed (Bird 2012, Colberg 2010, Hills 2010). Exercise promotes multiple physiological and cellular adaptations that may be beneficial to insulin sensitivity, including: increased expression of proteins within the insulin signalling cascade (Cao 2012, Cocks 2013, Stuart 2013); decreased formation of metabolites that interfere with protein phosphorylation (Anderson 2009, Bergman 2012, Boon 2013, Haus 2009, Kuzmenko 2016, Tirosh 1999); and expansion of tissue mass delivering and extracting glucose from the circulatory system (Baum 2015, Cocks 2013, Konopka 2010, Narici 2004).

Milk-protein supplementation has emerged as a simple dietary therapy that can also improve insulin sensitivity. Independently, twice-daily whey protein supplementation (27 g protein) for 12 weeks was shown to significantly improve homeostatic model assessment of insulin resistance (HOMA-IR) in overweight and obese adults compared to casein and a

glucose control (Pal 2010). Similarly, whey supplementation (54 grams 30 min before lunch) for 12 weeks significantly improved fasting blood glucose (FBG) in overweight and obese men compared to soy protein (Tahavorgar 2015). In animal models of T2D, whey protein consumed in drinking water for 11 weeks significantly increased glucose tolerance in mice (Shertzer 2011) and insulin sensitivity was significantly improved after 6 weeks of a high whey protein compared to a high red meat diet in rats (Belobrajdic 2004). These findings suggest that there may be potential to enhance glycaemic control in individuals with T2D by combining therapies.

2.3.1 Vascular Tissue

The restoration of glycaemia after eating is largely dependent upon the appearance rate of glucose at myocellular membranes where uptake occurs. Insulin increases glucose transport to muscle cells by initiating a signalling cascade at pre-capillary vascular epithelium that promotes formation of the vascular-relaxing gas nitric oxide (NO) (Figure 2.1). Current evidence suggests that NO-mediated relaxation in arterial tissue increases blood flow through the microvascular system (Barrett 2011), while relaxation at terminal arterioles promotes capillary recruitment (Vincent 2004). The subsequent increase in microvascular blood flow (mBF) and/or microvascular blood volume (mBV) raises the accessibility of glucose to muscle cells (Figure 2.2).

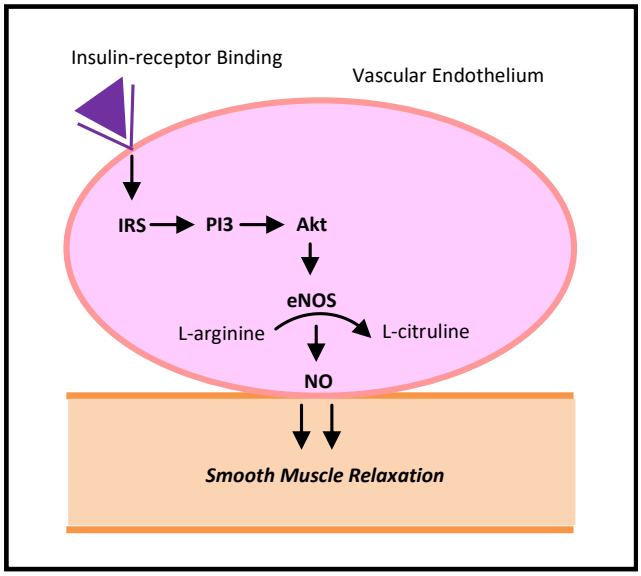


Figure 2.1 Insulin-mediated signal transduction at vascular epithelium. IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; Akt: protein kinase; eNOS: endothelial nitric oxide synthase; NO: nitric oxide.

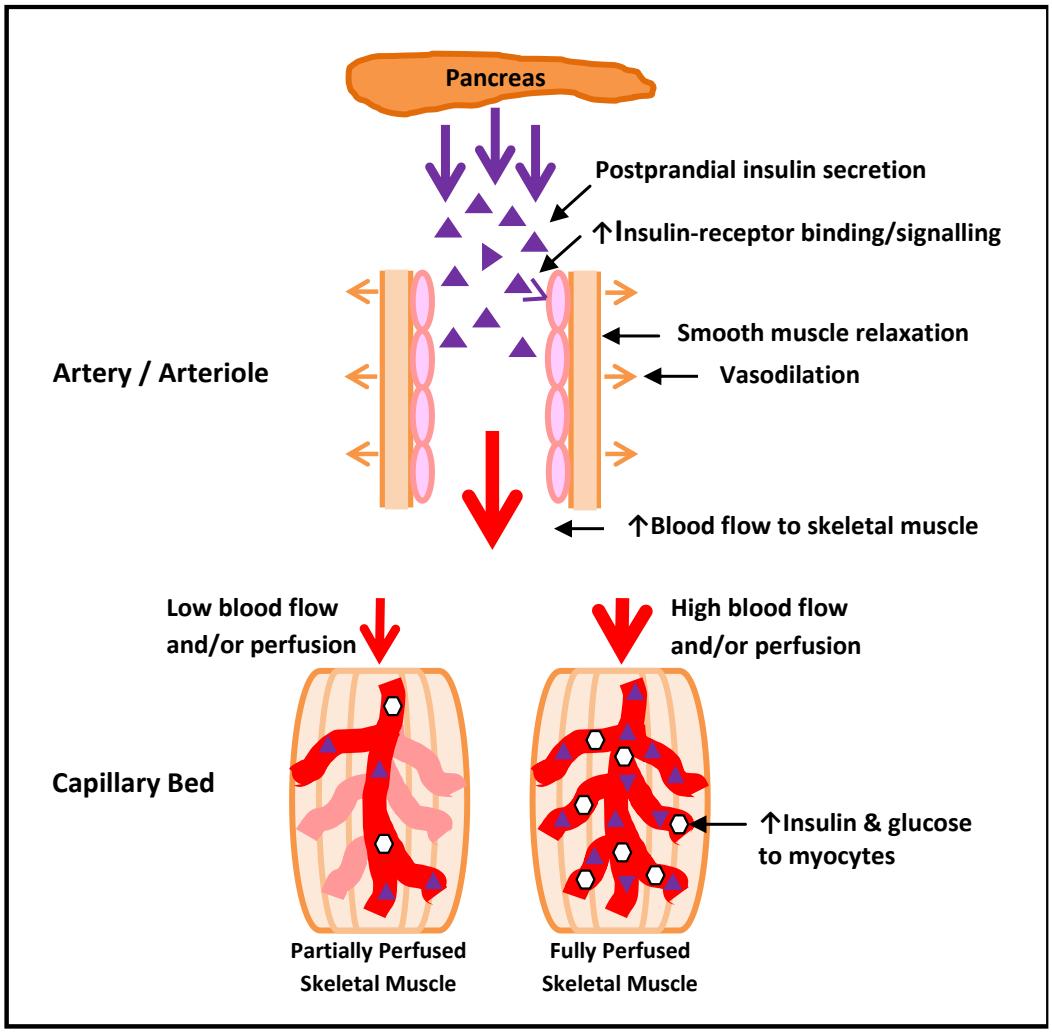


Figure 2.2 Pre-capillary vascular relaxation and microcirculation upregulation.

T2D is characterised by insulin resistance in vascular tissue and impaired blood flow (Laakso 1992, Reynolds 2017). In maurine models of the disease it has been well-documented that vascular dysfunction extends to the microvascular system. Lower levels of capillary recruitment (Padilla 2006, St-Pierre 2010, Wallis 2002) and microvascular blood flow following insulin infusion has been reported in diseased compared to healthy animals (Clerk 2007). Capillary rarefaction, a hallmark of T2D skeletal muscle (Benedict 2011, Marin 1994, Mathieu-Costello 2003, Murakami 2012, Prior 2009) is a likely contributor to the lower recruitment response, however, there is increasing evidence from animal models of the

disease showing that impaired formation of NO in pre-capillary vascular tissue mediates dysfunction through the microvascular system (Heitzer 2000, Khoo 2005).

Exercise and milk-protein supplementation have both been shown to independently improve NO-mediated vascular relaxation. In aged rats, 10 weeks of treadmill training has been shown to significantly improve relaxation at the arorta (Luttrell 2013) and at soleus muscle arterioles (Sindler 2009). Twice daily supplementation (28 grams) with whey or casein protein for 8 weeks significantly improved flow mediated dilation (FMD), a marker of NO-mediated vascular relaxation, in healthy adults compared to a carbohydrate control (!!! INVALID CITATION !!! (Fekete 2016, Fekete 2016)). In a study of combined therapies, FMD was significantly improved in postmenopausal women after 8 weeks of aerobic exercise (3-5 d/week) and milk-protein supplementation (2.8 g/day of casein hydrolysate) compared to exercise or supplementation alone (Yoshizawa 2010).

Upregulation of eNOS expression appears to be the likely mediator of improved vascular relaxation following milk-protein supplementation or exercise training. Significant increases in skeletal muscle eNOS concentration and reductions in arterial stiffness were reported following 6 weeks of cycle training in previously sedentary young men (Cocks 2012). In hypertensive rats, casein hydrolysate supplementation for 6 weeks was shown to improve eNOS expression and vasodilatory relaxation at the aorta (Sanchez 2011). Increased eNOS expression may also improve mBV via capillary expansion as skeletal muscle eNOS concentration has been previously linked to angiogenesis in animals (Tamarat 2002). Taken together, these findings suggest that adjunct milk-protein supplementation may enhance eNOS expression during exercise training which could lead to better insulin-mediated microcirculation.

2.3.2 Skeletal muscle

Skeletal muscle is the primary tissue of glucose disposal, accounting for ~75-80% of postprandial glucose uptake (DeFronzo 1985, Thiebaud 1982). Insulin mediates glucose disposal in skeletal muscle tissue by initiating a signalling cascade at myocellular membranes (Figure 2.3) that promotes glucose transporter 4 (GLUT4) translocation from the cytosol to the cell surface to increase active intracellular glucose transport (Figure 2.4). T2D is characterised by insulin resistance at the myocellular level evidenced by impaired GLUT4 translocation (Garvey 1998) and lower glucose extraction rates (Melvin 2017).

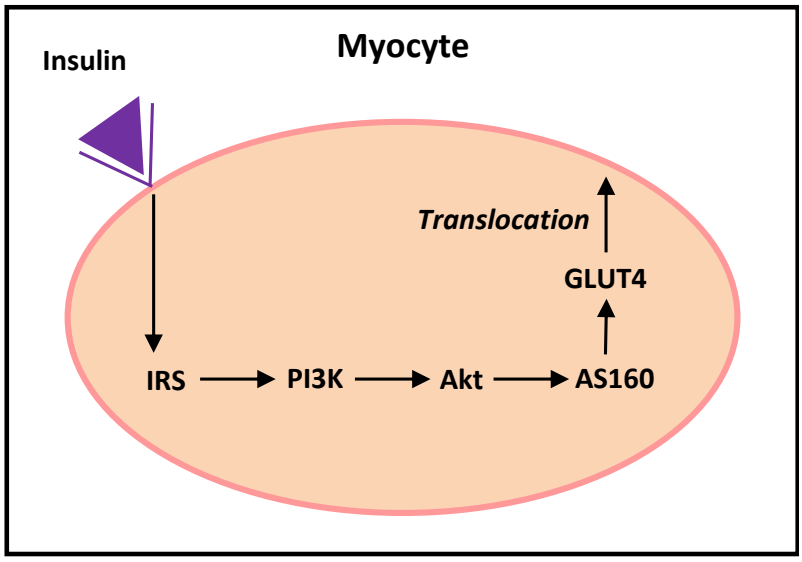


Figure 2.3 Insulin-mediated signal transduction at myocytes. IRS: insulin receptor substrate; PI3K: phosphoinositide 3-kinase; Akt: protein kinase; B AS160: Akt substrate of 160 kDa; GLUT4: glucose transporter 4.

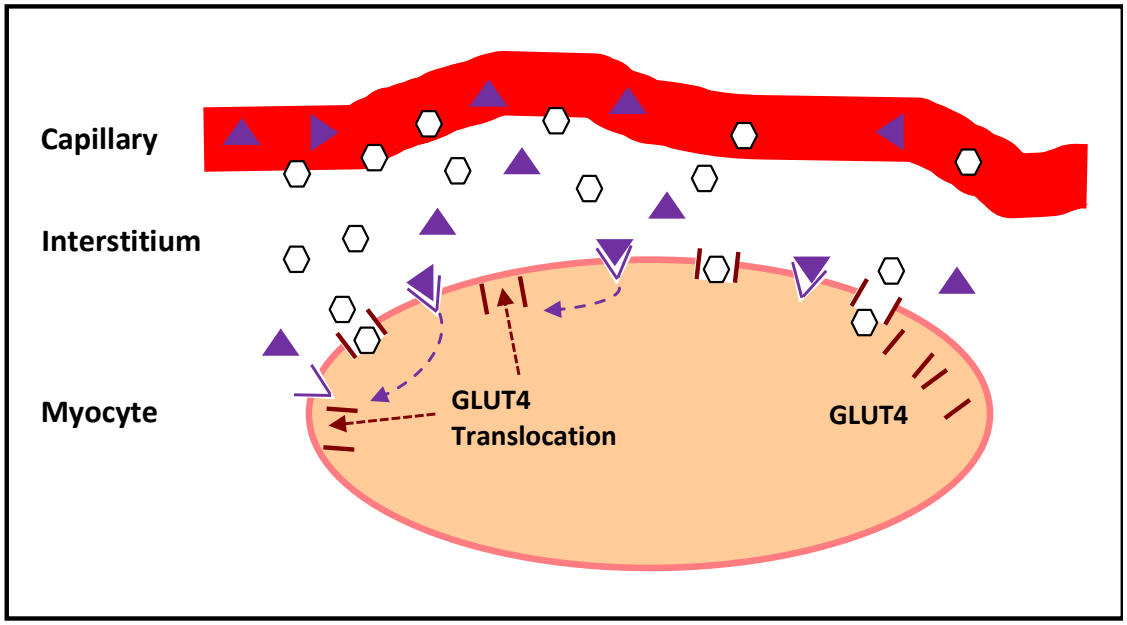


Figure 2.4 Insulin-mediated upregulation of active glucose transport. GLUT4: glucose transporter 4.

Mitochondrial dysfunction is foremost amongst a number of mechanistic theories of myocellular insulin resistance. It is well-documented that mitochondrial density and enzyme concentration are substantially decreased in T2D compared to healthy skeletal muscle tissue (Chomentowski 2011, Hsieh 2011, Kelley 2002, Meex 2010, Mogensen 2007). A leading theory has been that mitochondrial rarefaction reduces the capacity for normal lipid oxidation, increasing the accumulation of lipid and lipid derived metabolites that interfere with insulin-mediated molecular signalling (Boon 2013, Haus 2009, Kuzmenko 2016, Tirosh 1999). An investigation comparing the respiration rates of isolated mitochondrial in the skeletal muscle of obese diabetic and non-diabetic men found that respiration through the electron transport chain was significantly lower in the diseased population (Mogensen 2007). Furthermore, increased levels of lipid-derived reactive oxygen species (ROS) (Schrauwen 2010, Wohaieb 1987) and lipid substrate species ceramide (Boon 2013, Haus 2009, Kuzmenko 2016) and diacylglycerol (Bergman 2012) have been observed in T2D skeletal muscle and shown to interfere with insulin-mediated molecular signalling in cell studies

(Anderson 2009, Boon 2013, Tirosh 1999). However, it is unclear whether myocellular lipid accumulation mediates these irregularities as increased lipid density has been observed in some (Patti 2010, Peterson 2004), but not all populations with the disease (Anderson 2009, Chomentowski 2011, Patti 2010, Peterson 2004) and further investigation is warranted.

Positive associations between mitochondrial density and glucose disposal rates after insulin infusion have been observed in both healthy and insulin resistant populations (Chomentowski 2011) suggesting that mitochondrial expansion should be a target of T2D therapy. It has been well-documented that exercise upregulates mitochondrial biogenesis in T2D skeletal muscle leading to increased mitochondrial volume and enzyme content. 6 high-intensity interval cycling sessions were shown to significantly increase mitochondrial enzyme concentration at the *vastus lateralis* (VL) muscle of adults with T2D (Little 2011). Similarly, 12 weeks of mixed-mode training (2 cycling + 1 resistance session each week) was shown to raise mitochondrial function assessed via ATP resynthesis rates using magnetic resonance spectroscopy at the VL muscle of men with T2D to levels comparable to that of healthy men (Meex 2010).

Supplemental strategies to enhance mitochondrial biogenesis during exercise training have produced mixed findings. As an individual therapy amino acid infusion has also been shown to increase molecular signalling for mitochondrial biogenesis in healthy young men and adding branch chain amino acids to drinking water was shown to significantly reduce the age-related decline in mitochondrial volume in aging mice after 30 days (D'Antona 2010) and increase CS and COX enzyme concentration and mitochondrial density in rats (Corsetti 2008). When combined with treadmill training, amino acid supplementation was shown to significantly increase skeletal muscle mitochondrial density compared to either therapy alone. In athletic populations, consuming whey protein after endurance cycling was shown to significantly upregulate the transcriptome driving mitochondrial biogenesis and oxidative

metabolism at 48 hours in the VL muscle of trained cyclists (Rowlands 2011) and consuming whey protein (1.2 grams per kg bodyweight added to a sports drink) each day for 2 weeks during cycle training was shown to significantly increase VL muscle expression of PGC-1a mRNA, a key regulator of mitochondrial biogenesis, compared to carbohydrate alone (Hill 2013). In contrast, mitochondrial synthetic rates at the VL muscle were not increased after 4 hours in trained men who consumed a whey-carbohydrate (10g-25g) supplement immediately and 30 minutes after an endurance cycling session (Breen 2011) or by consumption of a whey supplement (25 g) immediately after a bout of combined leg resistance and moderate intensity cycling (30 minutes) (Camera 2015). Furthermore, consuming mixed milk protein (27 grams) after treadmill training for 6 weeks exercise did not increase mitochondrial DNA content in skeletal muscle in previously sedentary men compared to a carbohydrate control (Robinson 2011). These mixed findings show that further investigation of this phenomenon, particularly in a population such as T2D where mitochondrial rarefaction is present, is needed to confirm whether milk protein supplementation can be beneficial to mitochondrial expansion during exercise training.

2.4 Muscle Wasting

T2D is characterised by a 2 to 3-fold greater rate of age-related muscle wasting than healthy populations (Kim 2010, Lee 2011, Lee 2010, Park 2009). As skeletal muscle is the major tissue of postprandial glucose uptake (DeFronzo 1985, Thiebaud 1982) loss of tissue mass is likely to contribute to and/or exacerbate the disease. Lean mass has also been negatively associated with glycaemia in populations with T2D (Lee 2011), suggesting that increasing muscle mass could lower blood glucose concentrations.

Muscle growth has been the primary focus of the majority of studies investigating the effect of the effect of milk-protein supplementation as an adjunct to exercise. While it is well-

established that consumption of a rich source of mixed amino acids proximal to resistance training increases muscle protein synthetic rates (Bechshoeft 2013, Beelen 2008, Dideriksen 2011, Howarth 2009, Moore 2011) there has been mixed findings on the benefits of chronic supplementation. A meta-analysis of the effect of milk-protein supplementation on lean mass during chronic resistance training found that supplementation provided minimal benefit to muscle growth during resistance training in healthy populations (Reidy 2016). In contrast, a meta-analysis of studies investigating only the effect of whey protein supplementation, found that lean mass was significantly improved in healthy populations during chronic resistance training (Miller 2014). Investigations of clinical and aged populations have also produced mixed findings. Consumption of a protein supplement drink (10 g protein, 7 g carbohydrate, 3 g fat) 3 times a week for 12 weeks immediately after low-moderate intensity resistance training significantly increased the quadriceps fermoris cross-sectional area by 7% compared to no change with delayed supplementation (+2h) in elderly men (Esmarck 2001) . In contrast, ingestion of 20 g of whey protein immediately after moderate-high intensity resistance exercise three times per week, did not improve lean body mass in elderly men and women compared to a carbohydrate control (Arnarson 2013). The different findings in investigations of this nature are likely explained by the large variability in the exercise and supplementation modes that have been employed. Further investigation is warranted to determine if an ideal exercise-supplement mode can be employed to improve lean mass in populations with T2D.

2.5 Mood

Emotional status has emerged in recent years as a co-morbidity and potential mediator of hyperglycaemia in T2D. Epidemiological studies have revealed that the prevalence of mood disorders is substantially increased amongst individuals with T2D (Almawi 2008, Bener 2011, Kaur 2013, Khuwaja 2010); in some countries reaching as high as ~40-60% of

the population (Khuwaja 2010, Tovilla-Zarate 2012). Mortality risk has been shown to be substantially increased and quality of life decreased in depressed populations who also have T2D (Ali 2010, Zhang 2005). It is currently unclear whether mood disturbances underlie or are the result of hyperglycaemia, however, a meta-analysis of available literature a decade ago found that while depression was a strong predictor of T2D risk (Mezuk 2008), T2D was a weak predictor of depression.

Heightened emotional states, such as depression, anxiety, and stress, have also been linked to elevated secretion of the glucose-releasing hormone cortisol (Adam 2010, Evans 2008, Stetler 2011) . Elevated cortisol levels have been documented in populations with T2D (Manenschijn 2013, Shirzaii 2016), and to a greater extent in T2D individuals who were depressed (Alvarez 2013), suggesting that mood disorders may underlie hyperglycaemia. More recently, associations have been reported between survey rated depression and HbA1c (Papelbaum 2011) and survey rated depression and stress and fasting glycaemia (Alipour 2012, Kaur 2013, Tovilla-Zarate 2012), indicating that further investigation of the impact of mood on glycaemia is warranted.

Exercise is a well-documented therapy for improving mood status in populations with mood disorders (Archer 2014, Heinzel 2015, Lincoln 2011). In populations with T2D exercise training has been shown to improve quality of life ratings including ratings of physical function (Baptista 2017, Dede 2015, Dincer 2016, Liu 2016). As physical capacity measures such as aerobic fitness, muscle strength, and mobility are commonly better following exercise training (Barrile 2016), greater physical function may contribute to improvements in mood status.

Milk-protein supplementation has shown some potential as an adjunct to exercise for enhancing physical function. In healthy populations consumption of milk-proteins has been shown to increase 1RM strength in resistance trained men (Cribb 2006), strength and agility

in female college basketball players (Taylor 2016), and VO2max in sedentary men during treadmill training (Robinson 2011). In elderly adults with chronic obstructive pulmonary disease, twice daily supplementation with a whey-based nutritional drink during a 3-month low-intensity exercise program was reported to significantly improve a 6-minute walk test and health related quality of life, compared to exercise alone (Sugawara 2012). Furthermore, grip strength, leg strength, gait speed and agility was significantly improved by daily whey protein supplementation during a low-intensity exercise program in aged individuals (Niccoli 2017). Others have reported no benefit of milk-protein supplementation during exercise training on strength (Chale 2013, Denysschen 2009), or aerobic fitness (Weinheimer 2012) indicating that further investigation of this phenomena is needed. The effect of milk-protein supplementation during exercise training on either mood or functional capacity has not been tested in a population with T2D.

2.6 Exercise and Supplemental Regimens

While exercise guidelines for improving glycaemia in populations with T2D are well-established (Colberg 2010), ongoing investigation to optimise exercise modes has led to some refinement in recent years. Aerobic and resistance training have been shown to be equally effective for improving glycaemic control (Abd El-Kader 2011, Bacchi 2012), with increasing evidence indicating that a combination of exercise modes provides the best glycaemic outcomes (Church 2010, Sigal 2007). High-intensity interval training (HIT) has emerged as an attractive alternative to traditional low-moderate intensity exercise therapy due to the lower time investment required and potentially greater adaptive responses (Bird 2012). Six high-intensity cycling sessions (10 x 1 min intervals at 90% of max HR) was shown to significantly improve 24 h blood glucose concentration by 14% and postprandial glycaemia by 30% in middle-aged men with T2D (Little 2011). HIT on a treadmill for 12 weeks was

shown to significantly reduce HbA1c (-10%) after 12 weeks in adults with T2D compared to oxygen consumption matched continuous training (-3%) (Mitranun 2014). Similarly, performing three 20-second sprints on a cycle ergometer, three times each week for 12 weeks, were shown to improve insulin sensitivity during a glucose tolerance test to similar magnitude (52%) as a 45-minute moderate intensity cycling protocol (34%) (Gillen 2016). HIT training has also been shown to be more effective for improving flow mediated dilation (FMD) and nitric oxide (NO) concentration in blood compared with continuous exercise (Mitranun 2014). Taken together, these findings suggest that a combination of high-intensity interval training utilising a mixed-mode exercise regimen could be an optimal approach to exercise therapy for improving glycaemia in populations with T2D.

Establishing an optimal supplementation regime for improving glycaemia is difficult as there have been no investigations of this nature in a population with T2D and dosage studies have largely focused on acute hypertrophic responses. In healthy populations consumption of ~20 grams of milk-protein after exercise training has been shown to maximally stimulate MPS during the acute recovery phase (Moore 2009). Others have shown that hypertrophic improvements can be obtained via both pre- and/or post-exercise supplementation, with delayed consumption lowering the benefit of supplementation on MPS (Esmarck 2001, Tipton 2001, West 2011). Some evidence indicates that whey protein supplementation induces significantly greater MPS than casein protein (Aoi 2011, Cribb 2006). Whey protein supplementation has also been shown to be more effective for improving vascular relaxation (Fekete 2016) and mood (Markus 2008) compared to casein protein. From these findings it appears that a pre- and post-training whey supplement that supplies ~20 grams of protein could enhance the effect of high-intensity exercise on multiple outcomes important to glycaemia in populations with T2D.

2.2 Summary of Findings and Purpose of Thesis

As described above, whey-protein supplementation during exercise therapy has the potential to improve glycaemia in populations with T2D via multiple mechanisms, including: enhanced insulin-signal transduction in skeletal muscle and vascular tissue, increased skeletal muscle mass, and decreased mood-driven hypercortisolaemia. However, there have been no investigations of these phenomena in a population with T2D. Therefore, the purpose of this thesis (herein detailed in 5 separate chapters) was to determine if consumption of whey protein supplementation before and after intense exercise training for 10 weeks would improve glycaemia in a population with T2D and to explore potential mediators of improvement that have been previously linked to better glycaemic control.

It was hypothesised that whey-protein supplementation would improve glycaemic control and that specific tissue and clinical outcome enhancement would include: vascular insulin sensitivity, mitochondrial expansion, muscle growth, and mood.

CHAPTER 3

METHODS

This section describes the methods that were used throughout the clinical trial and the experimental thesis chapters that follow.

3.1 Participants

Men with T2D (n=24) were recruited from local medical centres in Wellington, NZ (Appendix A and B) and screened for health complications prior to inclusion (Appendix C). Inclusion characteristics were age 40-65 y, BMI<40, not requiring insulin therapy, and not meeting the ACSM guidelines for exercise for T2D (Colberg 2010). Figure 3.1 shows the number of recruited participants who entered and were excluded from the study. The study was approved by the Northern B Health and Disability Ethics Committee, Ministry of Health, Wellington NZ (13/NTB/69). Participants provided written informed consent (Appendix A).

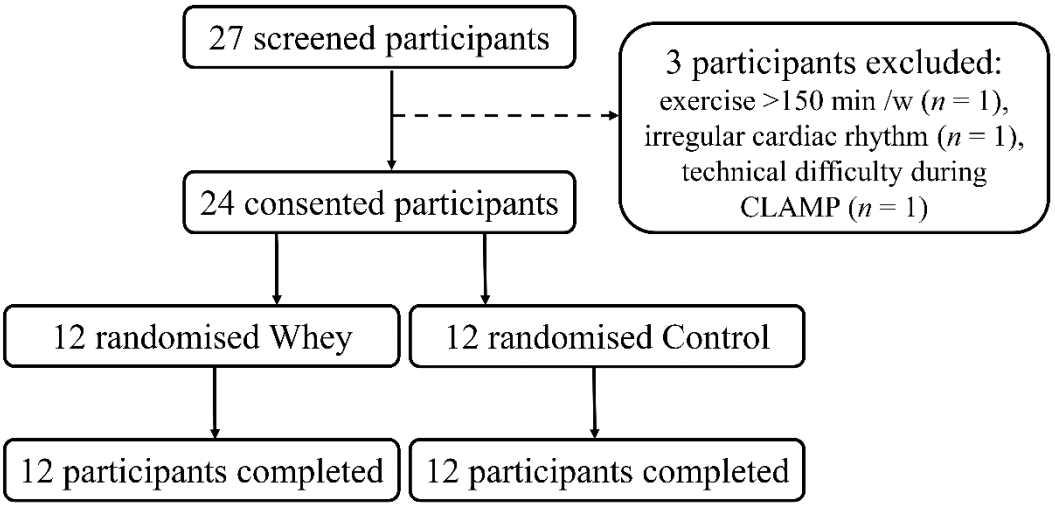


Figure 3.1 Recruitment Flowchart.

3.2 Experimental Design

The design was a double blind, randomized (Research Randomizer, Version 4.0, http://www.randomizer.org), placebo controlled trial (http://www.anzctr.org.au/, Registration number ACTRN12613000340730). At early stages of data collection, the original intended third group: whey without mixed-mode interval training (MMIT), was removed from the study design because recruited eligible participants declined to participate if not randomised to an exercise group creating sampling bias. In the two-group design, participants consumed a whey-protein beverage or carbohydrate placebo before and after 45 early-morning MMIT sessions over 10 weeks.

Participants were encouraged to maintain dietary and medication habits throughout the experimental period and not to participate in strenuous activity within 2 days of testing sessions (Appendix D). Participants were familiarised with all testing procedures except the euglycaemic insulin clamp prior to baseline testing. Cardiac screening via ECG was performed at familiarisation during a VO_{2peak} cycling test. Baseline testing occurred 5-10 days prior to commencement of the intervention with post-testing 2 days after 45 exercise sessions. The post glucose clamp was performed 48 hours after maximal cycling and strength tests to provide a washout period that would allow for the bulk of the acute effects of intense exercise on glycaemia to return to pre-exercise levels without inducing a period of deconditioning (Black 2010, Marliss 2002).

3.3 Exercise Protocol

Participants completed 27 cycling and 18 resistance training sessions (4-5 sessions each week) over 10 weeks. Sessions included a 5-minute warm-up at low intensity on a cycle ergometer or rowing machine followed by 20 minutes of 1-minute interval style cycling or resistance exercise. Pre-programmed cycling sessions (VeloTron Racer Mate, Seattle, WA)

included 10 intervals at 70%-90% (increased 5% every 2 weeks) of the participants' peak oxygen consumption volume (VO_{2peak}) obtained from baseline and fortnightly cycle testing (SensorMedics Vmax, YorbaLinda, CA), with 1-minute active recovery intervals at 40% of peak workload. Resistance training included 5 sets of 30 repetitions of each exercise (Day 1: bench press and seated rows), and (Day 2: lateral pulldowns and barbell upright rows) with 1 minute of crunches on a fitball (Hart Sport, Auckland, NZ) as active recovery. Intensity was set at 20% of 1-repetition maximum (1RM) (Appendix E) during weeks 1-2 and increased to 25% of 1RM to elicit a high-intensity workload, for the remainder of the intervention based upon baseline and fortnightly testing. If participants were unable to maintain a set cycling or strength workload for a full minute the subsequent interval was reduced by 10%. All exercise and testing sessions were supervised by the researchers.

3.4 Supplement

Participants appeared each morning to the exercise laboratory in a fasted state. A chocolate flavoured whey protein isolate (WPI-895, Fonterra, Auckland, New Zealand [Appendix F]) beverage (20 grams protein/10 grams carbohydrate/3 grams milk-fat) or an identically-flavoured but non-protein formulated isocaloric beverage (30 grams carbohydrate/3 grams milk-fat) was consumed immediately before and after each exercise session. Each drink contained 175 calories (731 kilojoules). To reduce hunger and provide opportunity for a clear peri-training whey compared to carbohydrate consumption effect to be observed, each participant consumed a low-protein snack bar (Nature Valley, General Mills, Auckland, NZ) 1 hour after exercise and resumed normal eating habits after 2 hours.

3.5 Glycaemic Measures

3.5.1 Euglycaemic Insulin Clamp

Glucose disposal rate (GDR) for each individual was determined via a modified euglycaemic insulin clamp as described previously (Matsuda 1999). Briefly, participants appeared for testing between 7 and 9 am after an overnight fast and at least 48 hours after the last exercise testing session. A catheter was placed at the antecubital vein for insulin and glucose infusion, and dorsally at the hand for blood draws. Arterialised blood was obtained by placing the hand in a heater box at 50 °C. Participants received priming insulin doses of 160 $mU \cdot m^2 \cdot min^{-1}$ for 4 minutes and 80 $mU \cdot m^2 \cdot min^{-1}$ for 3 minutes, after which the dosage was reduced to 40 $mU \cdot m^2 \cdot min^{-1}$ for the remainder of the clamp. A 25% glucose infusion was initiated at 15 minutes or sooner if fasting blood glucose levels were below 6.5 $mmol \cdot L^{-1}$and adjusted after 5-minute blood glucose readings until stabilised at 5 $mmol \cdot L^{-1}$. As this method elevated blood insulin concentrations within a physiological rather than a supraphysiological range, the time to stabilisation was variable between participants. GDR was calculated from the average rate of glucose infused ($mg \cdot kg^{-1} \cdot min^{-1}$) during a 60 minute stabilisation phase.

3.5.2 Fasting Blood Glucose

Fasting blood samples were obtained at rest prior to each clamp. Blood glucose concentration (mmol/L) was determined via enzymatic reaction in an automated glucose analyser (YSI Instruments, Yorba, CA).

3.5.3 Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Insulin concentration in fasting blood samples was analysed by a third party (Massey University Nutrition Laboratory, Palmerston North, NZ) and used to calculate HOMA-IR via the HOMA2 online calculator (https://www.dtu.ox.ac.uk/homacalculator/).

3.6 Physical Exercise Capacity Tests

3.6.1 Aerobic Capacity

Participants completed a continuous ramp protocol to volitional exhaustion on a cycle ergometer commencing at 40 Watts for 3 minutes and increasing 1 Watt every 4 seconds. Participants were encouraged to maintain a cadence of 70 rpm during the ramp. Peak oxygen consumption (VO_2peak) was measured (SensorMedics Vmax, YorbaLinda, CA) as the average of the highest 30-second consumption rate ($mL \cdot kg^{-1} \cdot min^{-1}$) during the test. Acceptance of a maximal effort was dependent upon the participant achieving a maximal Borg Scale (1-20) rating and/or an RER>1.15.

3.6.2 1RM Strength

Estimated 1 repetition-maximum (1RM) tests were completed at baseline and every 2 weeks for smith machine bench press, lateral pulldown, seated row and barbell upright row during a maximal-effort of 3-6 repetitions and predicted via the Brzycki Formula (Brzycki 1999).

3.7 Body Composition

Body composition measures including weight (kg), BMI ($kg.m^{-2}$) and waist circumference (cm) were taken in a fasted state prior to exercise testing. *Vastus Lateralis* (VL) muscle thickness (Figure 3.2) and subcutaneous adipose tissue (SAT) (Figure 3.3) were measured after lying supine for 15 minutes via B-mode ultrasound (Terason T32000, Teratech Corp., Burlington, MA) using previously validated protocols (Thomaes 2012, Toomey 2011) modified to include measurement of SAT at the biceps and cross-section diameter at the VL muscle. Measurements were taken in a supine position after participants

had been lying relaxed for 15 minutes and then analysed using ImageJ software (National Institute of Health, Bethesda, Maryland). SAT was determined from the sum of adipose thickness at 4 standard calliper sites: thigh, calf, biceps, and triceps and VL thickness from the maximal cross-sectional diameter measured at 1/3 the distance from the centre of the patella to the tubercle of the anterior superior iliac spine.

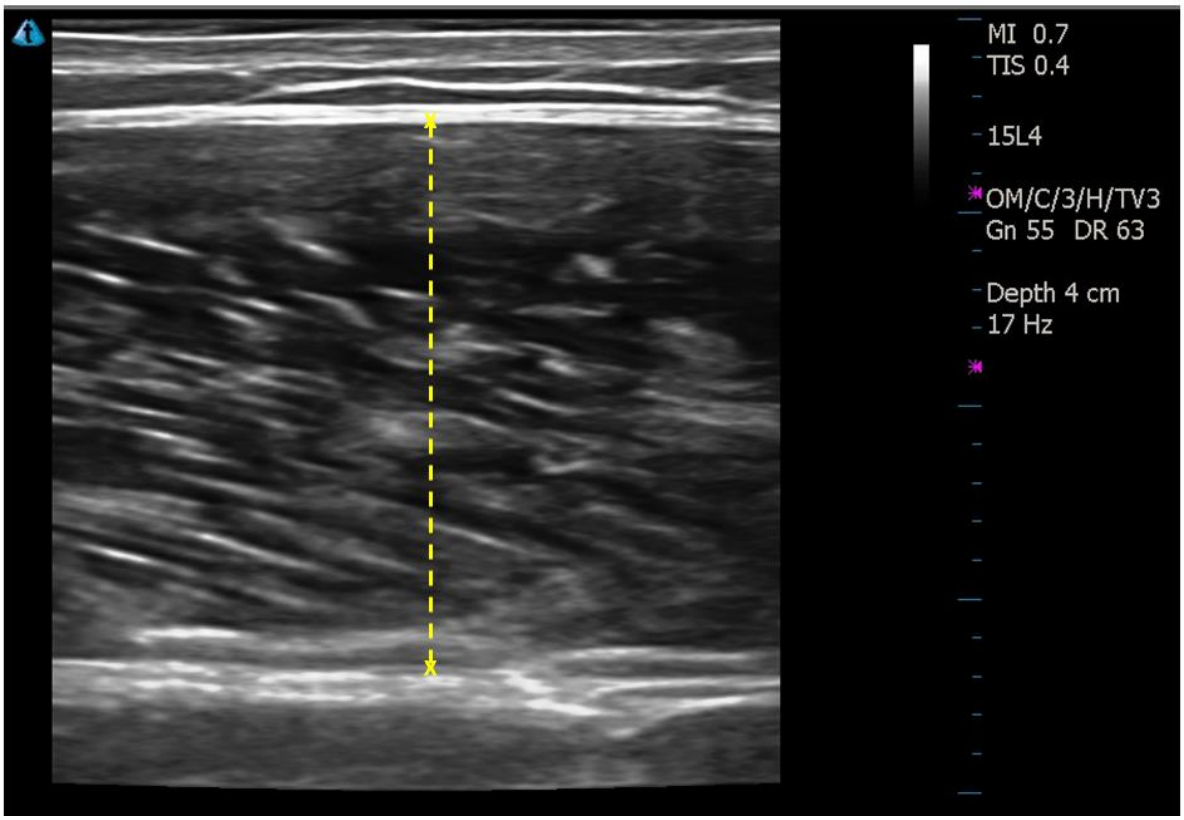


Figure 3.2 *Vastus lateralis* muscle thickness via B-mode ultrasound.

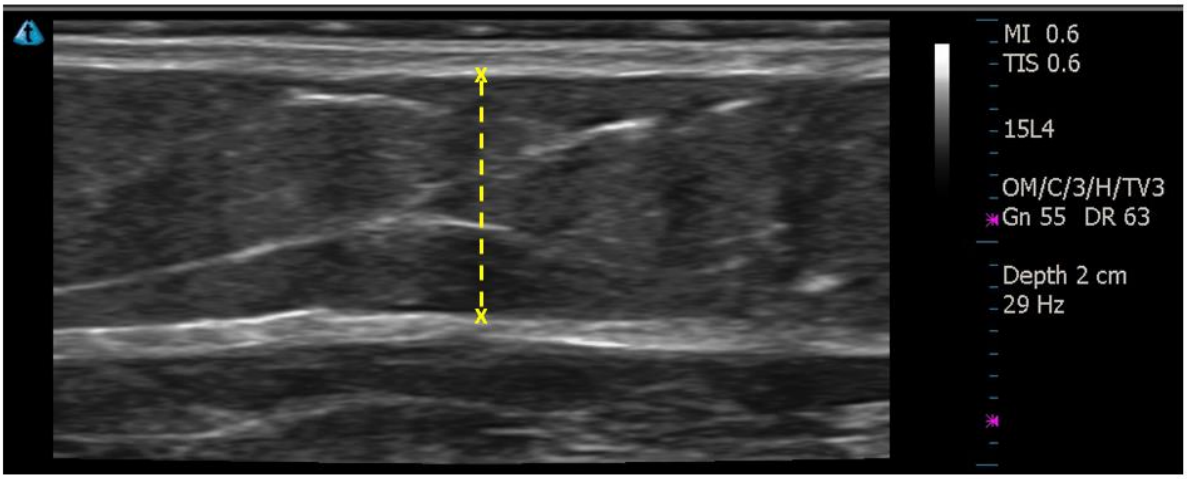


Figure 3.3 Subcutaneous adipose thickness via B-mode ultrasound.

3.8 Muscle Biopsies

Muscle biopsies were obtained from the VL muscle under local anaesthesia using a Bergstrom needle (Bergstrom 1975) with suction. Samples were collected 45 minutes after lying supine and then 30 minutes after glucose stabilisation was achieved during the euglycaemic insulin clamp. Muscle samples were immediately freed from blood, visible fat, and connective tissue. 5 mg of tissue was placed in Karnovsky's fix (Sheehan 1980) for 2 hours and then stabilised in a buffer solution for electron microscopy analysis. The remaining tissue ~30 mg was immediately frozen in liquid nitrogen after extraction, then transferred to cryovials for storage at -80^{o}C.

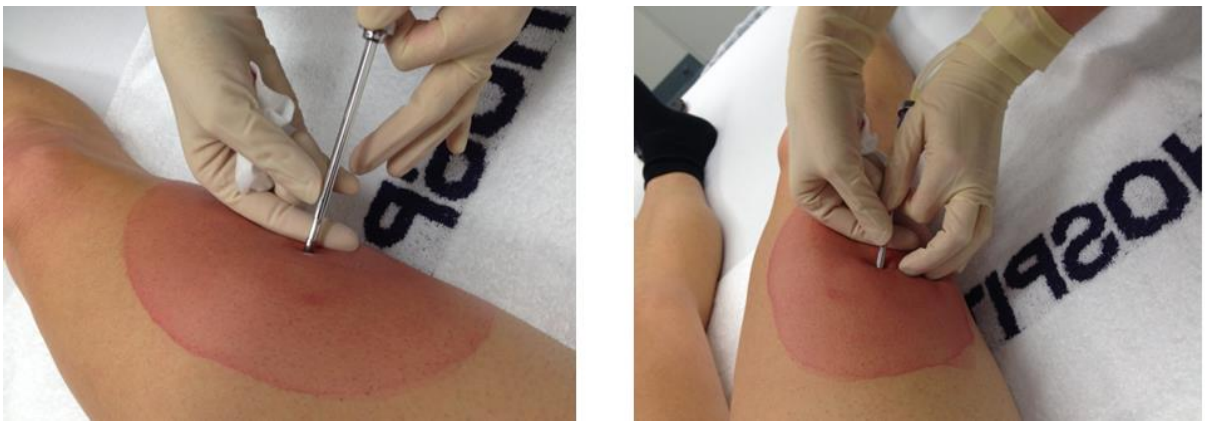


Figure 3.4 *Vastus Lateralis* Muscle biopsy.

3.9 mRNA

There was sufficient tissue for RNA extraction in 15 samples (control *n*=7; whey *n*=8). ~10 mg of thawed tissue was homogenised in lysis buffer (Norgren RNA/DNA/Protein Purification Plus Kit (#47700). RNA was extracted and sent to a third party (Environmental Science Research, Wellington, NZ) for mRNA analysis (qPCR) using previously described methods (Rowlands 2011). Included genes were associated with mitochondrial biogenesis

(*PPARG C1A [PGC1-α], CS, NRF1*), angiogenesis (*VEGFA, VEGFR2, NOS3* and NO-mediated signal transduction (*NOS3*).

3.10 Microcirculation

3.10.1 Near Infrared Spectroscopy

Near infrared spectroscopy (NIRS) was used to assess microvascular blood volume (mBV) and microvascular blood flow (mBF) at the VL muscle using previously validated methods (Lucero 2018). Briefly, a NIRS probe was secured over the belly of the VL muscle about two-thirds from the top of the muscle and parallel to the muscle fibres (Figure 3.5). VL 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. 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 [HbO2] and deoxygenated haemoglobin [HHb], respectively, as well as the haemoglobin concentration in the total blood volume ([tHb] = [HbO2] + [HHb]) (Figure 3.6). mBV was determined from the average total haemoglobin (t[Hb]) concentration (μM) over 5 minutes, at rest before (basal), then 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) of the femoral vein, and converted to $mL \cdot min^{-1} \cdot 100\ mL^{-1}$ using the equation:

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

where [Hb] was determined from a resting blood draw, and Δt[Hb] averaged over 5 measurements taken 1 minute apart.

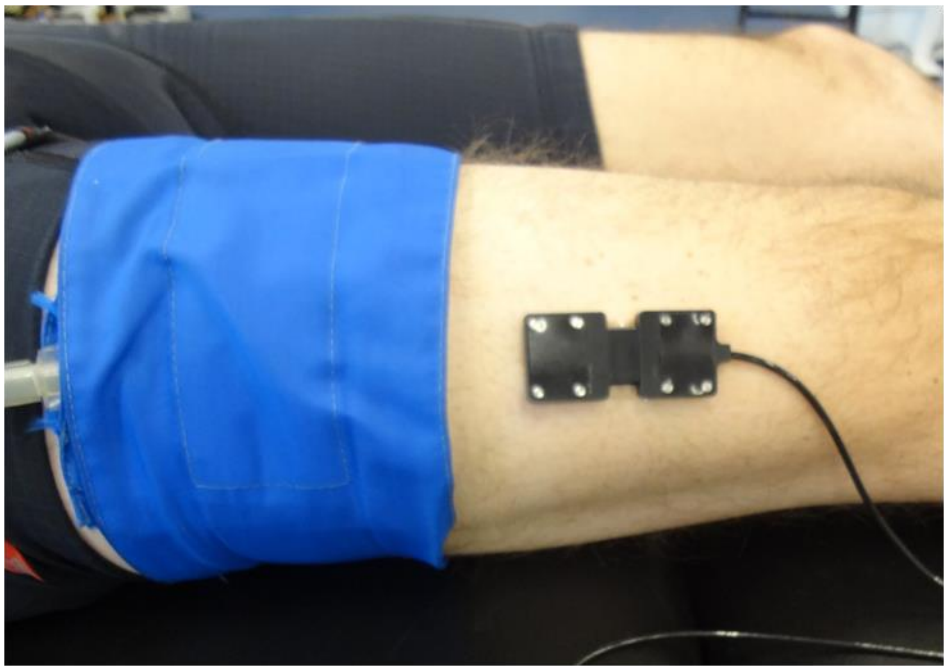


Figure 3.5 Placement of NIRS sensor and occlusion cuff.

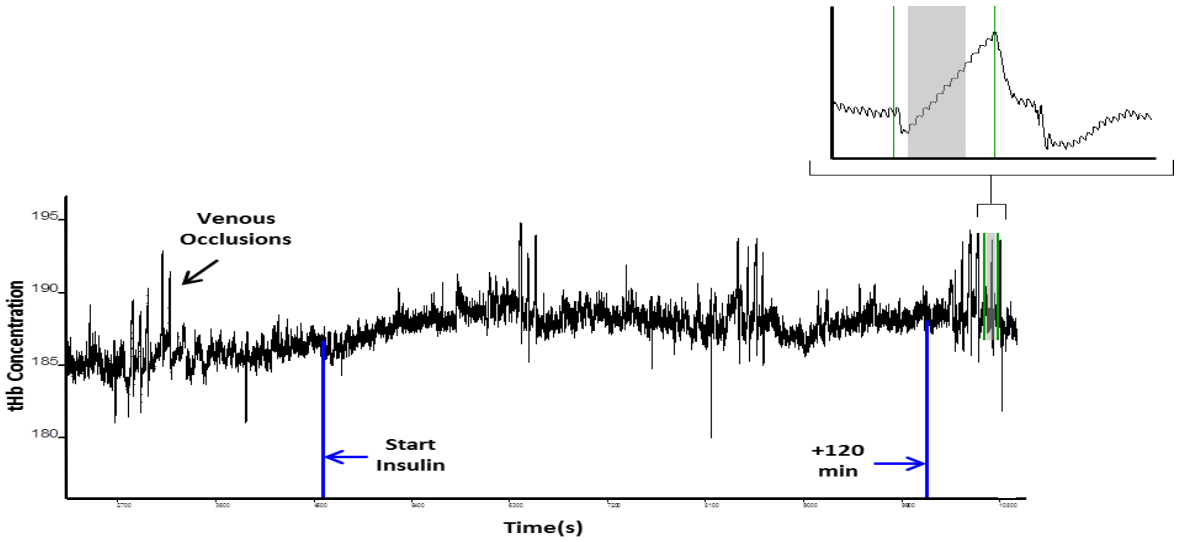


Figure 3.6 NIRS trace during clamp (total haemoglobin concentration).

3.10.2 Capillarity

A section of muscle tissue was selected using a dissecting microscope and placed in cassettes in a Tissue Tek VIP 5 processor overnight. The tissue was orientated into cross sections, embedded and cut into 4 micron sections. Sections were collected on adhesive slides and dried for 1 hour at 60 degrees Celsius. Sections were stained with Periodic Acid Schiff. Capillary counts were taken from sections of 100-150 cells using light microscopy and digital photos taken at x400 magnification (Figure 3.7). Capillarisation was expressed as the capillary to myofibre ratio (C/F ratio).

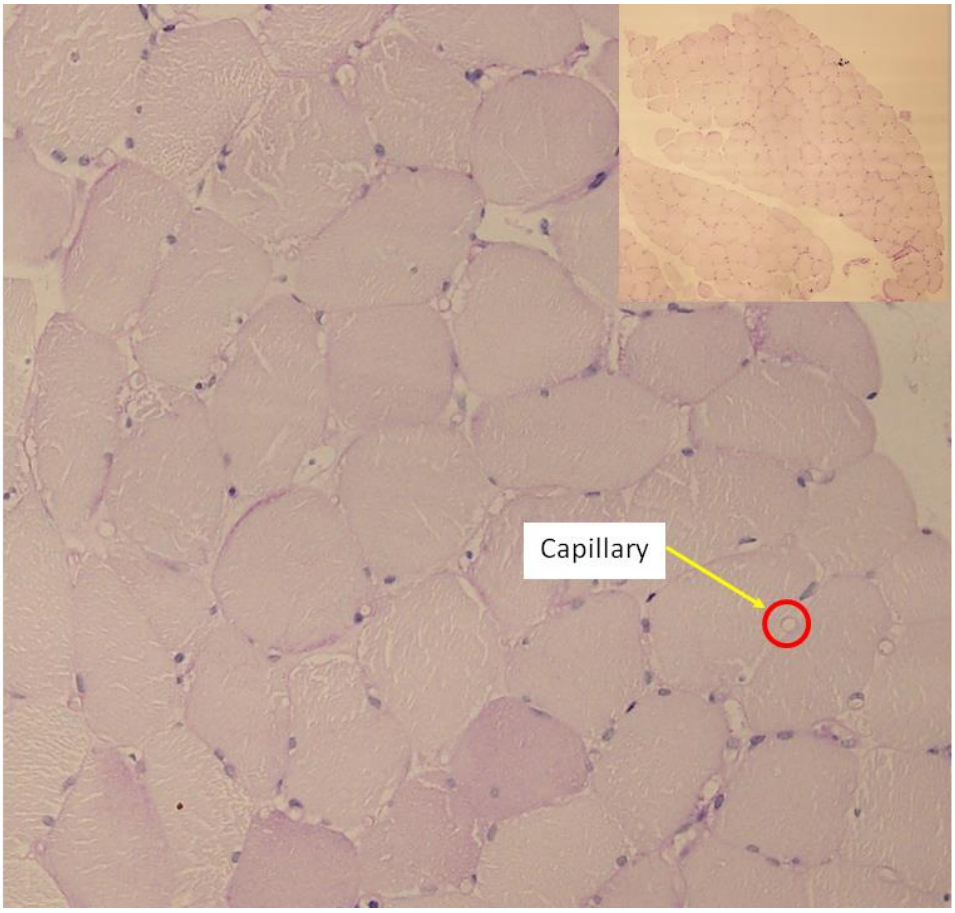


Figure 3.7 Stained skeletal muscle cross-section for counting capillary to fibre ratio.

3.11 Mitochondria and Lipid

3.1.1 Density

Baseline and post-intervention muscle biopsies (control n=11, whey n=12) were taken at the VL muscle. For electronmicroscopy 5 mg of tissue was prepared for imaging analysis using previously described methods (Hayat 2013). Intermyofibrillar sections were viewed under a Philips CM100 TEM at a magnification of 5800X. Photos were taken using the Morada soft imaging system. A minimum of 10 and up to 14 intermyofibrillar images of a muscle cell were taken for each muscle tissue sample. Mitochondrial and lipid density for each component were determined using Image J software (version 1.48v, National Institutes of Health, Bethesda, MD) by manually tracing only clearly discernible outlines of mitochondria and lipid droplets on each image using a graphic tablet (MP1060-HA60, Monoprice, California) as shown in Figure 3.8. Density was quantified as the average percentage of traced pixels to the number of pixels within the whole images.

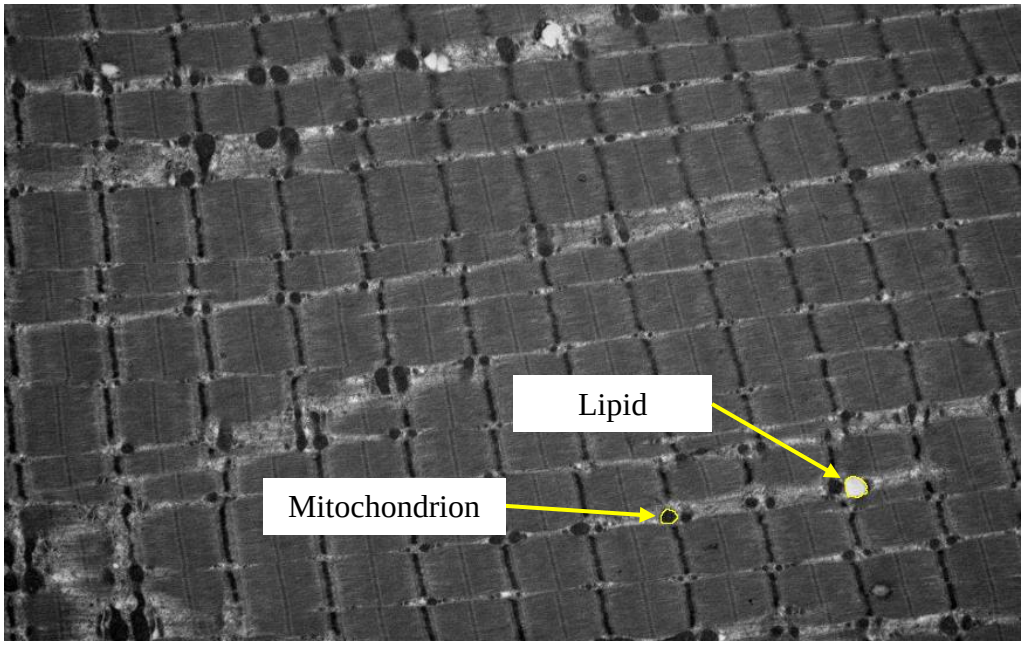


Figure 3.8 Representative electron microscopy image of myocellular mitochondrial and lipid density tracings.

3.11.2 Enzymes

Mitochondrial enzyme concentration for citrate synthatase (CS) and cytochrome c oxidase (COX) was inferred from the rates of enzymatic activity as previously described (Hayat 2013). For full methods see Appendix G.

3.12 Psychometric Surveys

3.12.1 Depression, Anxiety, Stress Scale

The DASS42 is a 42 item questionnaire (Appendix H) used to identify levels of depression, anxiety and stress over the previous week (Antony 1998). Scores can be grouped to provide a single score of general mood with larger scores indicating heightened emotional status.

3.12.2 Short Form-36 Questionnaire

The Short Form-36 (SF-36) is a 36 item questionnaire (Appendix G) which measures Quality of Life (QoL) across eight domains, which can be grouped into physical and mental function subscales (Ware 1992).

3.13 Statistical Analysis

Sample size estimation was based upon the primary outcome GDR using the test-retest values reported by Defronzo et al (Defronzo 1979) in a healthy adult population and upon sample size estimations for magnitude based clinical inference (Hopkins 2016, Hopkins 2009). The typical error of measurement was doubled to allow for uncertainty in variability in a T2D population and *n* was increased by 10% to allow for potential dropouts, which brought the required sample to 24. The threshold for smallest worthwhile clinical change in GDR was

5.4% based upon the effect of 3 months of hypoglycaemic therapy (Metformin) on naïve Type-2 diabetics (Derosa 2009).

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) and reported as the percentage change from baseline. All continuous data not including negative values were log transformed prior to analysis to manage heteroscedasticity. Uncertainty was presented as 90% confidence limits or *P* value. Magnitude-based inference was employed to infer clinical and mechanistic outcome effects (Burton 1998, Hopkins 2009). The probability that a contrast was at least greater than the clinical threshold or smallest Cohen's *d* standardized difference (0.2 × baseline SD) was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain (Hopkins 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 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, inconclusive* refers to outcomes where the likelihood of both benefit and harm exceeded 5%. The likelihood of a clinical benefit of intervention was expressed as the benefit:harm odds ratio, with 66:1 the smallest adoption threshold (Hopkins 2009). Pre- and post-intervention scores are presented in figures as raw means and standard deviations.

Linear regression was used to determine associations between outcome variables. The likelihood that there was a small, moderate, large, very large or extremely large association using thresholds of 0.10, 0.30, 0.50, 0.70 and 0.90 was predicted using a published spreadsheet (Hopkins 2010).

CHAPTER 4

NIL WHEY PROTEIN EFFECT ON GLYCAEMIC CONTROL AFTER INTENSE MIXED-MODE TRAINING IN TYPE-2 DIABETICS.

Full Publication

Gaffney, K. A., Lucero, A., Stoner, L., Faulkner, J., Whitfield, P., Krebs, J., & Rowlands, D. (2018). Nil Whey Protein Effect on Glycemic Control after Intense Mixed-Mode Training in Type 2 Diabetes. *Medicine & Science In Sports & Exercise*, *50*(1), 11-17. doi:10.1249/MSS.0000000000001404

Abstract

Introduction: While intense endurance and resistance exercise training and whey protein supplementation have both been shown to independently improve glycaemic control, no known studies have examined the effect of high-intensity mixed-mode interval training (MMIT) and whey supplementation in adults with Type-2 diabetes (T2D). The purpose of this investigation was to determine if peri-training whey protein supplementation combined with MMIT can improve glycaemic control.

Methods: In a double-blind randomised controlled trial, 24 men (55.7±5.6 y) with T2D performed MMIT with whey (20 grams) or placebo control for 10 weeks. Glycaemic control was assessed via glucose disposal rate (GDR) during a euglycaemic insulin clamp, fasting blood glucose concentration (FBG), and HOMA-IR. Changes in peak oxygen consumption (VO_{2peak}), 1-repetition maximum strength (1RM), Vastus lateralis (VL) muscle and subcutaneous adipose thicknesses (SAT), and waist circumference (WC) were also assessed.

Results: 10-weeks of MMIT substantially improved GDR by 27.5% (90%CI 1.2%, 60.7%) and 24.8% (-5.4%, 64.8%) in the whey and control groups, respectively. There were likely and possible reductions in FBG by -17.4% (-30.6%, -1.6) and HOMA-IR by -14.1% (-25.3%, 1.08%) in the whey group, however, whey effects were not clearly beneficial to glycaemic outcomes, relative to control. MMIT also clearly substantially improved 1RM by 20.6% (16.3%, 24.9%) and 22.7% (18.4%, 27.2%), VO_{2peak} by 22.6% (12.0%, 26.2%) and 18.5% (10.5%, 27.4%), VL muscle thickness by 18.9% (12.0%, 26.2%) and 18.6% (10.5%, 27.4%) and possibly reduced WC by -2.1% (-3.1%, -1.0%) and -1.9% (-3.7%, -0.1%) in the control and whey groups respectively, but the whey-control outcome was trivial or unclear.

Conclusion: A clinically-meaningful enhancement in glycaemic control following 10-weeks of MMIT was not clearly advanced with peri-training whey protein supplementation in middle-aged men with Type-2 diabetes.

4.1 Introduction

A central pathology of type-2 diabetes (T2D) is impaired glycaemic control, a condition characterised by a diminished capacity to restore postprandial blood glucose concentrations to homeostatic levels. Skeletal muscle is the major tissue of postprandial glucose disposal (Thiebaud 1982), and a well-established site of dysfunction in T2D (Bjornholm 2005). It is well-documented that T2D skeletal muscle displays low expression of proteins contributing to glucose uptake and metabolism, including: contractile, glucose transporter, and mitochondrial proteins (Hwang 2010, Kampmann 2011, Mullen 2010). Exercise has been shown to upregulate the expression of these proteins (Little 2011, Son 2012), and it is well-established that the improvements in aerobic capacity, lean mass and strength that follow progressive aerobic or circuit resistance training are also associated with better glycaemic control (Gillen 2016, Kang 2009). High-intensity interval training has emerged as an effective low-volume and time-efficient exercise mode for rapidly improving glycaemic control. In middle-aged men with T2D, 2 weeks of high-intensity cycle interval training was shown to significantly increase the expression of glucose transporter 4 (GLUT4) and mitochondrial proteins in the *vastus lateralis* muscle and lower 24-hour blood glucose concentrations (Little 2011).

Milk protein supplementation has shown promise as a complementary therapeutic agent to exercise for improving glycaemic control. Milk proteins are rich in amino acids that stimulate protein synthesis in skeletal muscle (Bohe 2003), which may, like exercise training, lead to better glycaemic control. Independently, whey supplementation was shown to improve glucose tolerance and FBG after 8 weeks in insulin resistant rats (Belobrajdic 2004, Tong 2014) and HOMA-IR after 12 weeks in overweight and obese adults (Pal 2010). As an adjunct therapy to exercise and compared to carbohydrate consumption alone, milk-protein supplementation for 6 weeks was reported to improve VO_{2max} in treadmill trained sedentary

men (Robinson 2011) and lean mass and 1RM bench press strength after 8 weeks in mixed-mode trained female college basketball players (Taylor 2016). As each of those outcomes has been previously associated with improved glycaemic control (Bacchi 2012, Kang 2009, Larose 2011) combined treatments may also provide better therapeutic outcomes than exercise alone in populations with T2D.

The aim of this study was to determine whether whey supplementation for 10 weeks would improve glycaemic control in a population with T2D performing high-intensity mixed-mode interval training. We hypothesised that whey supplementation would enhance glycaemic control to a greater extent than exercise alone. If effective, this may provide a practical adjunct therapy to exercise for improving T2D rehabilitation outcomes.

4.2 Methods

Refer to chapter 3.1-3.4 for design, 3.5-3.8 for analytical methods, and 3.13 for statistical analysis and inferential approach.

4.3 Results

Twenty-four men with T2D were recruited to the study (Figure 4.1). There were no clear differences between group characteristics at baseline (Table 4.1). All participants completed the 45 exercise sessions within the 10-week period. The glycaemic control outcomes and statistical summary for all parameter measures are in Figure 4.2 and Table 4.2, respectively. Ten weeks of MMIT produced a clinically meaningful enhancement in GDR in the whey and control groups, respectively, relative to the smallest threshold change (5.4%); however, the whey-control difference was unclear. The secondary outcome measures of

glycaemic control (FBG and HOMA-IR) showed a likely and possible benefit of whey supplementation on FBG, and possible and unclear benefits on HOMA-IR in the whey and control groups respectively, reaching the adoption threshold (OR>66:1) only in the Whey group; however, there was also no clear difference in the Whey-Control contrast. Very likely and almost certain improvements in VO_{2peak}, 1RM strength and VL muscle thickness in response to 10-weeks of MMIT in both the Whey and Control groups were observed (Figure 4.3), but the whey-control differences were also negligible and unclear. There was a possible decrease in WC in both groups and a possible decrease in SAT in the whey group only (Table 4.2).

Table 4.1. Baseline characteristics of the control and whey groups

	Control ***n*=12**	**Whey** ***n*=12**
Parameter	Mean ± SD	Mean ± SD
Age (y)	57.8 ± 5.2	53.5 ± 5.6
Height (cm)	174.6 ± 7.1	177.1 ± 8.7
Weight (kg)	91.9 ± 15.5	92.8 ± 11.0
BMI ($kg \cdot m^2$)	30.1 ± 4.9	29.6 ± 2.7
VO_{2peak} ($mL \cdot kg^{-1} \cdot min^{-1}$)	26.9 ± 10.2	28.7 ± 4.9
FBG ($mmol \cdot L^{-1}$)	9.4 ± 2.9	10.2 ± 3.6
GDR ($mg \cdot kg^{-1} \cdot L^{-1}$)	2.11 ± 1.4	1.93 ± 0.8
Time to euglycaemia (min)	106.3 ± 67.2	106.7 ± 53.9

Data are presented as means and standard deviations.

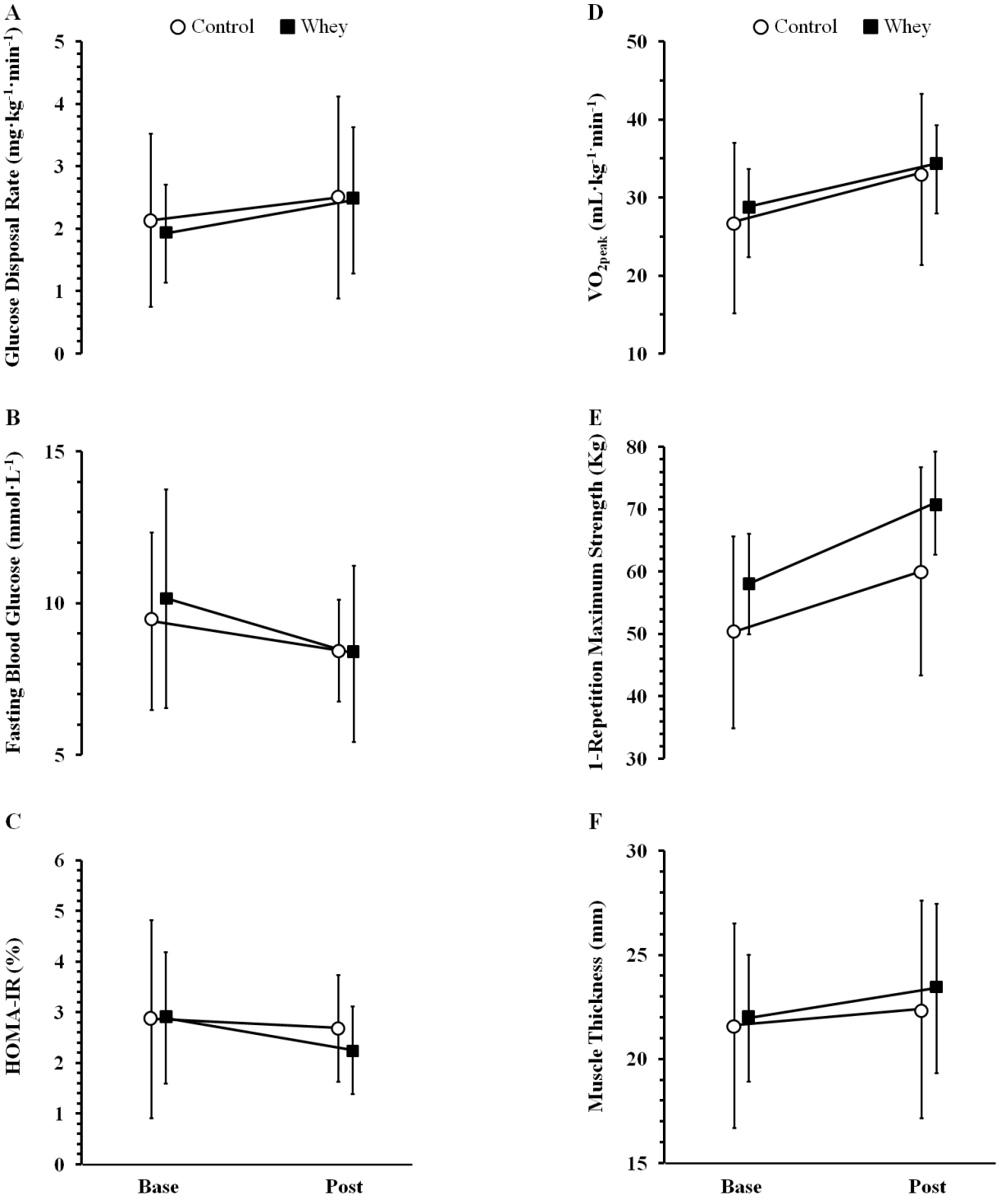


Figure 4.1. Effect of 10 weeks of peri-training whey supplementation on: A) glucose disposal rate; B) fasting blood glucose concentration; C) HOMA-IR; D) VO_{2peak}; E) 1RM strength (the back log-transformed average of 4 log-transformed lift scores); and, F) *vastus lateralis* muscle thickness. Data are raw means and SD for the Pre (baseline) and Post testing time points.

Table. 4.2. The effect of 10-weeks peri-training whey-protein supplementation on established clinical measures of glycaemic control, exercise performance, and body composition.

Contrast[a]	% Change	Upper CI	Lower CI	Likelihood (%) benefit/trivial/harm[b]	Qualitative[b]	Benefit odds[b]
Glucose Disposal Rate						
Control	24.8	64.8	-5.4	90.1/7.1/2.8	Benefit likely	318
Whey	27.5	60.7	1.2	95.6/3.5/0.9	Benefit very likely	2424
Whey-Control	2.7	44.8	-28.0	42.6/24.4/33.0	Unclear	2
Fasting Blood Glucose						
Control	-8.1	10.7	-23.7	50.4/45.8/3.8	Benefit possible	26
Whey	-17.4	-1.6	-30.6	88.8/11.0/0.2	Benefit likely	3291
Whey-Control	-10	15.3	-29.8	57.3/35.9/6.8	Unclear	19
HOMA-IR						
Control	-5.3	28.3	-30.1	23.7/68.8/7.6	Unclear	4
Whey	-14.1	1.08	-25.3	42.0/58/0.0	Benefit possible	3331
Whey-Control	9.2	25.4	-34.2	35.0/59.1/6.0	Unclear	8
VO_{2peak}						
Control	22.6	26.2	12.0	99.8/0.2/0.0	Benefit almost certain	5.05E+07
Whey	18.5	27.4	10.5	99.1/0.9/0.0	Benefit very likely	2.81E+06
Whey-Control	-3.3	9.07	-8.75	4.4/69.1/26.5	Trivial possible	0
1-Repetition Maximum Strength[c]						
Control	20.6	24.9	16.3	100/0.0/0.0	Benefit almost certain	3.29E+31
Whey	22.7	27.2	18.4	100/0.0/0.0	Benefit almost certain	7.80E+35
Whey-Control	1.8	7.1	-3.2	0.1/99.8/0.0	Trivial almost certain	11
Muscle Thickness						
Control	18.9	26.2	12.0	100/0.0/0.0	Benefit almost certain	1.78E+09
Whey	18.6	27.4	10.5	99.89/0.02/0.0	Benefit almost certain	6.62E+07
Whey-Control	-0.2	9.1	-8.8	13.6/70.6/15.9	Unclear	1
Waist Circumference						
Control	-2.1	-1.0	-3.1	41.0/59.1/0.0	Benefit possible	7.44E+05
Whey	-1.9	-0.1	-3.7	28.6/71.4/0.0	Benefit possible	2888
Whey-Control	0.1	2.1	-1.8	0.3/99.5/0.2	Trivial very likely	2
Subcutaneous Adipose Tissue[d]						
Control	-1	6.9	-8.3	6.7/90.8/2.5	Trivial likely	3
Whey	-6.9	3.5	-16.2	43.7/55.8/0.5	Benefit possible	151
Whey-Control	-6.0	6.7	-17.1	40.1/57.9/2.0	Benefit possible	32

[a] Data for each contrast are post-pre. [b] The threshold for smallest clinical effect for glucose disposal rate was 5.4% (28); and for all other measures the smallest standardised difference (0.2xSD). The likelihood that a contrast was at least greater than the clinical threshold was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain. Unclear refers to outcomes where the likelihood of both benefit and harm exceeded 5%. The clinical adoption threshold was expressed as a benefit: harm odds ratio >66:1. [c] Total 1-Repetition Maximum strength was expressed as the back log-transformed average of 4 log-transformed lift scores. [d] Subcutaneous Adipose Tissue was expressed as the sum of 4 sites.

4.4 Discussion

4.4.1 Main Findings

The current study showed that consumption of 20 grams of whey protein before and after MMIT for 10 weeks did not enhance glycaemic control in a T2D population assessed via measures of glucose disposal rate, fasting blood glucose, and HOMA-IR. Similarly, whey supplementation did not enhance any of the exercise performance adaptations accruing in response to MMIT, including VO_{2peak}, 1RM strength, and muscle thickness. While previous evidence indicates that whey supplementation and high-intensity interval training independently improve glycaemic control (Little 2011, Pal 2010), no clear benefit of combined therapies was observed.

Previously, consumption of 10 grams of whey protein hydrolysate before and after resistance training for 10 weeks was shown to significantly increase quadriceps cross-sectional area in healthy trained men (Farup 2014). In addition, consumption of a single-dose of a mixed milk-protein (20 grams) carbohydrate beverage after treadmill training for 6 weeks significantly increased VO_{2max} in sedentary middle-aged men compared to an isocaloric carbohydrate control (Robinson 2011). As both the increase in mid-thigh muscle cross-sectional area and VO_{2peak} following exercise intervention have been previously associated with improved HbA1c in populations with Type-2 diabetics (Kang 2009, Larose 2011), we predicted that peri-training whey supplementation for 10 weeks would lead to better glycaemic control than the MMIT alone. Our observation that whey supplementation did not clearly increase muscle thickness at the VL or VO_{2peak} suggests that adaptive responses previously seen in exercising healthy populations may be lost with the development of T2D and may explain why we saw no effect of whey protein on GDR, FBG or HOMA-IR.

It is possible that adults with T2D require a larger dose of milk-protein to induce clinically meaningful outcomes. 20 grams of protein has been reported to be the optimal dosage for improving protein synthetic responses in the skeletal muscle of healthy young men (Moore 2009). While we also provided a total of 40 g of protein as 20 g before and 20 g after MITT, in another study in healthy elderly individuals (71±4 y), 40 grams of whey protein increased muscle protein synthesis after resistance training compared to a 20 gram dose (Yang 2012). The cohort in the current study was middle-aged (55.6±5.7 y), however, T2D skeletal muscle has been shown to display characteristics of aged tissue, including: accelerated muscle wasting (Lee 2010); lower contractile strength to muscle volume (Park 2006), and decreased mitochondrial density (Chomentowski 2011). Future investigations should test dosage effects on muscle protein synthetic responses in a population with T2D.

4.4.2 Secondary Findings

While we saw no clear benefits of whey supplementation on glycaemic control in this study, there was some evidence that the protein exposure produced a more pronounced effect on each of the glycaemic measures compared to exercise alone, as suggested through the observation of a substantially larger clinically-beneficial odds ratios for GDR, FBG, and HOMA-IR in the whey compared to the control group. We also observed that the adoption threshold (odds ratio >66) was reached for FBG, HOMA-IR and SAT only in the whey group, suggesting that the magnitude of the improvements in those secondary outcomes was sufficient to justify treatment use only when therapies were combined. It is important to acknowledge, however, that the full placebo-control adjusted outcome (whey-control), which takes into account the on-study effect, left a statistically unclear whey-protein effect. We suggest that a longer intervention or a larger cohort (to increase study power) may have

clarified whether whey supplementation was enhancing the pattern for improvement in clinical outcomes.

4.4.3 Limitations

An inherent potential confounder of investigations with control of energy intake was that the control group was consuming substantially more carbohydrate each training morning than the whey group (60 compared to 20 grams). We reasoned that while there was potential for the control group to be consuming more carbohydrate than their normal dietary intake, which could be deleterious to glycaemic control, we expected that the metabolic demands of 20 minutes of MMIT would obviate any effect on post-exercise blood glucose concentration in a previously sedentary population. In addition, 6 x 20 minute sessions of high-intensity interval cycling was previously shown to significantly improve postprandial and 24-hour blood glucose regulation in middle-aged adults with T2D (Little 2011). Our findings confirm that chronic intense interval training is effective for improving glycaemic control in populations with T2D. We also found that the 5-days per week, mixed-mode training regime was well-adhered to by a previously sedentary, middle-aged T2D population, improved glucose disposal rates by a 4-5-fold greater magnitude than an equivalent duration of pharmacotherapy (Metformin) alone (Derosa 2009), and negated the potentially deleterious impact of consuming 2 × 30 grams of a carbohydrate beverage each morning. Therefore, the MMIT mode of exercise training may prove to be highly effective for improving T2D health outcomes in long-term rehabilitation programs where high intensity exercise is appropriate.

4.4.4 Conclusion

In conclusion, consumption of 20 grams of whey protein before and after high-intensity mixed-mode interval training for 10 weeks, compared to isocaloric non-protein control, did not clearly enhance glycaemic control, VO_{2peak}, 1RM strength, or VL muscle cross-section diameter in middle-aged men with T2D. These findings suggest that over short-term interventions, populations with T2D may be resistant to nutritional stimulation of this nature. However, recent dose response data, and patterns for greater gains in some clinical parameters in the whey group support further investigation of the nutritional intervention, possibly increasing the supplement dose or the intervention period.

CHAPTER 5

WHEY SUPPLEMENTATION IMPROVES MICROCIRCULATION AFTER 10 WEEKS IN EXERCISING MEN WITH T2D.

Abstract

Introduction: Type-2 diabetes (T2D) is characterised by vascular insulin resistance, which impairs the normal upregulation of microcirculation after eating. Milk-protein supplementation and exercise have been shown to independently improve vascular function, but the effect of combined therapies on microcirculation is unknown. Therefore, the purpose of this investigation was to test the effect of adjunct whey protein supplementation with exercise on insulin-mediated microcirculation in T2D skeletal muscle.

Method: In a double blind, randomized clinical trial, 24 non-insulin dependent middle-aged men with T2D were allocated to a peri-training whey-carbohydrate (20g-10g) supplement or carbohydrate-only control. Participants completed 45 high-intensity morning exercise sessions (27 cycling, 18 resistance) over 10 weeks. Microvascular blood flow (mBF) and blood volume (mBV) were measured at the Vastus lateralis muscle via near-infrared spectroscopy at rest and in response to insulin during the plateau stage of a euglycaemic insulin clamp at baseline and after 10 weeks. Changes in capillarity were determined from the ratio of capillaries to muscle cells from sections of biopsied muscle tissue under light microscopy, and the angiogenic environment from relative mRNA expression of eNOS, VEGF and VEGFR2 (RT-PCR).

Results: 10 weeks of exercise training had no substantial effect on mBF or mBV at rest or in response to insulin; however, the whey-control contrast showed likely and possible improvements in mBV of 16.8% (90%CI -4.3%, 42.6%) and mBF of 5.9% (-3.7%, 16.3%) respectively at rest and likely improvements in insulin-stimulated mBV of 17.5% (-3.7%, 43.5%) and mBF of 10.2% (0.3%, 21.1%) following whey supplementation. There was no difference in the magnitude of the insulin-mediated change in mBF or mBV between groups. There was a likely increase of 24.5% (-0.1%, 55.0) and a very likely increase of 26.3%

(1.9%, 56.6%) in capillary to fibre ratio in the control and whey groups respectively, with no clear group difference. There were possible, likely, and very likely reductions in the mRNA expression (RT-qPCR reaction cycles) of *VEGF* by -0.2 Cq (-0.6%, 0.1), *VEGFR2* by -0.4 (-0.8, 0.1), and *eNOS* expression by -0.8 (-1.4, -0.2) in the whey compared to the control groups.

Conclusion: Whey protein supplementation elevated skeletal muscle microcirculation in exercising men with T2D; however, there was no evidence that vascular insulin sensitivity was improved. Whey-associated angiogenic plasticity was not supportive of an additive effect beyond that of exercise alone. Further research is warranted on the possibility of adjunct therapy with whey protein ingestion to modulate microvascular haemodynamics following chronic exercise training in T2D men.

5.1 Introduction

Insulin resistance in vascular tissue has been identified as a major barrier to glucose disposal in populations with T2D. In health, insulin is excreted into the circulatory system after eating to promote vascular relaxation, the mechanism that elevates blood kinetics through the microvascular system where glucose extraction occurs (Baron 1995, Fugmann 2003, Vincent 2004). Circulatory regulation has been estimated to account for up to 40% of the normal increase in postprandial glucose disposal (Fugmann 2003); however, in populations with T2D vascular relaxation responses are diminished (Bosevski 2010) and circulatory haemodynamics in response to insulin are reduced in both arterial (Laakso 1992, Reynolds 2017) and microvascular tissue (Clerk 2007, Padilla 2006, St-Pierre 2010, Wallis 2002). Exercise and milk protein supplementation have been shown to independently improve vascular relaxation and circulatory responses to insulin (Luttrell 2013, Mikus 2011, Sindler 2009) and combined therapies could lead to better glucose delivery to disposal tissues. However, this has not been tested in a population with T2D and investigation is warranted.

Skeletal muscle is the primary site of glucose disposal and a tissue critical to glycaemic control (Bogan 2012). The volume of glucose and insulin reaching myocellular membranes is regulated by an insulin-mediated response at vascular epithelium that promotes formation of the vasodilator nitric oxide (NO) in arterial tissue to increase microvascular blood flow (mBF) (Barrett 2011) and at terminal arterioles to increase microvascular blood volume (mBV) via capillary recruitment (Vincent 2004, Zhang 2004). In maurine models of T2D both responses have been shown to be impaired (Clerk 2007, Padilla 2006, St-Pierre 2010, Wallis 2002) with decreased formation of NO as the likely mediator of dysfunction (Khoo 2005, Laakso 1992, Russo 2008, Shankar 2000, Williams 1996). Capillary rarefaction has also been well-documented as a characteristic of T2D skeletal muscle (Benedict 2011,

Marin 1994, Mathieu-Costello 2003, Murakami 2012, Prior 2009) that lowers the capacity for capillary recruitment (Murakami 2012). Taken together, these findings indicate that increasing circulatory responses to insulin through the microvascular system may be critical to improving glycaemic control in populations with T2D.

Exercise is a well-established therapy for improving vascular relaxation and postprandial circulation. Investigations with aging rats, a model of vascular dysfunction, has demonstrated that treadmill training significantly improved vascular relaxation at both the arterial (Luttrell 2013) and arteriolar (Sindler 2009) level of the vascular tree. Furthermore, in populations with T2D, 7 days of mixed treadmill and cycle training was shown to significantly increase femoral artery blood flow during a glucose tolerance test (Mikus 2011) and 6 weeks of resistance training was found to significantly increase forearm capillary blood flow during an oral glucose challenge (Russell 2017). Circulatory improvements after exercise training are likely mediated by an improved capacity to produce NO. 12 weeks of treadmill interval training was shown to significantly increase plasma NO and FMD, a measure of vascular relaxation, in adults with T2D (Mitranun 2014) and 12 weeks of water-based cycle training was reported to significantly increase plasma NO and microvascular blood flow at the forearm in aged adults with T2D (Saowaluck 2017). These findings suggest that increasing the capacity to produce NO in T2D vascular tissue could lead to better insulin-mediated circulation.

Milk protein supplementation has shown potential as a therapy for increasing NO formation and vascular relaxation. In hypertensive rats, casein hydrolysate supplementation for 6 weeks was shown to significantly increase the concentration of the NO-forming enzyme eNOS in arterial tissue and vascular relaxation at the aorta (Sanchez 2011). As an adjunct to exercise, casein hydrolysate supplementation (2.8 g/day) for 8 weeks during aerobic exercise training was reported to significantly improve FMD in postmenopausal women (Yoshizawa

2010). If combined therapies can be utilised to improve arterial relaxation in response to insulin, then circulatory responses through the microvascular system and glucose disposal rates may also be enhanced.

Therefore, the purpose of this investigation was to determine whether consuming a peri-training milk protein supplement for 10 weeks would improve insulin-mediated microcirculation in a population with T2D and whether improvements in microvascular function would correspond with changes in glycaemic control. We hypothesised that whey protein supplementation would improve insulin-mediated mBF and mBV to a greater extent than exercise training alone.

5.2 Methods

Refer to chapter 3.1-3.4 for design, 3.8-3.10 for analytical methods, and 3.13 for statistical analysis and inferential approach.

5.3 Results

There were no substantial differences between group characteristics at baseline (Table 5.1). The 10-week change in mBV, mBF, capillary to fibre ratio (C/F ratio), and transcript expression and the statistical summary for all parameter measures are shown in Table 5-2. Exercise alone (the within-group 10-week effect) had no substantial effect on ΔmBF or ΔmBV under basal or insulin-mediated conditions, but whey protein supplementation produced a possible and likely increase in ΔmBF during basal and insulin-mediated conditions (Figures 5.1 A and B), and a likely increase in ΔmBV during both basal and insulin-mediated conditions compared to the control group (Figures 5.1 D and E). There was no difference in the magnitude of the insulin-mediated change in mBF or mBV between

groups (Figures 5.1 C and F). Regression analysis of the pooled 10-week change scores, revealed negative associations between basal mBV and FBG (r=.27) and HOMA-IR (r=.30); and a negative association between basal mBF and HOMA-IR (r=.48). No associations were observed between any of the changes in microvascular measures and GDR. There was a very likely and likely increase in C/F ratio in the whey and control groups respectively, with no clear group difference (Table 5.1). There were no associations found between the change in C/F ratio, and the change in GDR, mBF or mBV.

There was a very likely increase and an unclear decrease in skeletal muscle *eNOS* expression in the control and whey groups, respectively, with an almost certain whey-control decrease. There was an unclear increase and possible decrease in the expression of *VEGFA* and *VEGFR2* and a likely decrease and almost certain decrease in the expression of *VEGFA* and *VEGFR2* in the control and whey groups respectively and a possible and likely decrease in *VEGFA* and *VEGFR2* expression in the whey-control contrast.

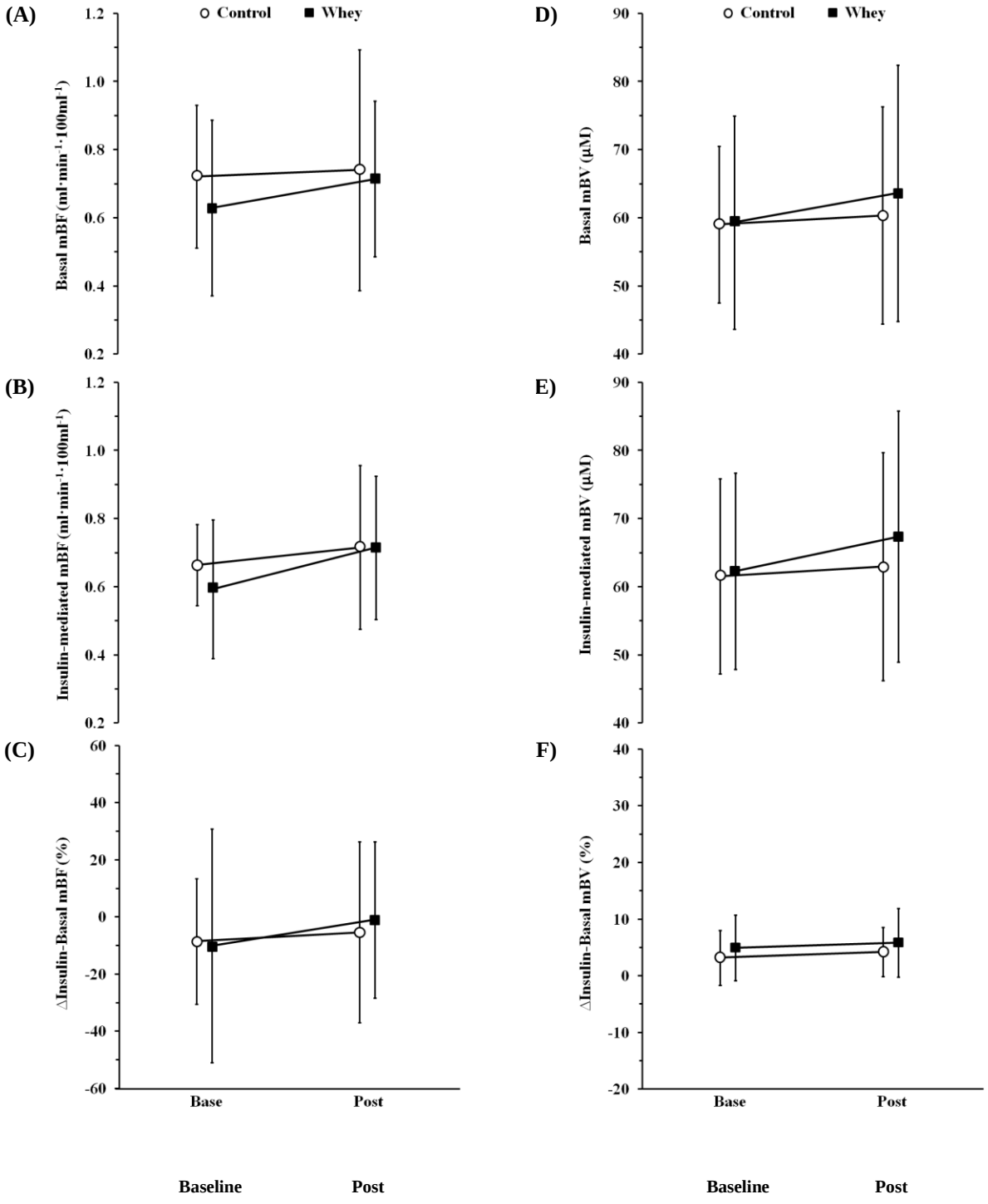


Figure 5.1. The effect of 10-weeks of peri-training whey-protein supplementation on mBF (A-C) and mBV (D-F) under basal and insulin-mediated conditions, and the percent change in insulin-mediated minus basal respectively. Data are raw means and SD for the pretesting (baseline) and post-testing time points.

Table 5.1. The effect of 10-weeks peri-training whey-protein supplementation on basal and insulin-mediated skeletal muscle mBV and mBF, NOS3, VEGFA, and VEGF2R mRNA expression, and C/F ratio in *vastus lateralis* muscle.

Contrast [a]	% Change	Upper CI	Lower CI	Likelihood (%) benefit/trivial/harm[b]	Qualitative[b]
Skeletal muscle microvascular blood volume					
			Basal		
Control	-1.3	14.6	-15.0	17.9/55.9/26.2	Unclear
Whey	15.3	31.7	0.9	83.6/16.0/0.5	Likely increase
Whey-Control	16.8	42.6	-4.3	77.6/19.0/3.4	Likely increase
			Insulin		
Control	3.9	20.6	-10.5	36.8/51.3/11.9	Unclear
Whey	22.1	39.4	6.86	95.7/4.3/0.1	Very likely increase
Whey-Control	17.5	43.5	-3.72	79.3/11.7/3.0	Likely increase
			Insulin-Basal		
Control	1.0	10.0	-7.3	21.7/65.8/12.5	Unclear
Whey	5.19	14.56	-3.42	47.9/49.2/2.9	Possible increase
Whey-Control	4.13	17.49	-7.71	42.6/47.1/10.3	Unclear
Skeletal muscle microvascular blood flow					
			Basal		
Control	0.4	6.7	-5.5	8.5/86.2/5.4	Likely trivial
Whey	6.3	14.4	-1.2	57.8/41.8/0.5	Possible increase
Whey-Control	5.9	16.3	-3.7	52.8/44.7/2.6	Possible increase
			Insulin		
Control	1.5	7.8	-4.5	13.8/83.2/3.0	Likely trivial
Whey	11.8	20.4	3.9	91.7/8.3/0.0	Likely increase
Whey-Control	10.2	21.1	0.3	79.0/20.6/0.4	Likely increase
			Insulin-Basal		
Control	5.2	29.9	-14.8	21.7/65.8/12.5	Unclear
Whey	5.9	30.7	-14.3	47.9/49.2/2.9	Possible increase
Whey-Control	0.7	35.6	-25.3	42.6/47.1/10.3	Unclear
Angiogenesis					
			Capillary to Fibre Ratio		
Control	24.5	55	-0.1	94.2/4.7/1.1	Likely increase
Whey	26.3	56.6	1.9	95.9/3.4/0.7	Very likely increase
Whey-Control	1.5	38	-25.3	40.7/26.0/33.2	Unclear
Angiogenic expression					
	(Cq)		***NOS3***		
Control	0.71	0.21	1.22	99.0/0.9/0.0	Very likely increase
Whey	-0.10	-0.50	0.30	13.5/41.2/45.3	Unclear
Whey-Control	-0.82	-1.40	-0.23	0.2/1.5/98.3	Very likely difference
			VEGFA		
Control	0.14	-0.17	0.45	59.3/34.7/6.0	Unclear
Whey	-0.08	-0.25	0.08	1.9/58.5/39.6	Possible decrease
Whey-Control	-0.22	-0.55	0.11	3.2/22.4/74.3	Possible difference
			VEGFR2/KDR		
Control	-0.37	-0.75	0.00	0.7/6.1/93.2	Likely decrease
Whey	-0.73	-1.01	-0.44	0.0/0.0/100.0	Almost certain decrease
Whey-Control	-0.35	-0.78	0.08	3.0/10.6/86.4	Likely difference

[a] Data for each contrast are post-pre. [b] The threshold for smallest clinical effect was the smallest standardised difference (0.2xSD). The probability that a contrast was at least greater than the clinical threshold was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain. Unclear refers to outcomes where the likelihood of both increase and decrease exceeded 5%.

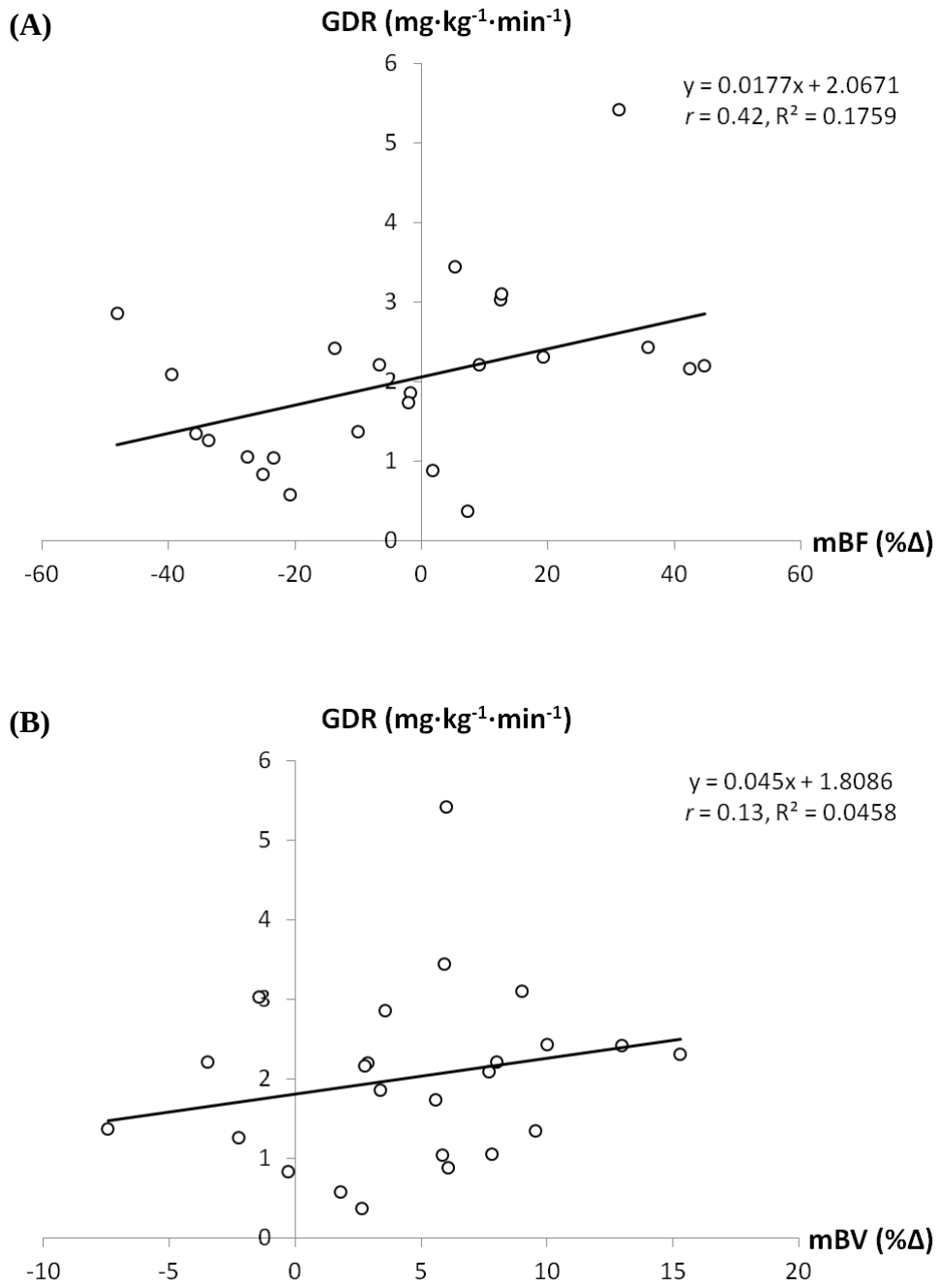


Figure 5.2. Associations between baseline GDR and the percent change in mBF (A) and mBV (B) during a euglycaemic insulin clamp.

5.4 Discussion

5.4.1 Main Findings

The main finding of this investigation was that 10 weeks of peri-training whey protein supplementation increased microvascular blood flow and volume in exercising middle-aged men with T2D; however, the changes did not reflect a greater sensitivity to insulin but rather an increase in basal microcirculation. The improvement in basal microcirculation may be beneficial to 24-hour glycaemia supporting further exploration of whey protein supplementation as an adjunct to exercise in T2D therapy.

Milk-protein supplementation has been previously shown to significantly increase FMD in aging women after aerobic exercise training (Yoshizawa 2010), suggesting that NO-mediated mechanisms may be enhanced by combined therapies. We predicted that whey protein supplementation during intense exercise training would similarly increase insulin-mediated NO formation with a corresponding improvement in vascular relaxation that would upregulate blood flow and volume through skeletal muscle capillary beds. In contrast, the research hypothesis was not supported and after 10 weeks of intense exercise training there was no clear improvement in the insulin-mediated upregulation of mBF or mBV in the whey compared to the control group. While, mBF and mBV were elevated at 10 weeks in the whey group after insulin infusion, this was likely due to an increase in basal blood kinetics. The likely increase in basal mBV and possible increase in basal mBF in the whey group may underlie the observation of much higher odds ratios that supplementation produced a clinical effect on FBG and HOMA-IR (Chapter 4) compared to exercise alone which did not meet the adoption threshold. While there was no clear benefit of whey protein supplementation on glycaemia, it is reasonable to speculate that elevated blood kinetics throughout the day would enhance the delivery of glucose to disposal tissues. An assessment of 24-hour glycaemia should be considered in future investigation of this phenomenon.

5.4.2 Secondary Findings

This was the first investigation to examine both microvascular blood flow and volume under insulin-mediated conditions in a human population with T2D. Previously, capillary recruitment has been identified as the primary microvascular mechanism regulating glucose disposal in healthy populations (Coggins 2001, Eggleston 2007); however, studies of animal models of T2D suggest that changes to both blood flow and volume contribute to the upregulation of glucose disposal rates (Clerk 2007, Padilla 2006, St-Pierre 2010, Wallis 2002). In this investigation it was shown that mBV was elevated under both basal and insulin stimulated conditions after 10 weeks in the whey group, indicative of greater capillary recruitment; however, the increase did not produce a clear increase in glucose disposal rates compared to the control group. Regression analysis of the pooled data showed a positive association between the 10-week change in insulin-mediated microvascular blood flow and the change in glucose disposal rate but no association was observed between the change in microvascular blood volume and glucose disposal rate. Taken together, the findings suggest that to improve postprandial glycaemia in populations with T2D therapies should aim to elevate microvascular blood flow.

It is a well-established phenomenon that consuming a rich source of amino acids proximal to exercise increases the synthesis of skeletal muscle proteins during exercise recovery (Beelen 2008, Dideriksen 2011, Howarth 2009, Moore 2011) with some evidence from animal studies indicating that the phenomenon may extend to proteins that promote angiogenesis.

In this study, there was a downregulation of angiogenic transcript expression in the whey compared to the control group after 10 weeks; however, there was no clear difference in the increase in capillary to fibre ratio between groups. Furthermore, while the changes in

capillary to fibre ratio indicate that whey protein supplementation did not enhance capillary expansion, the clear elevation of blood volume during basal and insulin-mediated states that was not attributed to greater insulin sensitivity, suggest an increase in capillarisation may have occurred and that more extensive exploration of this phenomenon is required to reveal the vascular regulating mechanism that was improved by whey protein supplementation.

4.4.3 Study Limitations/Strengths

A limitation of this study was that microvascular regulation during an insulin clamp, where the participant remains prone for a number of hours, may be indicative of vascular insulin sensitivity, but not accurately reflect true glycaemic regulation under normal daily conditions where some physical activity would likely increase microcirculation and the rate of glucose delivery to muscle cells for disposal. An investigation of longer duration, assessing the change in 24-hour blood glucose concentration may have better reflected our observations of a therapeutic benefit of whey protein supplementation on glycaemic control.

This study, for the first time has provided a global assessment of microcirculation in T2D by encompassing changes in blood flow, blood volume and capillarisation. The observation that capillary expansion did not increase the insulin-mediated recruitment response in the control group and that greater recruitment did not lead to an increase in glucose disposal in the whey group demonstrates the multi-component complexity of microvascular dysfunction in the diseased state and that single-measure assessments of microvascular function may poorly reflect dysfunction or therapeutic outcomes in populations with T2D. The observation that whey protein supplementation improved microcirculation is also likely to be clinically relevant to the treatment of several vascular diseases where impaired microcirculation contributes to the pathology of the disease and the

findings provide a basis for further investigation of this phenomenon in a variety of other clinical populations.

5.4.4 Conclusion

In conclusion, 10 weeks of adjunct whey protein supplementation produced a beneficial effect on microvascular blood flow and volume that would likely enhance the delivery of glucose to skeletal muscle tissue and which was not seen following exercise alone. However, as no clear improvement in vascular insulin sensitivity or glycaemic control was observed, the supplement strategy employed in this study is not supported in the treatment of T2D. It is likely that the outcomes are clinically relevant to the treatment of several vascular diseases where impaired microcirculation contributes to the pathology of the condition and has provided a basis for further exploration of the utility of whey protein supplementation as an adjunction to exercise in a wider variety of clinical populations.

CHAPTER 6

THE EFFECT OF 10 WEEKS OF PERI-EXERCISE WHEY PROTEIN SUPPLEMENTATION ON MITOCHONDRIAL CONTENT IN MEN WITH TYPE-2 DIABETES.

Abstract

Introduction: Mitochondrial rarefaction and associated lipid accrual have been identified as potential mediators of myocellular insulin resistance in type-2 diabetes (T2D). Exercise is known to increase mitochondrial content, and evidence from non-diabetic populations indicates that adjunct whey protein supplementation may upregulate mitochondrial biogenesis to a greater magnitude. The purpose of this investigation was to test whether 10 weeks of peri-training whey-protein supplementation enhanced skeletal muscle mitochondrial biogenesis in middle-aged men with T2D.

Method: 24 non-insulin dependent middle-aged men with T2D were randomly allocated to consume a peri-training whey protein-carbohydrate supplement (20-10 grams) or carbohydrate placebo (30 grams) in conjunction with 10 weeks of high intensity interval training (3 cycling and 2 resistance sessions each week). Muscle biopsies were taken at the *vastus lateralis* (VL) to determine changes in mitochondrial and lipid volume, mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1-α*), nuclear respiratory factor 1(*NRF1*), and citrate synthase (CS); and CS and cytochrome c oxidase (COX) enzyme concentration.

Results: There was an almost certain increase in mitochondrial volume of 24.3% (90%CI 13.8%, 35.8%) and 26.7% (16.8%, 37.5%) in the control and whey groups respectively, with no clear group difference. CS and COX enzyme concentration were substantially increased by 40% and 31% in the control group and by 55% and 41% in the whey group respectively, with no clear group differences. There was a likely decrease in mRNA expression of *PGC1-α* of -0.20% (-0.01%, -0.40%) in the whey compared to the control group; but differences in *NRF1* and *CS* were unclear or trivial. There was a very likely and likely increase in lipid volume of 68.3% (15.9%, 144.3%) and 39.8% (-5.3%, 106.4%) in the whey and control groups respectively, with unclear group differences.

Conclusion: 10 weeks of peri-training whey protein supplementation did not increase mitochondrial expansion above exercise training alone and is not supported as an adjunct therapy for this purpose in populations with T2D. Intense exercise training increased intramyocellular lipid content and glucose disposal rates together in both groups demonstrating that lipid accrual was not detrimental to insulin sensitivity.

6.1 Introduction

Mitochondrial rarefaction is a well-documented characteristic of type-2 diabetic myocytes (Chomentowski 2011, Hsieh 2011, Kelley 2002, Meex 2010, Mogensen 2007, Toledo 2007)) that has been associated with insulin resistance and disease severity (Chomentowski 2011, Hsieh 2011, Kelley 2002, Meex 2010, Mogensen 2007, Toledo 2007); however, the mechanism linking the irregularity to hyperglycaemia has not been fully resolved. An enduring theory has been that the decrease in mitochondrial volume in T2D myocytes reduces the capacity to oxidise lipid (Patti 2010, Peterson 2004) leading to an increase in the formation of lipid substrates and reactive oxygen species that interfere with insulin-mediated molecular signalling (Coen 2012, Furler 2001, Goodpaster 2001, Peterson 2004). Exercise training is a well-established therapeutic mode for promoting mitochondrial expansion in T2D skeletal muscle (Little 2011, Meex 2010) and has been well-reviewed (Lumini 2008). Emerging evidence suggests that consuming milk-proteins proximal to exercise upregulates molecular signalling for mitochondrial biogenesis during exercise recovery (Hill 2013), which could be beneficial to mitochondrial expansion; however, this has not been tested in a population with T2D and further investigation is warranted.

Skeletal muscle is the primary tissue of glucose disposal, accounting for ~75-80% of postprandial glucose uptake (DeFronzo 1985, Thiebaud 1982). Observations of decreased mitochondrial density and lipid accumulation in T2D myocytes has led to considerable investigative scrutiny to determine whether the irregularities are mechanistically linked to the disease (Patti 2010, Peterson 2004). Mitochondrial rarefaction has been associatively linked to elevated levels of myocellular lipid in T2D skeletal muscle (Coen 2012, Goodpaster 2001, Peterson 2004) and increased lipid-derived reactive oxygen species (ROS) (Patti 2010, Schrauwen 2010, Wohaieb 1987) and lipid substrates including ceramide and diacylglyerol species (Bergman 2012, Boon 2013, Haus 2009, Kuzmenko 2016) have been shown to

interfere with normal insulin signal transduction in cells studies (Anderson 2009, Boon 2013, Tirosh 1999). However, lipid-mediated theories of insulin resistance have also been disputed as elevated lipid density has not been observed in all populations with T2D (Anderson 2009, Chomentowski 2011, Patti 2010, Peterson 2004).

Milk protein supplementation and intense interval training have emerged as potentially complementary therapies for enhancing mitochondrial expansion in skeletal muscle tissue. Independently, 6 high-intensity cycling sessions (10 x 60 second intervals; 90% heart rate maximum) was shown to significantly increase the expression of several mitochondrial enzymes at the *Vastus Lateralis* (VL) and 24-hour glycaemia in adults with T2D (Little 2011). In maurine models of age-related mitochondrial rarefaction, it has been shown that consumption of branch chain or mixed amino acids that are found richly in milk proteins, significantly increased intermyofibrillar mitochondrial density (Corsetti 2008) and to a greater extent when combined with exercise training (D'Antona 2010). Investigations of this kind in human populations have largely utilised a proximal-to-exercise supplement regimen, a well-established protocol for enhancing muscle protein synthetic rates during exercise recovery; but findings have been mixed. Acutely, consumption of whey protein after endurance cycling was shown to significantly upregulate the transcriptome driving mitochondrial biogenesis and oxidative metabolism at 48 hours in the VL muscle of trained cyclists (Rowlands 2011) and consumption of whey protein (1.2 grams per kg bodyweight added to a sports drink) during and after prolonged daily cycling sessions for 2 weeks significantly increased PGC-1α mRNA, a central signalling protein for myocellular mitochondrial biogenesis in trained cyclists 3 hours after a cycling session compared to a sports drink alone (Hill 2013). In contrast, mitochondrial synthetic rates at the VL muscle were not increased after 4 hours in trained men who consumed a whey-carbohydrate (10g-25g) supplement immediately and 30 minutes after an endurance cycling session (Breen

2011) or by consumption of a whey supplement (25 g) immediately after a bout of combined leg resistance and moderate intensity cycling (Camera 2015). Furthermore, consumption of a mixed-milk protein supplement after treadmill training three times each week for six weeks did not enhance mitochondrial DNA content in sedentary (Robinson 2011) older men. Taken together, it is unclear whether mitochondrial expansion can be enhanced by combined therapies and further exploration is needed. It is also pertinent to test the phenomenon in a population with T2D where enhanced mitochondrial expansion could lead to greater insulin sensitivity.

Therefore, the purpose of this study was to determine whether consuming whey protein before after intense exercise training for 10 weeks would increase mitochondrial expansion in T2D skeletal muscle and whether changes would be associated with reductions in myocellular lipid volume and improvements in insulin sensitivity.

6.2 Methods

Refer to chapter 3.1-3.4 for design, 3.9 and 3.11 for analytical methods, and 3.13 for statistical analysis and inferential approach.

6.3 Results

Table 6.1 shows the baseline tissue characteristics for each group. There were no clear group differences between participant characteristics at baseline. The change in mitochondrial and lipid density, and statistical summary for all parameter measures of mitochondrial biogenesis are shown in Table 6.2 and Figure 6.1 respectively. Ten weeks of intense exercise produced an almost certain increase in mitochondrial density in both groups

and a likely and very likely increase in lipid volume in the control and whey groups respectively, but group differences were trivial or unclear.

Table 6.1. Baseline skeletal muscle tissue characteristics of the control and whey groups.

	Control *n*=11	**Whey** *n*=12
Parameter	Mean SD	Mean SD
Mitochondrial Density (% of total cell volume)	4.0 ±1.1	4.1 ± 1.1
Lipid Density (% of total cell volume)	0.5 ± 0.3	0.6 ± 0.3
Citrate Synthatase (enzymatic activity)	0.13 ± 0.07	0.13 ± 0.09
Cytochrome C Oxidase (enzymatic activity)	0.11 ± 0.03	0.09 ± 0.03

Data are presented as means and standard deviations.

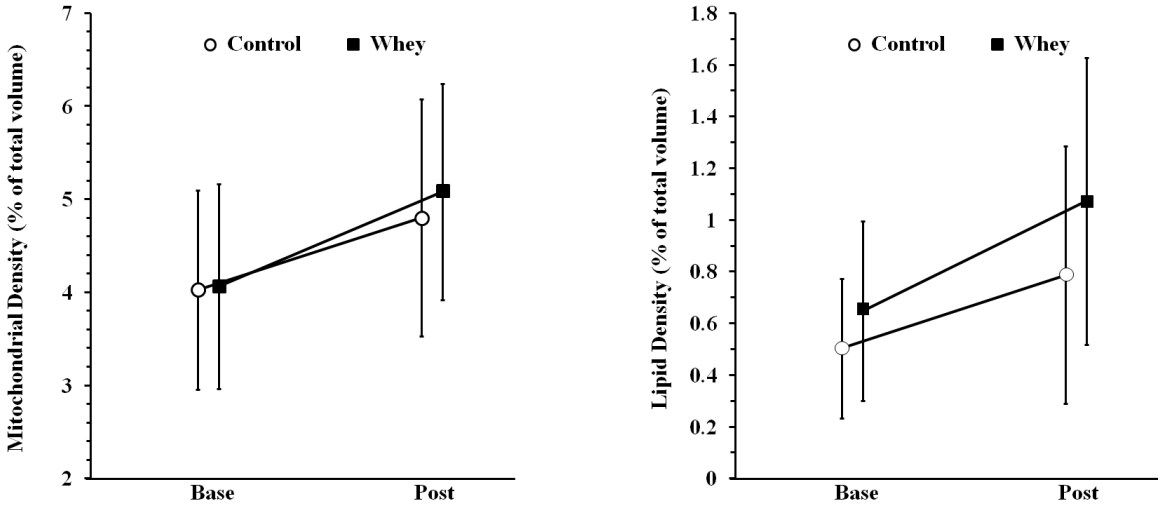


Figure 6.1. Effect of 10 weeks peri-training whey protein supplementation on VL intermyofibrillar mitochondrial and lipid density.

There was a possible increase in CS enzyme concentration in both groups and a likely and very likely increase in COX concentration in the control and whey groups respectively, but group differences for enzyme concentrations were unclear. Analysis of transcription regulators of mitochondrial biogenesis revealed a possible increase and a possible decrease in PGC1-α expression in the control and whey groups, respectively, and a likely decrease in whey-control contrast. The change in CS mRNA was trivial in both groups; NRF1 showed a very likely increase in both groups; but group differences for CS and NRF-1 were trivial or unclear.

Regression analysis of the pooled 10-week changes in mitochondrial density, lipid density and GDR (Chapter 4) revealed a positive association between lipid density and GDR (r = 0.29) but no associations were observed between mitochondrial and lipid density or mitochondrial density and GDR.

Table 6.2. The effect of 10-weeks peri-training whey-protein supplementation on myocellular mitochondrial and lipid density, mitochondrial enzymes and expression of mRNA associated with mitochondrial biogenesis.

Contrast[a]	% Change	Upper CI	Lower CI	Likelihood (%) benefit/trivial/harm[b]	Qualitative[b]
Density analysis					
			Mitochondria		
Control	24.3	35.8	13.8	100.0/0.0/0.0	Almost certain increase
Whey	26.7	37.5	16.8	100.0/0.0/0.0	Almost certain increase
Whey-Control	1.9	15.0	-9.7	5.0/93.3/1.7	Likely trivial
			Lipid		
Control	39.8	106.4	-5.3	87.4/11.2/1.4	Likely increase
Whey	68.3	144.3	15.9	98.5/1.3/0.1	Very likely increase
Whey-Control	20.4	106.4	-29.8	58.7/29.0/12.3	Unclear
Enzyme Concentration					
			Citrate Synthase		
Control	40.05	131.1	-15.1	58.4/41.3/0.3	Possible increase
Whey	54.88	275.5	-36.1	65.0/30.7/4.3	Possible increase
Whey-Control	10.59	193.5	-58.3	31.2/50.9/17.9	Unclear
			Cytochrome-c Oxidase		
Control	31.07	91.27	-10.2	87.5/10.3/2.2	Likely increase
Whey	40.83	80.45	9.9	98.9/1.1/0.0	Very likely increase
Whey-Control	7.45	66.78	-30.8	47.9/29.3/22.8	Unclear
mRNA Expression					
	(*Cq*)		***PPARG C1A (PGC1-α)***		
Control	0.10	-0.06	0.25	48.4/50.7/0.9	Possible increase
Whey	-0.11	-0.26	0.04	0.5/44.3/55.2	Possible decrease
Whey-Control	-0.21	-0.4	-0.01	0.3/16.9/82.8	Likely decrease
			CS		
Control	0.05	-0.04	0.14	13.8/86.1/0.1	Likely trivial
Whey	-0.04	-0.17	0.10	2.5/82.2/15.3	Likely trivial
Whey-Control	-0.09	-0.24	0.06	0.9/62.4/36.7	Possibly trivial
			NRF1		
Control	0.57	0.29	0.84	99.9/0.1/0.0	Very likely increase
Whey	0.47	0.24	0.70	99.7/0.3/0.0	Very likely increase
Whey-Control	-0.10	-0.42	0.23	11.0/44.8/44.2	Unclear

[a] Data for each contrast are post-pre. [b] The threshold for smallest clinical effect was the smallest standardised difference (0.2xSD). The likelihood that a contrast was at least greater than the clinical threshold was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain. Unclear refers to outcomes where the likelihood of both benefit and harm exceeded 5%.

6.4 Discussion

6.4.1 Main Findings

The main finding of this study was that while 10 weeks of high-intensity exercise substantially increased myocellular mitochondrial density in middle-aged men with T2D, whey protein supplementation did not accentuate the effect. Similarly, whey protein had no effect on lipid density, which, novel to this investigation was clearly increased by 10 weeks of intense exercise training and not detrimental to insulin sensitivity. The findings suggest that chronic whey protein supplementation produced no therapeutic benefit to mitochondrial expansion above that provided by intense mixed-mode interval training alone.

Previously, whey protein supplementation for 2 weeks was shown to upregulate transcript expression of *PGC1-α*, a central mediator of mitochondrial biogenesis, at the VL muscle, 6 hours after endurance cycling in well-trained men (Hill 2013). We predicted that whey protein supplementation would similarly upregulate molecular signalling for mitochondrial biogenesis after intense exercise training leading to an increase in mitochondrial volume and enzyme concentration after 10 weeks. The findings showed that while intense exercise substantially increased mitochondrial density and the concentration of CS and COX enzymes, whey protein supplementation did not enhance the effect. Analysis of several transcripts associated with mitochondrial biogenesis showed that 48 hours after exercise there was a sustained increase in NRF1 expression in both groups, but the expression of *CS* and *PGC1-α* transcripts were dampened in both groups and *PGC1-α* expression may have been substantially decreased to a greater extent in the whey group. While the endurance cycling modality used in the previous investigation may have produced a different pattern of signalling for mitochondrial biogenesis compared to the intense training regimen utilised in the current study, it is also possible that the effect of whey protein supplementation is transient and therefore potentially more effective when muscles are trained on a daily basis.

As daily walking is a common recommendation for individuals with T2D, this line of inquiry is likely worth further study. It is also possible that molecular signalling for mitochondrial biogenesis is altered by age. Similar to our findings, decreased expression of *PGC1-α* was observed at the VL muscle of older women after 12 weeks of progressive cycle training despite a significant increase in COX IV enzyme (Konopka 2010). As T2D skeletal muscle shows multiple characteristics of aged tissue including: accelerated muscle wasting (Lee 2010); lower contractile strength to muscle volume (Park 2006), and decreased mitochondrial density (Chomentowski 2011), the dampening of *PGC1-α* concentration in the whey group may have not have prevented mitochondrial expansion, but impaired any potential whey-induced increase in the synthesis of new mitochondrial proteins.

Elevated lipid density in T2D skeletal muscle has been debated as a potential mediator of cellular lipotoxicity, a state that is detrimental to insulin-mediated molecular signalling (Patti 2010, Peterson 2004). We predicted that mitochondrial expansion would improve lipid oxidation leading to a decrease in lipid density and a subsequent improvement in insulin sensitivity. In contrast, the findings showed that despite a substantial increase in mitochondrial density and insulin sensitivity throughout the cohort, lipid density was elevated after 10 weeks of intense exercise. Previously, lipid accrual has been identified as a pro-adaptation to endurance training in athletes who also display high mitochondrial function and insulin sensitivity (Goodpaster 2001, Kelley 2000, van Loon 2004). Similarly, a significant increase in intramuscular triglyceride was observed in type-1 muscle fibres of obese T2D after 6 months of endurance training; however, in that study there was no control group for comparison (Shaw 2012). Furthermore, a trend towards increased intramyocellular lipid at the VL muscle and an improvement in insulin sensitivity was also observed in men with T2D after 12 weeks of mixed-mode exercise (Meex 2010) and more recently an investigation comparing myocellular lipid accretion after exercise in athletic, overweight and insulin

resistant men showed that insulin sensitivity was positively associated with lipid synthetic rates after exercise (Bergman 2012). The current findings provide additional evidence that in the trained state, lipid accrual is not necessarily detrimental to insulin sensitivity and while the scope of this study does not allow for mechanistic inferences, it is reasonable to speculate that the increase in mitochondrion would lower the formation of potentially detrimental lipid metabolites. As regression analysis of the pooled data revealed that the change in lipid density after 10 weeks of intense exercise was positively associated with the change in insulin sensitivity, lipid accretion may prove to be favourable to insulin sensitivity in the trained state, an observation that may better guide further investigation of this kind.

6.4.2 Study Limitations

As the current study was the first to assess whey protein supplementation-exercise interactions in a population with T2D, a limitation in this study was predicting the duration required for exercise adaptations to be enhanced. Previously, consumption of mixed milk-protein after 18 sessions of moderate intensity continuous treadmill training did not increase the expression of mitochondrial DNA (Robinson 2011). However, high-intensity interval training (HIT) has been shown to increase adaptation rates compared to continuous training modes (Cocks 2012, Gillen 2016) and substantial improvements in mitochondrial enzyme concentration has been demonstrated after 6 cycling HIT sessions in sedentary (Hood 2011) and T2D adults (Little 2011). We estimated that 27 intense cycling sessions would be sufficient to demonstrate a nutrition effect on mitochondrial function. However, we saw no effect of whey protein supplementation on mitochondrial expansion apart from a dampening of transcript signalling for mitochondrial biogenesis. It is possible that whey protein supplementation would have better sustained signalling for mitochondrial biogenesis at the

VL muscle if we had utilised a daily cycling regimen rather than including days of upper-body resistance training between cycling sessions.

6.4.4 Conclusion

In conclusion, this investigation found that whey protein supplementation was an ineffective adjunct to intense interval training for enhancing mitochondrial expansion in T2D myocytes. A novel observation was that lipid accretion at the intermyofibrillar region of T2D myocytes was found to be a pro-adaptive response to intense exercise and possibly favourable to insulin sensitivity, and could be an important target in T2D therapy.

CHAPTER 7

NIL WHEY PROTEIN EFFECT ON MOOD AFTER 10 WEEKS OF EXERCISE IN TYPE-2 DIABETES.

Abstract

Introduction: Populations with type-2 diabetes (T2D) have a higher incidence of mood disorders including depression, anxiety and stress. Heightened emotional states have been associated with hyperglycaemia, suggesting that improving mood could also lower glycaemia. Exercise and whey protein supplementation have been independently shown to improve mood, however, the effect of combined treatments on mood in a population with T2D is unknown.

Methods: In a double-blind randomised controlled trial, 24 men (55.7±5.6 y) with T2D performed intense mixed-mode exercise with peri-training whey protein (20 grams) or placebo control for 10 weeks. Changes in mood and quality of life were determined via the short form Depression, Anxiety, Stress Survey (DASS-42) and SF-36 questionnaire respectively. Linear regression was used to determine associations between changes in mood and physical function and mood and fasting blood glucose (FBG).

Results: Exercise training substantially lowered survey-rated mood by -9.0 scale units (90%CI -13.8, -4.2) and -8.9 scale units (-15.8; -4.2) and improved quality of life rated physical function by 9.7 (1.1; 18.2) and 10.0 (1.5; 18.6) and mental function by 8.0 (0.0; 15.9) and 8.2 (0.2; 16.2) scale units in the control and whey groups respectively, but whey protein supplementation provided no clear additional benefit. There was a very likely association (r = -0.54) between the pooled 10-week changes in mood and mental function but no association between mood and physical function outcomes. There was a likely association (r = 0.39) between the change in mood and the change in FBG.

Conclusion: The findings show that 10 weeks of exercise training improved mood in middle-aged men with T2D with enhanced mental function being a likely mediator; however, whey protein supplementation provided no additional benefit. Improvements in mood were

beneficial to glycaemia, indicating that mood may be an important target for improving glycaemia in populations with T2D.

7.1 Introduction

Mood disorders have emerged in recent years as a co-morbidity and potential mediator of hyperglycaemia in T2D. The prevalence of mood disorders is substantially increased amongst individuals with T2D (Almawi 2008, Bener 2011, Kaur 2013, Khuwaja 2010); in some countries reaching as high as ~40-60% of the population (Khuwaja 2010, Tovilla-Zarate 2012). In addition to being associated with decreased quality of life (Ali 2010, Seppala 2013) and increased mortality risk (Zhang 2005), populations with mood disorders have been shown to display higher tissue levels of the glucose releasing hormone cortisol (Alvarez 2013, Stetler 2011) which could promote or exacerbate hyperglycaemia. It has also been shown that populations with T2D and to a greater extent, adults with both T2D and depression have elevated tissue cortisol levels (Manenschijn 2013, Shirzaii 2016).

Exercise is a well-documented therapy for improving emotional status in populations with mood disorders and T2D. In older adults with T2D, resistance training for 16 weeks was shown to improve survey-rated depression and the mental health subscale in a quality of life survey (Lincoln 2011). Similarly, mixed-mode training for 24 months was shown to improve survey-rated mood and the mental health subscale in a quality of life survey in older adults with T2D (Baptista 2017). A recent burgeoning interest in the effect of exercise on quality of life in populations with T2D has revealed that people with T2D report higher ratings of both mental and physical function after exercise training (Baptista 2017, Dede 2015, Dincer 2016, Liu 2016). As improved physical capacity, including: aerobic fitness, muscle strength, and increased mobility are common outcomes of exercise training in populations with T2D (Barrile 2016, Barry 2009, Mitranun 2014), increased physical function may mediate or contribute to mood improvements.

Consumption of milk-proteins proximal to exercise has been shown to enhance physical function during exercise training. Strength, agility and lean mass were significantly

improved after 8 weeks in female college basketball players who consumed 24 grams of whey protein before and after anaerobic and resistance training but not in players consuming a carbohydrate placebo (Taylor 2016). VO_2max was significantly improved in sedentary men who consumed 20 grams of mixed milk protein after treadmill training for 6 weeks compared to a carbohydrate placebo (Robinson 2011). In elderly adults with chronic obstructive pulmonary disease, twice daily supplementation with a whey-derived peptide (10 grams) during a 3-month low-intensity exercise program was reported to significantly improve a 6-minute walk test and health related quality of life, compared to exercise alone (Sugawara 2012). Furthermore, grip strength, leg strength, gait speed and agility were significantly improved by daily whey protein supplementation during a low-intensity exercise program in aged individuals (Niccoli 2017). However, others have reported no benefit of milk-protein supplementation during exercise training on strength (Chale 2013, Denysschen 2009) or aerobic fitness (Weinheimer 2012) indicating that further investigation of these phenomena is warranted. The effect of milk-protein supplementation during exercise training on mood has not been tested in a population with T2D.

Therefore, the purpose of this investigation was to test whether peri-training milk-protein supplementation improved mood status above exercise alone in a population with T2D; and if so, to determine whether better physical or mental function was the likely mediator. Additionally, we assessed whether changes in mood status were associated with changes in glycaemia. These findings may lead to better therapies for treating T2D.

7.2 Methods

Refer to chapter 3.1-3.4 for design, 3.12 for analytical methods, and 3.13 for statistical analysis and inferential approach.

7.3 Results

Table 7.1 shows the baseline characteristics (DASS total and SF-36) for each group. There were no substantial differences in Total DASS-42 or SF-36 scores between groups at baseline. Table 7.2 shows the statistical summary for total and each subscale change in DASS-rated mood and the physical and emotional component of the SF-36 quality of life survey after 10 weeks in the control and whey groups.

Table 7.1. Baseline DASS-42 total and SF-36 quality of life physical and mental subscale ratings.

	Control *n*=12	Whey *n*=12
Parameter	Mean ± SD	Mean + SD
DASS-42 total score	17 ±10.5	30 ± 26.1
SF-36 Physical	67.3 ± 17.8	71.9 ± 18.3
SF-36 Mental	75.3 ± 18.3	67.1 ± 19.3

Data are presented as means and standard deviations.

Mood

There were possible and likely improvements in total DASS mood scores in the control and whey groups respectively, with unclear group differences. No clear benefit of whey protein supplementation on mood was observed in the depression, anxiety or stress subscales.

Quality of Life

There were very likely improvements in the SF-36 physical health and mental health subscales in both groups with no clear group difference.

Mechanistic Associations

When the group data was pooled, there was no association between the change in total DASS scores and the change in the physical health subscale scores of the SF-36 (Figure 7.1 A) or any of physical capacity outcomes; VO_2peak or 1RM strength (Chapter 4), but there was a very likely negative association (r = -0.54) between the 10-week change in total DASS and the change in the mental health subscale of the SF-36 (Figure 7.1 B). There was a likely positive association (r = 0.39) between the 10-week change in total DASS scores and the change in FBG (Figure 7.1 C).

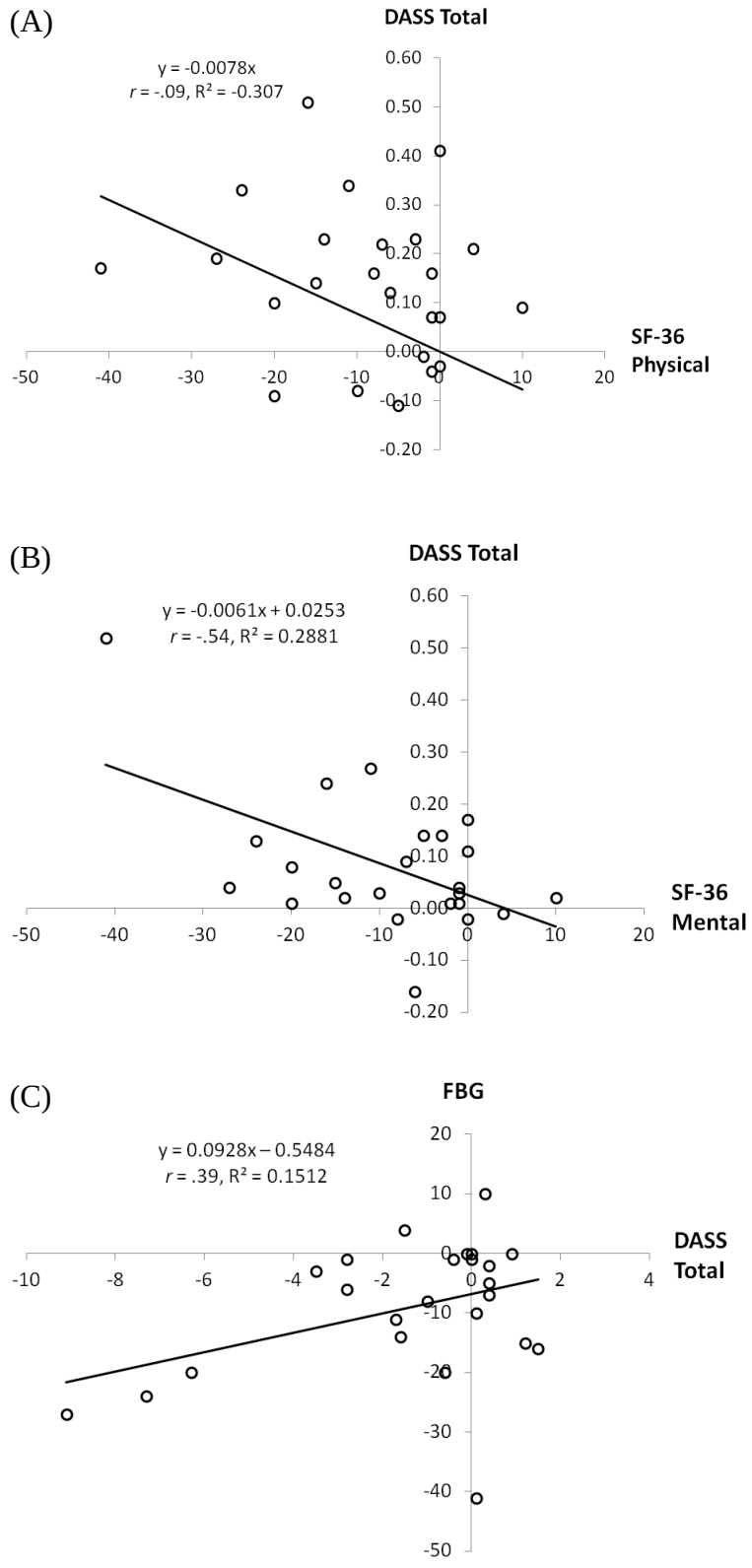


Figure 7.1. Associations between 10-week changes in total DASS-421 scores and SF-36 rated (A) physical function, (B) mental function, and the association between FBG and (C) total DASS-21 scores.

Table 7.2. The effect of 10 weeks of peri-training whey supplementation on DASS-42 mood and SF-36 quality of life ratings.

Contrast [a]	Change (Scale Units)	Upper CI	Lower CI	Likelihood (%) benefit/trivial/harm[b]	Qualitative[b]
DASS-42					
			Total Score		
Control	-9.0	-13.8	-4.2	70.1/29.5/0.5	Possible benefit
Whey	-8.9	-15.8	-3.8	83.5/16.4/0.1	Likely benefit
Whey-Control	0.1	4.0	-4.2	33.6/51.9/14.5	Unclear
			Depression		
Control	-2.3	0.7	-5.4	70.1/29.5/0.5	Possible benefit
Whey	-3.0	0.1	-6.1	83.5/16.4/0.1	Likely benefit
Whey-Control	-0.7	3.7	-5.0	33.6/51.9/14.5	Unclear
			Anxiety		
Control	-2.3	0.1	-4.6	84.3/15.5/0.2	Likely benefit
Whey	-2.3	0.1	-4.6	84.3/15.5/0.2	Likely benefit
Whey-Control	0	3.3	-3.3	0	No benefit
			Stress		
Control	-4.4	-1.1	-7.7	94.4/5.6/0	Likely benefit
Whey	-3.9	-0.6	-7.2	90.0/10.0/0	Likely benefit
Whey-Control	0.5	5.2	-4.2	15.0/57.5/27.6	Unclear
SF-36					
			Physical		
Control	9.7	18.2	1.1	93.3/6.6/0.1	Very likely benefit
Whey	10.0	18.6	1.5	94.5/5.4/0.1	Very likely benefit
Whey-Control	0.4	12.5	-11.7	30.3/44.2/25.5	Unclear
			Mental		
Control	8.0	15.9	0.0	86.4/13.5/0.1	Very likely benefit
Whey	8.2	16.2	0.2	87.3/12.6/0.1	Very likely benefit
Whey-Control	0.1	11.5	-11.1	25.7/50.4/23.8	Unclear

[a] Data for each contrast are post-pre. [b] The threshold for smallest clinical effect was the smallest standardised difference (0.2xSD). The likelihood that a contrast was at least greater than the clinical threshold was: 25-75% possible, 75-95% likely, 95-99.5% very likely, >99.5% almost certain. Unclear refers to outcomes where the likelihood of both benefit and harm exceeded 5%.

7.4 Discussion

7.4.1 Main Findings

The main findings in this study were that 10 weeks of exercise training substantially improved mood and quality of life in middle-aged men with T2D, but the effects were not enhanced by peri-training whey-protein supplementation. Improvements in mood were associated with improvements in glycaemia (FBG). The findings indicate that mood status is an important target of T2D rehabilitation and that exercise was an effective therapeutic mode for improving both mood and glycaemia, without additional benefit from whey protein supplementation.

Milk protein supplementation during exercise training has been previously shown to increase several components of physical function, including: strength, aerobic fitness and agility (Robinson 2011, Sugawara 2012, Taylor 2016). Higher ratings of physical function have also been accompanied by better ratings of mood in populations with T2D after exercise training (Baptista 2017, Dede 2015, Dincer 2016, Liu 2016) suggesting that milk protein supplementation could produce additional beneficial effects on mood. We predicted that whey protein supplementation would enhance both physical function and mood; however, the research hypothesis was not supported. While total and subscale mood ratings and several measures of physical function (VO_2peak and 1RM strength [Chapter 4], and SF-36 physical function) were substantially improved after 10 weeks of intense exercise, whey protein supplementation did not accentuate the effect and no associations were observed between the magnitudes of the pooled change in total mood or any measure of physical function. In contrast, there was a very likely association between the change in DASS scores and SF-36 mental function, suggesting that exercise improves mood via improved mental function. If so, then identifying exercise modalities that best improve mental function in populations with T2D is an area for further exploration.

7.4.2 Secondary Findings

Previously, the relationship between mood and glycaemia in populations with T2D has come largely from cross-sectional studies (Levinger 2012). A novel finding in this study was that the improvement in mood status after exercise training was associated with the improvement in fasting glycaemia, indicating that the two irregularities may be related. In an investigation of older adults with T2D, mixed-mode exercise for 24 months was shown to significantly improve mood and mental and physical subscales of the health related quality of life survey (HRQoL); however, HbA1c was used to assess glycaemic control and no associations were observed (Baptista 2017). It is possible that FBG is a more effective measure for revealing the transient effects of mood on glycaemia than HbA1c which is influenced by a variety of other factors such as physical activity and diet. Although we did not assess cortisol secretion in this study, it has been previously shown that exercise training lowered plasma cortisol during stressful states in sedentary adults (Zschucke 2015) and a rat model of depression (He 2012). In order to provide a clear mechanistic target for therapeutic intervention, future investigations should clarify whether cortisol was the mediator of the observed relationship between mood and glycaemia following exercise training. It has also been argued that the relationship between mood disorders such as depression and cortisol elevation is bi-directional (Lopresti 2012) and it is currently unclear whether mood disturbances underlie or are the result of hyperglycaemia, however, a meta-analysis of available literature a decade ago found that while depression was a strong predictor of T2D risk, T2D was a weak predictor of depression (Mezuk 2008).

7.4.3 Limitations

While adjunct whey protein supplementation to exercise training has been shown to induce improvements in physical function after interventions of similar duration (Cribb 2006, Robinson 2011), this was the first study to investigate these phenomena in a population with T2D. Therefore our observation that no markers of physical function (VO2peak, 1RM, SF-36 Physical Health) were enhanced after 10 weeks, may have been due to the treatment duration being too short and limited the investigations capacity to demonstrate a clear nutrition effect on mood. Others have shown that larger whey protein supplement doses may be more effective for improving skeletal muscle remodelling in older populations (Yang 2012), which should be an additional consideration in future investigations of this kind.

7.4.4 Conclusion

In conclusion, this study showed that mood was improved by exercise training, but the effect was not enhanced by whey protein supplementation. The observed relationship between the change in mood and FBG suggests that interventions that benefit mood may also improve glycaemia in populations with T2D. Future studies should explore mental function as a potential target for improving hyperglycaemia in populations with T2D.

GENERAL DISCUSSION

Milk-protein supplementation has shown promise as an adjunct therapy to exercise training for improving health and physical capacity outcomes in non-diabetic populations. In this study, we hypothesised that peri-training whey protein supplementation would enhance several skeletal muscle adaptations in middle-aged men with T2D during 10 weeks of intense exercise training, producing better insulin sensitivity and glycaemic control. The main finding was that whey protein supplementation produced a clear upregulation of microvascular blood kinetics that while not clearly seen to be beneficial to insulin sensitivity in a population with T2D, could be clinically meaningful to the treatment of vascular diseases where microcirculation is impaired. The intense mixed-mode interval training regimen substantially improved glycaemia, insulin sensitivity, and several skeletal muscle tissue characteristics that have been previously identified as sites of dysfunction in insulin resistance (skeletal muscle mass, mitochondrial volume, and capillarity); but whey protein supplementation did not accentuate the effect. The exercise regimen also led to better emotional status and quality of life without any additional benefit from whey protein supplementation. Taken together the findings support the inclusion of milk protein supplementation as an adjunct to exercise therapy for elevating microcirculation; however, further exploration is necessary to determine whether this phenomenon can be utilised to improve glycaemic control in populations with T2D.

Microvascular Function

The contribution of microvascular dysfunction to hyperglycaemia in populations with T2D is far from resolved. While insulin-mediated upregulation of macrovascular blood kinetics has been estimated to account for ~40% of the increase in glucose disposal after eating (Fugmann 2003), haemodynamics through the microvascular system where glucose

extraction occurs has not been well-described. Milk protein supplementation has been shown to be an effective adjunct to exercise therapy for improving arterial relaxation (Yoshizawa 2010), a mechanism that is important to insulin-mediated upregulation of blood kinetics and impaired in T2D (Bosevski 2010). We predicted that consuming a peri-training whey protein supplement during intense exercise training for 10 weeks would increase the arterial relaxation response to insulin in a population of middle-aged men with T2D leading to heightened blood kinetics through skeletal muscle capillary beds. We also predicted that elevated microcirculation would increase the rate of glucose disposal during a euglycaemic insulin clamp.

A novel finding in this study was that 10 weeks of peri-training whey protein supplementation clearly elevated microvascular blood volume at rest and microvascular blood flow and volume after insulin infusion. Unexpectedly, there was no clear evidence that exercise alone elevated any measure of blood kinetics within the microvascular system. The current findings demonstrated a clinical effect of peri-training whey protein supplementation on microvascular blood kinetics that could be beneficial to a variety of clinical populations where microcirculation is impaired; however, there was no clear evidence that the effect led to better glycaemic control. The pooled 10-week changes in resting microvascular blood flow and volume were both negatively associated with the change in FBG and HOMA-IR, suggesting that basal microvascular blood kinetics do effect glycaemia and should be therapeutic targets of T2D rehabilitation and may explain why there were much larger odds ratios that supplementation had a clinical effect on FBG and HOMA-IR in the whey group. Further exploration of this phenomenon is justified; however, 24-hour assessment of glycaemia may be a better tool for quantifying the clinical benefit of petri-training whey protein supplementation on glycaemia and should be considered in future investigations.

Mitochondrial Function

Amino acid supplementation has been shown to increase molecular signalling for mitochondrial biogenesis (*PGC1-α* and *NRF1* mRNA), and mitochondrial density in exercised maurine skeletal muscle (D'Antona 2010). We predicted that consuming whey protein, which provides a rich source of amino acids, immediately before and after intense exercise would upregulate signalling for mitochondrial biogenesis during exercise recovery leading to a substantial increase in mitochondrial density after 10 weeks. In contrast, the findings showed that there was no clear improvement in mitochondrial density or the concentration of mitochondrial enzymes (CS and COX) in the whey group. Analysis of transcript signalling for mitochondrial biogenesis indicated there was no effect from whey protein on *NRF1* or *CS*; and there was a likely down-regulation of *PGC1-α* in the whey group. Down-regulation of *PGC1-α* has been previously seen in older women after exercise training despite evidence of mitochondrial expansion and it is possible that molecular signalling for mitochondrial biogenesis is similarly altered in populations with T2D where skeletal muscle characteristics reflect aged tissue (Chomentowski 2011, Lee 2010, Park 2006).

While it has been well-established that consuming milk proteins proximal to exercise increases protein synthetic rates during exercise recovery, the current findings indicate that chronic peri-training whey protein supplementation did not elevate the synthesis of mitochondrial proteins above exercise alone and its use as an adjunct to exercise for this purpose is not supported. It is possible that additional doses of whey protein throughout the day may be necessary to produce a therapeutic effect. In aging mice, ad libitum consumption of amino acids during treadmill training for 30 days was shown to increase mitochondrial density (D'Antona 2010) and some evidence indicates that increasing total daily protein

intake may be necessary to optimise protein synthesis (Cermak 2012, Schoenfeld 2013) which should also be considered in future investigations of this kind.

Myocellular lipid accumulation has received extensive investigative scrutiny as a possible consequence of reduced mitochondrial oxidative capacity in T2D and a potential mediator of myocellular insulin resistance (Patti 2010, Peterson 2004, Schrauwen 2010). It was predicted that an increase in mitochondrial density would improve lipid metabolism leading to a reduction in lipid density that would be accentuated in the whey group. In contrast it was observed that despite a substantial and equivalent increase in mitochondrial density and insulin sensitivity in both groups, lipid density was also elevated after exercise training. The increase in lipid density was also found to be positively associated with the improvement in insulin sensitivity suggesting that lipid accrual was a pro-adaptation to intense exercise and possibly favourable to glycaemic control. Lipid accumulation has been previously considered to be a pro-adaptation to endurance training in athletic populations who have high myocellular mitochondrial development and insulin sensitivity (Goodpaster 2001). Endurance training was also reported to increase intramuscular triglyceride in an obese population with T2D (Shaw 2012). This was the first study to show an increase in myocellular lipid density following intense interval training in a population with T2D and adds further support that myocellular lipid accrual was a pro-adaptation to exercise.

While the scope of this study does not allow for mechanistic explanation of why lipid accumulation was favourable to insulin sensitivity, it is reasonable to speculate that mitochondrial expansion may have reduced the formation of lipid metabolites detrimental to insulin sensitivity. However, a recent investigation demonstrated that insulin sensitivity was positively associated with the capacity to synthesize myocellular lipid after exercise in healthy, overweight and T2D populations (Bergman 2012), which taken together with the current findings shows that lipid accumulation during exercise training is an adaptation that

may be favourable to glycaemic control and requires further exploration in populations with T2D.

Physical Function and Mood

Elevated prevalence of mood disorders amongst populations with T2D suggest that glycaemic control and mental health may be pathologically linked to the disease. It has been demonstrated that exercise training improves functional capacity and mood together (Baptista 2017, Dede 2015) suggesting that therapies that enhance physical function may lead to improvements in glycaemia. Milk-protein supplementation has previously been shown to improve physical function, including: muscle mass, 1RM strength, and aerobic capacity (VO2max), each of which has been previously linked to better glycaemic control (Ibanez 2005, Lee 2011, O'Gorman 2006) during exercise training in healthy and clinical populations (Sugawara 2012, Taylor 2016). It was predicted that whey protein supplementation would enhance skeletal muscle remodelling during intense exercise training in middle-aged men with T2D, leading to increased muscle growth and strength, aerobic fitness and mood status. We observed that the high-intensity training mode utilised in this investigation substantially improved VL muscle thickness, 1RM strength, VO_2peak, mood and quality of life together in a population with T2D; however, whey protein supplementation provided no additional benefit and its use as a therapeutic adjunct to intense exercise to improve mood is not supported.

A novel finding was that the magnitude of the pooled improvement in mood after 10 weeks was positively associated with the reduction in fasting glycaemia, supporting the use of intense exercise for improving both irregularities together in T2D and demonstrating that elevated mood and hyperglycaemia may be pathologically linked. Cortisol release may be the

underlying mechanism and further inquiry including additional analysis of cortisol secretion after intense exercise training is warranted.

High-intensity Mixed-mode Interval Training

High-intensity interval training HIT) has gained increasing attention as an alternative to other forms of exercise due to the lower time investment required and potentially greater adaptive responses (Bird 2012). In T2D populations HIT has largely taken the form of cycling or treadmill training and has been shown to effectively improve markers of glycaemic control (Gillen 2016, Little 2011, Mitranun 2014). In this study a mixed-mode interval training program with both cycle and resistance training sessions 4-5 times each week for 10 weeks was utilised for the first time in a population with T2D. Previously treadmill HIT 3 times each week for 12 weeks reduced FBG by ~1 mmol/L (Mitranun 2014). The 10-week exercise regimen utilised in this study produced a 100% adherence rate and reductions in FBG by ~1-1.8 mmol/L and an increase in insulin-mediated glucose disposal rate by ~ 25-28% in the control and whey groups, demonstrating that the training mode was both practical and effective in a group of middle-aged men with T2D.

Conclusion and Future Perspectives

In conclusion, this study found no clear evidence that whey protein supplementation produced a therapeutic benefit on glycaemic control during intense exercise training in a population with T2D. An elevation of resting and insulin-stimulated microvascular blood kinetics was observed after whey protein supplementation which could be beneficial for treating several vascular diseases where microcirculation is impaired occurs and warrants further investigation. No clear additional benefit from whey protein supplementation was observed in any of the measures of physical capacity or mood, or in any of the tissue and

cellular adaptations, including: muscle and adipose thickness, mitochondrial and lipid density and capillarisation and the findings do not support its use as a therapeutic agent for enhancing these outcomes above intense exercise alone. However, some evidence suggests that a larger supplement dose or an increase in the dosage frequency could be necessary to produce a meaningful effect on these measures and should be considered in future investigations of this kind.

APPENDICES

APPENDIX A PARTICIPANT INFORMATION & INFORMED CONSENT

Participant Information Sheet and
Consent Form

Study title:	Can Milk Protein in Combination with Exercise Training Improve Metabolic Flexibility and Cardiovascular Health in Type-2 Diabetics?		
Locality:	Wellington	Ethics committee ref.:	13/NTB/69
Lead investigator:	Mr Kim Gaffney	Contact phone number:	[redacted]

You are invited to take part in a study on the effect of milk protein supplementation around exercise on insulin sensitivity and cardiovascular health in Type-2 diabetics.
Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. We expect this will take about 30 minutes. You may also want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 8 pages long, including the Consent Form and the Muscle Biopsy Information Sheet. 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 of this project is to determine whether milk-protein sup plementation can be used to improve glucose control. An additional purpose is to determine whether milk protein supplementation combined with short-duration exercise results in greater improvements in glucose control, compared to milk protein or exercise alone, which have both shown the potential to be effective treatments for lowering blood glucose. Approximately 7% of the New Zealand population has Type 2 diabetes. Diabetes results from an inability of the body's tissue to take up and use glucose.

Most of the glucose in our bodies is taken up and used by skeletal muscle. An impaired capacity for skeletal muscles to take up and use glucose eventually leads to increased risk for cardiovascular disease, retinopathy, neuropathy, and other diseases. If we can improve glucose control we can potentially decrease disease risk and improve quality of life and life-span. The study will provide an opportunity to test two practical, inexpensive, and promising interventions for diabetic therapy.
As part of this study we will measure the effect of treatment on insulin sensitivity, cardiovascular health, and skeletal muscle health. Participants will be randomly allocated to one of three groups 1) exercise only, 2) milk protein supplement only, or 3) exercise plus milk protein supplement. Random allocation is important to ensure that the three groups are comparable. All Participants will be asked to consume unmarked supplement drinks containing either milk protein or a similar tasting placebo.
In recent research with Type-2 diabetics, there has been no adverse effects from consuming milk-proteins, however, there is some risk associated with exercise. Prior to beginning the study you will be screened by experienced clinical exercise physiologists to ensure exercise risk is minimized. Current research suggests that participants are likely to experience positive health outcomes following each of the therapies we will be testing.

The study is being conducted by researchers at The College of Health, Massey University Wellington Campus and the Endocrine, Diabetes, and Research Centre at Wellington Hospital. The study is currently being funded by research grants from Massey University.

The project coordinator is Mr Kim Gaffney, a clinical exercise physiologist and PhD candidate. A number of researchers will assist with this study. Dr David Rowlands who is Senior Lecturer and practiced researcher in exercise, nutrition and Type-2 diabetes. Dr Lee Stoner from Massey University will provide expertise on assessment of cardiovascular health. Dr James Faulkner from Massey University is a clinical exercise physiologist with experience in exercise prescription for a range of populations. Professor Jeremy Krebs and Dr Patricia Whitfield are research endocrinologists at Wellington Hospital.

The study has been approved by Northern B 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 2 weeks of preliminary testing, a 10-week study period, and a final day of testing. We will test insulin sensitivity, cardiovascular health, and skeletal muscle health at the start and end of the 10 week study.

Preliminary Testing
Two weeks prior to the start of the 10-week treatment period, you will be asked to attend the Massey University Exercise Laboratory on 2 mornings to become familiar with and undertake testing. Visits may occur on weekends if that is convenient. The week before the start of the study you will also be asked to visit Wellington Hospital Diabetes Clinic in a fasted state for approximately 4-5 hours of testing.

Massey University Exercise Laboratory Assessments

Day 1

Familiarisation: You will be asked to complete a health history questionnaire and then have the opportunity to become familiar with all exercise laboratory testing methods.

Day 2
Body composition*:* Body fat will be measured by ultrasound and skinfold callipers. Height, girth and weight measurements will also be taken.

Psychometric testing: You will be asked to complete a simple survey common to exercise interventions assessing psychological wellbeing.

Fitness Tests: You will be asked to perform a cycling test at increasing workloads until you have reached or come close to your maximal heart rate. During the test you will be connected to an ECG to allow the electrical conductivity of the heart to be monitored, and will breathe through a mouthpiece for the measurement of respiratory gases. You will also be asked to perform 5 strength tests where you will complete approximately 5 repetitions of the heaviest loads you can achieve.

Cardiovascular Health: Blood pressure measures will be taken at the arm. Near infrared spectrophotometry (NIRS) will be used to measure muscle tissue blood flow and mitochondrial function at the thigh. NIRS sensors will also be placed on the thigh during the cycling test.

Assessment at the Diabetes Research Centre Wellington Hospital

Day 3
Euglycaemic-hyperinsulinemic clamp. This test is the standard measure for determining how well the body uses glucose and is commonly used in diabetic research. Insulin and glucose will be infused into the forearm and blood will be monitored for changes in glucose concentration. Blood samples will also be used to provide measures of fasting glucose, lipid haemoglobin, and hormone profile.

Near-infrared spectroscopy and ultrasound.
Before and during the clamp procedure blood flow to your leg will be measured by NIRS sensors at the thigh after a series of light blood pressure cuff inflations.

Muscle biopsy. A small muscle tissue sample will be taken from the outer thigh, before and after the clamp procedure to determine changes in muscle cell structure, function and molecular signalling in order to explain the effects of treatment on glucose metabolism. The sample will be taken under local anaesthetic, sealed with a butterfly strip and does not require stitches. A detailed explanation of the biopsy procedure and associated risks is provided with this information sheet.

Tissue samples will be stored in a swipecard access locked laboratory at Massey University, and later analysed at research laboratories in New Zealand, Canada and the USA.

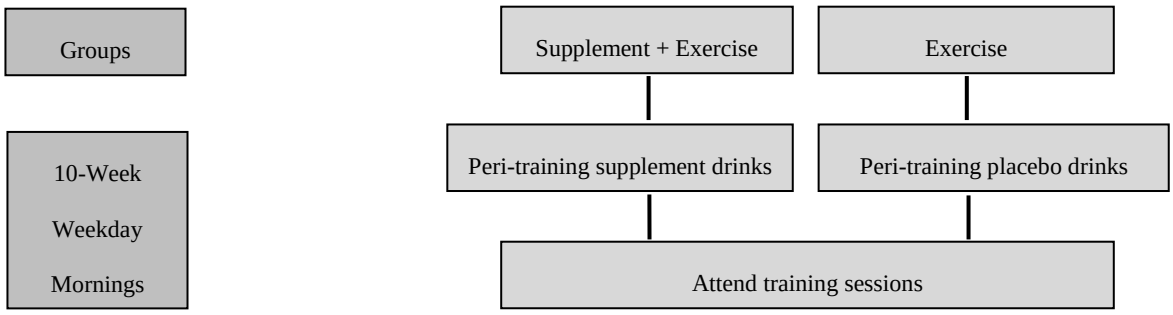
You will also be asked to perform some simple tests at home throughout the study including completing 3-day diet diaries and wearing activity monitors (similar to pedometers). You will also be asked to provide morning saliva samples.

Treatment

Following the above preliminary tests, you will be randomly allocated to one of three groups: 1) milk protein supplement only, 2) exercise only, or 3) milk protein supplement plus exercise. If you are randomised to the exercise-only or supplement plus exercise groups, you will be required to complete a 10-week exercise programme. The exercise programme will take place at the Massey University gymnasium on weekdays for 35 min at a suitable time between 6 and 8 am. You will be asked to come to the exercise session in the fasted state. Being fasted means not eating or drinking (other than water) from the time you wake up until the exercise session has finished. The exercise sessions are short and intensive and will include both cardiovascular and strength exercises. If you are unable to attend an exercise session you will be provided with an opportunity to make up the missed session on the weekend. Shower and change facilities will be available at the gymnasium.
As part of the 10 week study you will be asked to consume either a/milk-protein supplement dink or a similar tasting placebo drink before and after exercise. If you are in the supplement only group, you will be asked to consume your drinks at the same time each morning as the exercise groups. In addition to the drinks you will be provided with snack bars to be consumed 1 hour after supplement drinks, which together will provide similar energy values to a normal breakfast. Apart from the morning supplement drinks and snack bar, we want you to maintain normal eating and physical activity habits during the 10 weeks. The different treatments for each group are shown in Figure 1.

After 10 weeks, participants in the supplement-only group will have the option to join the 10-week exercise program.

Figure 1. Treatments of experiment groups.



To ensure that it is safe for you to participate, health information may be shared between the lead researchers and the health practitioner responsible for your health care.

What are the possible benefits and risks to you of participating?

Previous research has shown that both milk protein supplementation and exercise therapies improve insulin sensitivity and cardiovascular health. You may experience some, or all of these benefits. The exercise training program may result in some physical and psychological discomfort. Exercise risks also include musculo-skeletal injury and cardiac events. Experienced exercise physiologist will provide ongoing instruction and motivation, so that

exercise risks are minimized and benefits are maximized. All participants will receive a comprehensive report on their diabetic health shortly after the experiment and a copy of the group results once they become available.

What would happen if you were injured in the study?

If you are injured you will be able to apply for compensation from ACC, just like if you were injured in an accident at work or at home. If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

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 disadvantage.

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 and only researchers and their assistants will have access to personal health information or collected data.

What will happen after the study ends, or if you pull out?

At the completion of the study, data collected will be stored in the private office of a lead researcher for 10 years. Remaining tissue samples will be destroyed unless required for future cellular and tissue analysis that may contribute to the development of effective rehabilitation and treatment plans relating to diabetes mellitus. Stored samples will not be used for other research such as genetic inheritance, stem cell, or pharmaceuticals.
If requested, we can return the unused and processed tissue samples that are being analysed in New Zealand; due to logistics and cost we are unable to return samples that are being shipped overseas. Maori participants may request to have overseas laboratories blessed.

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:

Mr Kim Gaffney, Coordinating Investigator
Telephone number [redacted] mobile [redacted]
Email [redacted]

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 Information Sheet and
Consent Form

Study title:	Can Milk Protein in Combination with Exercise Training Improve Metabolic Flexibility and Cardiovascular Health in Type-2 Diabetics?		
Locality:	Wellington	Ethics committee ref.:	13/NTB/69
Lead investigator:	Mr Kim Gaffney	Contact phone number:	[redacted]

Declaration by participant:

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet. I have had the opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this study.

I have been given a copy of the Participant Information Sheet and Consent Form to keep.

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 study and has given informed consent to participate.

Researcher's name: _____

Signature: _____ Date: _____

MUSCLE BIOPSY INFORMATION SHEET

Introduction
You have volunteered to take part in a research study that requires you to undergo a muscle biopsy. This is a commonly performed procedure in medicine and research. The procedure will be performed by a doctor or senior researcher specially trained to perform muscle biopsies.

What is a muscle biopsy?
A muscle biopsy is a standard clinical procedure normally used to sample a small piece of tissue.

Why is a muscle biopsy performed?
In this research we require direct access to muscle tissue in order to measure changes in gene expression and protein synthesis.

In which muscle will the biopsy take place?
We will take a sample from your top/outer thigh muscle in a muscle called the Vastus lateralis.

How is the biopsy performed?
The procedure is performed under local anaesthetic. You will be asked to lie down, and a local anaesthetic will be given in the site from which the biopsy will be taken. A small cut (incision) of 4-5mm is made to access the muscle. The sample of muscle is then taken with a special needle designed for biopsies. The incision site will be sealed with a butterfly strip (or a stitch). The procedure takes around 15 minutes.

What do I do after the biopsy?
The incision will be closed with a suture strip and then covered with a good quality band-aid. Use an ice pack and bandage to reduce local inflammation for the first two (2) hours after the procedure. If possible, you should rest your leg for the first day if possible. Do your best to keep the site clean. First, wash it with warm water and soap, and then thoroughly dry the area with a clean towel. A topical antiseptic may be used as well. The site will heal on its own in line with a normal small cut to the skin.

What problems may there be with the biopsy?
The incision site may ooze a little, and this is normal. If there is excessive bleeding, you should contact the researchers. If you experience excessive redness, swelling, or infection around the biopsy site or excessive pain or stiffness in your leg, you should consult the researchers and your GP immediately. Treatment with antibiotics may be appropriate.

What are the side effects of a muscle biopsy?

As indicated above, there may be problems with bleeding or healing at the incision site. There will be a small scar at the site of the procedure, which will heal within a few weeks typically leaving a 3-4 mm hairline that is difficult to see after 1-2 months. If there is any pain or discomfort in the skin or muscle immediately following the procedure after the local anaesthetic has worn off, simple pain killing medication such as paracetamol or neurofen may be taken. The statistical probability of complications with the procedure from the laboratory of our collaborators (M.A. Tarnopolsky, McMaster University Hospital) is:
4/10000 with a superficial skin infection (possible risk of at least one infection)
6/10000 with a fibrous lump at the site of the biopsy (connective tissue), all of which disappeared within 1 week.
4/10000 with a small patch of numbness just past the incision (approximately the size of a 10 cent piece), due to the cutting of a small sensory nerve patch. In all cases complete recovery occurred within 3 months.
Most subjects experience transient dull muscle aching for 24-48 hours following biopsy, which is markedly reduced with ice and Neurofen.

The motor nerves are located on the medial/inside aspect of the quads. It is a real possibility that the biopsy could damage a small motor branch of the muscle on the outside of the thigh (vastus lateralis) and partially weaken the lower aspect of the muscle. This should not affect the function for this muscle (knee extension), as it is one of four muscles involved and other fibers adapt. This has never been observed by the researchers.

What happens to the muscle biopsy?
The muscle sample is immediately frozen and stored at -80°C. It will later be processed and evaluated for the effect of protein and/or exercise on tissue and cellular adaptation. Analysis will be conducted in New Zealand, Canada and the US.

Do you have any further questions?
When you come into the laboratory, the researchers will seek your consent to the biopsy and provide further information. This is also a chance to ask any further questions you may have.

Statement of Approval

This project has been reviewed and approved by the Northern B Health and Disability Ethics Committee. If you have any concerns about the ethics of this study, please contact 0800 4 ETHICS (438 442); email: hdecs@moh.govt.nz or write to Health and Disability Ethics Committees, No 1 The Terrace PO Box 5013 Wellington, 6011.

APPENDIX B RECRUITMENT PAMPHLETS

Mr Kim Gaffney
Massey University Wellington
Tel: [redacted]
Email: [redacted].

Dear Dr

The College of Health is seeking the services of a medical practitioner to provide medical assistance for two clinical research trials. The trials examine the effectiveness of exercise combined with novel dietary protein forms and intervention on insulin sensitivity and microvascular outcomes in Type-2 diabetics. The trials are being conducted within a joint collaboration between the CCDHB Endocrine and Research Centre and University of Otago at Wellington Hospital led by A/Prof Jeremy Krebs and Metabolic and Microvascular Research Unit at Massey University led by A/Prof David Rowlands.

The role will require setting up insulin and glucose infusions for a hyperinsulinemic-euglycemic clamp (gold standard method to assay insulin sensitivity) and collecting skeletal muscle biopsies for each testing session. The majority of other patient management and experimental procedures will be performed by the Massey University researchers, with back up support from Unit nurses if required.

The testing sessions will be conducted on weekday mornings (fasted participants) and last approximately 5 hours; the facility to work weekends can be investigated. We presently require support on a few testing days in 2014, with the majority of the project occurring in 2015. The research team has a degree of flexibility on testing days. Training and support for any aspect of the testing session will be provided where necessary.

This is an ideal opportunity to participate in meaningful clinical trials conducted by some of New Zealand's leading researchers.

Contract hours and remuneration are negotiable.

For more information please contact the coordinating investigator, Mr Kim Gaffney.

Yours sincerely

Kim Gaffney, MSc
College of Health
Massey University

Or, Assoc. Prof. David S. Rowlands, PhD
Metabolic and Microvascular Research Lab
College of Health, Massey University
63 Wallace St, Wellington
New Zealand
+64 27 2099 383
d.s.rowlands@massey.ac.nz

Capital & Coast District Health Board
ŪPOKO KI TE URU HAUORA

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Do You Have Type-2 Diabetes?

A New Non-Pharmacological Therapy for Improving Non-Insulin Dependent Type-2 Diabetes is Being Evaluated in an Exercise Research Study.

Benefits include, at no cost:

Access to gymnasium, guidance and support from exercise physiologists, comprehensive health evaluations and reports.

For more information contact the coordinating investigator,

Mr Kim Gaffney **Ph** [redacted] or email [redacted]

Can Whey Protein And Exercise Improve Insulin Sensitivity And Cardiovascular Health In Type-2 Diabetics?

Volunteers Wanted for a Research Study

Milk-proteins may improve insulin sensitivity and cardiovascular health. Better adaptations may be observed when milk-protein supplementation is combined with exercise. This research study will determine whether Type-2 diabetics experience improvement in insulin sensitivity and cardiovascular health following a 10-week combined whey protein supplement plus exercise program. If you are male with Type-2 diabetes, aged 40-60, BMI <35, HbA1c above 48, and not requiring insulin, you may be suitable for this study. Participants will have access to exercise facilities and be instructed and supported by exercise physiologists who specialise in diabetic rehabilitation at no cost for 10 weeks. Similar previous research suggests that participants are likely to experience significant improvements in diabetic and cardiovascular health.

APPENDIX C **PARTICIPANT SCREENING FORM**

GENDER: M / F

DESCENT: European, Maori, Pacific Islander, Asian, Indian, Other

TODAY'S DATE _____

NAME _____ AGE _____ DATE OF BIRTH _____

ADDRESS _____
Street City State Zip

TELEPHONE: HOME/CELL _____/ _____ E-MAIL ADDRESS _____

OCCUPATION/EMPLOYER _____/ _____ BUSINESS PHONE _____

MARITAL STATUS: (check one) SINGLE ☐ MARRIED ☐ DIVORCED ☐ WIDOWED ☐

PERSONAL PHYSICIAN _____ PHONE # _____

ADDRESS _____

Reason for last doctor visit? _____ Date of last physical exam: _____

Have you ever had any other exercise stress test? YES ☐ NO ☐ DATE & LOCATION OF TEST: _____

Have you ever had any cardiovascular tests? YES ☐ NO ☐ DATE & LOCATION: _____

Person to contact in case of an emergency _____ Phone _____ (relationship) _____

Please provide responses (YES or NO) to the following concerning family history, your own history, and any symptoms you have had:

FAMILY HISTORY

Have any immediate family members had a:

	YES	NO
heart attack	o	o
heart surgery	o	o
coronary stent	o	o
cardiac catheterization	o	o
congenital heart defect	o	o
stroke	o	o

Other chronic disease: _____

PERSONAL HISTORY

Have you ever had:

	YES	NO
High blood pressure	o	o
High cholesterol	o	o
Diabetes	o	o
Any heart problems	o	o
Disease of arteries	o	o
Thyroid disease	o	o
Lung disease	o	o
Asthma	o	o
Cancer	o	o
Kidney disease	o	o
Hepatitis	o	o

Other: _____

SYMPTOMS

Have you ever had:

	YES	NO
Chest pain	o	o
Shortness of breath	o	o
Heart palpitations	o	o
Skipped heartbeats	o	o
Heart murmur	o	o
Intermittent leg pain	o	o
Dizziness or fainting	o	o
Fatigue -- usual activities	o	o
Snoring	o	o
Back pain	o	o
Orthopedic problems	o	o

Other: _____

STAFF COMMENTS: _____

Have you ever had your cholesterol measured? Yes ☐ No ☐ If yes, value _____ Where: _____

Are you taking any prescription (include birth control pills) or nonprescription medications? Yes ☐ No ☐

For each of your current medications, provide the following information:

MEDICATION Dosage—times/day Time taken Years on medication Reason for taking

HOSPITALIZATIONS: Please list recent hospitalizations (Women: do not list normal pregnancies)

Year Location Reason

Any other medical problems/concerns not already identified? Yes ☐ No ☐ If so, please list: _____

LIFESTYLE HABITS

Do you ever have an uncomfortable shortness of breath during exercise or when doing activities?
Yes ☐ No ☐

Do you ever have chest discomfort during exercise? Yes ☐ No ☐
If so, does it go away with rest? Yes ☐ No ☐

Do you currently smoke? Yes ☐ No ☐ If so, what? Cigarettes ☐ Cigars ☐ Pipe ☐
How long have you smoked? _____ years
How much per day: <½ pack ☐ ½ to 1 pack ☐ 1 to 1½ packs ☐ 1½ to 2 packs ☐ >2 packs ☐
Have you ever quit smoking? Yes ☐ No ☐ When? _____
How many years and how much did you smoke? _____
Do you drink any alcoholic beverages? Yes ☐ No ☐ If yes, how much in 1 week? (indicate below)
Beer _____ (cans) Wine _____ (glasses) Hard liquor _____ (drinks)
Do you drink any caffeinated beverages? Yes ☐ No ☐ If yes, how much in 1 week? (indicate below)
Coffee _____ (cups) Tea _____ (glasses) Soft drinks _____ (cans)
Are you currently following a weight reduction diet plan? Yes ☐ No ☐
If so, how long have you been dieting? _____ months
Is the plan prescribed by your doctor? Yes ☐ No ☐
Have you used weight reduction diets in the past? Yes ☐ No ☐ If yes, how often and what type? _____

ACTIVITY LEVEL EVALUATION

What is your occupational activity level? Sedentary ☐ Light ☐ Moderate ☐ Heavy ☐
Do you currently engage in vigorous physical activity on a regular basis? Yes ☐ No ☐
If so, what type(s)? _____ How many days per week? _____
How much time per day? <15 min ☐ 15–30 min ☐ 31–60 min ☐ >60 min ☐
How long have you engaged in this type of activity? <3 months ☐ 3–12 months ☐ >1 year ☐

Do you engage in any recreational or leisure-time physical activities on a regular basis? Yes ☐ No ☐
If so, what activities? _____
On average: How often?_____ times/week; for how long? _____ time/session
How long have you engaged in this type of activity? <3 months 3–12 months >1 year

Your fitness goals and objectives are: _____

STAFF COMMENTS: _____

APPENDIX D TESTING PROCEDURES

INFORMATION FOR TESTING DAYS

To ensure our pre- and post-program measures are accurate and reliable it is important to follow specific guidelines on testing days. Tests at the hospital and exercise laboratory will commence between 6 and 8 am. Please follow these guidelines:

5. Do not take normal diabetic medication on the night before or morning of hospital testing
6. Do not perform strenuous exercise within 48 h of test days.
7. Report fasted – no food or drinks (except water) on the morning of test days.
8. Ensure you are well hydrated. Drink 500ml of water the evening prior to, and another 300ml upon rising on test days.
9. Please bring your water bottle.
10. If you are asthmatic please bring your inhaler.
11. If you require reading glasses please bring them.
12. Clothing needs to be comfortable for exercise and allow us to take measures at your arms– eg t-shirt or singlet. We will provide suitable shorts.
13. We may need to shave some sites where we will be attaching sensors to your skin.
14. We recommend that you take blood glucose readings upon rising and report those findings to us. It is also a good idea to take readings after exercise to ensure levels have not become low. You should eat shortly after exercise.

APPENDIX E ESTIMATED 1 REPETITION MAXIMUM CONVERSION CHART

Repetitionss	1	2 to 3	4	6	8 to 10	11 to 14	15	16 to 25
%1RM	100	95	85	80	75	70	65	50

APPENDIX F SUPPLEMENT NUTRITIONAL PROFILE

WHEY PROTEIN NUTRITIONAL INFORMATION & AMINO ACID PROFILE

ALACEN™ 895 NZ WHEY PROTEIN ISOLATE

Typical Nutritional Information

Natural	Per 30g	Per 100g
Energy (kJ)	477	1590
Calories	114	380
Protein (dry basis)	28.4g	98.1g
Protein (as is)	27.12g	93.5g
Fat	0.3g	0.4g
Carbohydrates	.02g	0.9g
- sugars	0.27g	0.9g
Sodium	39mg	130mg

Amino Acids per 100g of Protein

Branch Chain Amino Acids		Other Amino Acids	
Isoleucine	7.2g	Arginine	2.4g
Leucine	11.3g	Cystine	2.9g
Valine	6.4g	Glycine	1.9g
Other Essential Amino Acids		Proline	6.4g
Lysine	10.3g	Tyrosine	3.4g
Methionine	2.4g	Aspartic Acid	11.3g
Phenylalanine	3.3g	Serine	5.1g
Threonine	7.4g	Glutamic Acid	19.0g
Tryptophan	1.9g	Alanine	5.6g
		Histidine	1.9g

TREATMENT DRINK & PLACEBO: INGREDIENTS AND NUTRITIONAL PROFILE

Ingredients	
Treatment Drink	Placebo
20 g Whey	20 g Maltodextrin
12 g Sucrose	12g Sucrose
13 ml Cream	13 ml Cream
3 g Chocolate flavour	3 g Chocolate flavour

Macronutrients	Protein	CHO	Fat	kJ
Whey (WPI895) / g	0.935	0.009	0.004	15.9
Cream / ml	0.02	0.033	0.37	14.6
Choc flavour / g	0.196	0.112	0.11	9.68
Sucrose / g		0.999		16.18
Maltodextrin / g		0.952		15.9

Total Macronutrients	Treatment Drink	Placebo
kJ	731	731
Protein	19.55	0.85
CHO	12.75	31.8
Fat	5.21	5.13

APPENDIX G MITOCHONDRIAL ENZYME ANALYSIS

CITRATE SYNTHASE

Massey University

TITLE: **Citrate Synthatase Activity in Muscle Extracts**
PROCEDURE NO: xxxxx
TYPE: Method VERSION: 1.0
PAGE: 1 of 4

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1. PURPOSE

1.1. To describe the method for measuring Citrate Synthatase activity in muscle extracts.

2. PRINCIPLE:

2.1. Citrate Synthatase catalyses the conversion of oxaloacetate and acetyl-CoA into citrate and thiol-CoA. This reaction is linked in the assay by the reaction of thiol-CoA with DNTB to form the yellow coloured TNB, with can be measured at 412nm. Thus the activity of Citrate Synthatase is proportional to the increase in ABS_{412nm}.

3. EQUIPMENT

3.1. Gloves/labcoat/eye protection.
3.2. Suitable bench space
3.3. Microtitre plate reader capable of reading at 412nm and 25°C incubation
3.4. Autoclave
3.5. Variable single and multichannel pipettes

4. MATERIALS

4.1. Pipette tips, microtubes and 15ml tubes
4.2. 96 well, clear, flat bottom microtitre plates
4.3. Acetyl coenzyme A sodium salt. 809.57g/mol (Sigma Cat #A2056-5MG)
4.4. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB). 396.35g/mol (Sigma Cat #D8130-500MG)
4.5. Tris (Tris(hydroxymethyl)aminomethane).121.14g/mol
4.6. Oxaloacetic acid 132.07g/mol. (Sigma Cat #O4126-1G)
4.7. Ultrapure water

5. BUFFERS

5.1. 50mM Tris buffer pH 8.0

Tris	3.03g
Adjust pH with HCl	
Ultrapure Water to	500mL

5.2. Acetyl-CoA Stock (0.1M)

Acetyl-CoA	5mg (whole bottle)
50mM Tris buffer to	62µL
Freeze at -80°C in 6µL aliquots	

CYTOCHROME C OXIDASE

Massey University

TITLE: **Cytochrome C Oxidase Activity in Muscle Extracts**
PROCEDURE NO: xxxxx
TYPE: Method

VERSION: 1.0
PAGE: 1 of 4

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1. PURPOSE

1.1. To describe the method for measuring Cytochrome C Oxidase activity in muscle extracts.

2. PRINCIPLE:

2.1. Cytochrome C absorbs strongly at 550 nm in the reduced state. Upon oxidation by cytochorme c oxidase (COX) the absorbance weakens. Thus the activity of COX is proportional to the decrease in ABS_{550nm}.

3. EQUIPMENT

3.1. Gloves/labcoat/eye protection.
3.2. Suitable bench space
3.3. Microtitre plate reader capable of reading at 550nm and 25°C incubation
3.4. Autoclave
3.5. Variable single and multichannel pipettes

4. MATERIALS

4.1. Pipette tips, microtubes and 15ml tubes
4.2. 96 well, clear, flat bottom microtitre plates
4.3. Cytochrome C (Sigma Cat # C7752-100MG)
4.4. DTT (Dithiothreitol) 154.25 g/mol
4.5. Potassium dihydrogen phosphate (KH_2PO_4 136.09g/mol)
4.6. Dipotassium hydrogen phosphate (K_2HPO_4 174.18g/mol)
4.7. Muscle extract (see muscle extraction protocol)
4.8. Ultrapure water

5. BUFFERS

5.1. 0.05M phosphate buffer pH 7.4

KH_2PO_4	1.50g
K_2HPO_4	6.78g
Ultrapure Water to	1L

5.2. 0.1 M DTT.

DTT	0.154g
Ultrapure water up to	10mL

Make 500ul aliquots and freeze at -20°C

APPENDIX H PSYCHOMETRIC SURVEYS

DASS42

DASS

Name: *Date:*

Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you *over the past week*. There are no right or wrong answers. Do not spend too much time on any statement.

The rating scale is as follows:

0 Did not apply to me at all
1 Applied to me to some degree, or some of the time
2 Applied to me to a considerable degree, or a good part of time
3 Applied to me very much, or most of the time

1	I found myself getting upset by quite trivial things	0	1	2	3
2	I was aware of dryness of my mouth	0	1	2	3
3	I couldn't seem to experience any positive feeling at all	0	1	2	3
4	I experienced breathing difficulty (eg, excessively rapid breathing, breathlessness in the absence of physical exertion)	0	1	2	3
5	I just couldn't seem to get going	0	1	2	3
6	I tended to over-react to situations	0	1	2	3
7	I had a feeling of shakiness (eg, legs going to give way)	0	1	2	3
8	I found it difficult to relax	0	1	2	3
9	I found myself in situations that made me so anxious I was most relieved when they ended	0	1	2	3
10	I felt that I had nothing to look forward to	0	1	2	3
11	I found myself getting upset rather easily	0	1	2	3
12	I felt that I was using a lot of nervous energy	0	1	2	3
13	I felt sad and depressed	0	1	2	3
14	I found myself getting impatient when I was delayed in any way (eg, lifts, traffic lights, being kept waiting)	0	1	2	3
15	I had a feeling of faintness	0	1	2	3
16	I felt that I had lost interest in just about everything	0	1	2	3
17	I felt I wasn't worth much as a person	0	1	2	3
18	I felt that I was rather touchy	0	1	2	3
19	I perspired noticeably (eg, hands sweaty) in the absence of high temperatures or physical exertion	0	1	2	3
20	I felt scared without any good reason	0	1	2	3
21	I felt that life wasn't worthwhile	0	1	2	3

Reminder of rating scale:

0 Did not apply to me at all
1 Applied to me to some degree, or some of the time
2 Applied to me to a considerable degree, or a good part of time
3 Applied to me very much, or most of the time

22	I found it hard to wind down	0	1	2	3
23	I had difficulty in swallowing	0	1	2	3
24	I couldn't seem to get any enjoyment out of the things I did	0	1	2	3
25	I was aware of the action of my heart in the absence of physical exertion (eg, sense of heart rate increase, heart missing a beat)	0	1	2	3
26	I felt down-hearted and blue	0	1	2	3
27	I found that I was very irritable	0	1	2	3
28	I felt I was close to panic	0	1	2	3
29	I found it hard to calm down after something upset me	0	1	2	3
30	I feared that I would be "thrown" by some trivial but unfamiliar task	0	1	2	3
31	I was unable to become enthusiastic about anything	0	1	2	3
32	I found it difficult to tolerate interruptions to what I was doing	0	1	2	3
33	I was in a state of nervous tension	0	1	2	3
34	I felt I was pretty worthless	0	1	2	3
35	I was intolerant of anything that kept me from getting on with what I was doing	0	1	2	3
36	I felt terrified	0	1	2	3
37	I could see nothing in the future to be hopeful about	0	1	2	3
38	I felt that life was meaningless	0	1	2	3
39	I found myself getting agitated	0	1	2	3
40	I was worried about situations in which I might panic and make a fool of myself	0	1	2	3
41	I experienced trembling (eg, in the hands)	0	1	2	3
42	I found it difficult to work up the initiative to do things	0	1	2	3

SF-36 HEALTH SURVEY

SF36 Health Survey

INSTRUCTIONS: This set of questions asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. Answer every question by marking the answer as indicated. If you are unsure about how to answer a question please give the best answer you can.

1. In general, would you say your health is: (Please tick **one** box.)
   - Excellent ☐
   - Very Good ☐
   - Good ☐
   - Fair ☐
   - Poor ☐

2. Compared to one year ago, how would you rate your health in general now? (Please tick **one** box.)
   - Much better than one year ago ☐
   - Somewhat better now than one year ago ☐
   - About the same as one year ago ☐
   - Somewhat worse now than one year ago ☐
   - Much worse now than one year ago ☐

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? **(Please circle one number on each line.)**

	Activities	Yes, Limited A Lot	Yes, Limited A Little	Not Limited At All
3(a)	**Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports	1	2	3
3(b)	**Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	1	2	3
3(c)	Lifting or carrying groceries	1	2	3
3(d)	Climbing several flights of stairs	1	2	3
3(e)	Climbing **one** flight of stairs	1	2	3
3(f)	Bending, kneeling, or stooping	1	2	3
3(g)	Waling more than a mile	1	2	3
3(h)	Walking several blocks	1	2	3
3(i)	Walking one block	1	2	3
3(j)	Bathing or dressing yourself	1	2	3

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	(Please circle one number on each line.)	Yes	No
4(a)	Cut down on the **amount of time** you spent on work or other activities	1	2
4(b)	Accomplished less than you would like	1	2
4(c)	Were **limited in the kind** of work or other activities	1	2
4(d)	Had **difficulty** performing the work or other activities (for example, it took extra effort)	1	2

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (e.g. feeling depressed or anxious)?

	(Please circle one number on each line.)	Yes	No
5(a)	Cut down on the **amount of time** you spent on work or other activities	1	2
5(b)	Accomplished less than you would like	1	2
5(c)	Didn't do work or other activities as **carefully** as usual	1	2

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