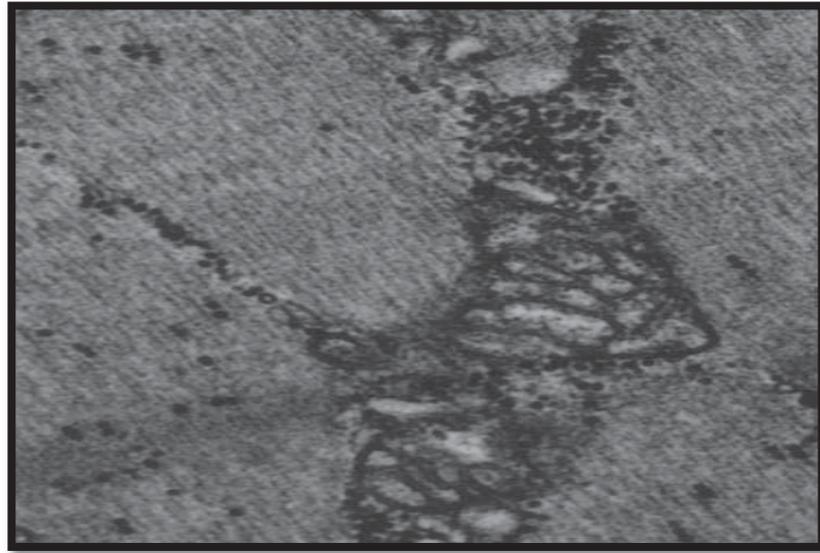


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*THE SOUTH PACIFIC ISLANDS RESIST DIABETES WITH
INTENSE TRAINING (SPIRIT) STUDY*

**Investigation of obesity markers and
morphological, functional and genetic changes
in the skeletal muscle**



A thesis presented in partial fulfilment of the requirements for the
degree of Doctor of Philosophy in Health Sciences

Institute of Food, Nutrition and Human Health

College of Health

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ABSTRACT

The skeletal muscle (SM), the major tissue for disposal of excess blood glucose, plays a big role in development of insulin resistance leading to type 2 diabetes mellitus (T2DM). Lipid accumulation and decline in mitochondrial activity in SM has been observed in people with T2DM. Several studies have demonstrated that exercise has the ability to increase SM lipid oxidation and mitochondrial activity and hence is effective as a treatment strategy for people with T2DM for improving blood glucose control and insulin sensitivity.

The SPIRIT study was the first clinical randomised exercise trial involving a cohort of Polynesian New Zealanders with T2DM. The uniqueness of this study is that it is the first clinical trial in Polynesian population with grade 3 obesity ($n=18$; BMI $43.8 \pm 9.5 \text{ kg/m}^2$) and T2DM. The SPIRIT cohort underwent 16 weeks of progressive resistance training (PRT) or aerobic exercise (AER) training. The cohort showed no changes in HbA1c levels after 16 weeks of exercise and hence no improvement in their blood glucose control. This was an unexpected result and led to the following hypothesis which underlines this PhD study – “In skeletal muscle of SPIRIT cohort, metabolic adaptation to exercise is delayed due to metabolic inflexibility”.

To investigate this hypothesis, mitochondrial function and morphology, lipid droplet content and changes in gene expression pre and post exercise intervention were examined in the SM. Since the SPIRIT cohort showed no changes in weight, waist circumference and BMI, examination of the concentration of specific obesity markers pre and post exercise training also occurred.

Mitochondrial function was examined pre and post 16 weeks exercise intervention by measuring the SM activity of three key mitochondrial enzymes; citrate synthase (CS) involved in Krebs cycle, beta-hydroxyacyl-CoA dehydrogenase (BHAD) involved in fat oxidation and cytochrome c oxidase (COX) involved in electron transport chain. The PRT cohort showed statistically significant increases in activity for COX ($P=0.005$) and CS ($P=0.007$) with very large effect size (2.3 ± 1.3 and 1.8 ± 1.3 respectively). AER exercise led to significant increases in the activity for all three enzymes COX ($P=0.01$), CS ($P=0.03$), BHAD ($P=0.03$) with moderate effect size for both COX and CS activity but very large effect for BHAD (6.7 ± 1.2). For all three enzymes there were statistically significant differences ($P<0.05$) between the AER and PRT groups. These results demonstrate increased mitochondrial activity and functioning after 16 weeks of PRT or AER exercise.

To further investigate the morphology of pre and post SM tissue the electron microscope images were examined for quantification of intramyocellular triglyceride (IMTG) content. There was a 48% statistically significant decrease ($P=0.007$) in IMTG (lipid droplets) in the AER group and there was a 28% statistically significant decrease ($P=0.04$) in IMTG content in the PRT group. The reduction in lipid droplet accumulation in the SM and associated increase in skeletal muscle BHAD activity (enzyme involved in oxidation of fatty acids in the mitochondria) demonstrates the benefit of exercise for the SPIRIT cohort.

The Ingenuity Pathways Analysis software was used to investigate the microarray gene data obtained for the SM of the SPIRIT cohort. The results indicate changes in gene expression associated with early phase connective tissue remodelling, for both forms of exercise by upregulation of genes like IGF-1, TGFBR2, PDGFRB in the resistance training group and COL4A1, COL3A1, MYH11, BGN, ACTA2, CD300LG, A2M, GPR116 in the aerobic training group. The AER training group also showed significant changes in SM mRNAs associated with glucose and lipid handling. Two key mRNAs that had increased expression after 16 weeks of AER exercise were *PPARGCIA* (gene encodes for PGC1- α , a regulator of energy metabolism) and *PPARG* (gene encodes for protein peroxisome proliferator-activated receptor gamma) regulates fatty acid storage and glucose metabolism.

Of the specific markers related to obesity that were examined only sex hormone binding globulin (a marker of insulin sensitivity) showed a statistically significant increase ($P=0.01$) in the PRT group. Seven of the nine PRT participants had an increase in SHBG levels, indicating a possible improvement in insulin sensitivity for these individuals. Statistically significant positive correlation of SHBG and statistically significant negative correlation of cortisol were established with number of exercise sessions attended in both groups meaning that greater exercise sessions may have positive impact on altering the metabolic profile of the individual.

The results of this PhD study have shown that exercise has induced changes in the skeletal muscle of the SPIRIT cohort. The increased mitochondrial enzyme activity and function, decreased IMTG content, increased fat oxidation and improved functional plasticity of the skeletal muscle are changes occurring at the functional, structural and genetic levels which denounce the hypothesis that metabolic adaptation in the SPIRIT cohort was delayed due to the SM being metabolically inflexible. These findings have demonstrated that exercise enhances metabolic flexibility in tissue that could be metabolically inactive e.g. tissue such as SM tissue in grade 3 obese individuals with T2DM.

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LIST OF ACRONYMS

1RM	1 Repetition Maximum
ACSM	American College of Sports Medicine
AER	Aerobic exercise
ANOVA	Analysis of Variance
BHAD	Beta hydroxyacyl-CoA dehydrogenase
BMI	Body Mass Index
COX	Cytochrome Oxidase
CS	Citrare Synthase
CV	Coefficient of Variability
FFA	Free Fatty Acids
GLUT4	Glucose Transporter 4th isoform
HbA1c	Haemoglobin A1c (glycated haemoglobin)
HOMA2-IR	Homeostasis Model Assessment Insulin Resistance
IPA	Ingenuity Pathway Analysis
PRT	Progressive Resistance Training
QOL	Quality of life
T2DM	Type 2 diabetes Mellitus
IMTG	Intramuscular Triglyceride
IMCL	Intramyocellular lipid
LD	Lipid Droplet
GWR	Greater Wellington Region
SPIRIT	South Pacific Islands Resist Diabetes with Intense Training

CHAPTER 1

INTRODUCTION

1.1 Introduction

Type 2 diabetes mellitus (T2DM) is a condition in which the uptake of blood glucose into cells becomes impaired [1]. It has become a major public health problem over the last 20 years around the world as well in New Zealand [2]. Skeletal muscle has an integral role in regulating whole body glucose homeostasis as 70-80% ingested glucose will be taken up by skeletal muscle cells, with the help of insulin, to either be stored as glycogen or to be oxidised [3]. In people with T2DM, insulin [1] and other mechanisms become inefficient to transport glucose into the cell resulting in a state of high blood glucose (hyperglycaemia) and high insulin levels (hyperinsulinaemia). Recent research suggests that in insulin resistant conditions such as T2DM and obesity, skeletal muscle is loaded with increased amounts of lipids which cannot be fully oxidised [4]. Exercise has been shown to enhance utilization of glucose at the skeletal muscle level thereby increasing insulin sensitivity and improving glycaemic control (assessed by glycosylated haemoglobin (HbA1c) a measure to identify average plasma glucose concentration over prolonged periods of time) in people with T2DM [5].

The intent of this PhD study is to look at the effects of exercise on skeletal muscle metabolism and morphology of Pacific Island and Maori individuals with T2DM and obesity. Skeletal muscle biopsy tissue from the clinical exercise trial SPIRIT (South Pacific Island Resist diabetes with Intense Training) study participant (section 1.2) who underwent two different types of exercise modalities i.e. aerobic and resistance training were available for this PhD study.

The purpose of this chapter is threefold:

- (1) To provide details regarding the SPIRIT study, a study focusing on Polynesian participants with T2DM and grade 3 obesity, using exercise to try and improve their glycaemic control (section 1.2),
- (2) To outline the research objectives of my PhD study (section 1.3) and
- (3) To provide an overview of the PhD thesis chapters (section 1.4)

1.2 SPIRIT study

The SPIRIT study (South Pacific Islands Resist diabetes with Intense Training) was carried out in the Greater Wellington Region (GWR) in 2008. The SPIRIT study was initiated by Bill Sukala, an exercise physiologist, in collaboration with researchers at Massey University, Wellington Capital Coast Health hospital, and participants from the Pacific Islands community. All individuals were interested in improving health outcomes of Polynesian individuals and it was by discussion and consultation between all interested parties that led to this first clinical exercise trial in a cohort of Polynesian New Zealanders diagnosed with T2DM and visceral adiposity [6-9].

The recruitment, exercise trial, biological sample collection and analysis were performed by Bill Sukala and formed the basis of his PhD [10]. Twenty-six adults of Polynesian descent (20 females, 6 males; 47 ± 8 years; 116.3 ± 27.5 kg, waist circumference 124.0 ± 17.8 cm) were recruited through established liaisons with Pacific Island church leaders in GWR and randomised to either 16 weeks of aerobic (AER) or progressive resistance (PRT) training. Inclusion criteria for the SPIRIT study are outlined in Table 1.1.

Table:1.1: Inclusion criteria for SPIRIT participants [8, 10]

Individuals were selected for participation in the trial if they met the following inclusion criteria:

- self-identified as of Māori or Pacific Islands descent;
 - a clinical diagnosis of type 2 diabetes mellitus;
 - waist circumference of ≥ 88 cm for women and ≥ 102 cm for men;
 - physically inactive for \geq six months;
 - no change in diabetes medications for previous two months; and
 - no acute or chronic medical conditions for which exercise would be contraindicated as outlined by the American College of Sports Medicine (ACSM) [11]. All protocols and safety procedures were developed in partnership with consulting diabetes specialists and were in accordance with established international safety guidelines for exercise set forth by the American Diabetes Association [12] and ACSM [11]
-

Details of the aerobic and resistance training carried out in the training schedules (Figure 1.1) are discussed in Bill Sukalas' PhD thesis [10] and included as appendix A. In summary, the PRT group progressed from 65% to 85% of their extrapolated IRM (repetition maximum) in phase 1, two sets at 85% during phase 2, three sets at 85% during phase 3 and a continuation of three sets until the end of intervention as presented in Figure 1.1(a).

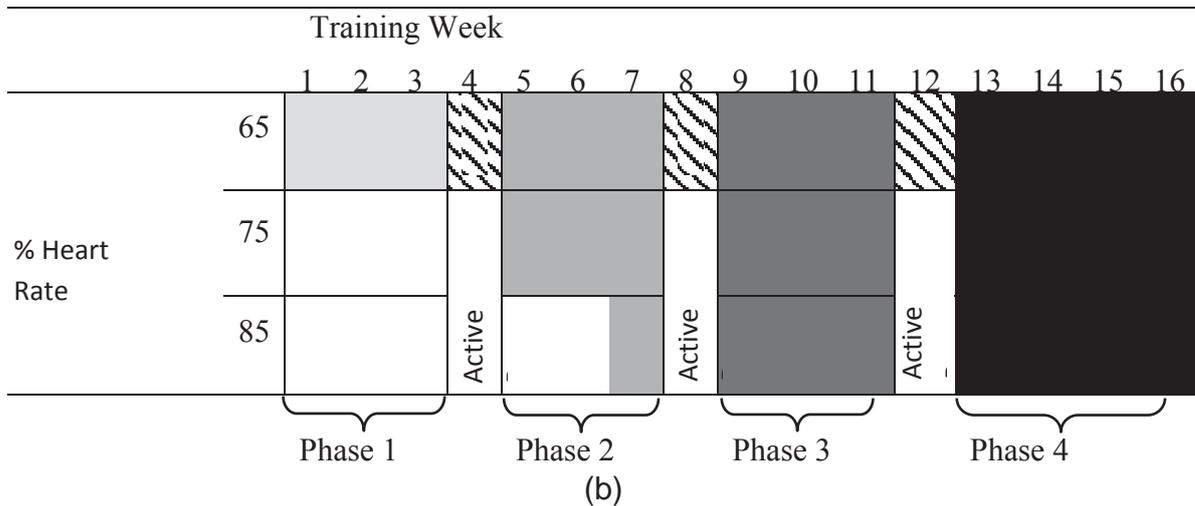
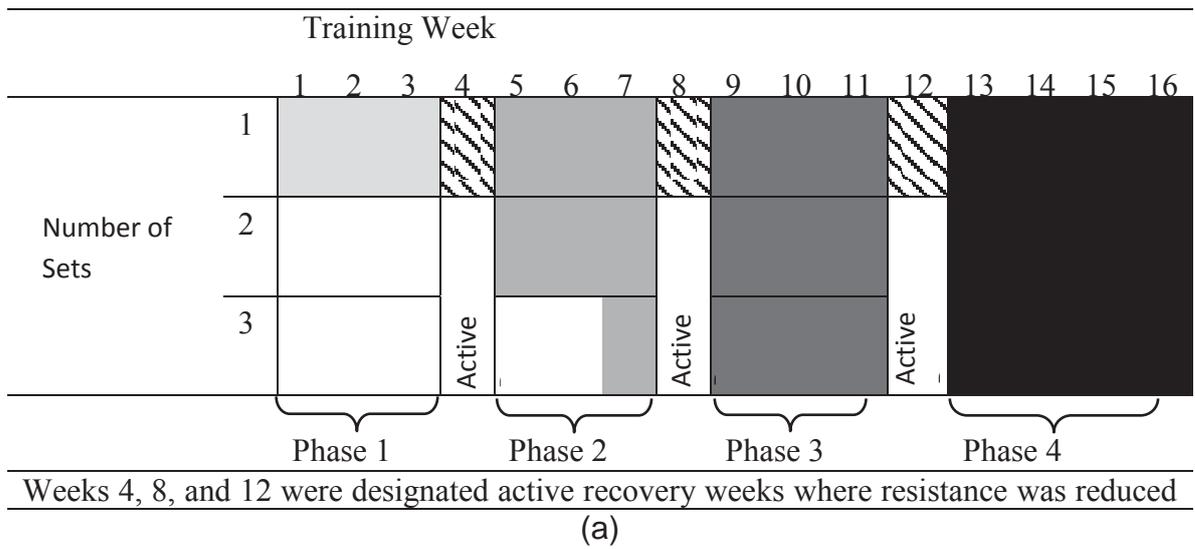


Figure 1.1: Exercise training schedules for SPIRIT study participants [21]. (a) represents training schematic for progressive resistance training and (b) represents aerobic training schematic.

The participants randomised to the AER group (Figure 1.1(b)) performed a graduated cycle ergometry (Life Fitness, Schiller Park, IL, USA) protocol in parallel with the PRT group. An equivalent training frequency, duration, and intensity were prescribed to evaluate the training response between modalities [10] and details of the aerobic intervention are in Appendix A.

A total of eighteen (9 per group) participants (13 females, 5 males; 49 ± 5 years; 122.7 ± 29.6 kg, 43.8 ± 9.5 kg/m²) completed the study and were included in the protocol analyses. Eight participants (5 AER, 3 PRT) completed $\geq 75\%$ of the exercise training and 6 patients (3 per group) completed $\leq 60\%$ of the training program. The baseline characteristics of the eighteen participants that completed the exercise intervention are outlined in Table 1.2. The majority of the participants (13/18) were using oral hypoglycaemic drugs (Table 1.2). The rest of the participants were controlling their blood glucose either by diet (2/18) or/and by insulin (3/18). BMI of cohort was 43.8 ± 9.5 (kg/m²) which is regarded as grade 3 obesity according to WHO [13].

The primary outcome measure for Bill Sukalas' PhD study was glycaemic control (examination of HbA1c levels) but he also included a wide variety of anthropometric, metabolic and physiological secondary outcome measures (Table 1.3) [7, 8, 10]. Details of the methods of detection for the primary and secondary outcome measures assessed are included in Appendix A.

The biological samples collected pre and post 16 weeks exercise were blood, urine and muscle biopsy tissue to allow measurement of specific biomarkers. The health and well being of the participants as determined via SF36 medical questionnaire was also examined pre and post 16 weeks exercise [9]. The results from the SPIRIT study showed improved scores for physical and mental components of health and wellbeing of the participants after 16 weeks [9] but there were no improvements in HbA1c levels, fasting glucose and fasting insulin levels, homeostasis model of insulin resistance (HOMA-IR), and total cholesterol (Table 1.3). In the aerobic training group a reduction in systolic and diastolic blood pressure was observed however, there were no improvements in weight or any other physiological parameters measured.

Table 1.2: Baseline subject characteristics for subjects completing the protocol (n = 18)

Characteristic	PRT Group	AER Group	Total Cohort
<i>N</i>	9	9	18
Age (years)	48 ± 6	51 ± 4	49 ± 5
Sex (female/male)	6/3	7/2	13/5
Height (cm)	166.2 ± 8.2	167.9 ± 5.0	167.1 ± 6.7
Weight (kg)	118.6 ± 46.0	126.8 ± 18.6	122.7 ± 29.6
Body Mass Index (kg/m ²)	42.7 ± 15.5	45.0 ± 6.5	43.8 ± 9.5
Waist Circumference (cm)	125.4 ± 39.8	131.9 ± 13.5	128.7 ± 18.7
Systolic Blood Pressure (mmHg)	123.2 ± 19.4	147.3 ± 16.1 [†]	135.3 ± 21.3
Diastolic Blood Pressure (mmHg)	85.7 ± 13.8	90.4 ± 5.7	88.1 ± 10.6
Self-identified ethnicity			
New Zealand Māori	6	4	10
Cook Islands Māori	1	2	3
Samoan	1	1	2
Fijian	-	1	1
Tokelauan	1	-	1
Tongan	-	1	1
Diabetes duration (years) [range]	2.6 ± 1.8 [0.5 – 5]	3.9 ± 4.3 [0.5 – 13]	3.3 ± 3.3 [0.5 – 13]
Glycosylated hemoglobin (HbA _{1c}) (%)	10.7 ± 3.2	8.9 ± 1.9	9.8 ± 2.1
Diabetes management regimen			
Diet only (<i>n</i>)	1	2	3
Oral hypoglycemics (<i>n</i>)	7	6	13
Oral hypoglycemics and insulin (<i>n</i>)	1	1	2
Blood pressure lowering medications (<i>n</i>)			
ACE inhibitors (<i>n</i>)	7	4	11
Diuretics (<i>n</i>)	4	0	4
β – blockers (<i>n</i>)	2	1	3
Angiotensin II receptor antagonist (<i>n</i>)	1	0	1
Lipid lowering medication medications (<i>n</i>)	5	3	8
Current smoker (<i>n</i>)	3	2	5

Data expressed as mean ± SD. Baseline comparisons determined by independent sample t test. †Statistically significant difference observed between groups at baseline ($P \leq 0.05$). ACE—Angiotensin converting enzyme.

These results raised the question “Why have we not seen an improvement in glycaemic control when so many other randomised exercise trials have shown the benefits of exercise in management of diabetes and reducing HbA1c levels?” The major physiological and biochemical changes such as improved glucose uptake, insulin sensitivity and muscle structural and functional adaptations to exercise are governed by metabolic changes at cellular and molecular levels [14-16]. Exercise improves skeletal muscle plasticity via improving muscle fibre architecture [17], mitochondrial function [18, 19], increasing lipid oxidation [20] and glucose metabolism [21]. Furthermore exercise especially AER is capable of inducing some key transcriptional factors involved in energy metabolism and capable of inducing structural and functional changes in the skeletal muscle [22-25]. Even though the SPIRIT cohort demonstrated no major physiological and biochemical changes such as reduced HbA1c, it could be proposed that 16 weeks of exercise training is inducing metabolic, molecular and genetic changes in the skeletal muscle cell. On this premise it was deemed essential to look for the skeletal muscle cellular, metabolic and genetic changes in the SPIRIT study cohort that has T2DM and grade 3 obesity.

The purpose of this PhD project has been to examine the SPIRIT participants skeletal muscle tissue, the main tissue for glucose disposal [3], and to investigate the metabolic markers related with skeletal muscle structure and function energy and lipid metabolism. The hypothesis, research questions and specific research objectives for my PhD study are outlined in section 1.3.

Table 1.3: Summary of within and between group differences at 16 weeks for primary and secondary outcome measures

Outcome Measures	PRT				AER				P ^{***} (between groups)
	Week 0	Week 16	Change	P	Week 0	Week 16	Change	P	
	Primary outcome								
HbA1c (%)	10.7 ± 2.1	10.6 ± 2.4	-0.1 ± 1.1	0.86	8.9 ± 1.9	8.8 ± 2.1	-0.1 ± 0.6	0.60	0.92
Secondary outcomes									
Body weight (kg)	118.6 ± 38.5	118.9 ± 37.5	+0.2 ± 3.4	0.85	126.8 ± 18.6	125.5 ± 19.7	-1.3 ± 3.6	0.30	0.35
BMI (kg/m ²)	42.7 ± 12.1	42.7 ± 11.7	0.0 ± 1.1	0.91	45 ± 6.5	44.5 ± 6.9	-0.5 ± 1.3	0.32	0.37
Waist circumference (cm)	125.4 ± 23.2	124.3 ± 23.2	-1.1 ± 2.8	0.30	131.9 ± 13.5	131.1 ± 14.7	-0.8 ± 5.1	0.64	0.91
Hip circumference (cm)	130.8 ± 16.4	129.2 ± 16.3	-1.7 ± 2.2	0.05	139.3 ± 16.6	136.8 ± 16.2	-2.5 ± 2.5	0.02	0.46
Waist to hip ratio	0.96 ± 0.1	0.96 ± 0.1	0.0 ± 0.0	0.65	0.95 ± 0.1	0.96 ± 0.1	0.01 ± 0.04	0.43	0.58
Body fat (%)	50.2 ± 7.6	49.8 ± 6.1	-0.4 ± 2.5	0.68	49.6 ± 5.2	48.8 ± 6	-0.7 ± 2.6	0.43	0.76
Lean body mass (kg)	56.7 ± 8.2	57.8 ± 11.2	+1.2 ± 3.9	0.40	63.4 ± 8.6	63.6 ± 9.6	+0.1 ± 3.0	0.92	0.53
Fat mass (kg)	61.9 ± 31.3	61.0 ± 27	-0.9 ± 5.7	0.63	63.4 ± 13.3	61.9 ± 14.5	-1.5 ± 4.3	0.33	0.82
SBP (mmHg)	123.2 ± 19.4	125.6 ± 17	+2.3 ± 16.9	0.69	147.3 ± 16.1	131.1 ± 9.1	-16.2 ± 13.0	0.006	0.02
DBP (mmHg)	85.7 ± 13.8	83.1 ± 8	-2.6 ± 11.2	0.51	90.4 ± 5.7	85.8 ± 4.6	-4.7 ± 4.9	0.02	0.61
Fasting glucose (mmol/L)	9.5 ± 3.5	11.4 ± 4*	+1.9 ± 3.2	0.17	10.2 ± 3.3	10.4 ± 2.9	+0.2 ± 1.6	0.72	0.19
Fasting insulin (pmol/L)	140.7 ± 100.1	134.1 ± 103.1*	-6.6 ± 24	0.50	177.4 ± 82.5	134.8 ± 64.1	-42.7 ± 65.8	0.09	0.19
HOMA2-IR Index	2.9 ± 2	2.9 ± 1.9*	0.0 ± 0.5	1.00	3.9 ± 1.9	2.9 ± 1.3	-0.9 ± 1.6	0.13	0.17
McAuley Index	5.2 ± 1.5	5.2 ± 1.3	0.0 ± 0.5	0.85	5.2 ± 0.8	5.3 ± 0.8	+0.1 ± 0.7	0.59	0.95
C-peptide (nmol/L)	1.6 ± 1.1	1.6 ± 1	+0.1 ± 0.5	0.69	1.4 ± 0.3	1.5 ± 0.7*	+0.1 ± 0.5	0.52	0.85
Free Fatty Acids (mEq/L)	0.5 ± 0.3	0.6 ± 0.3	+0.1 ± 0.2	0.40	0.7 ± 0.2	0.7 ± 0.2*	0.0 ± 0.2	0.86	0.69
Total cholesterol (mmol/L)	4.9 ± 1.5	4.5 ± 1	-0.4 ± 0.9	0.21	4.5 ± 0.4	4.7 ± 0.4	+0.3 ± 0.6	0.22	0.08
HDL cholesterol (mmol/L)	1.3 ± 0.4	1.3 ± 0.5	0.0 ± 0.1	0.37	1.1 ± 0.2	1.1 ± 0.2	0.0 ± 0.1	0.80	0.64
Total cholesterol/HDL ratio	4.2 ± 1.9	3.9 ± 1.1	-0.3 ± 1.0	0.40	4.1 ± 1.0	4.2 ± 0.8	+0.2 ± 0.6	0.35	0.22
LDL cholesterol (mmol/L)	2.7 ± 1.4	2.4 ± 0.7	-0.3 ± 0.8	0.27	2.6 ± 0.6	2.7 ± 0.4	+0.1 ± 0.5	0.71	0.23
Triglycerides (mmol/L)	2.2 ± 1.2	2.0 ± 1	-0.2 ± 0.6	0.35	1.6 ± 0.5	1.9 ± 0.6	+0.3 ± 0.2	0.004	0.03
Adiponectin (µg/ml)	5.6 ± 1.9	5.6 ± 2.2	0.0 ± 1.4	0.96	6.7 ± 3.3	6.7 ± 3.2	+0.1 ± 2.2	0.94	0.93
Capillary Density (px/fibre)	748.9 ± 605.4	689.2 ± 411.2	-59.7 ± 674.8	0.80	386.9 ± 238.6	648.9 ± 241**	+262.1 ± 319	0.05	0.24
C-reactive protein (mg/L) [†]	4 (×/± 3.1)	2.8 (×/± 3.4)	-1.2 (± 3)	0.41	6.4 (×/± 2.5)	4.2 (×/± 2.4)	-2.2 (± 3.7)	0.22	0.90
GLUT4 (px/fibre) [†]	496.1 (×/±)	495.4 (×/± 5.1)	-0.8 (± 385.2)	1.00	190.3 (×/±)	348.7 (×/±)	+158.5 (±133.6)	0.02	0.17

Data expressed as mean ± SD unless otherwise noted. *N = 7. **N = 8. † = C-reactive protein and GLUT4 were non-normally distributed and was log transformed. Data at weeks 0 and 16 are expressed as back-transformed means ×/± factor standard deviation. Change scores are presented as the difference in back transformed means (95% confidence limit). Abbreviations: PRT—Progressive Resistance Training; AER—Aerobic Exercise; HbA_{1c}—Hemoglobin A_{1c}; BMI—Body Mass Index; SBP—Systolic Blood Pressure; DBP—Diastolic Blood Pressure; HOMA2-IR—Homeostasis Modeling Assessment for Insulin Resistance; LDL—Low Density Lipoprotein; HDL—High Density Lipoprotein; GLUT4—Glucose Transporter (fourth isoform). ***P (between group) is obtained by comparing the change.

1.3 Research Questions for my PhD

With the SPIRIT study participants having BMI $> 40 \text{ kg/m}^2$ and mean waist circumference greater than 88 cm for females and more than 102 cm for the males, the following hypothesis has been proposed.

Hypothesis: In skeletal muscle of Pacific island individuals with T2DM and grade 3 obesity, SPIRIT study cohort, metabolic adaptation to exercise is delayed due to metabolic inflexibility.

The skeletal muscle is the most important site related with the pathogenesis of T2DM. Diabetes induces diverse functional [26], metabolic [27] and structural changes [28] in the skeletal muscle. Reduction in glucose cellular uptake [29] and disturbances in fatty acid oxidation [30] lead to structural changes like lipid deposition in the myocytes and production of intramyocellular triglycerides (IMTG) [31], which adversely affect mitochondrial function [32]. These metabolic, cellular and functional events could be the consequence of obesity a well known recognised risk factor for T2DM. Regular exercise has been recommended as an effective strategy for enhancing insulin sensitivity and improving blood glucose control in people with T2DM [33-35]. Evidence suggests that both aerobic and resistance exercises have their own benefits [36]. Aerobic exercise seems to improve metabolic activity [37], insulin resistance [16] and cardiovascular fitness [38] whilst resistance training has been shown to improve body composition [39], lipolysis [40] and muscle mass [41]. However the mechanisms underlying such benefits are not fully understood [42]. Randomised trials with exercise have been performed on T2DM participants with BMI up to 32 kg/m^2 [44, 45]. The average BMI of the SPIRIT cohort was $43.8 \pm 9.5 \text{ kg/m}^2$ which is regarded as grade 3 obesity by WHO [13]. The SPIRIT study is the first randomised exercise trial and only exercise trial at present where grade 3 individuals with T2DM are participants. There is also an associated lack of evidence examining cellular, molecular and morphological adaptations in the skeletal muscle resulting from exercise [46, 47] in T2DM individuals with grade 3 obesity. The majority of exercise control trials with T2DM subjects did not mention any ethnic differences. In a recent systematic review by Sukala *et al.* [48] the key differences in the exercise trials with different ethnic populations were highlighted. Considering the important factors of ethnicity, grade 3 obesity and lack of HbA1C improvement in the SPIRIT study cohort after

16 weeks exercise, the purpose for undertaking this PhD project is as follows. Although the preliminary results from the SPIRIT study show no improvement in glycaemic control [7, 8, 10] there could be early functional, morphological and/or metabolic changes occurring in the skeletal muscle [42], the main tissue involved in insulin resistance [3]. Examination of skeletal muscle metabolism could provide better understanding of impact of exercise in people with T2DM and obesity. Therefore my research questions are as follows:

1. What are the effects of 16 weeks of exercise training on biomarkers associated with obesity and T2DM in SPIRIT study cohort?
2. Do changes occur in skeletal muscle mitochondrial activity and lipid metabolism in response to 16 weeks exercise training in Polynesian adults diagnosed with T2DM and visceral obesity?
3. Do morphological changes occur in skeletal muscle tissue 16 weeks exercise training in Polynesian adults diagnosed with T2DM and visceral obesity?
4. Are there any changes in the skeletal muscle gene expression relevant to structure and metabolic function?
5. Is there any therapeutic benefit of resistance training over aerobic training in Polynesian adults diagnosed with T2DM and grade 3 obesity?

To address these research questions the specific research objectives of my PhD study are as follows.

Specific Research Objectives:

1. To examine the effect of exercise on four specific obesity markers i.e. 24-hours urinary cortisol, serum cortisol binding globulin (CBG), serum sex hormone binding globulin (SHBG) and serum leptin, in the SPIRIT study cohort.
2. To investigate three key mitochondrial enzymes (citrate synthase (CS); cytochrome oxidase c (COX); beta-hydroxyacyl-CoA dehydrogenase (BHAD) to assess the skeletal muscle mitochondrial activity and lipid metabolism (BHAD) after 16 weeks of exercise training in the SPIRIT study cohort.
3. To examine intramuscular triglyceride (IMTG) density and mitochondrial morphology before and after the exercise intervention in the SPIRIT study cohort.

4. To investigate the effect of 16 weeks exercise training on changes in skeletal muscle mRNA expression in the SPIRIT study cohort.
5. To compare and contrast the functional, morphological and genetic changes induced by the two exercise training regimes in the SPIRIT study cohort.

An overview of my PhD thesis chapters are provided in section 1.4 below.

1.4 Thesis Chapter Outline

The thesis is divided into 7 chapters as outlined below.

1.4.1 Chapter 1: Introduction

This chapter provides a brief introduction of the SPIRIT study, the research questions and objectives of my PhD thesis and an outline of the chapters in my thesis.

1.4.2 Chapter 2: Literature Review

This chapter describes the pathophysiology of T2DM and discusses obesity as a risk factor of T2DM. The importance of the mitochondria in the skeletal muscle and overloading of intramuscular triglycerides in the skeletal muscle is highlighted in the pathogenesis of T2DM. The role of exercise in management of T2DM, with particular attention being paid to skeletal muscle metabolism is reviewed and the potential benefits of exercise in relation to muscle metabolism are discussed.

1.4.3 Chapter 3: Materials and Methods

Chapter 3 describes the experimental methods for collection of blood, urine and muscle biopsy samples and the detailed account of methods used for measuring activity of different mitochondrial enzymes in the skeletal muscle, examination of morphological changes in the skeletal muscle and investigation of changes in gene expression in the skeletal muscle.

1.4.4 Chapter 4: Investigation of Impact of 16 weeks of Exercise on Obesity Markers in New Zealand Pacific Peoples with Type 2 Diabetes Mellitus and Grade 3 Obesity

This chapter is the first results chapter and examines the effect of exercise on some obesity markers (leptin, cortisol, cortisol binding globulin (CBG) and sex hormone binding globulin (SHBG) in the SPIRIT study participants.

1.4.5 Chapter 5: Effect of aerobic and resistance training on the skeletal muscle mitochondrial function, IMTG content and fat metabolism in the SPIRIT study cohort

This chapter is the second results chapter and examines the effect of 16 weeks aerobic and resistance exercise on skeletal muscle mitochondrial function and morphology and intramuscular triglyceride (IMTG) density in the SPIRIT study participants.

Chapter 5 presents results and discussion of the three key mitochondrial enzymes citrate synthase, cytochrome c oxidase, and beta-hydroxyacyl-CoA dehydrogenase as well as examining the changes in mitochondrial morphology. The IMTG data showing number and density before and after 16 weeks exercise is also presented and discussed.

1.4.6 mRNA expression changes in the skeletal muscle of SPIRIT participants after 16 weeks of AER and PRT

This chapter is the third results chapter and examines the effect of exercise training on the mRNA expression in the skeletal muscle tissue of the SPIRIT study participants. This chapter presents and discusses the results on mRNA profiling in SPIRIT study participants skeletal muscle tissue pre and post 16 weeks exercise training. The chapter describes

- a) Effect of exercise training on key regulatory pathways related with energy and lipid metabolism and
- b) Identification of other primary pathways altered in the skeletal muscle tissue after 16 weeks of exercise.

1.4.7 Chapter 7: Conclusion

This chapter presents an overall discussion of the cellular, metabolic and genetic changes after 16 weeks of aerobic and resistance training in the SPIRIT study cohort and summarises key findings, study limitations and provides recommendations for future research in this area.

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CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

According to the International Diabetes Federation [1], the prevalence of diabetes is estimated at 336 million people in the world. The scientific study of type 2 diabetes mellitus (T2DM) has dominated the world in the last thirty years. Lifestyle induced diseases such as T2DM are determined mostly by lifelong interaction between multiple genetic and environmental factors. However, the underlying molecular basis for the development and progression of lifestyle diseases still remains unclear. Decreased physical activity, obesity, and associated defects in glucose and lipid metabolism have been identified as mechanisms having a role in the pathogenesis of T2DM [2]. This metabolic disease is characterised by insulin resistance, relative beta-cell dysfunction leading to hyperglycaemia inducing both microvascular and macrovascular complications which can lead to significant impairments such as end-stage renal disease, blindness, ischaemic heart disease, stroke and early mortality [3].

T2DM is often associated with visceral obesity and dyslipidemia and insulin resistance is considered to be the major contributing factor to diabetes. Insulin resistance is characterised by reduced insulin-stimulated glucose disposal mainly within the skeletal muscle. Recent data suggest that the excess deposits of lipid within the skeletal muscle are responsible for this resistance [4]. Likewise obesity is also correlated with insulin resistance and transformed morphology and functionality of skeletal muscle [5]. This obesity induced insulin resistance is frequently linked with the pathogenesis of T2DM.

Despite an overwhelming body of evidence [6-9] that supports the use of exercise as the treatment modality for improving insulin resistance, the exact cellular mechanisms and pathways that can be improved with various modes of exercise training in people with T2DM remain to be established.

The current objective of exercise physiology and biochemistry in the field of diabetes is to better understand the molecular mechanisms involved in lipid and glucose turnover and their regulation during exercise. The recent publications in this area focus on identifying how acute exercise signals may initiate responses that form the basis of adaptations to regular exercise in patients with metabolic disorders such as T2DM and obesity [10-13].

It is interesting to note that most pharmacological interventions for the management of T2DM are directed at the pathophysiological defect of this disease and better pharmacological interventions are compared on their ability to modify the physiological processes back

towards normal homeostasis at the cellular level to mitigate disease-related symptoms (e.g. hyperglycemia) [3]. However, adverse effects are associated with each pharmacological therapy. On the other hand, research in the field of exercise in people with T2DM is focusing to discover how exercise improves various physiological pathways to mitigate or reverse this disease. Notably, exercise has recently been proposed by the American Diabetes Association (ADA) as the first line of treatment in adults diagnosed with T2DM [3]. The New Zealand health system is also following the same guidelines proposed by ADA as outlined in New Zealand Primary Care Handbook 2012 [14].

Considering the paramount role of exercise in the management of T2DM, the purpose of this literature review is three-fold.

- a) To review the current exercise research in T2DM with particular emphasis on the metabolic and cellular pathways and mechanisms within the skeletal muscle of individuals with obesity.
- b) To discuss the potential benefits of exercise in relation to muscle metabolism that could be targeted to prevent and manage T2DM associated with grade 3 obesity.
- c) To identify the gaps in the literature in relation to T2DM, obesity and exercise to provide the rationale for this current PhD project.

This literature review will focus mainly on changes in skeletal muscle metabolism and insulin resistance which can be a cause or consequence of obesity and T2DM and how exercise can improve glycaemic control in people with T2DM. The literature review will concentrate on the exercise induced cellular changes in muscle metabolism related to lipid and energy metabolism and will discuss the possible role of ethnicity in relation to exercise and T2DM.

2.2 Obesity and ethnicity as key risk factors for T2DM

The International Diabetes Federation [1] proposes a list of risk factors associated with the development of T2DM (Table 2.1). It is evident from this list that obesity, insulin resistance, physical inactivity and ethnicity are the major risk factors that need to be considered in relation to the application of appropriate intervention therapies.

Table 2.1: Risk factors associated with type 2 diabetes mellitus (T2DM)

Several risk factors associated with T2DM include:

- **Obesity**
 - **Diet and physical inactivity**
 - **Increasing age**
 - **Insulin resistance**
 - **Family history of diabetes**
 - **Ethnicity**
-

Sourced from International Diabetes Federation [1]

Over the last 20 years, the prevalence of obesity has increased in both developing and developed countries [16,17]. In 1997 World Health Organization (WHO) formally recognised obesity as a global epidemic and currently, the WHO data suggests that 1.54 billion adults are obese worldwide [16]. Using the WHO criteria for overweight ($BMI > 25 \text{ kg/m}^2$) and obese ($BMI > 30 \text{ kg/m}^2$), the highest levels are observed among Pacific Islanders, with obesity rates as high as 79% in the adult population [15]

Being one of the major risk factors, the increased prevalence of obesity has been accompanied by an increased prevalence of T2DM [2] and according to the WHO the number of individuals with known T2DM will exceed 550 million within the next 10 years [16]. The global prevalence of T2DM is increasing with the majority occurring in developing countries around the world [17]. Evidence shows that the prevalence of T2DM is elevated in many ethnic groups as compared to people with Caucasian-European origin [17, 18]. Evidence-based guidelines for the prevention of T2DM in recent European consensus statement has included ethnicity as a non-modifiable risk factor citing the prevalence of the disease highest amongst individuals of Hispanic, African-American, and Asian-Indian descent compared to Caucasians [18]. Approximately in Asia 38 million adult population is diagnosed with T2DM however data of undiagnosed cannot be estimated due to lack of proper diagnosis. On the other hand in USA 26 million adult population is diagnosed with T2DM [17]. This data reflects the global burden of T2DM to be approximately 7-10% of the total adult population of the world. The future global burden of T2DM will be largely determined by the current lifestyle modifications [19]. Many developed countries in the world have focused on

addressing the burden of this disease by early diagnosis and public awareness for the risk factors such as obesity and lifestyle disorders such sedentary behavior, eating disorders and stress [20].

It is estimated that the increase in prevalence for T2DM will be especially high in the Asia-Pacific region [21]. Polynesians (Maori and Pacific Islanders) are among the major ethnic groups at the higher risk of developing T2DM [22]. The Pacific Ocean has estimated 20,000 - 30,000 islands, which are often called Oceania (which include Australia and New Zealand). The Pacific Island populations represent a diverse group of people which are divided into three geo-ethnic groups: Polynesia, Micronesia and Melanesia. The term 'Polynesia' means 'many islands'. Polynesia includes the nation states of Samoa, American Samoa, Tonga, Niue, Tuvalu, Tokelau, the Cook Island, the Midway Island and Easter Island. Polynesians are the most homogeneous in culture, language and physical appearance compared to Micronesia and Melanesia. Recent epidemiological studies [21] have shown that in New Zealand there are significant differences in T2DM incidence, prevalence and health outcomes between Polynesians and those of European descent. The recent National Health Survey in New Zealand, in 2008, has reported that the prevalence of diagnosed diabetes was 4.3% in Caucasians and 10.1% in Polynesians [23]. It is estimated that by the year 2021, assuming no change in risk factors, one in twenty two Europeans (4.5%) as compared to one in six Polynesian adults (17.6%) will have T2DM in New Zealand [23]. New Zealand is included in the top ten countries in the world with the highest rate of obesity (i.e. 20.9% of the adult population) and subsequently rates of T2DM in this country are also extremely high, particularly among people of Maori and Pacific Islands descent [15, 23, 24].

The recent National Health Survey in New Zealand (2008) has reported (Table 2.2) that the prevalence of diagnosed diabetes was 4.3 % in Caucasians and 10.0 % in Pacific Island People [23].

Table 2.2: Diagnosed T2DM in adults, by ethnic group [15]

Ethnic group	Prevalence (95% CI)	Number of adults
European/ Other	4.3 (3.8–4.8)	109200
Māori	5.8 (4.9–6.7)	20800
Pacific	10.0 (8.1–11.8)	16400
Asian	6.5 (5.4–7.7)	18100

Source: 2006/07 New Zealand Health Survey

Note: Total response standard output for ethnic groups has been used.

Furthermore, by examining the prevalence of associated risk factors i.e. obesity (Table 2.3: Maori = 41.7%, Pacific Islanders = 63.7%) and physical inactivity (Table 2.4: Maori = 14%, Pacific Islanders = 19.4%) it is evident that the Polynesian population is at the greater risk of developing T2DM which could potentially lead to enormous burden on the health care system in the coming years. These statistics warrant the need for better planning and preventive measures for obesity and T2DM in this population.

Table 2.3: Obesity for adults, by ethnic group [15]

Ethnic group	Prevalence (95% CI)	Number of adults
European/ Other	24.3 (23.1–25.5)	619200
Māori	41.7 (39.8–43.7)	148300
Pacific	63.7 (60.0–67.5)	104900
Asian	11.0 (9.0–13.0)	30800

Source: 2006/07 New Zealand Health Survey

Table 2.4: Sedentary behaviour for adults, by ethnic group [15]

Ethnic group	Prevalence (95% CI)	Number of adults
European/ Other	13.8 (12.8–14.7)	351900
Māori	14.0 (12.4–15.7)	49900
Pacific	19.4 (16.6–22.2)	31900
Asian	23.0 (20.4–25.5)	64000

Source: 2006/07 New Zealand Health Survey

Note: Total response standard output for ethnic groups has been used.

2.3 Mechanisms linking obesity to insulin resistance and T2DM

Generally, chronic metabolic disorders are based on the theory of polygenic interactions with environmental factors with diet and physical inactivity playing an important role in development and progression of T2DM. The aetiology of T2DM is characterised by insulin resistance of the major tissues, including the skeletal muscle, liver, and adipose tissue. The skeletal muscle is the major site of glucose utilisation in response to insulin therefore it is the major site of insulin resistance in T2DM [25-27]. At present, the exact mechanisms underlying insulin resistance in skeletal muscle are not fully understood.

The insulin signaling pathway is responsible for the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and insulin mediated glucose transport from the blood into the cell (Figure 2.1). Insulin binds to the insulin receptor (IR) which causes phosphorylation of insulin receptor substrate-1 (IRS-1) leading to activation of phosphatidylinositol PI-3 kinase. Activated PI-3 kinase regulates the activation of protein kinase C (PKC) with protein kinase B (Akt). The activated Akt can stimulate production of AKT substrate 160 k DA (AS-160). In insulin resistant states, the phosphorylation of IR is hampered which leads to reduced activation of PI-3 kinase and possible impaired GLUT 4 translocation. When this mechanism is affected, it affects protein, lipid, glycogen metabolism and glycolytic activity in the mitochondria by decreasing cellular respiration and increasing glycolysis, decreasing amino acid, nucleotide and fatty acid metabolism and hence weakening the metabolic flexibility of the skeletal muscle tissue (Figure 2.1). The resultant metabolic derangement leads to increased levels of intramyocellular triglycerides (IMTG) which accumulate within

the muscle cells. It has been speculated that the accumulation of lipid intermediates such as long-chain fatty acyl CoA, diacylglycerol (DAG) and ceramide are metabolically active forms, and can produce further disturbances in the GLUT4 mediated glucose transport through stimulating and inhibiting some PKCs. To date, 12 isoforms of PKCs have been identified and the isoform PKC ξ has been linked with glucose transport in human skeletal muscle [25].

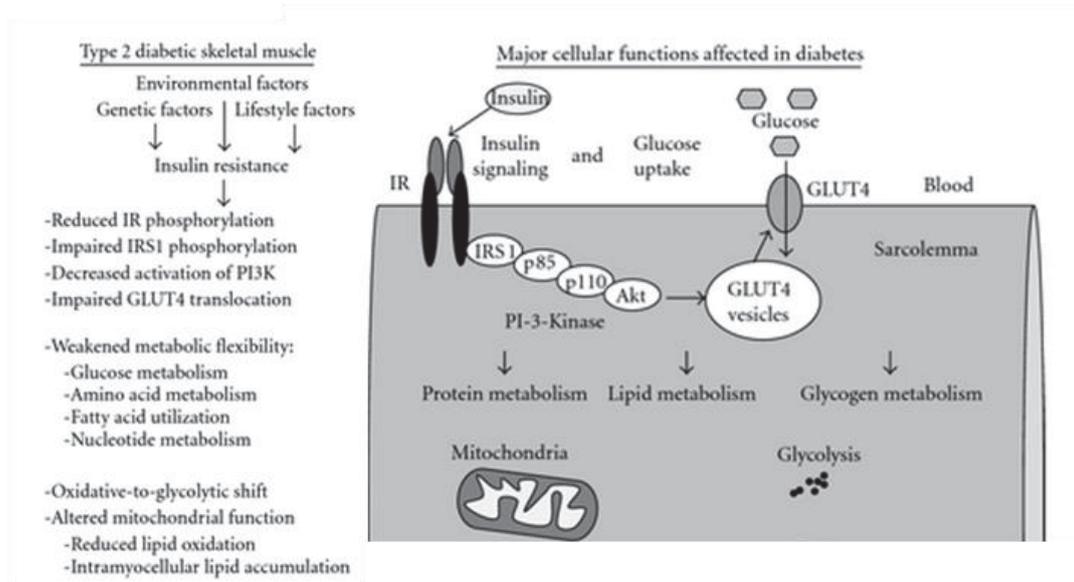


Figure 2.1: Major cellular dysfunctions in type 2 diabetic skeletal muscle. Reduced Insulin Receptor Substrate1 (IRS1) phosphorylation, decreased PI-3Kinase activation, impaired GLUT4 translocation lead to impaired glucose entry into the muscle cell. This leads to reduced glucose, amino acid, fatty acid and nucleotide metabolism representing metabolic flexibility of the skeletal muscle. Reduced lipid oxidation lead to intramyocellular lipid accumulation and impaired mitochondrial function. Adapted from Maassen et al. [25]

Studies with obesity and insulin resistance have shown increased expression of cell membrane and mitochondrial membrane transporter proteins such as fatty acid binding transporter protein (FABP) and carnitine palmitoyltransferase (CPT1) [2, 28]. The results from these studies support the theory that muscle overloaded with lipids can adapt and start to preferentially use fatty acid oxidation over glucose oxidation for the generation of ATP [29]. This alteration in the muscle metabolism initially tries to protect the myocyte against insulin resistance, and it does this by promoting increased entry of fatty acids into the cell and then into the mitochondria. With increasing levels of fatty acids within the muscle cell, lipids start accumulating, ultimately leading to decreased mitochondrial activity [25-27, 30].

More recent research has focused on the contribution of skeletal muscle mitochondrial activity to the development of insulin resistance [25-27, 30]. Kelley *et al.* [31] have shown that in people with T2DM, there is less activity of key regulatory mitochondrial enzymes such as beta-hydroxyacyl-CoA dehydrogenase involved in fatty acid beta oxidation, citrate synthase in the Krebs cycle, and cytochrome oxidase an important protein in the electron transport chain. It is also proposed that in obese individuals and individuals who are insulin resistant, low activity of mitochondrial enzymes in skeletal muscle mitochondria can contribute to producing a state of metabolic inflexibility or reduced metabolism of fatty acids and glucose [5, 32, 33]. Metabolic flexibility is defined as the ability of the body to shift the use of substrate (carbohydrate/lipid/protein) according to the tissues needs during the periods of energy supply and expenditure [34]. The features associated with metabolic flexibility are the use of carbohydrate in the times of abundance, lipids in the periods of scarcity and the ability to shift between the two. Weakened metabolic flexibility (Figure 2.1) is a condition associated with disturbances in the normal glucose, lipid, amino acid and nucleotide metabolism within the skeletal muscle. The environmental, genetic and lifestyle factors can contribute to weakening the metabolic flexibility within the skeletal muscle, impairing insulin signaling, and GLUT 4 translocation and ultimately leading to decreased glucose uptake by the muscle cell (Figure 2.1) resulting in sustained increased blood glucose levels (hyperglycemia) and development of T2DM [35-37].

Earlier research in this area has suggested that in insulin resistant conditions, the skeletal muscle is presented with increased amounts of free fatty acid (FFA) that it cannot oxidise (Figure 2.1). It has been speculated that increased levels of FFA produces a state of substrate competition with glucose within the skeletal muscle cell and may lead to inhibition of glucose metabolism and increased production of triglycerides as discussed previously. However, it has been suggested that an elevated level of FFA is not the only factor responsible for insulin resistance [25].

A critical issue with obesity is the expansion of adipose tissue which leads to an increase in lipolysis resulting in increased free fatty acids (FFA) in the blood. These FFA are then taken up into the skeletal muscle cells and used for forming lipids called intramuscular triglycerides (IMTG) (Figure 2.2). Activation of skeletal muscle NF-kB and I-kB kinase by the proinflammatory cytokines (e.g. IL-6, IL-10 and TNF-alpha) in the blood [38], along with increased formation of intramuscular triglycerides [4, 39] and increased free fatty acids within

the skeletal muscle, cause the release of inflammatory cytokines within the skeletal muscle. These cytokines can lead to an impaired insulin signaling cascade [32, 40] which can progressively lead to insulin resistance (Figure 2.2) [32]. This model of obesity-induced insulin resistance [32] proposes that in obesity, increased lipolysis and increased secretion of proinflammatory cytokines from the adipose tissue enhances lipid accumulation in the skeletal muscle and perturbation of the skeletal muscle metabolic homeostasis. Obesity and the oversupply of lipids within the myocytes activate formation of inflammatory cytokines within the skeletal muscle which leads to impairment in insulin signaling and potential development of insulin resistance.

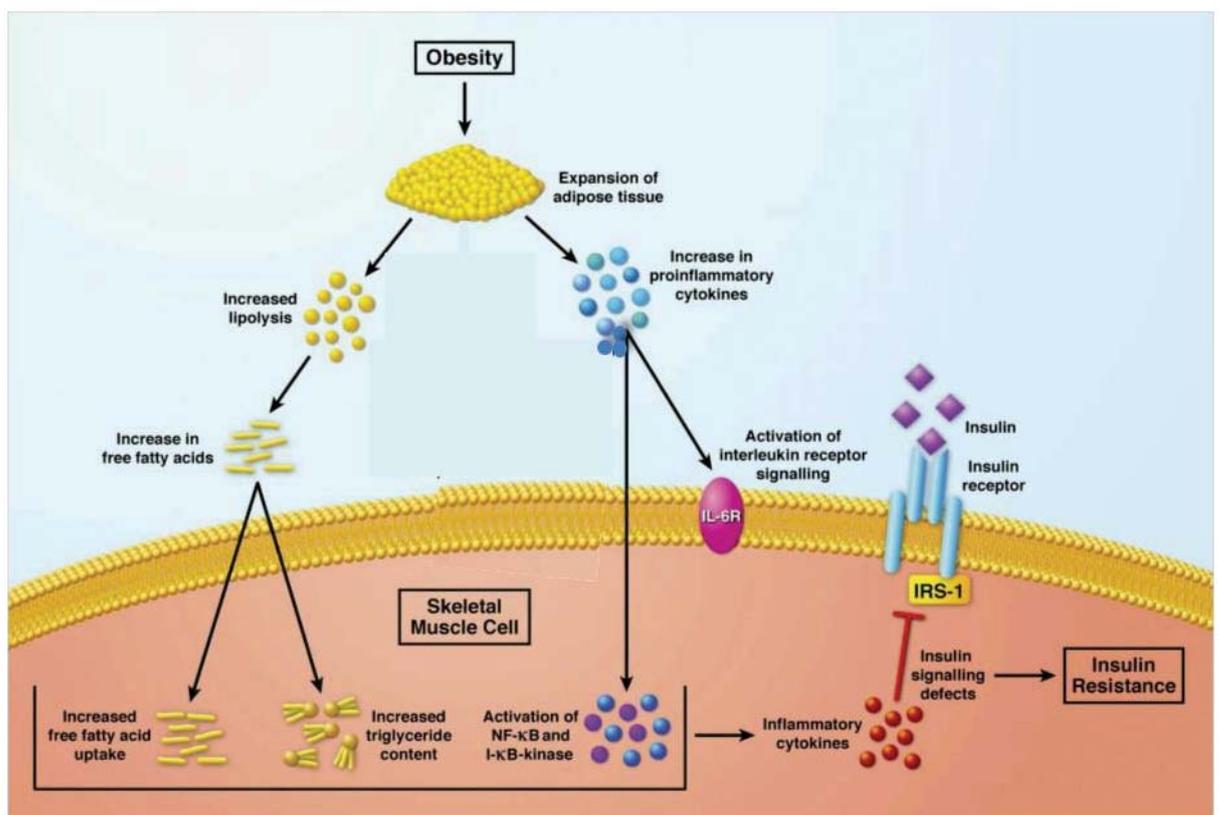


Figure 2.2: Sources of inflammation associated with insulin resistance in skeletal muscle. *IRS-1 is insulin receptor substrate-1. Sourced from Coletta and Mandarino [32]*

Coletta and Mandarino [32] further suggest that inflammation can contribute to mitochondrial dysfunction which is frequently observed in skeletal muscle of T2DM (Figure 2.3). Mitochondrial dysfunction refers to decreased mitochondrial activity [30, 31] with associated changes in morphology [28]. Martins *et al.* [26] further extends the Coletta and Mandarino

obesity-induced insulin resistance model [32] by adding that mitochondrial dysfunction is an important part of insulin resistance. In summary, mitochondrial dysfunction could be the cause of impaired fatty acid metabolism instead of the consequence. As mitochondria is the site of fatty acid metabolism and mitochondrial abnormalities are observed in people with T2DM, there could be other mechanisms involved that can cause mitochondrial defects. Coletta and Mandarino [32] indicate that inflammation induces extracellular matrix remodelling in the muscle which causes reduced gene expression in proteins related with structure and function of the skeletal muscle. These changes are critical as they lead to a decreased number of mitochondria per myocyte coupled with reduced activity (Figure 2.3).

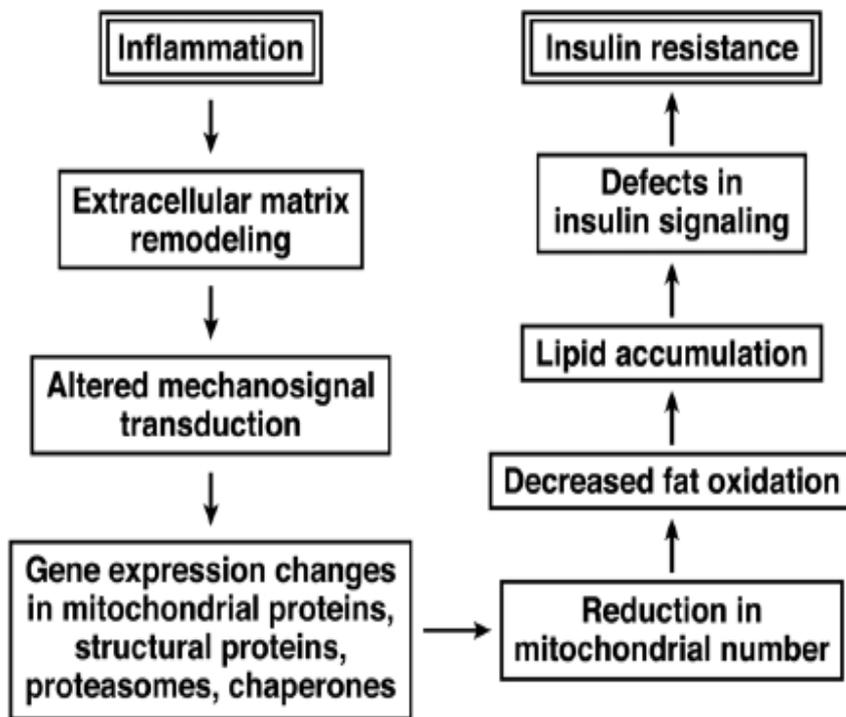


Figure 2.3: Proposed Model showing relationship between inflammation, extracellular matrix, gene expression changes and mitochondrial function in the skeletal muscle leading to insulin resistance. Sourced from Coletta and Mandarino [32].

Figure 2.3 illustrates that a reduction in the number of mitochondria leads to their reduced function which in turn will lead to an associated reduction in fat oxidation. Reduced number of mitochondria has been associated with reduced transcription of mitochondrial genes. Several reports suggest that reduced mitochondrial content may result from the reduced

expression of some transcriptional factors such as PGC1- α [41-43], although little direct evidence has been offered to support this conclusion. Holloway *et al.* [44] suggest that reduced physical activity can cause decreased gene expression of transcriptional factors other than PGC1- α which leads to decreased oxidative phosphorylation, lipid oxidation and lipid accumulation and insulin resistance (Figure 2.4).

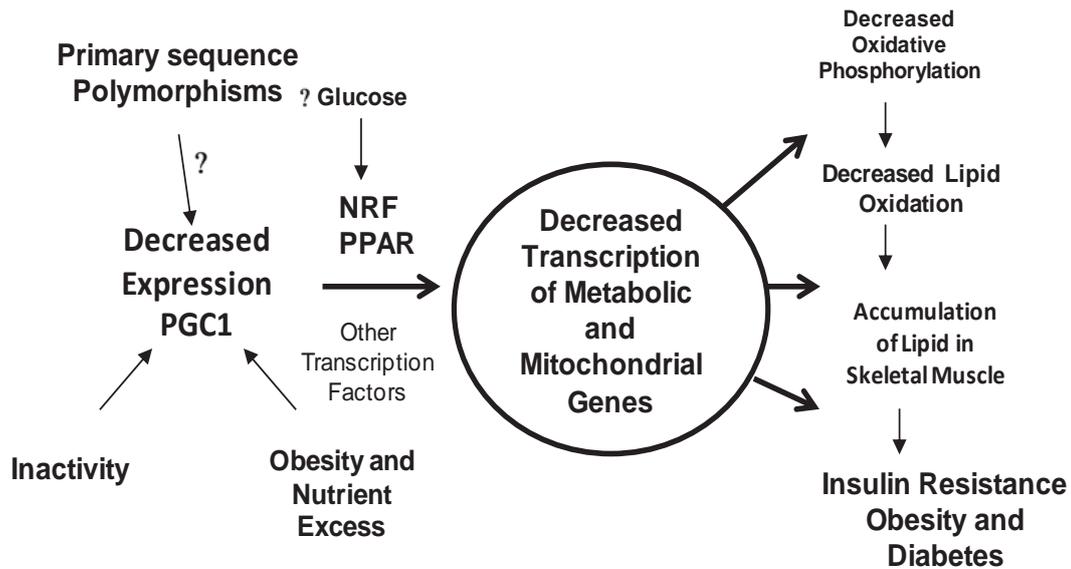


Figure 2.4: Potential role of decreased peroxisome proliferator-activated receptor- γ coactivator-1 (PGC1). PGC1 expression in skeletal muscle as it relates to the development of metabolic phenotype of insulin resistance and T2DM. Sourced from Patti *et al.* [41].

This theory by Holloway *et al.* [44] further supports the thrifty phenotype which is associated with physical inactivity leading to inhibition of health promoting proteins. Physical inactivity causes a state of cellular starvation that disturbs the homeostatic signalling of gene expression at the nuclear level. These further signals inhibit the health promoting proteins and activate the disease promoting proteins. At this level, the system tries to maintain its balance and suffers from a metabolically challenging phase. If these risk factors remain to persist, they can exceed the physiological limits causing the pathophysiological states with disturbed metabolic functions leading to overt clinical symptoms of disease.

This phenomenon of metabolic perturbations is explained in a recent review by Wells and Sievro [45] proposing that obesity induced metabolic inflexibility produces a state which

further deteriorates the situation by producing lethargy and hyperphagia. This problem produces a condition which makes the cells of the body insulin resistant to such an extent that all the protective mechanisms fail to protect that body from the lipid overload with this metabolic trauma leading to pathophysiological conditions. However, there is insufficient data available in the literature on morbidly obese humans to evaluate this theory of metabolic inflexibility.

These models of lipid induced mitochondrial dysfunction do not include the role of adipokines and their involvement in further ameliorating this process. Literature shows that there are several hormonal biomarkers that play a role in obesity and insulin resistance [46-48]. These biomarkers include adipokines such as leptin and adiponectin, the stress hormone cortisol, with associated binding protein (cortisol binding globulin). Leptin is a protein hormone secreted from adipocytes and acts on receptors in the hypothalamus of the brain to suppress food intake and stimulate energy expenditure [49]. Leptin also acts on liver cells and skeletal muscle cells to increase fatty acid oxidation [50], decrease fat accumulation [50], trigger up-regulation of uncoupling proteins 1 and 2 [51] and improve glucose utilisation through pathways independent of insulin signaling [52]. Leptin also decreases secretion of insulin and can improve insulin sensitivity [53]. The physiological functions of leptin appear to be protective mechanisms against development of diabetes. However, several studies have shown circulating leptin levels are elevated in individuals who are obese [54] and also in obese individuals with T2DM [55]. Current research demonstrates a positive correlation between serum leptin levels and percentage body fat [56]. Also the rate of insulin resistance and diabetes increases as the body fat content increases. The question remains as to what role leptin plays in the development of diabetes. It has been proposed that being overweight can lead to leptin resistance, hyperleptinaemia [57] and results in dysregulation of leptin function in the hypothalamus, liver and skeletal muscle cells. This could lead to lipotoxicity in the cells, decreased glucose utilisation, promote insulin resistance, and ultimately result in T2DM.

When blood glucose levels are low, cortisol can cause an increase in blood glucose by stimulating gluconeogenesis and an increase in FFA by stimulating lipolysis. In obese individuals it has been shown that levels of free cortisol are elevated which can lead hyperglycaemia and altering the lipid profiles [58-60]. The similarities between hypercortisolism and characteristics of obesity, such as weight gain in depressed persons and

changes in the food intake as a result of stress proposes a strong link between stress, obesity and T2DM [61]. Cortisol binding globulin (CBG) is a specific plasma transport protein for cortisol that regulates cortisol activity (cortisol must be in free form not bound to CBG to be active) by affecting its bioavailability [62]. Cortisol and CBG may be markers of obesity [63, 64] but it is not still clear as to whether they are involved in the pathogenesis of insulin resistance resulting in T2DM or that free cortisol is the result of insulin resistance [62, 65, 66]. Studies with weight reduction in obese people with T2DM have shown improvement in the levels of cortisol and cortisol binding globulin [67]. Currently there are no studies that have looked at impact of exercise in people who are obese and have T2DM, and the effect of exercise on cortisol levels and CBG levels.

Sex hormone binding globulin is a glycoprotein that binds the sex hormone [68]. It is produced by liver and it has been proposed that its plasma levels are controlled by many factors and insulin is one of them [69]. Furthermore certain insulin resistant conditions like obesity, polycystic ovarian syndrome, cushing syndrome and T2DM are associated with reduced levels of this glycoprotein [68, 70, 71]. Laaksonen *et al.* [72] have shown that in obesity and T2DM there are low levels of sex hormone binding globulin (SHBG) in spite of normal sex hormone levels, thus suggesting that SHBG can be potentially used as a marker for insulin resistance in obese individuals. SHBG may also be another marker of insulin resistance [73] as suggested by the reduced levels of SHBG in insulin resistant states [74], reduced levels of SHBG with higher BMI [68] and low high density lipoprotein cholesterol level [75].

To sum up this section, the pathophysiology related to T2DM is a multitude of mechanisms involving some key metabolic pathways associated with risk factors such as obesity, physical inactivity and stress. Obesity plays an important role by deteriorating the metabolic flexibility in skeletal muscle tissue. Adipose tissue expansion leads to free fatty acid release, influx of fatty acids into the myocytes and deposition of IMTGs. Adipose tissue also releases proinflammatory cytokines leading to release of inflammatory substances in the skeletal muscle that interfere with the insulin signalling pathway inducing insulin resistance in skeletal muscle. The skeletal muscle overloaded with lipid (IMTG) contributes to causing a metabolically challenged state within the tissue which leads to the mitochondria becoming dysfunctional due to decreased mitochondrial activity (downregulation of some transcriptional factors related with energy metabolism and production of mitochondrial

enzymes). Due to the overwhelming influx of fatty acids into the mitochondria through CPT1, skeletal muscle gradually loses metabolic flexibility or plasticity that leads to a metabolic inflexibility state. Gradually and progressively the skeletal muscle in its metabolic inflexible state becomes unable to take up glucose through insulin-mediated or GLUT-4 mediated pathways. This condition can lead to insulin resistance and potential development of T2DM.

Furthermore along with release of adipocytokines and proinflammatory cytokines from excessive adipose tissue, an increase in release of the stress hormone, cortisol, occurs. The increased levels of cortisol systemically aided with all the other factors mentioned above produce a state of glucotoxicity (very high levels of glucose that could damage the normal cells in different tissues such as retina, nephrons and peripheral nerves), lipotoxicity (very high levels of lipids in the body), oxidative stress, low-grade inflammation, insulin resistance and potentially aid in development of metabolic disorders such as T2DM.

This PhD is unique in that the cohort are grade 3 obese with T2DM and no-one has examined the impact of exercise on the adipocytokines, proinflammatory cytokines and other obesity markers as well as IMTG deposition and mitochondrial activity in the skeletal muscle of this cohort.

2.4 Exercise as therapeutic intervention

Exercise along with diet and medication has been recommended as a cornerstone in the management of T2DM. In the 1990's the American Diabetes Association recommended aerobic training as the type of exercise that people with T2DM should perform in order to help improve their blood glucose levels [76]. Boule *et al.* [80] undertook a meta-analysis of the inter-relationship among exercise intensity, exercise volume, changes in cardiorespiratory fitness and changes in HbA1c. This analysis was restricted to aerobic exercise studies and provided support for moderate intensity aerobic exercise for glycaemic control in people with T2DM. To date there are at least 16 randomised controlled trials (RCT) that have shown glycaemic improvement in T2DM patients undergoing aerobic exercise.

Progressive resistance training (PRT) is defined as exercise in which the resistance against which a muscle generates force is progressively increased over time and in which oxygen is used up more quickly than the body is able to replenish it inside the working muscle [77]. Aerobic training is defined as constant and moderate intensity work that uses up oxygen at a

rate in which the cardiorespiratory system can replenish oxygen in the working muscles. Examples of such activity are exercises like bike riding or walking [76]. Regular aerobic and resistance trainings have been recommended by ACSM and ADM [3] for the management of T2DM. Both serve as a means to improve insulin sensitivity and glucose homeostasis. They prevent the development of the metabolic syndrome and T2DM [76]. Both AER and PRT improve insulin sensitivity in part by reducing total body fat, increasing glycogen synthase activity and hence increasing formation of glycogen [77] and increasing GLUT4 levels and hence increasing amount of glucose entering the cell [78]. AER increases activity of insulin signalling protein receptor substrate (IRS-1) and PI-3 kinase [33] and hence improves insulin mediated uptake of glucose from the blood into the cell. Both exercise trainings lead to increased expression of the transcriptional factors such as PGC1- α and PPAR-gamma [79] which leads to a reduction in IMTG content, improved mitochondrial morphology and function [80] and overall an improvement in metabolic flexibility [5] (Figure 2.1 and 2.4). Therefore it can be proposed that the exercise stimulus either by aerobic or by resistance exercise can induce changes in the transcriptome (leading to activation of some transcriptional factors such as PGC1- α), muscle cell architecture, mitochondrial morphology, and muscle cell energy metabolism related with glucose and lipid. The exact mechanism(s) for inducing these changes by aerobic and resistance exercise is not clear therefore investigation of the metabolic pathways involved in energy metabolism, muscle morphology and mitochondrial function would be pertinent for comparing and contrasting the benefits and adverse effects of both trainings.

The first published control trial for use of resistance training in treatment of T2DM was in 1997 [78] and there have been 25 reported clinical trials since this study that provide support for the use of resistance exercise training in improving glycaemic control. A systematic review done by Gorden *et al.* [81] of 24 clinical trials includes 13 RCT, 8 non-randomised control trials, and 3 uncontrolled trials. The summary of the data from these 24 trials, show that resistance training is effective in improving glycaemic control and insulin insensitivity in people with T2DM who are likely to have co-morbidities. In 2004, the American Diabetes Association included resistance training in their recommendations as an adjunct therapy for the management of people with T2DM. They proposed that moderate to high-intensity resistance training is safe for people with T2DM. Sigal *et al.* [82] was the first study that involved comparing the effect of resistance, aerobic and combined aerobic and resistance

training in individuals with T2DM and sedentary people. The results showed that aerobic or resistance training prescribed in isolation could lead to significant improvements in glycaemic control, however, combining these two modalities resulted in superior adaptation as compared to prescribing either modality on its own.

According to the ADA treatment guidelines, the chronic illness of diabetes requires complex care that involves factors other than glycaemic control that need to be addressed [3]. The most recent position statement in 2011 of ADA and European Association for the study of Diabetes (EASD) [3] recommends that patients with HbA1c < 7.5% could be given the opportunity of engaging in exercise first to control blood glucose levels before embarking on pharmacological intervention. The recommendation for exercise is that it could be aerobic or resistance and of moderate intensity. The New Zealand recommendation is also in the same line [24].

Epidemiological data show that the prevalence and incidence of diabetes is high among various ethnic groups such as Asian-Indians, African Americans, Latino-Americans, Hispanics and Pacific Islanders [83]. There have only been a few clinical exercise trials looking at the effect of exercise training in populations with high prevalence of T2DM [84]. Winnick *et al.* [89] reported that eight weeks of resistance training had a positive effect on weight loss and glucose metabolism in African Americans compared to their Caucasian European counterparts. Misra *et al.* [85] also reported improvement in the insulin sensitivity, glycaemia and lipid profile in Asian Indian people with T2DM undergoing PRT. Similar results were observed for Hispanic older adults [77]. Two studies have shown that aerobic exercise is very effective in reducing HbA1c levels in Chinese with T2DM [86] and black females with T2DM [87]. The Polynesian population is a population which is also more prone to diabetes as discussed in section 2.2 of this chapter. There has only been one randomised clinical exercise trial conducted with individuals who have T2DM and are of Polynesian descent. This trial was called the SPIRIT study and has been discussed in Chapter 1 section 1.2. In the SPIRIT study a number of metabolic outcome measures were investigated in a New Zealand Polynesian population that had T2DM and grade 3 obesity (average BMI = 43.8 ± 9.5). For this study, 16 weeks of either aerobic or resistance training did not improve fasting glucose, insulin or HbA1c levels [84, 88]. The aerobic group did show a significant decrease in systolic blood pressure ($P=0.02$) which was also clinically significant. In previous studies by Winnick *et al.* [77], Misra *et al.* [85], Castaneda *et al.* [89], Brooks *et al.* [91] and trials

mentioned in the review by Gordon *et al.* [81] and Sukala *et al.* [84] participants BMI range were between 25kg/m²-35kg/m² which is grade 2 obesity. Thus a uniqueness regarding the SPIRIT study was that this cohort had BMI >40 kg/m² which is regarded as grade 3 obesity by WHO as discussed in chapter 1 section 1.2.

2.4.1 Cross roads between exercise, obesity, T2DM and the mitochondria

Lipid accumulation, inflammation and resistance to adiporegulatory hormones such as insulin and leptin are implicated in the pathogenesis of obesity and T2DM. There is a rapidly growing interest in exercise interventions to look for the pathways that could be targeted to manage this metabolic crisis. Exercise is reported to confer multiple health benefits protecting against conditions like insulin resistance, obesity, T2DM and metabolic syndrome. Both aerobic and resistance exercise modalities are now integral in the treatment strategy of T2DM [3, 24]. Exercise can stimulate muscle cells in T2DM individuals to inhibit lipid accumulation (Figure 2.5) by stimulating different pathways like PGC1- α and PPARG (Figure 2.4).

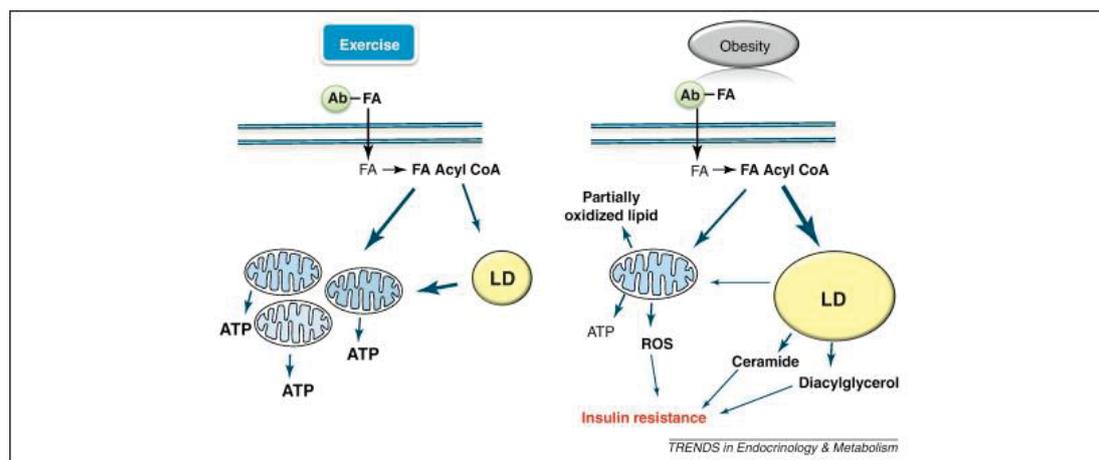


Figure 2.5: Role of intramyocellular lipid (IMCL) during exercise and in obesity. This schematic depicts the fate of the major IMCL lipid species within the context of exercise and obesity. During exercise, fatty acid (FA) acyl CoA is oxidised in mitochondria to synthesise ATP. FA acyl CoA is also partitioned to lipid droplets (LDs), where it is esterified to triglyceride (TG). TG can subsequently be lyophilised to release FAs for mitochondrial oxidation. In obesity, because of lower energetic demand, most FA acyl CoA is partitioned to LDs. IMCL in LDs can then act as a surrogate for ceramide and diacylglycerol. FA acyl CoA oversupply to the mitochondria during low energetic demand results in incomplete beta oxidation and production of reactive oxygen species (ROS). The size of the arrows represents the rate of flux. Ab represents albumin. Sourced from Coen and Goodpaster [39].

When fatty acids (FA) released into the cytosol exceed the β -oxidation capacity of mitochondria, the increased FA concentrations would induce mitochondrial toxicity due to incomplete beta oxidation and production of reactive oxygen species (ROS) and increased deposition of lipids in the mitochondria, along with resulting increased triacylglycerol deposits (LD in Figure 2.5) producing more ceramide and DAGS (Figure 2.5). The increased production of ROS, ceramide and DAG can lead to impaired insulin signalling, impaired translocation of GLUT4 transporter, and impaired antioxidant defense (Figure 2.5) with resulting insulin resistance [39, 92-94]. Teixeira De Lamos *et al.* [95] argues that exercise could also serve as a strategy to improve the inflammatory state (Figure 2.2 and 2.3) and reduce formation of the reactive oxygen species (ROS).

Exercise can induce mitochondrial biogenesis by stimulating PPAR and PGC1 α which leads to improvement in the function, morphology and content of mitochondria [95-97]. Increase in mitochondrial enzyme activity such as citrate synthase and beta-hydroxyacyl-CoA dehydrogenase [98] and GLUT4 transporter [91, 99] has been demonstrated in people with T2DM. These results show that exercise improves mitochondrial activity and fatty acid metabolism which can result in improved insulin sensitivity in the skeletal muscle. Hence this alteration in the skeletal muscle could be targeted for intervention and prevention of T2DM.

2.4.2. Skeletal muscle and intramuscular triglycerides (IMTGs)

Myofibrils, mitochondria, lipid, glycogen and t-system surrounding the muscle myofibril constitute the main components of skeletal muscle cell or myocyte. The intramyocellular triglycerides (IMTG) accumulation in skeletal muscle has been linked with T2DM pathophysiology (Figure 2.3 - 2.5) [32]. Some studies report that T2DM people have higher total IMTG content compared with BMI-matched controls further supporting a causal relationship between increased IMTG and development of T2DM. However this has been challenged. For instance aerobic-trained athletes have increased skeletal muscle IMTG [33, 94], and females appear to have greater IMTG content compared to males [100]. This suggests that IMTG accumulation may not be the principal factor leading to insulin resistance. The role of associated fatty acids metabolites such as diacylglycerol (DAG) and ceramide is crucial in determining the insulin sensitivity [4, 93]. Hence it seems more suggestive that high IMTG levels could represent a marker of deranged fatty acid metabolism [101].

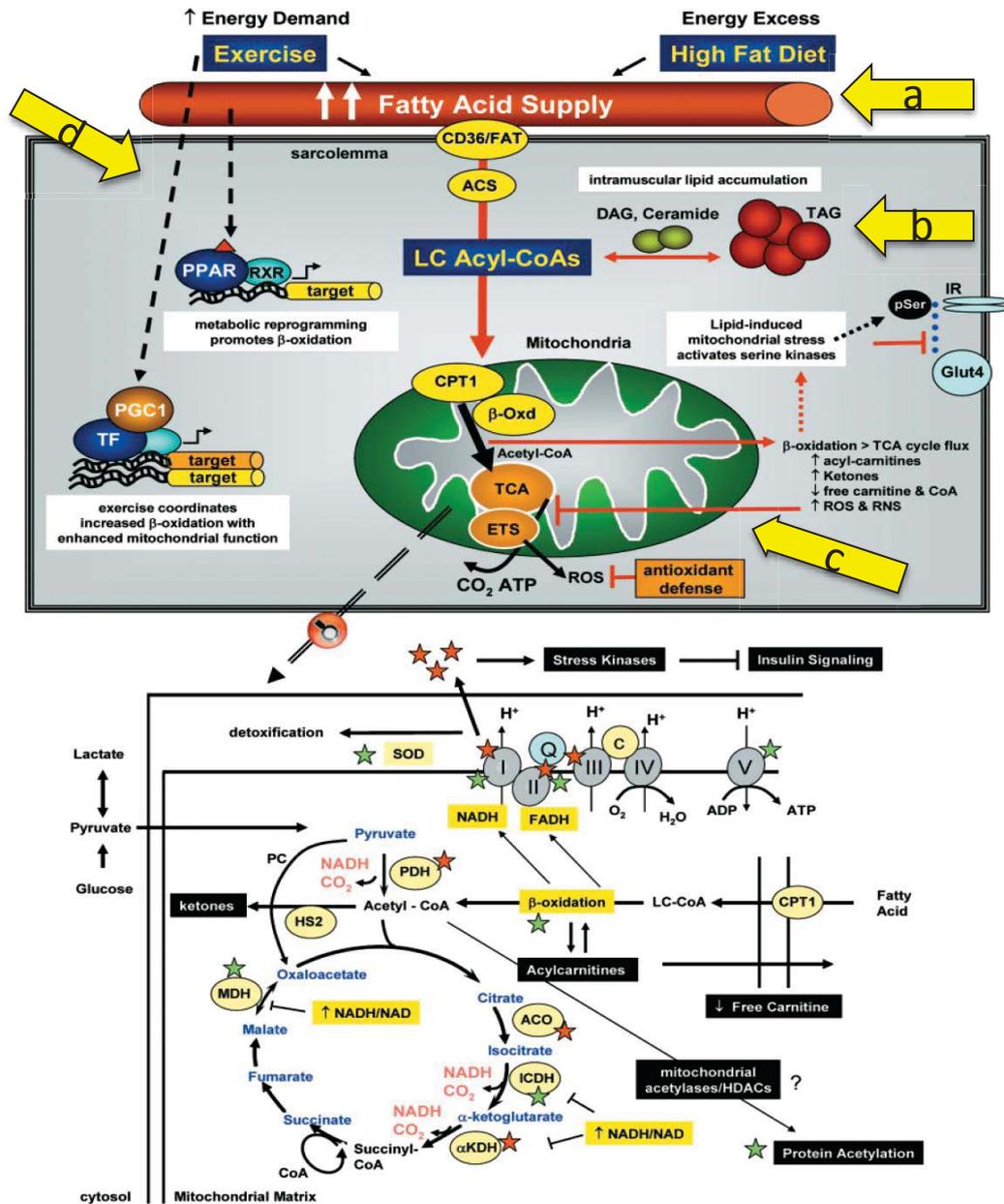


Figure 2.6: The schematic representation of pathophysiology involved in skeletal muscle overloaded with fatty acids and exercise induced improvements a) High fat diet leading to increased supply of fatty acid to the cell b) insufficient oxidation of fatty acids enhances production of lipid moieties like diacylglycerol (DAG) and ceramide leading to interference with insulin sensitivity c) increased mitochondrial burden leading to reactive oxygen species (ROS) formation d) exercise inducing transcriptional factors such as PPAR and PGC1 that stimulates mitochondrial metabolism such as beta-oxidation (β -oxid), Krebs cycle, electron transport chain. LC acyl-CoA represents long chain fatty acyl-CoA. Sourced from Coen and Goodposter [39]

Skeletal muscle takes up plasma free fatty acids (FFAs) by passive diffusion and by fatty acid translocase/cluster of differentiation 36 (CD36/FAT) (Figure 2.6) or plasma membrane fatty acid binding protein (FABPpm). The vast majority of FAs taken up by muscle are destined to be oxidised by mitochondria or they start accumulating within the muscle cell. The fatty acid enters the mitochondria in the fatty acyl CoA form via the action of carnitine palmitoyl transferase I (CPTI), which is expressed on the outer mitochondrial membrane (Figure 2.6). The unoxidised fatty acids start accumulating in the form of IMTG or triacylglycerols (TAG) as shown in Figure 2.6.

In obesity, because of lower energetic demand, most FA acyl CoA is partitioned to lipid droplets (LDs) (Figure 2.5 and 2.6). Intramyocellular lipids (IMCL) in LDs can then act as a surrogate for ceramide and DAG. These lipid moieties will hamper translocation of GLUT4 in the skeletal muscle which will lead to hyperglycaemia [102] (Figure 2.6). FA acyl CoA oversupply to the mitochondria during low energetic demand results in incomplete β -oxidation and reactive oxygen species (ROS) production (Figure 2.5 and 2.6). The released LDs are highly dynamic organelles with many roles, including cell signalling, vesicle trafficking, and a fuel source for mitochondria. LD is located proximal to muscle mitochondria and sarcoplasmic reticulum and contains predominantly TAG, but also DAGs, cholesterol esters, and free cholesterol.

A PAT family of proteins including perilipin (PLIN2-5) coat the phospholipid layer of the LD and mediate the function of TAG synthesis or breakdown, vesicle trafficking and cellular signalling [103]. A functional role for PAT proteins in skeletal muscle has yet to be described. Collectively, these studies suggest an important role for PLIN5 in regulating FA oxidation [104, 105]. The four main types of perilipin (PLIN2 (ADRP) PLIN4 (S3-12) PLIN3 (TIP47) PLIN5 (OXPAT) are expressed in skeletal muscle however, studies on other cell types suggest that PLIN2 may act as a scaffold protein and its expression seems to be reciprocal to TG content [106]. In skeletal muscle of endurance cyclists, PLIN5 is highly expressed in muscle, is localised to mitochondria and LDs, and is closely associated with IMTG content [107].

2.4.3 Transcriptional regulation of muscle cellular architecture

Exercise involves the up-regulation of gene transcripts for critical factors of lipid metabolism in skeletal muscle such as PPARA (Peroxisome Proliferator activated receptor A) and binding proteins. PPAR α triggers the up-regulation of leptin receptors and fatty acid binding proteins. Figure 2.6 shows the energy pathway of skeletal muscle. It depicts the steps of lipid metabolism producing energy for muscle work. It demonstrates that exercise can stimulate PGC1 and PPAR- γ leading to β -oxidation and mitochondrial biogenesis which are the key regulators of lipid metabolism in skeletal muscle.

The regulation of specific gene expression in response to changes of exercise has been studied in rodents [108] and humans [109]. Lipoprotein lipase (LPL), is a glycoprotein enzyme [110] that is the rate-limiting enzyme in the hydrolysis of triglycerides (TAG) [111], and plays an important role in the differentiation and maturation of adipose cells. Moreover, it controls the TAG partitioning between adipose tissue and muscles [112]. Fatty acid binding protein (FABP), is encoded by the *FABP4* gene [113], and supplies long-chain fatty acids as an important energy source for muscle growth and maintenance, forcing long chain fatty acids towards fat storage within muscle fibres [114]. AMP-activated protein kinase (AMPK) is a metabolic master switch regulating glucose and lipid metabolism [115] and has been implicated in the control of adipose tissue content [116]. In response to cellular metabolic stresses, AMPK is activated. In its activated form it phosphorylates and inactivates acetyl-coenzyme a carboxylase enzyme (ACC) [117]. AMPK is a heterotrimeric complex comprising a catalytic subunit ($\alpha 2$) and two regulatory subunits (β and γ). The catalytic subunit $\alpha 2$ induces having AMPK- kinase $\alpha 2$ subunit gene (*PRKAA2*) [2] Carnitine palmitoyltransferase I (CPT 1), encoded by the *CPT1B* gene, is part of the mitochondrial transport system and is a key enzyme in the control of long-chain fatty acid oxidation. It leads to fatty acid oxidation within the mitochondria [118]. Most of these key enzymes are subject to both acute and chronic control. Their gene expression is regulated by important transcription factors like peroxisome proliferating activated receptor alpha and gamma (PPAR- α , PPAR- γ). PPAR- α , also known as nuclear receptor subfamily 1, group C, member 1 (NR1C1), is a nuclear receptor protein that in humans is encoded by the *PPARA* gene. Activation of PPAR- α promotes uptake, utilisation, and catabolism of fatty acids by up-regulation of genes involved in fatty acid transport and peroxisomal and mitochondrial fatty acid β -oxidation. However, there has been no clinical trial or randomised controlled trial

reported to date regarding the investigation of impact of exercise on skeletal muscle gene expression changes especially transcriptional changes in people with T2DM and grade 3 obesity or in Pacific people with T2DM. The uniqueness of this PhD study is that the SPIRIT study [88] is the first and only clinical exercise trial to date where exercise as an intervention has been performed on Polynesian grade 3 obese individuals with T2DM. For this PhD, skeletal muscle tissue is available pre and post exercise intervention and examination of the impact of exercise on cellular, metabolic and genetic parameters can be done.

People with T2DM can benefit from exercise that can stimulate changes in metabolic pathways in the skeletal muscle related with reduced fibrosis and extracellular matrix remodelling as proposed in Figure 2.4. Studying these pathways and associated gene expression changes may provide insight into the exercise induced mechanisms involving muscle modifications associated with insulin resistance and could provide pertinent targets for therapeutic intervention.

2.4.4 Effect of exercise on adipocytokines

The concept of adipose tissue as an endocrine tissue originated in 1995 by the discovery of leptin which further led to the discovery of other adipocytokines such as IL-6, adiponectin and many others. However leptin and adiponectin have been widely regarded as key hormones related to energy homeostasis and appetite regulation as already discussed in section 2.3. Kieffer and Habener [119] were some of the first researchers who described the relationship between leptin and insulin by proposing that improving leptin sensitivity would aid in improving insulin sensitivity. A reduction in body fat either by exercise and/or by diet consequently leading to the reduction in leptin levels may improve leptin and insulin sensitivity [120]. Several studies [121-123] have shown that after chronic exercise (>12 weeks) of either resistance or aerobic there was a significant reduction in the serum leptin levels in people with T2DM. In some animal studies on diabetic rats, it has been shown that exercise improves leptin receptor mRNA expression levels in skeletal muscle [124]. This increase in leptin receptors in the skeletal muscle can also improve leptin sensitivity [49]. Furthermore, low levels of adiponectin, the other important adipocytokine, has been reported to be decreased in obese and insulin resistant states. Two case studies in obesity prone Pima-Indians [82] and one in Caucasians [125] suggest that weight loss, caloric restriction increases adiponectin levels and gene expression. However Sukala *et al.* [78] reported that there were

no changes in the adiponectin levels in Pacific islands people with T2DM and grade 3 obesity, after 16 weeks of either aerobic or resistance trainings.

2.5 Summary

The global escalation of the incidence of obesity is currently one of the world's largest health concerns. Obesity contributes to a chronic proinflammatory state and to deterioration of glucose and lipid metabolism which increases an individual's risk of developing T2DM and cardiovascular disease. Known contributing factors for the development of obesity include imbalances in pathways of glucose and lipid metabolism as a consequence of extrinsic and intrinsic factors such as quantity and quality of nutrition throughout lifetime, sedentary lifestyle, exercise and genetic predisposition. Visceral fat accumulation contributes to a pro-inflammatory and pro-oxidant state and to deterioration of glucose and lipid metabolism. Because muscle is the primary tissue contributing to whole-body insulin-mediated glucose disposal, insulin resistance in skeletal muscle is the hallmark feature of T2DM. T2DM is associated with increased intramyocellular lipid content, mitochondrial dysfunction and disturbed biochemical pathways that link chronic low grade inflammation and production of reactive oxygen species.

Exercise training improves metabolic processes and can be a useful tool for intervention in T2DM by improving multiple levels of associated pathophysiology including lowering of glucose levels (anti-hyperglycaemic Figure 2.7(a)), increasing break down of fatty acids (anti-dyslipidemic Figure 2.7(b)), antioxidant (decreasing production of reactive oxygen species (ROS) (Figure 2.7 (c)) and reduces cytokines (anti-inflammatory effects) (Figure 2.6 (d)). Exercise promotes mitochondrial biogenesis, improves mitochondrial function and reduces intramyocellular triglycerides (Figures 2.4 - 2.6).

Regular exercise training (aerobic or resistance) results in adaptations that improve muscular strength, cardiovascular function, mitochondrial density and oxidative capacity, and mobility. A number of clinical studies show improved glycaemic control in patients with insulin resistance following exercise training. Exercise prevents metabolic dysregulation and inflammation, and it stimulates protein synthesis and muscle plasticity. Few studies have assessed the role of exercise training in detail in Pacific Peoples. Accordingly [120], exploration of pathways related with aerobic and resistance exercises would be valuable to

determine the metabolic changes that may improve T2DM in this vulnerable population group.

There is increasing evidence from the body of literature that there is a need for exercise interventions that can address the following issues regarding the prevention of T2DM;

- a) Chiefly, T2DM is a disease of disturbed energy balance with the net effect of exceeding glucose influx. Most trials using either aerobic or resistance exercise have focused on HbA1c as the primary outcome measure to assess the effectiveness of interventions in people with BMI between 25 to 30. There is clearly a lack of trials with obese and morbidly obese populations which describe specific metabolic pathways in more detail.

- b) Although recent research has focused on the discovery of pathways that underpin the pathophysiology of obesity and T2DM, exercise trials exploring and concentrating on the effects of exercise on specific cellular and molecular changes in the skeletal muscle are needed to further our understanding of the benefit of exercise prescription for populations at higher risk.

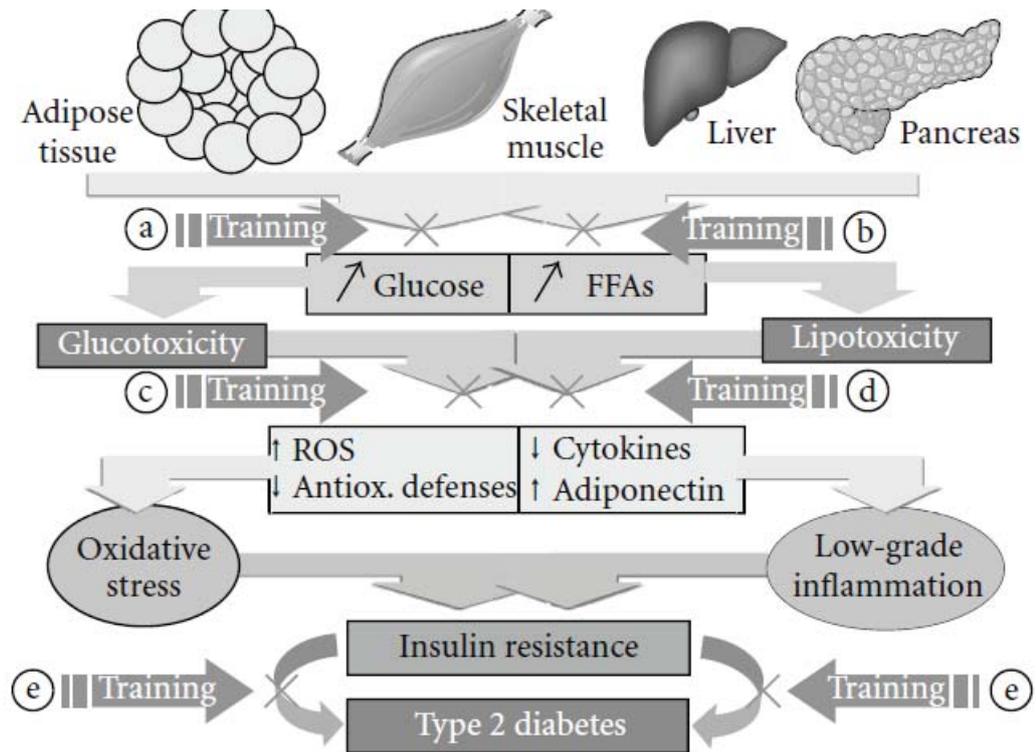


Figure 2.7: Schematic illustration of the proposed effects of regular physical exercise (training) in tissues of people with T2DM. Exercise will have antihyperglycaemic (a), antidyslipidaemic (b), antioxidant (c), and anti-inflammatory (d) effects and thus prevent the development of insulin resistance and T2DM or improvement of insulin sensitivity in a person with T2DM (e). FFA represents free fatty acids and ROS represents reactive oxygen species. Sourced from Holten et al. [91].

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CHAPTER 3

EXPERIMENTAL MATERIALS AND METHODS

3.1 Introduction

The SPIRIT study was carried out in 2008, in the Greater Wellington Region and is the first randomised clinical exercise trial that has investigated the effect of different types of exercise on Polynesian New Zealanders with type 2 diabetes (T2DM) and visceral obesity (section 1.2). Eighteen adults of self-described Polynesian descent were randomised and completed either 16 weeks of supervised progressive resistance training (PRT) exercise or aerobic training (AER) exercise. The SPIRIT study cohort were classified as grade 3 (BMI>40 kg/m²) obesity [1]. No improvement in glycaemic control (no change in HBA1c levels) or insulin resistance was observed (see Table 1.3). To investigate the effect of aerobic and resistance exercise at the molecular level different measures in the biological samples, blood, urine and skeletal muscle (SM) tissue of the SPIRIT study participants were examined.

The purpose of this chapter is to briefly describe and provide details of the experimental procedure for the SPIRIT study (section 3.2), the methods of collection of blood, urine and skeletal muscle (SM) biopsy samples and analysis of urine and blood markers (section 3.3), provide a detailed account of the methods used for determining activity of different mitochondrial enzymes (section 3.4), examination of IMTG density (section 3.5) and analysis of changes in mRNA expression in the SM (section 3.6).

3.2 Details on SPIRIT Study Experimental Procedures

Eighteen participants were recruited from the GWR and a detailed account of baseline characteristics of these participants and results of the assessed outcomes measures of the SPIRIT intervention has been discussed in chapter 1. The sample size, subject screening, recruitment, randomization, assessment of outcome measures, supervision and exercise intervention details of the SPIRIT study that was performed by Bill Sukala for his PhD project are included in Appendix A.

3.3 Biological Sample Collection

The following biological samples, blood (section 3.3.1), urine (section 3.3.2) and muscle tissues (section 3.3.3) were collected from the SPIRIT study participants (section 1.2) before and after the sixteen weeks of AER and PRT exercise.

3.3.1 Collection of blood samples and analysis

Fasting blood draws were performed by hospital phlebotomists. Participants were given a subject number and the samples were labeled according to that number. Each sample was de-identified to reduce the bias. An example of a medical laboratory result sheet for a participant is presented in Appendix C.

Once the blood samples were collected, they were sent to Canterbury District Health Board (CDHB) for measurement of serum leptin, serum cortisol binding globulin (CBG) and serum sex hormone binding globulin (SHBG). Serum leptin was determined by radioimmunoassay (Human leptin RIA kit HL81K; Linco Research Inc, USA). Serum SHBG and CBG levels were measured by electrochemiluminescence immunoassay (ECLIA, Roche diagnostics Cobas).

3.3.2 Collection of urine samples and analysis

All participants were provided with a labelled container to collect total 24-hour urine sample. The labelling for these containers was consistent with the designated number of the participant. Once the urine samples were collected, they were sent to laboratories for measurement of 24-hour cortisol level in urine. Cortisol in 24-hour urine samples was determined by ECLIA (Roche Diagnostics Cobas) using Roche diagnostics modular system.

3.3.3 Collection of skeletal muscle tissue

Skeletal muscle is a key tissue in the body related with glucose and lipid metabolism and as discussed in Chapter 2 section 2.2, lipid and mitochondrial abnormalities lead to the pathogenesis of T2DM. The skeletal muscle tissue was collected from the SPIRIT participants at baseline (0 weeks) and 16 weeks. One SPIRIT study participant declined having a muscle biopsy done. This participant was in the aerobic group. This meant that there were 16 muscle biopsy samples (eight at 0 weeks and eight at 16 weeks) for the aerobic group. All PRT group participants had muscle biopsies at 0 and 16 weeks.

The standard procedure for the collection of skeletal muscle tissue is obtaining a muscle biopsy from the vastus lateralis muscle [2]. Biopsies from the right vastus lateralis were collected under local anaesthesia (1% Xylocaine, Astra Zeneca Ltd, Auckland, New Zealand)

at 0 and 16 weeks using a 5 mm Bergstrom needle with applied suction. The 16 week muscle biopsy was collected ~72 hours following the final exercise session to avoid the confounding effect of the acute transcriptome and signalling response to acute exercise. The snap frozen tissue was broken into pieces by orienting longitudinally in Tissue-Tek optimal cutting temperature (OCT) embedding medium (Sakura Finetek Ltd, Tokyo, Japan) and snap frozen again in liquid nitrogen-cooled isopentane and then stored in Eppendorf cryotubes at -80°C until analysed.

The four sections of biopsied skeletal muscle tissue ranged from 15-25 mg. Three of the sections were reserved for protein, electron microscopy and mRNA gene expression analysis and the fourth section was designated as spare tissue. The skeletal muscle biopsy samples were stored in -80°C freezer in the Institute of Food, Nutrition and Human Health, Wellington Campus Biochemistry Laboratory.

The 15-25 mg muscle sample reserved for protein analysis was used for detecting the activities of three different enzymes: citrate synthase, cytochrome c oxidase and beta-hydroxyacyl-CoA dehydrogenase. The details of the methods used for extraction of protein and measuring enzyme activity in skeletal muscle are discussed in section 3.3 below.

The 15-25 mg skeletal muscle tissue reserved for electron microscopy work was used to determine intramuscular triglyceride (IMTG) density and mitochondrial morphology. The 0 week and 16 week skeletal muscle biopsy samples were fixed and examined under electron microscope in the Department of Pathology, University of Otago, and the quantification of the sectioned tissue was completed at Massey University. The detailed procedure for fixing the tissue and analysis of the tissue is discussed in section 3.4 below.

The 15-25 mg skeletal muscle tissue reserved for mRNA analysis was sent to Professor Eric Hoffmans' laboratory at National Child Medical Research Centre (NCMRC) Washington DC, USA. Ethics approval was obtained (No. CEN/07/08/054) for sending the SM tissue overseas for genetic analysis. Details of the mRNA microarray procedure and bioinformatic analysis are presented in section 3.5 below.

3.4 Determining Mitochondrial Enzyme Activity in Spirit Study Skeletal Muscle Tissue

Skeletal muscle (SM) protein homogenate (section 3.4.1) was required for determining the different mitochondrial enzyme activities (sections 3.4.2.1 - 3.4.2.3). Optimisation of the protein extraction method as well as the enzyme assays used to measure the three enzymes of interest are discussed in detail in Chapter 5 section 5.3. The final optimised method used for extraction of protein from SPIRIT study SM tissue is presented in section 3.4.1 below.

The final optimised enzyme assays used for measuring enzyme activity of three key mitochondrial enzymes are discussed in section 3.4.2.1 for citrate synthase, section 3.4.2.2 for cytochrome c oxidase and section 3.4.2.3 for beta-hydroxyacyl-CoA dehydrogenase.

3.4.1 Extraction of protein from skeletal muscle tissue

The method of protein extraction from the skeletal muscle (SM) was optimised by using rat SM and some spare human tissue from the subjects who dropped out of the study. The optimisation of this procedure is discussed in Chapter 5 (section 5.3.1). The final optimised method used for extraction of protein from SM is derived from previously used methods [3-6]. The pre-weighed muscle tissue reserved for protein work was removed from the -80 freezer and transferred to a 2 ml Eppendorf tube filled with extraction buffer (Appendix B) (25 times weight of the muscle). It was homogenised with tissue homogeniser (IKA-homogeniser, T-10, supplied by Bioscience Auckland, New Zealand) for 20 seconds three times with an interval of 10 seconds in between each cycle. The homogenate was centrifuged (Eppendorf centrifuge 5415D) at 600 g, 4°C, for 15 min. The supernatant was removed and kept on ice. The pellet was passed through a 1 mL syringe and 25 gauge needle 20 times by adding 200 µl of extraction buffer and centrifuged at 600 g, 4°C for 15 minutes. The supernatant from this spin was added to the supernatant being kept on ice and mixed. The protein concentration of this SM protein homogenate (combined supernatants – see Figure 5.4) was measured using the standard BCA assay (Merck Cat # 71285-3). The rest of the SM protein homogenate was stored as 40 µl aliquots in to 1.5 ml Eppendorf tubes at -80°C ready to be used for determination of different enzyme activities (section 3.4.2).

3.4.2 Enzyme activity

All enzyme assays were performed in flat bottomed 96 well plates (Greiner bio-one) by microplate spectrophotometry (Bio-Rad benchmarkplus). All reagents for the enzyme assays were ordered from Sigma-Aldrich (Auckland, New Zealand). Each SPIRIT study SM biopsy sample was assayed in triplicate for each enzyme measured.

The extraction buffer used for extraction of protein from SM tissue served as a negative control in all enzyme assays. Rat muscle tissue was used as a positive control for enzyme activity because it was used to aid in optimisation of the enzyme assays before the enzyme assays were trialled in human muscle tissue and then used to determine enzyme activity in the SPIRIT study participant SM tissue (see Chapter 5 section 5.3.5).

Details of the final optimised enzyme assay are outlined in sections 3.4.2.1 - 3.4.2.3 below.

3.4.2.1 Citrate Synthase Assay (EC 4.1.3.7)

Citrate synthase (CS) catalyses the first step in the Krebs cycle (or otherwise named as Citric acid cycle or Tricarboxylic acid cycle) and is a biomarker for mitochondrial activity. Citrate synthase catalyses the conversion of oxaloacetate and acetyl coenzyme A (acetyl-CoA) into citrate and thiol-CoA (CoA-SH) (Figure 3.1). To be able to determine CS activity in the mitochondria, a coupled assay is used (Figure 3.1). The thiol-CoA (CoA-SH) produced in the reaction catalysed by citrate synthase is then coupled with DTNB (5, 5'-dithio-bis-2-nitrobenzoic acid) to form a yellow product called 5-thio-2-nitrobenzoic acid (TNB). Production of TNB is directly associated with CS activity. TNB can be measured spectrophotometrically at 412 nm. The conditions of the CS assay used for this study were adapted from a previous study [3]. These methods were provided by the co-supervisor Prof Bernhard Brier through personal communications.

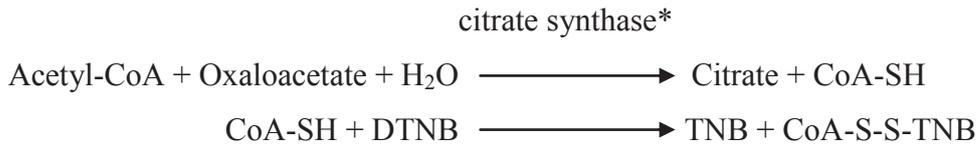


Figure 3.1: Coupled Assay for Determination of Citrate Synthase Activity. *citrate synthase in SPIRIT participant skeletal muscle protein homogenate.

The final optimised assay used for measuring CS activity in the SPIRIT study participants SM tissue is as follows. A master mix of 50 mM Tris-HCl buffer pH 8.1, 0.1 mM acetyl CoA and 0.2 mM DTNB was made and 200µl of the master mix was pipetted into wells of the 96 well plate. Ten µl of muscle protein homogenate (section 3.4.1) was then added to the 200µl master mix in the well and incubated for 5 minutes. Twenty µl of 5 mM oxaloacetic acid was then added to each well to start the reaction. The 96 well plate was inserted into the microplate spectrophotometer (Bio-Rad, Benchmark Plus (serial number; 10756, made in Japan, supplied by Bio-Rad Auckland, New Zealand) to run the cycle of 10 seconds of mixing and reading at every 30 seconds interval for 10 minutes at 412 nm wavelength and 25 °C. The repeated (30 seconds) absorbance values were then plotted against time to calculate the maximum change in absorbance/min. The CS activity was calculated by using the following equation:

$$\text{Citrate synthase activity } (\mu\text{mol}/\text{ml}/\text{min}) = \frac{(\Delta A_{412\text{nm}}/\text{min})(\text{Dil})}{\Delta \epsilon^{\text{mM}} * L(\text{cm}) * V_{\text{enz}}(\text{ml})}$$

One unit of CS is 1 µmol/ml/min; $\Delta A_{412\text{nm}}/\text{min}$ = Absorbance at 412 nm/minute (sample) – Absorbance at 412 nm/minute (blank); Dil - the dilution factor of the original sample; $V_{\text{enz}}(\text{ml})$ – the volume of the enzyme sample in ml, ϵ^{mM} (mM-1 cm-1) – the extinction coefficient of TNB at 412 nm is 13.6; $L(\text{cm})$ – path length for absorbance measurement: for 96 well plate $l = 0.552$ cm

For this assay the activity curves start at absorbance levels different to the blank. The blank activity is minused from the test activity.

3.4.2.2 Cytochrome c Oxidase (EC 1.9.3.1)

A colorimetric assay is used to determine cytochrome c oxidase (COX) activity in human skeletal muscles of SPIRIT study participant. Cytochrome c has a sharp absorption band at 550 nm in the reduced state. Upon oxidation, this band becomes weaker and broader. The COX enzyme is a large transmembrane protein located in the inner membrane of the mitochondria and is the terminal electron acceptor in the electron transfer chain, taking 4 reducing equivalents from cytochrome c and converting molecular oxygen to water. The observation of the change in colour by detecting a decrease in absorbance at 550 nm of ferrocytochrome c (pale purple red) caused by its oxidation to ferricytochrome c (pale orange red) by cytochrome c oxidase (Figure 3.2) is the basis of the colorimetric assay for detecting COX activity.

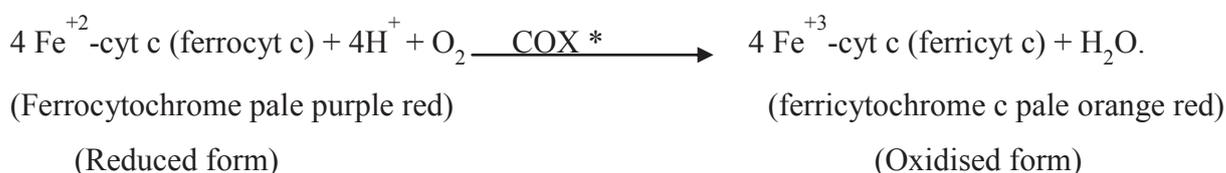


Figure 3.2: Colorimetric change for determining COX activity. *Cytochrome c oxidase in SPIRIT participant skeletal muscle protein homogenate.

The final optimised assay used for measuring COX activity in SM of the SPIRIT study participant followed what was outlined in Gauthier *et al.* [5] but adapted for microplate detection and analysis. Cytochrome c (0.2 mM) was made from 2.7 mg of cytochrome c dissolved in 1ml of ultrapure water containing 5 μ l of 0.1 M dithiothreitol (DTT). The solution was placed on the bench at room temperature overnight to form reduced cytochrome c. To continue with the COX assay, the A550/A565 ratio of the reduced cytochrome c must be between 10 and 20. For every assay, overnight prepared reduced cytochrome c was used in the assay and was only utilised if the A550/A565 ratio was between 10 and 20. The reduced cytochrome c was diluted four times with 0.05 mM potassium phosphate buffer. Ten μ l of the muscle protein homogenate (section 3.4.1) was placed in the wells of the 96 well plate. The diluted reduced cytochrome c was then pipetted in each well and the decrease in absorbance was recorded in the spectrophotometer for every 30 seconds for 10 minutes at 550 nm wavelength and 25 $^{\circ}$ C. The repeated (30 second) absorbance values were then plotted

against time to calculate the maximum change in absorbance/min value. The activity of the enzyme was calculated by using the following formula.

$$\text{COX activity } (\mu\text{mol/ml/min}) = \Delta A_{550\text{nm}/\text{min}} * \text{Dil} * V_{\text{enz}}(\text{ml}) * \Delta \epsilon^{\text{mM}}$$

One unit of COX is 1 $\mu\text{mol/ml/min}$, $\Delta A_{550}/\text{min}$ = Absorbance at 550 nm/minute (sample) – Absorbance at 550 nm/minute (blank); Dil = dilution factor of original sample; V_{enz} (ml) = volume of enzyme or sample in ml, $\Delta \epsilon^{\text{mM}}$ = 21.84 extinction coefficient between ferrocytochrome c and ferricytochrome c at 550nm

3.4.2.3. *Beta-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)*

Fatty acids undergo beta-oxidation in the mitochondria and beta-hydroxyacyl-CoA dehydrogenase (BHAD) is an enzyme involved in the beta-oxidation pathway. The substrate, S-Acetoacetyl-CoA is converted to β -Hydroxybutyryl-CoA in the presence of β -NADH and BHAD (Figure 3.3). The BHAD activity is measured by oxidation of β -NADH (β -nicotinamide adenine dinucleotide, reduced form) to β -NAD (β -nicotinamide adenine dinucleotide, oxidised form) which is detected at 340 nm (Figure 3.3).

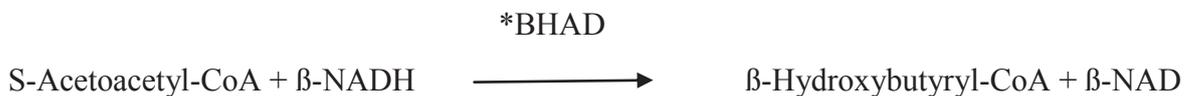


Figure 3.3: Conversion of Acetoacetyl-CoA by BHAD. **Beta-hydroxyacyl-CoA dehydrogenase in SPIRIT participant skeletal muscle protein homogenate.*

The conditions for this assay were from Kim *et al.* [6] but adapted to be performed in microplate reader. A master mix buffer containing 100 mM potassium phosphate buffer pH 7.3, 5.4 mM S-acetoacetyl Coenzyme A solution and 6.4 mM β -NADH was made. 200 μl of the master mix was pipetted into wells of the 96 well plate. 7 μl of muscle protein homogenate (section 3.4.1) was then added to the 200 μl master mix in the well and incubated for 5 minutes. The plate was inserted into the Bio-Rad microplate spectrophotometer to run the cycle of 10 seconds of mixing and reading at every 30 second interval for 10 min at 340 nm wavelength and 25 $^{\circ}\text{C}$. The extraction buffer instead of muscle protein homogenate was

used as control blank. The repeated (30 second) absorbance values were then plotted against time to calculate the maximum change in absorbance (ΔA) in both muscle protein homogenate at 0 or 16 weeks (Test samples) and blank at 340 nm. The activity of BHAD was calculated by using the following equation.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340 \text{ nm/min}} \text{ Test} - \Delta A_{340 \text{ nm/min}} \text{ Blank}) * \text{Dil} * V_{\text{enz}}(\text{ml})}{\Delta \epsilon^{\text{mM}} * V_{\text{enz}}(\text{ml})}$$

One unit of BHAD will convert 1 μ mol/ml acetoacetyl-CoA to β -hydroxybutyryl-CoA per minute; $\Delta A_{340}/\text{min}$ = Absorbance at 340 nm/minute (Test) – Absorbance at 340 nm/minute (blank); Dil = dilution factor of original sample; $V_{\text{enz}}(\text{ml})$ = volume of enzyme or sample in ml, $\Delta \epsilon^{\text{mM}}$ = 6.22 extinction coefficient of β -HAD at 340 nm

3.4.3 Statistical analysis

Statistical analysis was done using Microsoft Excel 2007 and SPSS (Statistical software package for social sciences version 20.0). Within group effect was analysed by paired t-test and between group effect was analysed by two-way analysis of variance (ANOVA). Enzyme activity and obesity marker results are presented as mean \pm SD and $P < 0.05$ was considered statistically significant. Data for the enzyme analysis was expressed as the effect of exercise training expressed as standardised difference (effect size), where the within group baseline standard deviation was used as the denominator. Effect size thresholds were calculated for small standardised difference, according to Cohen, 1986 and are as follows; 0.2= small, 0.6= moderate 1.2= large, 2.0= very large.

Further information on specific statistical analyses performed on the obesity marker data collected are described in detail in chapter 4 (section 4.2).

To determine reproducibility and repeatability of the enzyme assay, the intra and inter-assay co-efficient of variability (CV) was calculated for each enzyme assay [7]. For this PhD study the enzyme activity for each sample was measured in triplicate and each experiment was done in triplicate. The degree to which the triplicate sample results vary (% CV) can be determined by dividing the standard deviation (SD) of the triplicates by the triplicate mean of the enzyme activity and multiplying by 100. The average of the individual %CV is then determined to provide the intra-assay CV. Since each experiment was done in triplicate it was important to determine inter-variability between experiments (microplate to microplate variations). This

was done by determining the overall mean, standard deviation and %CV for the microplate first. Then the Overall %CV for the microplate is determined by dividing the SD of plate means by mean activity of the plate and multiplying by 100. The inter-assay CV for the three experiments is then determined by averaging the mean of the %CV means determined for each microplate. Good reproducibility and precision of an enzyme assay should have an intra-assay CV less than 10% and inter-assay CV less than 15% [7].

3.5 Determination of Intramuscular Triglyceride (IMTG) Density and Examination of Mitochondrial Morphology

Carbohydrates and fats are the main fuel sources for exercise in skeletal muscle. As discussed in Chapter 2 section 2.2 in a sedentary lifestyle and with obesity the muscle cell is loaded with lipid droplets in between the muscle fibres. These lipid droplets are called intramuscular triglycerides (IMTGs) or intramuscular fat droplets. For the determination of the density of lipid droplets in the SPIRIT study SM tissue, a portion of SM was fixed (section 3.5.1), viewed and photographed under the electron microscope (section 3.5.2) and quantified (section 3.5.3). SM mitochondrial morphology pre and post 16 weeks exercise was also examined (section 3.5.4) using SM tissue fixed for IMTG analysis.

3.5.1 Preparation of Skeletal muscle tissue

Intramyocellular lipid density and inspection of mitochondrial morphology in muscle tissue mixed with both type I and type II fibers was estimated by direct visualisation from electron microscopy using adaptation of the method for transmission electron microscope (TEM) validated by Tarnopolsky *et al* [8]. 10-25 mg of muscle was fixed in half strength Karnovsky's fixative (0.1M cacodylate buffer, 2% paraformaldehyde, 2% glutaraldehyde, 1 mM calcium chloride, 20 mM sucrose). Tissue was dehydrated in ethanol, embedded in an Epon type resin, then cut into thin, longitudinal sections (70 nm) on a microtome (Reichert-Jung Ultracut, Reichert-Jung Co., Heidelberg, Germany) [9]. The tissue was now ready for viewing under the electron microscope (section 3.5.2)

3.5.2 Examination under Electron microscope

Prepared muscle sections were viewed at x6500 using a TEM (Philips CM100, Philips/FEI Corporation, Eindhoven, Holland). Eight to 15 images per muscle sample were taken under light-standardised conditions using a film picture camera (Kodak 4489, Rochester, NY) from 2 randomly selected fibres, with 1/3 of images from the subsarcolemmal region near the nucleus, 1/3 from the subsarcolemmal region away from the nucleus, and 1/3 in the middle of the fibre. Electron microscopy and image capture were performed by a skilled pathologist (Dr St John Wakefield from Otago University).

3.5.3 Scanning and analysis using Adobe Photoshop CS5.5 and IPKT

Plates were digitised at 1200 dpi as Tiff file images. Total number of lipid droplets, mean lipid area, and percent lipid density was determined in Photoshop CS5.5 utilizing the analysis features provided by plugin (Fovea Pro, Reindeer Graphics, Asheville, NC, USA) as applied to quantification of features using immunocytochemistry [10]. Image areas were cropped to exclude non-myocellular space (Figure 3.4 (a)). Lipid droplets were identified using software tool function manual selection. Total lipid area was subsequently determined by filter feature function, with threshold filtering to exclude artefacts. Lipid features were recorded black (Figure 3.4 (c)). The non-lipid background was then removed from the image (converted to white space) followed by manual deletion of any non-lipid features, permitting subsequent quantification of lipid droplet number and lipid area relative to total image area as shown in Figure 3.4 (d). Image pixel data were standardised to 1 μm grid reference image taken per sample batch. Outcomes are reported as the muscle fraction (μm^2) occupied by lipid droplets, which yields values similar to grid-point counting estimates [8].

For the quantification purposes 8 -15 images taken for each muscle biopsy sample was processed as stated above and lipid droplets, mean lipid area, and percent lipid density for each image was determined then all results per images were combined to determine total lipid droplets for each muscle biopsy sample at 0 and 16 weeks for the participants. As one participant declined for the muscle biopsy so there were 8 participants in the AER group. For comparison purposes from the PRT group also 8 participants were selected randomly.

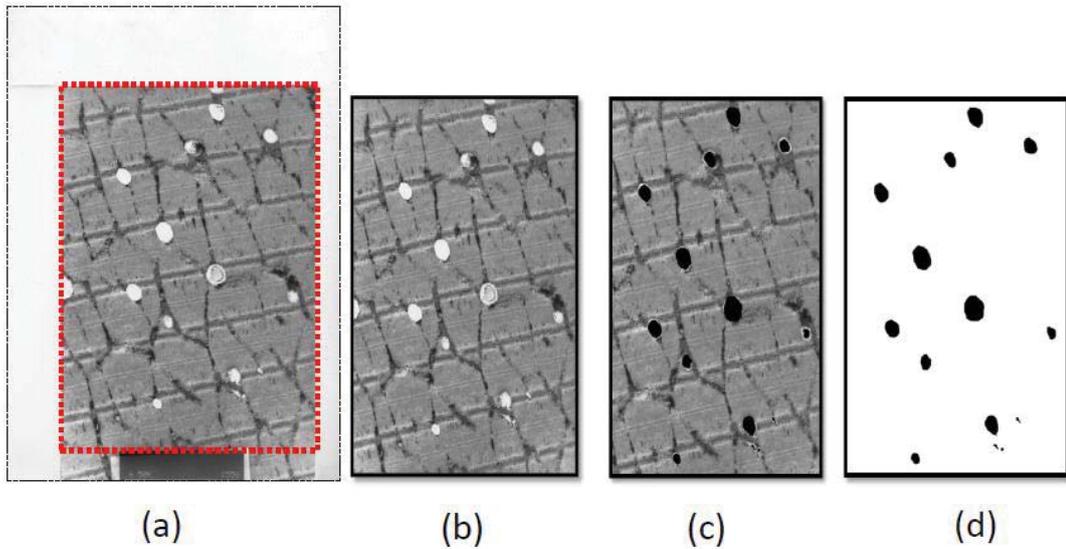


Figure 3.4: Steps of quantification of IMTG in SPIRIT participant skeletal muscle (a) a raw image which is cropped to select the quantified area (b) a copy of an image of a muscle fibre under electron microscope selected for quantification (c) black droplets represent IMTGs in that muscle fibre (d) final image area used for quantification of IMTG.

3.5.4 Examination of mitochondrial morphology

The mitochondrial morphology was also studied using the same captured images utilised for investigating lipid density (sections 3.5.3). Examination of the captured images was by observation of shape and structure and is presented in Chapter 5 section 5.5.3.

3.5.5 Statistical analysis

A two-way mixed model ANOVA (SAS 9.1, SAS, Cary, NC) was used with subject as the random effect to determine the muscle lipid outcomes. Intramyocellular lipid data were log-transformed prior to analysis to account for marked heteroscedasticity (non-normal variation of residuals). The standardised within-subject baseline score was included as a covariate. Inference was by standardised difference and confidence interval for mechanisms outcomes according to the method described by Hopkins *et al.* [11].

3.6 Determination of mRNA Expression in Skeletal Muscle Tissue Before and After 16 weeks exercise

To investigate the effect of 16 weeks exercise training on changes in skeletal muscle gene expression (Chapter 1 section 1.3), mRNA expression profiling was completed using Illumina gene microarray. The steps involved extraction of mRNA from SM of SPIRIT study participants at 0 and 16 weeks (section 3.6.1), mRNA gene expression profiling (section 3.6.2) and then statistical and bioinformatic analysis (section 3.6.3). RNA extraction and mRNA profiling (section 3.6.2-3) was performed in Washington DC by research technicians at the Children's National Medical Research Center (CNMRC). The normalisation and statistics were performed by the research center bioinformaticians whilst the statistical and bioinformatics analysis for the genes related with lipid and energy metabolism by using ingenuity software was performed in Massey University under supervision of Dr David Rowlands.

3.6.1 RNA extraction

mRNA was isolated from muscle tissue samples (~10 mg) using mirVana™ miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX). Concentration of RNA was determined by NanoDrop® spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE) and RNA quality was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). The RNA isolated was used for mRNA gene expression profiling (Section 3.6.2).

3.6.2 mRNA Expression Profiling

200 ng mRNA (Section 3.6.1) from each sample was used for mRNA gene expression profiling using Illumina® bead arrays (Illumina, Inc., San Diego, CA) for all 20,000 genes in the human genome. Reverse transcription and in vitro transcription amplification incorporating biotin-labeled nucleotides was performed with Illumina® TotalPrep™ -96 RNA Amplification Kit (Ambion, Austin, TX). 750 ng of the biotin-labeled IVT product (cRNA) was hybridised to HumanHT-12_v4_BeadChip (Illumina) for 16 h followed by washing, blocking, and streptavidin-Cy3 staining according to the Whole-Genome Gene Expression

Direct Hybridisation protocol (Illumina). Arrays were scanned using HiScanSQ System. Decoded images were analysed by GenomeStudio™ Gene Expression Module (Illumina).

3.6.3 Statistical analysis and Bioinformatics analysis

Illumina signal intensity were normalised to controls and in GenomeStudio and subject to hierarchical clustering for further quality control integration (Partek 6.6, St Louis, MO); accordingly mRNA data was background adjusted. Average signal data and all annotation files were exported for statistical analysis and integration (Partek 6.6, St Louis, MO). The mRNA datasets were quantile normalised followed by log₂ transformation. Principal components analysis plot revealed batch and sample bias in all arrays. Therefore, the effect of training was estimated using mixed model ANOVA with subject as the repeated-measures identifier and array chip as a random effect. A large number of methods for analysis of genomics data are available. To focus attention on outcomes with most likely biological relevance and to accommodate low sample size, we used Global Error Assessment (GEA) ANOVA adjustment to derive a robust p value (ROBP). We settled on a selection that included genes with a ROBP of <0.005. This data set is now ready for bioinformatics analysis that is described below.

Bioinformatics was conducted using Ingenuity pathway analysis to define molecular functions and physiological processes affected by training. The analysis comprised (a) analysis based on hypothesis (stated in chapter 1) on changes thought to affect metabolic fuel substrate handling and endomysium remodelling including vasculogenesis, and (b) an unbiased exploratory analysis driven by the statistical gene selection and Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) filter criteria.

Bioinformatics analysis involving network construction and functions analysis was performed in IPA software. Core analyses were run on probe selections filtered specific for skeletal muscle tissue in humans only but unfiltered for prediction state and relaxed filter. The top ranked transcriptome networks were extracted and the involved molecular functions and physiological systems guided biological interpretation made and compared between conditions (IPA Comparison Analysis). Top ranked were reported, and upstream regulators identified. Further specific details regarding bioinformatics are discussed in chapter 6 section 6.2.

3.7 References

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CHAPTER 4

INVESTIGATION OF THE IMPACT OF 16 WEEKS OF EXERCISE ON OBESITY MARKERS IN NEW ZEALAND PACIFIC PEOPLES WITH TYPE 2 DIABETES MELLITUS AND GRADE 3 OBESITY

4.1 Introduction

Obesity and Type 2 Diabetes Mellitus (T2DM) are closely interlinked. The body mass index (BMI) is commonly used as a measure of obesity with BMI of 18.5 - 24.9 kg/m² defining the desirable range [1]. Obesity can be defined as a metabolic disorder characterised by accumulation of excessive adipose tissue. Excessive adipose tissue accumulation is a key part of the pathogenesis of T2DM [2]. Indicators or markers of obesity include BMI, waist-to-hip ratio, and circulating levels of triglycerides. Some other markers may include hormones/pro-inflammatory cytokines released from adipose tissue and some binding proteins such as sex hormone binding globulin (SHBG). The critical issue with obesity is the accumulation of lipids in non-adipose tissue e.g. liver and muscle, initiating inflammatory processes and release of bioactive substances. As the fat mass increases in the body, it may cause dysregulation of metabolism related with glucose and fatty acid in the body [3]. The excessive adipose tissues secrete bioactive peptides, substances known as adipocytokines. The increased production of these adipokines has an impact on multiple functions; such as appetite, energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and homeostasis [4]. Some of the adipocytokines such as leptin and adiponectin are closely associated with obesity related insulin resistance [5]. The insulin resistance associated with type T2DM [6] has been positively correlated with leptin resistance [7] and metabolic inflexibility [8]. Exercise has a known integral role in regulating energy balance [9] and adaptations in response to exercise are reported to be linked with alterations in metabolic pathways related with glucose and fatty acid [10]. The changes in obesity biomarkers levels or concentrations can be used to examine the impact of interventions [11], alterations in adipose milieu [12] and changes in metabolism [13].

Exercise affects pathways related with energy regulation such as carbohydrate and lipid metabolism [14, 15]. Insulin and leptin are well known hormones related with glucose and lipid metabolism [16, 17]. Furthermore insulin and leptin resistance are often observed in both obesity and T2DM [18]. Exercise and weight loss targeting insulin resistance has been implicated as one of the main therapeutic strategies in people with T2DM, obesity and metabolic syndrome [19]. Exercise is known to improve insulin resistance and evidence shows it is also equally beneficial in improving leptin sensitivity [5].

Cortisol is a glucocorticoid hormone that has a well-known association with abdominal obesity [20]. Cortisol, a stress hormone stimulates the glucose and lipid pathways [21]. It antagonises the action of insulin at the cellular level, which can result in hyperglycaemia and hyperlipidaemia [4]. Research in this area also suggests that cortisol can suppress leptin hormonal action by centrally blocking neuropeptide Y in the hypothalamus thus inducing an increase in appetite coupled with hyperglycaemia and hyperlipidaemia [22]. It has been postulated that cortisol also affects immune function and the inflammatory response by altering actions of some interleukins which are co-morbid factors in association with obesity and T2DM [23]. Evidence suggests that acute exercise produces physical stress in the body and stimulates the release of cortisol by activating the Hypothalamic Pituitary Adrenal (HPA) axis [24] (Figure 4.1). On the other hand chronic aerobic exercise (i.e. > 12 weeks), cortisol levels under resting conditions are usually decreased [25].

The peripheral antagonistic actions of glucocorticoids especially cortisol can promote insulin resistance in the peripheral tissues and can lead to a hyperglycaemic condition known as Cushing's syndrome. The magnitude of the sensitivity of skeletal muscle to glucocorticoid actions is determined by its receptors (NR3C1, NR3C2) and also by the expressions of 11-beta –hydroxysteroid dehydrogenase 1 and 2 (11 β_1 HSD and 11 β_2 HSD). 11 β_1 HSD favours the conversion of cortisone to cortisol thus favouring more cortisol production whereas 11 β_2 HSD modulates conversion of cortisol to cortisone thus limiting its supply to the tissue. The levels of cortisol in the plasma are also regulated by its binding protein called cortisol binding globulin (CBG). Low levels of CBG have been reported in people with T2DM, obesity and insulin resistance suggesting its position as a useful indicator of disturbed metabolic state [26]. Another binding protein is sex hormone binding globulin (SHBG). This binding globulin and CBG are produced in the liver. SHBG is reported to be associated with insulin resistance [27, 28] and regarded as the surrogate indicator of insulin resistance [29, 30] as has been discussed in Chapter 2 section 2.3. Evidence has suggested that increases in serum SHBG levels indicate an improvement in insulin sensitivity [31].

Leptin, a polypeptide hormone, produced and secreted by adipocytes, is a vital link between cortisol, insulin and energy metabolism (Figure 4.1). Leptin circulates at levels directly proportional to total body fat [32]. Circulating leptin levels are also influenced by circulating cortisol levels [33,34]. Figure 4.1 shows the relationship between obesity, stress, leptin and insulin resistance ultimately leading to T2DM.

A wealth of evidence has suggested that both AER and/or PRT [19, 22-24] exercise training can produce significant changes in adipose tissue and adipocytokine regulation in obese individuals and in people with T2DM. However, none of the clinical trials have previously investigated the relationship of exercise with stress, adipo-insular axis and insulin resistance in a Polynesian population with obesity and T2DM. The main aim of this chapter is to examine the impact of 16 weeks of exercise (aerobic or resistance exercise) on the obesity markers serum leptin, adiponectin, CBG and SHBG and 24 hour urinary cortisol. The specific objectives of this chapter are to:

- a) Explore the association between exercise and obesity biomarkers leptin, adiponectin and stress hormone cortisol and its binding protein CBG
- b) Investigate the effects of exercise on SHBG, as a predictive marker of insulin resistance.

4.2 Methods

The methodology for collection of blood and urine samples and analysis of biomarkers was presented in Chapter 3 section 3.2.1 and section 3.2.2. The obesity marker concentrations were examined to see if they were within normal reference range. The statistical analysis for within group changes was performed using paired t-Test and between group differences was two-way ANOVA, further followed by post-hoc analysis using SPSS (Statistical software package for social sciences version 20.0). Data from participants whose sample were unavailable for post-16 week assessments (one participant did not provide urine sample for 24-hour cortisol examination) were excluded, as per protocol analysis. All data were visually inspected and statistically evaluated for normality (skewness and kurtosis between -1 and +1). Normally distributed data were described as mean \pm SD. Nonnormally distributed continuous variables were log-transformed before analysis via parametric models. Data are presented as back transformed means \pm factor standard deviation. Baseline differences between groups were compared using an independent t-test for continuous variables. Within and between group changes from weeks 0 to 16 were analysed by repeated measures ANOVA.

Pearson's correlation were performed using change scores (16-0 week) for each group to evaluate relationships between obesity markers and 1) changes in HbA1c, insulin, fat mass, triglycerides, waist to hip ratio and BMI, 2) the percentage of exercise sessions attended and

3) other obesity markers. A correlation of p-value < 0.05 and < 0.01 (two-tailed) was accepted as statistically significant.

The skeletal muscle mRNA expression for leptin (*LEP*), adiponectin (*ADIPOQ*), leptin receptor (*LEPR*), glucocorticoid receptors (*NR3C1* and *NR2C2*) and 11-beta-hydroxysteroid dehydrogenase 1 and 2 enzymes (*11 β 1 HSD* and *11 β 2 HSD*) were also examined and for details regarding analysis of the data see section 3.5.

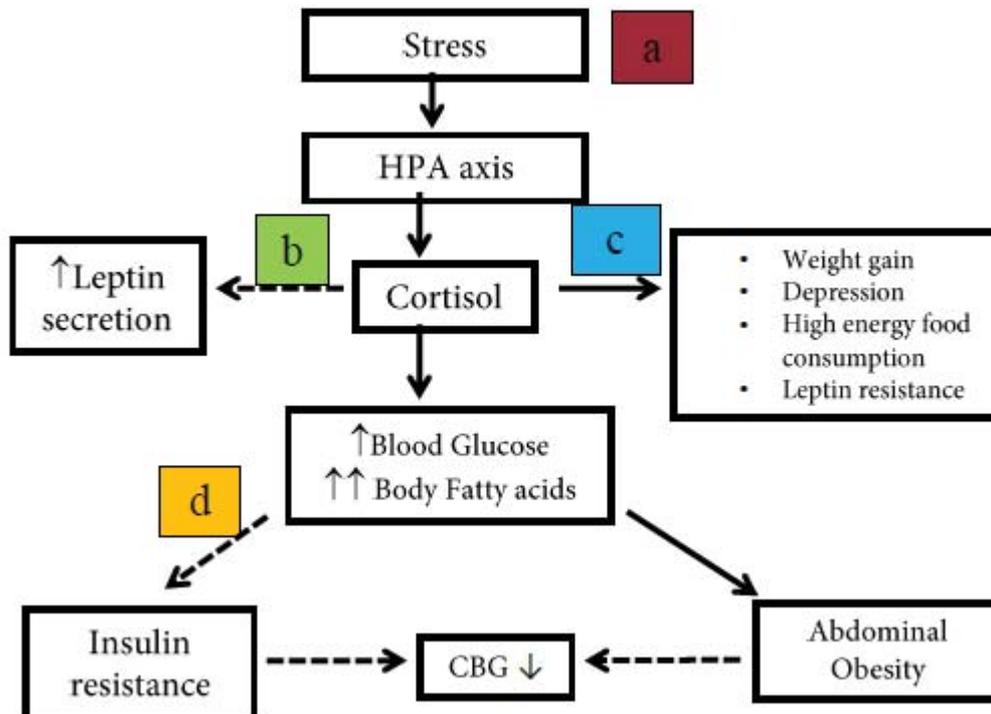


Figure 4.1: The relationship between obesity, stress, leptin and insulin resistance ultimately leading to type 2 diabetes. a) stress either physical or psychological activates the hypothalamic-pituitary-adrenal (HPA) axis resulting in glucocorticoid (cortisol) release b) cortisol blocks neuropeptide Y release thus favouring leptin release in the body c) The chronic stimulation of stress hormone promotes high energy food consumption, which leads to weight gain and leptin resistance. It is also associated with depression d) Cortisol released regulates stress response by increasing glucose levels, increasing body fatty acid leading to insulin resistance and favouring abdominal obesity. All the above factors are indirectly associated with low levels of CBG. These low levels indicate the presence of more free and less bound cortisol in the circulation.

4.3 Results

The results are presented as overall mean results for obesity markers (section 4.3.1), mRNA results for genes related to obesity markers (section 4.3.2), correlations associated with obesity markers and other related outcome measures (section 4.3.3) and pre and post exercise intervention individual results for obesity markers (section 4.3.4).

4.3.1 Overall mean results for obesity markers

Table 4.1 represents the normal reference ranges for the obesity makers being investigated in this study which were provided by Capital Coast and District Health Board laboratory. The mean urinary cortisol, serum CBG, SHBG, leptin and adiponectin concentrations for the SPIRIT study participants at 0 weeks and after 16 weeks of PRT or AER exercise are shown in Table 4.2.

Table 4.1: Normal Reference Ranges for Obesity Markers

Obesity Marker	Normal Reference Range*
Urinary Cortisol	10-100 mg/24 hour
Serum Cortisol binding globulin (CBG)	300-1800 nmol/L
Serum Sex Hormone Binding Globulin (SHBG)	26.1-110.0 nmol/L
Serum Leptin	Female: 7 - 35 mg/L Male: 2 - 10 mg/L
Serum Adiponectin	Interim range > 4 µg / ml

**Normal reference ranges were obtained from Capital Coast District Health Board laboratory.*

Mean values for the four obesity markers under investigation are in Table 4.2 and graphically represented in Figure 4.2. On examination of the mean values it can be seen that serum leptin levels at 0 and 16 weeks for both PRT and AER groups are below normal maximum leptin value of 35 mg/L for females. Seven of the 9 participants in the AER group were female and 7 of the 9 participants in the PRT group were female. Individual results are examined in

section 4.3.4. There was no statistically significant difference between 0 and 16 weeks for serum leptin concentration within the AER group or PRT group. There was also no statistically significant difference between AER or PRT groups ($P = 0.4$).

At baseline (0 week) and 16 weeks the PRT group had mean urinary cortisol above the maximum normal value of 100 mg/ 24 hr. The AER group mean urinary cortisol was above the maximum normal urinary cortisol. There was no statistically significant difference between 0 and 16 weeks for urinary cortisol within the PRT group ($P = 0.32$) or AER group ($P = 0.47$). There was also no statistically significant difference between AER or PRT groups ($P = 0.40$).

Table 4.2: Concentration of obesity markers at baseline and after 16 weeks of exercise

	PRT (n=9)			AER (n=9)		
	0 week	16 weeks	<i>P</i>	0 week	16 weeks	<i>P</i>
Leptin (mg/L)	24.6 ± 16.6	23.8 ± 13.6	0.64	29.9 ± 9.5	27.9 ± 13.6	0.37
Cortisol (mg/24 hour)	303 ± 303.0	105 ± 113.2	0.32	*108 ± 79.2	93.5 ± 44.5	0.47
CBG (nmol/L)	642 ± 137.2	622 ± 145	0.07	588 ± 151.6	468 ± 122.9	0.09
SHBG (nmol/L)	31 ± 11.14	34.2 ± 9.7	0.01	37 ± 15.2	35.6 ± 15.1	0.56
Adiponectin# (µg / ml)	5.6 ± 1.9	5.6 ± 2.2	0.96	6.7 ± 3.3	6.7 ± 3.2	0.94

*Values represent mean ± SD. $P < 0.05$ is considered statistically significant. *P* values in the Table are within group. Baseline represents 0 weeks. * represents $n=8$ as one participant did not provide 24 hours collected urine sample for investigation. Leptin, adiponectin, CBG and SHBG were measured in serum. Cortisol measured in 24 hour collected urine. # serum adiponectin was measured in Bill Sukalas' PhD project [35]*

Serum CBG values for 0 and 16 weeks for both PRT and AER groups were within the normal reference range. There was a trend towards a decrease in the CBG levels in both PRT and AER groups with P values of 0.07 and 0.09 respectively (Table 4.2). Between groups revealed no statistical significance ($P= 0.46$). Serum SHBG levels within the PRT group showed a statistically significant increase (Table 4.2) after 16 weeks of exercise. There was no statistically significant change within the AER group (Table 4.2). The SHBG concentrations are within normal reference range for both PRT and AER groups. There was also no statistically significant difference between AER or PRT groups ($P= 0.09$). Serum adiponectin levels were above the 4 $\mu\text{g}/\text{ml}$ interim range for both PRT and AER group. There was no statistically significant change within the PRT or AER group (Table 4.2) or between exercise groups ($P= 0.93$) (see chapter 1 Table 1.3).

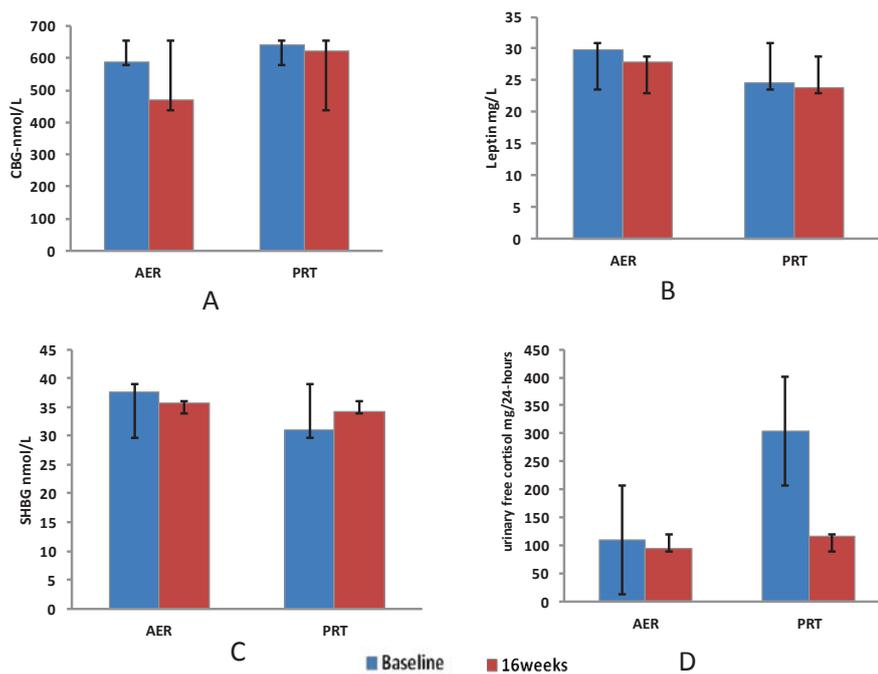


Figure 4.2: Mean values for the obesity markers in AER and PRT groups at baseline and at 16 weeks after intervention. A) serum levels of CBG (nmol/L) B) serum leptin levels (mg/L) C) serum SHBG(nmol/L) and D) urinary cortisol (mg/24hours). The blue bars show baseline (0 weeks) and red bars after 16 weeks respectively. Data are expressed as mean \pm SD and $n=9$ for AER and $n=9$ for PRT except for the urinary cortisol where $n=8$ in the AER group.

4.3.2 mRNA expression of specific obesity marker related genes

Investigation of skeletal muscle leptin, leptin receptor, adiponectin, glucocorticoid receptors, 11-betahydroxysteroid dehydrogenase 1 and 2 enzyme mRNA levels before and after 16 weeks exercise was performed by interrogating the mRNA profile data (obtained from microarray data). The fold change in mRNA expression and significance (P-value) for the PRT group and AER group are shown in Table 4.3. For microarray data 1.0 fold change represents no change in mRNA level. There was no statistically significant change in mRNA expression observed for the obesity associated genes investigated (Table 4.3).

Table 4.3: Fold change and P-values within the group for skeletal muscle mRNA levels

Gene	PRT		AER	
	Fold change	P value	Fold change	P value
<i>ADIPOQ</i>	1.01	0.56	1.00	0.87
<i>LEP</i>	1.05	0.76	1.07	0.06
<i>LEPR</i>	1.03±0.01	0.32	1.02	0.17
<i>11 b₁HSD</i>	-1.01	0.68	-1.01	0.51
<i>11 b₂HSD</i>	1.02	0.43	1.00	0.82
<i>NR3C1</i>	-1.02	0.37	-1.03	0.18
<i>NR3C2</i>	1.01	0.38	1.02	0.43

mRNA fold change data was obtained from microarray data (section 3.5). P < 0.05 is considered significant. ADIPOQ gene is adiponectin gene, LEP and LEPR genes encode for leptin and leptin receptor. 11 b₁HSD and 11 b₂HSD genes represent 11-betahydroxysteroid dehydrogenase 1 and 2 respectively. NR3C1 and NR3C2 genes encode for glucocorticoid receptors. The microarray data presented in the Table is from one micro-array probe except for the leptin receptor where data is representative of 5 probes (mean±SD)

4.3.3 Examination of Correlations between obesity markers, related outcome measures and exercise sessions attended

Table 4.4 represents correlations between change in serum leptin levels with related outcome measures. A positive and statistically significant correlation was found between leptin change and change in the BMI and fat mass in the AER group. A positive correlation was observed in the PRT group between change in leptin and change in the fat mass after 16 weeks of training.

Table 4.4 Correlation between changes in serum leptin levels (week 16 – week 0) and change score for metabolic outcome measures, exercise sessions attended and other obesity markers

Outcome measure	AER		PRT	
	r	P	r	p
HbA1c (%)	0.59	0.091	0.01	0.976
Insulin (pmol/L)	0.25	0.504	0.45	0.219
BMI (kg/m ²)	0.93*	0.001	0.55	0.122
Waist-to-Hip ratio	0.005	0.98	0.345	0.363
Fat Mass (kg)	0.696*	0.037	0.786*	0.016
Total Cholesterol (mmol/L)	-0.136	0.72	-0.25	0.513
Triglyceride (mmol/L)	-0.207	0.59	-0.04	0.376
Exercise Sessions Attended	0.174	0.654	-0.19	0.6
CBG (nmol/L)	-0.176	0.651	-0.551	0.124
SHBG (nmol/L)	0.393	0.17	-0.2	0.45
urinary cortisol (mg/24hours)	0.159	0.68	-0.45	0.22

* p<0.05 statistically significant ;** p< 0.01 statistically significant; r= regression coefficient

Table 4.5 represents correlated change in serum SHBG levels with related outcome measures, exercise sessions attended and other obesity markers. A positive and statistically significant correlation between change in SHBG levels with total cholesterol and number of exercise sessions attended by the participant in PRT was found. A negative and statistically significance correlation with urinary cortisol was also observed in the PRT group. A positive and statistically significant correlation between change in serum SHBG levels and sessions attended by the participant in AER was found. A statistically significant negative correlation existed in AER group with change in waist-to-hip ratio and urinary cortisol.

Table 4.5 Correlation between changes in serum SHBG levels (week 16 – week 0) and change score for metabolic outcome measures, exercise sessions attended and other obesity markers

Outcome measure	AER		PRT	
	r	p	r	p
HbA1c (%)	0.24	-0.28	-0.30	0.42
Insulin (pmol/L)	-0.09	0.80	-0.40	0.37
BMI (kg/m ²)	0.19	0.609	-0.372	0.32
Waist-to-Hip ratio	-0.716*	0.03	-0.65	0.05
Fat Mass (kg)	0.17	0.05	-0.52	0.14
Total Cholesterol (mmol/L)	0.11	0.77	0.78*	0.013
Triglyceride (mmol/L)	-0.559	0.40	0.39	0.36
Exercise Sessions Attended	1**	0.001	1**	0.001
Leptin (mg/L)	0.17	0.64	-0.2	0.606
CBG (nmol/L)	0.393	0.296	-0.06	0.87
urinary cortisol (mg/24hours)	-0.94**	0.0001	-0.965**	0.0001

* p<0.05 statistically significant ;** p< 0.01 statistically significant; r= regression coefficient

Table 4.6 represents correlation of change in urinary cortisol levels with some outcome measures and other obesity markers. It can be seen that cortisol change has a positive and statistically significant correlation with waist-to-hip ratio in AER and PRT groups. Cortisol showed a statistically significant negative correlation with exercise sessions attended in both AER and PRT groups. There was a statistically significant positive correlation with fat mass and a statistically significant negative correlation with SHBG in the PRT group.

Table 4.7 represents correlation between CBG change with some outcome measures and other obesity markers. No statistically significant correlations were observed between CBG and some metabolic outcome measures, exercise sessions attended and other obesity markers.

Table 4.6 Correlation between changes in urinary cortisol levels (week 16 – week 0) and change score for metabolic outcome measures, exercise sessions attended and other obesity markers

Outcome measure	AER		PRT	
	r	P	r	P
HbA1c (%)	-0.04	0.908	0.283	0.46
Insulin (pmol/L)	0.18	0.6	0.495	0.17
BMI (kg/m ²)	0.11	0.77	0.773	1.015
Waist-to-Hip ratio	0.72*	0.02	0.688*	0.040
Fat Mass (kg)	0.06	0.870	0.682*	0.043
Total Cholesterol (mmol/L)	0.51	0.156	-0.77	0.013
Triglyceride (mmol/L)	-0.18	0.68	-0.328	0.388
Exercise Sessions Attended	-0.94*	0.001	-0.96**	0.001
Leptin (mg/L)	0.15	0.68	0.45	0.22
SHBG (nmol/L)	0.17	0.64	-0.965**	0.0001
CBG (nmol/L)	-0.45	0.22	-0.094	0.811

* p<0.05 statistically significant ;** p< 0.01 statistically significant; r= regression coefficient

Table 4.7 Correlation between changes in serum CBG levels (week 16 – week 0) and change score for metabolic outcome measures, exercise sessions attended and other obesity markers

Outcome measure	AER		PRT	
	r	P	r	P
HbA1c (%)	0.594	0.091	-0.085	0.829
Insulin (pmol/L)	0.157	0.68	0.057	0.884
BMI (kg/m ²)	-0.035	0.92	-0.61	0.079
Waist-to-Hip ratio	-0.026	0.94	-0.052	0.89
Fat Mass (kg)	-0.068	0.86	-0.633	0.068
Total Cholesterol (mmol/L)	-0.062	0.87	-0.136	0.727
Triglyceride(mmol/L)	0.328	0.389	-0.105	0.789
Exercise sessions Attended	0.39	0.291	-0.062	0.874
Leptin (mg/L)	-0.176	0.651	-0.551	0.124
SHBG (nmol/L)	0.393	0.296	-0.2	0.6
urinary cortisol(mg/24hours)	-0.453	0.221	0.45	0.22

* p<0.05 statistically significant ;** p< 0.01 statistically significant; r= regression coefficient

4.3.4 Individual participant results

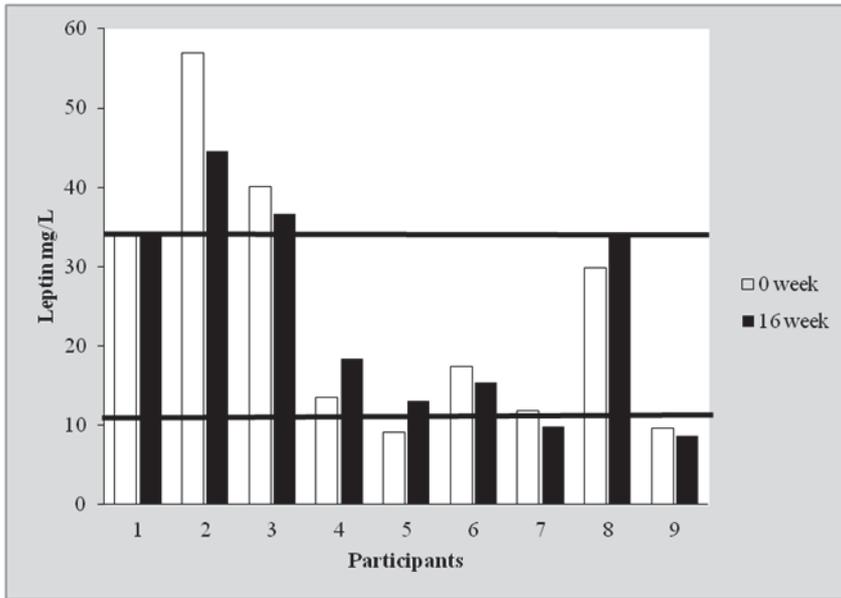
Due to the limited number of participants that completed the intervention in the SPIRIT study (n=18), individual participant results for the serum obesity markers were also analysed in order to determine if group changes presented previously (section 4.3.1) were driven by large intra- and/or inter-individual variability at baseline or 16 weeks. Individual participant results for leptin (Figure 4.3, Table 4.8), cortisol (Figure 4.4, Table 4.8), CBG (Figure 4.5; Table 4.8) and SHBG (Figure 4.6, Table 4.8) are presented below

Table 4.8 Individual participant values for obesity biomarker concentrations before and after 16 weeks of AER and PRT

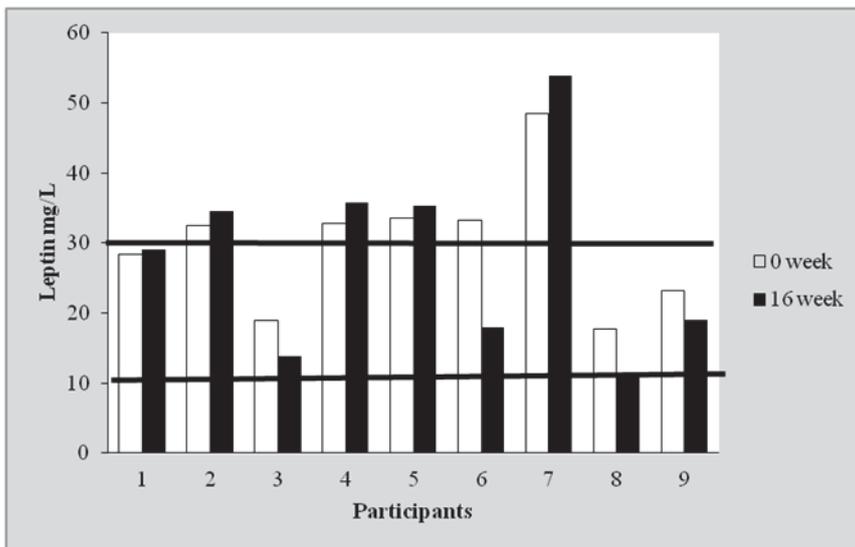
n	Leptin (mg/L)				SHBG (nmol/L)				Cortisol (mg/24 hours)				CBG (nmol/L)			
	AER		PRT		AER		PRT		AER		PRT		AER		PRT	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	28	29	34	33	20	22	29	36	42	75	46	44	659	686	824	856
2	32	34	57	44	67	45	24	31	36	106	97	116	1003	479	521	564
3	19	14	40	36	24.2	27	36	42	86	40	1796	353	606	722	661	684
4	33	36	13	18	45.5	46	54	51	203	136	63	5	544	588	835	563
5	33	35	9	13	36.1	31	25	28	114	***	212	170	711	343	650	395
6	33	18	17	15	22.7	29	17	20	28	42	42	27	574	485	605	571
7	48	54	11	9	51.4	39	21	23	74	88	152	186	537	449	940	714
8	17	11	29	34	37.4	46	32	36	192	90	17	25	723	491	776	768
9	23	19	9	8	34.1	32	38	39	209	171	64	42	514	431	600	483

*** represents no urinary sample provided for cortisol measurement in AER participant 5, n= designated participant number

Four of the 9 participants in the PRT group (participant 2, 3, 6, 9) and 4 of the 9 participants in AER group (participants 3, 6, 8, 9) experienced a drop in the leptin levels after 16 weeks of exercise training (Figure 4.3). One female participant in the PRT group (participant 3) and one male participant in the AER group (participant 7) had leptin levels outside the reference range. All four males in the study had leptin levels greater than the maximum leptin concentration of 10 mg/L for males (Figure 4.3).

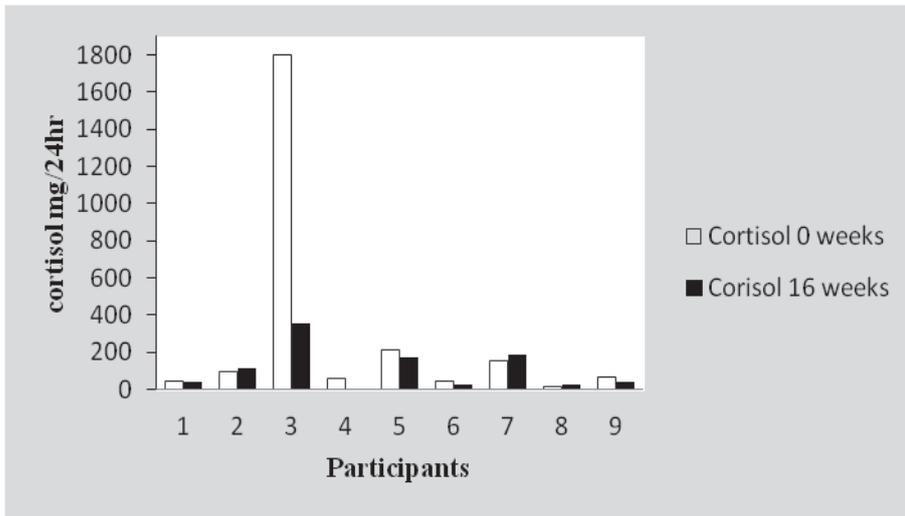


(a)

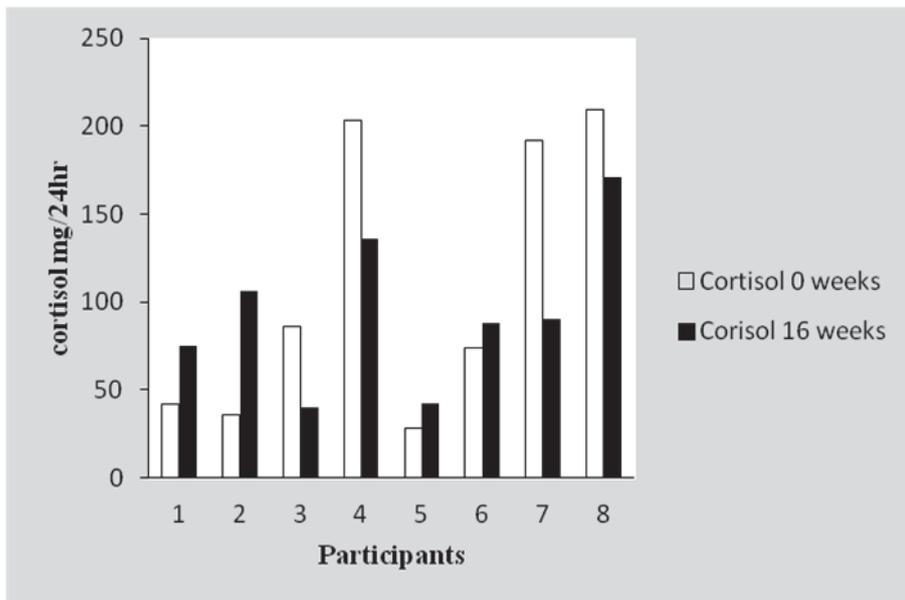


(b)

Figure 4.3: Serum leptin levels in (a) PRT group and (b) AER group at 0 week and 16 weeks training. The black line represents the maximum leptin value of 35 mg/L for females and 10 mg/L for males. Participants 2 and 8 in PRT group are males. Participants 4 and 7 in AER group are males



(a)



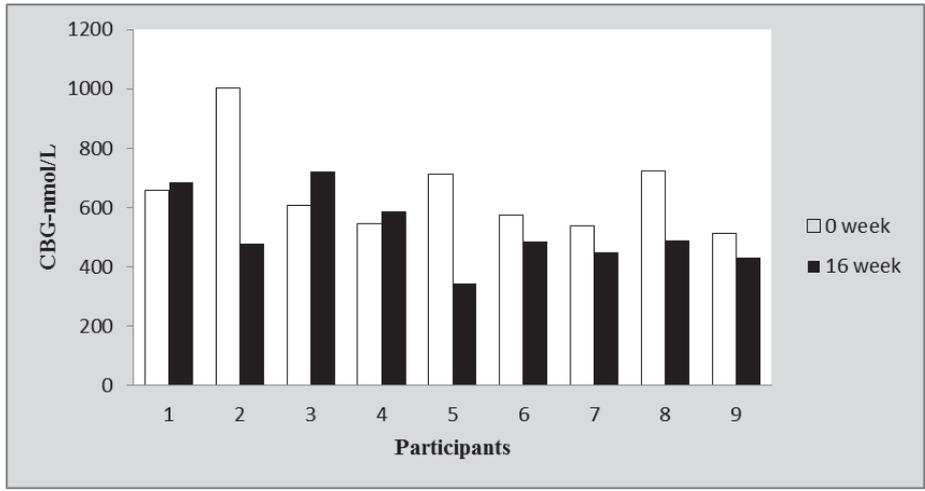
(b)

Figure 4.4: Urinary cortisol levels in (a) PRT group and (b) AER groups at 0 week and 16 weeks training. Participants 2 and 8 in PRT group are males. Participants 4 and 7 in AER group are males. There are eight participants in AER group as one participant did not provide 24-hour urine sample at 16-week

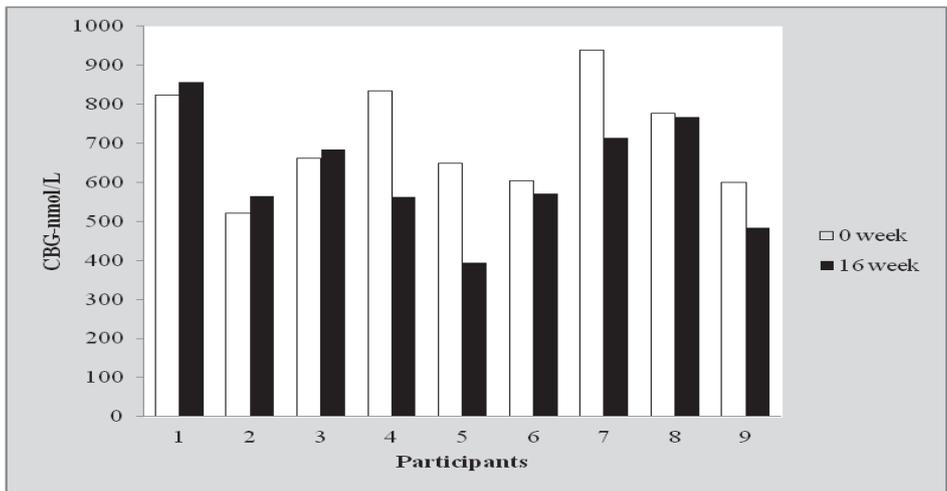
The cortisol levels were within the normal reference range (Table 4.1) except for participant 3 in the PRT group who had extraordinary levels of urinary cortisol at baseline. Data was re-checked to ensure that this was not an outlier. Cortisol was re-measured, standards were all correct so it was assumed that cortisol concentration provided was correct. Participants 4, 7 and 8 in the AER group (Figure 4.4(b)) also had higher than normal cortisol levels. Participant 3 in the PRT showed a 5-fold decrease after the 16 weeks of training. Participants 3, 4, 6, 9 in the PRT group and Participants 3, 4, 7 and 8 in the AER group showed decreased levels of cortisol after 16 weeks of exercise training (Figure 4.4). Four out of 8 individuals in the AER group showed an increase in cortisol levels (participant 1, 2, 5 and 6) after 16 weeks of exercise. Only one individual, participant 7, in the PRT group showed an increase in the cortisol levels after 16 weeks.

All SPIRIT study participants had serum CBG levels within the normal reference range (Table 4.1) of 300-1800 nmol/L. Six of the 9 participants in the PRT group (Figure 4.5(a)) showed a decrease in the CBG level (participants 2, 5, 6, 7, 8 and 9) after 16 weeks of exercise. Whilst 5 out of the 8 participants in the AER group (Figure 4.5(b)) showed a decrease in CBG level (participants 4, 5, 6, 7 and 9) after 16 weeks of exercise.

All SPIRIT study participants had serum SHBG levels lower than the maximum SHBG normal reference value (Table 4.1) of 110 nmol/L. Three individuals (participant 6 and 7 in PRT group and participant 1 in AER group) had SHBG levels lower than the minimum SHBG normal reference value of 26 nmol/L (Table 4.1). One individual in the PRT group (participant 4) showed a drop in SHBG levels (Figure 4.6(a)) and four participants in the AER group (participants 2, 5, 7 and 9) showed a decrease in SHBG levels (Figure 4.5(b)) after 16 weeks of exercise.

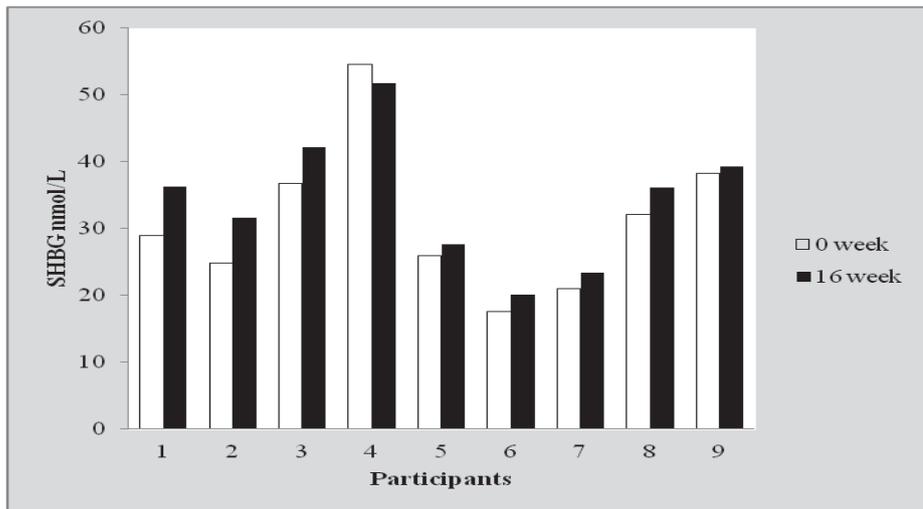


(a)

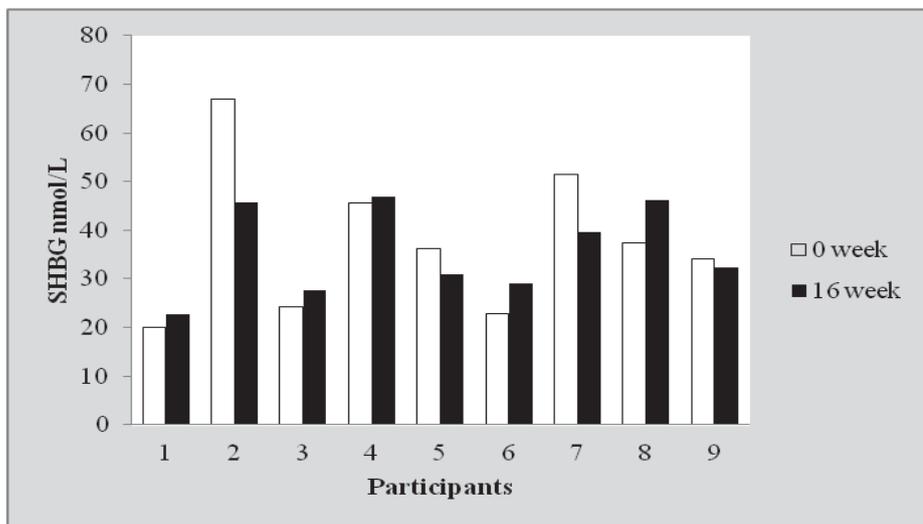


(b)

Figure 4.5: Serum cortisol binding globulin (CBG) levels in (a) PRT group and (b) AER groups at 0 week and 16 weeks training. Participants 2 and 8 in PRT group are males. Participants 4 and 7 in AER group are males.



(a)



(b)

Figure 4.6: Serum sex hormone binding globulin (SHBG) levels in (a) PRT group and (b) AER groups at 0 week and 16 weeks training. Participants 2 and 8 in PRT group are males. Participants 4 and 7 in AER group are males.

4.4 Discussion

This study investigated the independent effects of PRT and AER exercise, each prescribed for 16 weeks on serum leptin, CBG, SHBG and 24-hour urinary cortisol levels in Polynesian adults with T2DM and grade 3 obesity. The PRT group significantly increased SHBG levels after 16 weeks of exercise ($P = 0.01$) whilst the AER group showed no change in SHBG levels after 16 weeks of exercise (Table 4.2). There were no other statistically significant within group changes for the obesity markers leptin, cortisol, CBG and adiponectin, in the AER and PRT exercise groups (Table 4.2, Figure 4.2). There was a trend towards significance for a decrease in CBG levels in both the PRT ($P = 0.07$) and AER ($P = 0.09$) group. There were no statistically significant between group changes for the obesity markers investigated in this study. The post-hoc analysis followed by one-way ANOVA also revealed no significant changes or trends in the obesity markers under investigation. The 16 weeks of either AER or PRT training was unable to show any significant change in skeletal muscle mRNA gene expression for leptin, leptin receptor, adiponectin, glucocorticoid receptors and 11-beta-hydroxysteroid dehydrogenase 1 and 2.

In the AER group there were a number of positive statistically significant correlations associated with the obesity markers investigated. An increase in SHBG levels was associated with increased number of sessions attended, increased leptin levels were associated with increased BMI and fat mass, and increased cortisol levels were associated with increased waist-to-hip ratio. These results suggest that attendance at the available sessions for the exercise was a key factor in relation to change in the SHBG levels, a surrogate marker of insulin resistance as previously described [31]. So, this may furnish the idea that the more exercise sessions the SPIRIT participant attended the greater the possibility they had in improving their insulin sensitivity. Furthermore higher leptin levels were positively correlated with BMI and fat mass. High leptin levels are known to be associated with increased fat mass, obesity and insulin resistance [36]. Leptin, an appetite controlling hormone, released from fat mass ultimately can lead to leptin resistance [37]. Studies with reduction in fat mass have shown to lower serum leptin levels [38]. From the correlation results in this PhD study it could be surmised that a drop in the BMI could induce a drop in serum leptin levels. This has been previously shown by Ozcelik et al [39] where after 12 weeks of exercise in obese females a drop in leptin was significantly correlated with reduction of fat mass ($r = 0.899$, $P=0.001$) showing that leptin levels could be reduced by reducing the BMI and fat mass.

In the AER group in this PhD study, cortisol levels were statistically negatively correlated with the number of exercise sessions attended meaning that if the participant attend more exercise sessions this should lead to decreased cortisol levels. In our AER group 4 out of 9 participants had decreased cortisol levels after 16 weeks aerobic exercise. This concurs with a study by Rosa *et al* [40] which included 10 participants for two sessions of aerobic exercise for 10 weeks. A drop in the Cortisol levels, a stress hormone associated with obesity and insulin resistance (Figure 4.1) [41] could be beneficial for this grade 3 obese cohort in the SPIRIT study. A lowering of cortisol levels could be beneficial for T2DM subjects as higher levels of cortisol has been associated with hyperglycaemia, hyperlipidemia and hyperleptinemia aiding in the development of metabolic complications that occur in T2DM [42]. These results may suggests that increase attendance at exercise sessions as shown by the positive correlation with exercise sessions may have positive impact on altering the metabolic profile of the individual.

In the PRT group there were a number of positive statistically significant correlations associated with the obesity markers investigated. Increase in SHBG levels was associated with increased number of exercise sessions attended, an increase in leptin levels was associated with increased fat mass, and decreased cortisol levels were associated with increased SHBG. These results may illustrate that the number of exercise sessions could be important to induce any change in the SHBG levels. The decreased levels of cortisol, well-known stress hormone, with improved SHBG levels, an important marker of insulin resistance could be a positive outcome of resistance exercise as cortisol has been associated with metabolic complications in obese individuals [43]. As higher levels of cortisol are associated with disturbed lipid profiles in people with T2DM as reported by Reynolds *et al* [44], the decrease cortisol levels in people with more attendance at exercise sessions could be beneficial for the SPIRIT study cohort.

As previously mentioned (chapter 2 section 2.3) leptin resistance and insulin resistance are closely associated with high levels of cortisol [22, 41]. Cortisol interferes with the action of these hormones (Figure 4.1) and exercise training for more than 12 weeks may help to regulate the HPA axis [45, 46]. There is some evidence in the literature regarding how exercise affects the HPA axis and adipose tissue function (section 2.3 in Chapter 2). To date, there has been no data gathered from Pacific peoples with or without grade 3 obesity in regards to their cortisol levels. The SPIRIT study is the first clinical trial to investigate the

effect of exercise on a Polynesian population and this study was set out with the aim of assessing the effect of exercise on well known obesity markers related with T2DM in NZ Polynesian population.

As cortisol is an indicator of stress and closely linked with obesity, it was interesting to investigate the total 24-hour urinary cortisol levels as the marker for improvement in stress levels. A drop in the cortisol levels after 16-weeks exercise training in both groups was expected as previously reported [40, 47, 48]. However it was noted that all participants in the SPIRIT study were within the normal reference range for urinary cortisol (Table 4.6 ; Figure 4.4) except participant number 3 in PRT group (Fig 4.3 (a)) and participants 4,7, and 8 in the AER group (Figure 4.3 (b)). Participant 3 in the PRT showed a 5-fold decrease in cortisol levels and participants 3, 4, 7, 8 in the AER group also showed overall a decrease in cortisol levels; however, no statistically significant decrease was noted. A negative correlation was observed with the exercise sessions attended (Table 4.6) which could indicate that cortisol levels can be affected with exercise of longer durations. It was proposed that after 16 weeks of exercise training the PRT and AER groups will show improvement in insulin resistance and obesity by studying markers such as leptin, adiponectin, SHBG, cortisol and CBG. The results of this PhD study indicate that there was a significant improvement in the SHBG levels in PRT group only and there was a trend towards a decrease in CBG in spite of no change in the cortisol levels. This investigation did not find any significant alterations in adipocytokines such as leptin and adiponectin. However correlations of cortisol with other obesity markers and outcome measures revealed that cortisol was positively correlated with waist-to-hip ratio in both groups and had a negative correlation with SHBG a surrogate marker of insulin resistance. In this study there was no statistically significant drop in urinary cortisol levels for both AER or PRT groups. However there was a positive correlation cortisol noted with number of exercise sessions attended. So it could be proposed that for this grade 3 obese cohort, with T2DM, a longer duration >16 weeks of exercise could lead to a greater effects such as greater drop in cortisol levels and associated changes in body composition e.g fat mass and wist-to-hop ration.

Previous studies have found an improvement in the leptin levels after exercise [49, 50]. Recently Abidi and colleagues [51] showed improvements in leptin levels after AER exercise in obese females ($BMI=30\pm 5 \text{ kg/m}^2$). A relationship has been established between leptin and weight loss by aerobic and resistance exercise [50]. The exercise and subsequent weight loss

decreases the leptin levels and improves the leptin resistance at the cellular level. In the SPIRIT participants the leptin levels were not significantly elevated before the exercise training. In this study participants did not show weight loss after sixteen weeks (Table 1.3) [52]. The lack of weight loss in this study can be related to the lack of improvement in leptin levels and its receptors levels in the skeletal muscle. A positive correlation was observed between change in BMI and fat mass with leptin in both exercise groups (Table 4.4). A reduction in leptin levels with weight loss has been reported in previous research studies in this field [33, 49, 50]. A recent review by Bouassida *et al.* [34] investigating factors that improved leptin and adiponectin function included a number of studies that showed decreased levels of these hormones after exercise training of more than 12 weeks [39, 53]. The finding from these studies suggest that acute exercise duration of more than 60 min or exercise expending energy more than 800 kcal is capable of decreasing leptin levels. Also exercise training more than 12 weeks appears to be associated with decreasing leptin levels if the exercise is associated with reduced fat mass and body weight. In SPIRIT study the participants performed exercise of high intensity (60min/ 3 times a week) expending around 800 calories for 16 weeks yet there was no significant weight loss or change in BMI was observed in the SPIRIT study cohort hence the associated levels of leptin did not improve (Table 4.2). There is limited data available regarding the studies on adiponectin levels with exercise. However, the few exercise trials that have investigated this show that adiponectin levels improve with exercise training [34]. This study showed no significant change in serum adiponectin levels after 16 weeks of either PRT or AER exercise (Table 4.2).

The body fat, waist-hip ratio and abdominal diameter are reported to be highly dependent on the endocrine status of a person [54]. Cortisol along with other hormones is involved in this fat accumulation. Drapeau *et. al* suggests that cortisol activates lipoprotein lipase which activates fat accumulation [41]. There is an interesting relationship between cortisol and leptin resistance which is shown in Figure 4.7. It is postulated that excess cortisol stimulates neuropeptide Y (NPY) [55] and promotes leptin resistance at the tissue level [20]. The cortisol levels have been reported to be higher in obese people as compared to skinny individuals [56]. It has also been reported that cortisol may be involved in the pathogenesis of T2DM [44] and evidence suggests that people with T2DM with or without obesity have high levels of cortisol that produces a barrier for proper glucose control [22]. 24 hour cortisol levels have been shown to decrease after exercise training of more than 12 weeks duration

[22, 57]. Recently Cruz *et al.* [58] showed marked improvement in the cortisol and leptin levels after AER exercise. However, there is less data available in relation to the effect of exercise on the improvement in the cortisol level in people with T2DM and visceral obesity. The SPIRIT study is the first study to examine these changes in cortisol, its' binding globulin, the mRNA expressions of enzymes 11 β_1 HSD, 11 β_2 HSD, and glucocorticoid receptors (NRC31 and NRC32). The mRNA levels for 11 β_1 HSD enzyme which converts cortisone to cortisol, 11 β_2 HSD which converts cortisol to cortisone were not affected after 16 weeks exercise and along with no change in CBG levels. These results do not support that 16 weeks of exercise is starting to have an effect in the SPIRIT study participant and leading towards a decrease in cortisol levels. However, with the small number of participants in the intervention group and the small changes noted in the cortisol and associated markers, these results need to be further validated. However a negative correlation with exercise session in both groups, a positive correlation with waist-to-hip ratio in both exercise groups could suggest the longer duration of exercise for this cohort (>16weeks) in improving the cortisol levels.

SHBG is a binding protein that is reported to be affected by metabolic status of the body [17, 59, 60]. Low levels of SHBG has been reported to be associated with insulin resistance [59, 60]. This binding globulin is closely associated with improvement in insulin sensitivity [40]. The statistically significant increase in SHBG levels in the PRT group is consistent with previous research in this area [59, 61-64] and may indicate possible improvement in insulin sensitivity. It was also positively correlated with number of exercise sessions attended reflecting that longer duration of exercise or more exercise sessions could a bring positive change in the individuals.

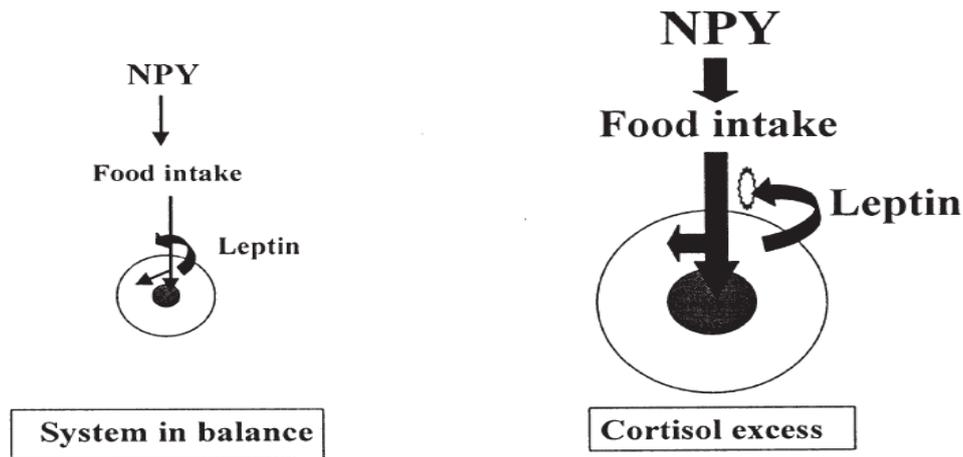


Figure 4.7: Neuroendocrine background to abdominal obesity. With cortisol excess (right panel) the secretion of neuropeptide Y (NPY) is stimulated and leptin effects blunted producing leptin resistance. The left panel in the absence of excessive cortisol the system is at balance. Sourced from Leal-Cero et. al [55]

A number of reasons may explain the lack of improvement in leptin, cortisol and CBG levels in the present study. Firstly, although subjects were 100% compliant with exercise protocols when present for training sessions, low attendance rates reduced overall training volume and may have blunted the effectiveness of the exercise stimulus. Only eight (PRT=3, AER=5) of 18 participants that completed the entire 16 week intervention attended at least 36 of 48 (75%) available exercise training sessions. As we can observe from the results described by Sukala *et al.* [66, 67] there was a tendency towards a reduction in body weight and fasting plasma insulin and an increase in triglycerides in the SPIRIT study cohort, hence the improvements in leptin and cortisol levels could be delayed. Future exercise studies composing of longer duration for people with grade 3 obesity may yield more appreciable changes in cortisol, CBG and serum leptin levels.

Secondly, the level of obesity seen in this cohort (BMI = $43.8 \pm 9.5 \text{ kg/m}^2$; n = 18) was considerably higher than in subjects in previous exercise interventions for T2DM management (BMI ≤ 36) [68, 69]. No improvements in the obesity status could have delayed the effect of the exercise stimulus or the external stimulus was below threshold to bring change in the obesity levels of these individuals. Thus, future exercise investigations of longer

duration, more frequent and intense training sessions, may provide the necessary stimulus to improve obesity related parameters such as improvement in the insulin resistance and leptin resistance in people with T2DM and grade 3 obesity.

Thirdly, as previous research studies [32, 36, 69] have suggested that insulin resistance preceded the leptin resistance, thus a reduction in CBG [22] and an increase in SHBG levels during a longer duration of exercise may serve as markers for improved insulin sensitivity and leptin resistance.

The increase in SHBG in the PRT group potentially suggests a link between resistance exercise and improvement in insulin resistance. There is strong evidence showing support for SHBG as a surrogate marker of insulin resistance [17, 59, 60] The finding in this study is in agreement with previous research findings where an improvement in SHBG levels was observed for participants who exercised [61, 62]. The other interesting finding was the trend towards a decrease in CBG levels, and no significant change or concomitant increase in the cortisol levels was observed. This may be caused by the adaptive mechanism of the body with exercise, so that by decreasing the cortisol binding globulin, more cortisol would be made available to the tissue in the chronic stress of exercise which would not affect the overall levels of cortisol levels. So it is possible to hypothesise that obesity could be a major factor, leading to resistance to metabolic adaptations with exercise. It is therefore assumed that lack of improvement in the physiological markers in the SPIRT study participant after 16-weeks of exercise is due to the overwhelming fat mass and associated adipocytokines.

One limitation of the SPIRT study as reported by Sukala [70] was low subject numbers (n = 9 for each group) and, consequently, this has reduced the extent to which statistically significant inferences could be made. Due to the small sample size in this study and the inherent potential for large or small intra- and inter-subject variability in some parameters, the acceptance or rejection of the null hypothesis based on *P* value alone may inadvertently expose or mask clinically meaningful physiological effects. For this reason, individual subject data were visually inspected to determine if large or small changes in the mean after 16 weeks of training were influenced by a single outlier or other aberrant findings. Accordingly, the results of the present investigation must be considered preliminary and await corroboration by future studies with a larger number of subjects.

These findings have important implications for developing further studies in investigating the effects of exercise training in the Polynesian population, especially in grade 3 obese individuals. An implication of this is the possibility that exercise duration should be of a longer duration. One of the issues that emerged from these findings was that obese individuals with T2DM may be resistant to exercise adaptations. Some of the concerns emerging from this finding could be related to the thrifty phenotype responsible for obesity epidemic. This phenotype is reported to be resistant to the lipid-lowering effects of exercise. Both researchers and exercise prescriptionists should be aware that there are ethnic factors that likely make it impossible in a few ethnic populations to see the affects of exercise even if they adhere properly to the exercise program. For such specific populations, goals should be targeted first to increase fitness as it is evident from research that trained overweight individuals' exhibit better metabolic profiles than their sedentary counterparts [5].

In conclusion, the application of 16 weeks of supervised high-intensity resistance or aerobic exercise to Polynesian people with T2DM and grade 3 obesity did not show any improvements in obesity markers such as serum leptin, adiponectin, CBG and total 24 hour urinary cortisol. 16 weeks of PRT training produced a statistically significant increase in SHBG levels which could reflect a move towards improvement in insulin sensitivity. The statistically significant correlations of SHBG and cortisol with number of sessions attended, for both AER and PRT groups, indicate the importance of duration of exercise in observing metabolic changes in the individuals. However, these results must be interpreted with caution due to low subject numbers. Future investigations of longer duration with a greater number of participants, and improved social intervention to enhance attendance may be required to manage and successfully improve obesity and T2DM outcomes in this SPIRIT study cohort.

4.5 References

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CHAPTER 5

EFFECT OF AEROBIC AND RESISTANCE TRAINING ON THE SKELETAL MUSCLE MITOCHONDRIAL FUNCTION, IMTG CONTENT AND FAT METABOLISM IN THE SPIRIT STUDY COHORT

5.1 Introduction

It is well known that the production of the energy molecule, adenosine triphosphate (ATP) and oxidation of fatty acids are chief functions of the mitochondria, the power house of a cell [1]. There is evidence that mitochondria in the muscle cells are capable of getting rid of non-esterified fatty acids by oxidation and can protect against fatty acid induced insulin resistant disorders such as type 2 diabetes mellitus (T2DM), obesity and metabolic syndrome. Mitochondria perform the oxidation of fatty acids by a process called β -oxidation. The by-products of the β -oxidation metabolic pathway are recycled by the electron transport chain (ETC) in mitochondria (Chapter 2). Studies have shown that fatty acid oxidation in mitochondria of individuals with fatty acid/lipid associated disorders is hampered gradually and progressively to an extent that lipid moieties start accumulating inside the muscle cytosol in the form of intramuscular triglycerides (IMTG) [2] and inside the mitochondria [3]. Accumulation of lipids within the muscle has gained considerable attention over the past 10 years because of their association with insulin resistance and related disorders (Chapter 2 section 2.4.3). Low mitochondrial enzyme activity and the accumulation of IMTG within the muscle cell are hallmark features of insulin resistant disorders such as T2DM, obesity and metabolic syndrome.

The rate at which the muscle cell can convert ADP to ATP is directly proportional to the metabolic activity of the cell. The metabolic activity of a muscle cell can be defined as, “A process by which the cell utilises glucose or fatty acids to produce energy in the form of ATP” [4]. Thus the activities that can enhance the metabolic activity of a cell such as exercise can be helpful in diseases associated with cellular metabolic derangements like T2DM, obesity and metabolic syndrome. The role of mitochondria in energy metabolism has been discussed in Chapter 2 section 2.4.3. Briefly Figure 5.1 demonstrates the importance of the mitochondria and shows the cellular disturbances that can occur in the muscle cell that lead to an insulin resistant state. Exercise has been reported to be associated with improvements in insulin resistance by stimulating mitochondrial biogenesis, increasing mitochondrial enzyme activity [5] and decreasing production of FFA derivatives in the muscle cell [6]. These effects clearly strengthen the argument for the clinical application of exercise for the management of T2DM; however, the availability of data demonstrating mitochondrial adaptations to exercise is still limited. As discussed in Chapter 2 section 2.4 the majority of clinical exercise trials

have been conducted on populations having a BMI <30 and in most of the trials the ethnicity of the population has not been specified. Globally there are ethnic variations in the prevalence of obesity and T2DM so the response to exercise in terms of metabolic adaptations in particular at-risk ethnic populations such as Pacific Islanders still needs to be investigated.

As discussed in Chapter 1 the SPIRIT study is the first clinical exercise trial that investigated the impact of exercise on glycaemic control in a NZ Pacific Islands population that had T2DM and grade 3 obesity (BMI>35). The SPIRIT study cohort had no improvement in insulin sensitivity as measured by HOMA-IR (Chapter 1 section 1.3). HOMA-IR is not the gold standard method for determining insulin sensitivity however, it was clear that 16 weeks of exercise training whether it was aerobic or resistance training, did not seem to have much impact on this cohort. As mentioned previously, and also shown in Figure 5.1, lipid metabolism [7] and mitochondrial function [4] is disturbed in people with insulin resistance states such as T2DM, obesity and metabolic syndrome and exercise has shown to improve insulin signalling [8], lipid oxidation [9] and mitochondrial oxidative capacity [3] yet these cellular and metabolic pathways have not been examined specifically in a Polynesian population with T2DM and obesity. Therefore the molecular mechanisms underpinning the exercise physiology in the skeletal muscle, the major tissue for glucose disposal [10] and thus vulnerable to insulin resistance [7], needed to be examined in the SPIRIT study cohort. The questions that needed answering in regards to this cohort were; Is there any improved beta-oxidation in the mitochondria after 16 weeks of exercise? Does IMTG reduce after 16 weeks of exercise? Does mitochondrial enzyme activity increase after 16 weeks of exercise? Does aerobic exercise provide more changes metabolically and cellularly compared to resistance training?

Mitochondrial activity can be examined by measuring the activity of three well-known targeted key mitochondrial enzymes COX [11], CS [12] and BHAD [13]. The impact of exercise on fat metabolism in the muscle cell can be determined by examining the activity of BHAD [6], one of the key enzymes in the beta-oxidation pathway and by determining IMTG content [14]. Therefore the purpose of this chapter is two-fold:

- 1) To investigate the three key mitochondrial enzymes related with energy metabolism; COX, enzyme in electron transport chain, CS, an enzyme of citric acid cycle and BHAD a key enzyme of β -oxidation before and after 16 weeks of AER and PRT exercise and

2) To examine 0 week and 16 week skeletal muscle tissue of SPIRIT study cohort by inspecting the IMTG content and mitochondrial morphology via electron microscopy.

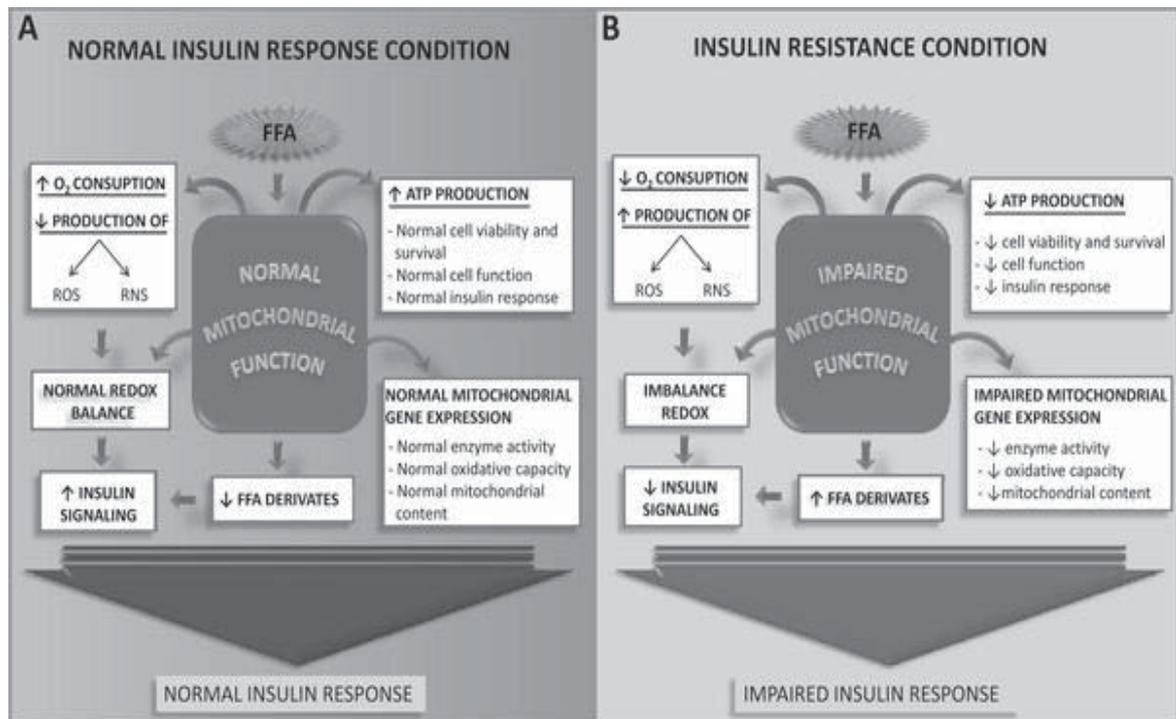


Figure 5.1: Mitochondrial changes that occur with insulin sensitive (A) and insulin resistant (B) states in the muscle cell. In the insulin resistance condition the mitochondrial function is impaired and there is less production of ATP, decreased activity of mitochondrial enzymes and reduced mitochondrial content which in turn leads to the formation of fatty acid derivatives and reactive oxygen species (ROS). This derangement leads to impaired insulin response. Sourced from Martin et al.[15].

5.2 Methods

The method for the collection and storage of the muscle biopsy tissue that was used for measuring mitochondrial enzyme activity and determining IMTG and mitochondrial content has been discussed in Chapter 3 section 3.3. The statistical analysis for skeletal muscle enzyme activity and quantification of IMTG has been discussed in chapter 3 see section 3.4.3 and 3.5.5 respectively.

5.3 Results

To perform the enzyme assays for three mitochondrial enzymes; CS, COX and BHAD on the SPIRIT study muscle biopsy samples, optimisation of the three enzyme assays had to occur.

The standard enzyme assays [11-13] for each mitochondrial enzyme being investigated had separate protein extraction methods which all used different detergents. Since the human

muscle biopsy sample from the SPIRIT study participant was very precious and in small quantity, optimisation of the three enzyme assays occurred using rat skeletal muscle tissue first and then some of the spare human skeletal muscle tissues from the dropout participants (Figure 5.2). Optimisation of the protein extraction method was performed first with identification of one detergent suitable for extraction and non-interference in all three enzyme assays. Protein extraction is discussed in section 5.3.1. The optimisation of the COX assay was done in conjunction with optimising the protein extraction method. The individual enzyme assays were then optimised using rat skeletal muscle tissue and spare human tissue and are discussed in sections 5.3.2 - 5.3.4. The final enzyme assay results using SPIRIT study skeletal muscle tissue is shown in section 5.3.5.

Investigation of the morphological changes in the SPIRIT participants skeletal muscle after 16 weeks AER or PRT exercise are discussed in sections 5.3.6 - 5.3.7.

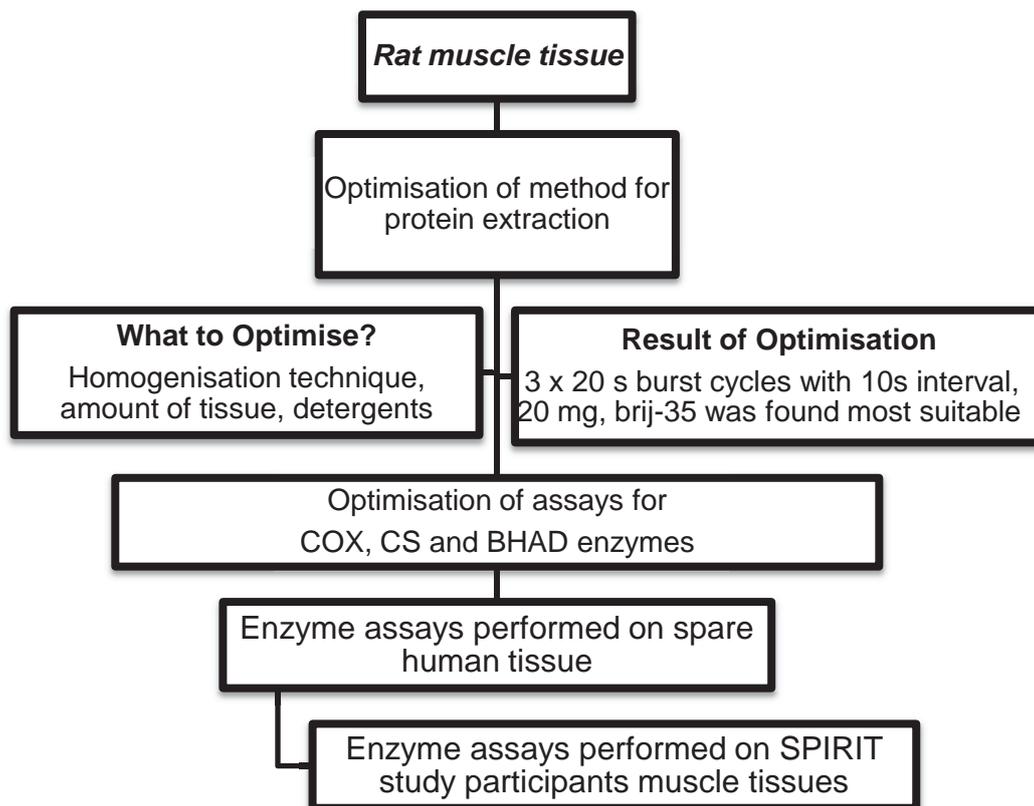


Figure 5.2: Flow diagram showing the optimisation process for extraction of protein and detection of enzyme activity in the skeletal muscle. Rat tissue was used first and then spares human muscle tissue before determination of COX, CS and BHAD enzyme activity in the SPIRIT study skeletal muscle samples.

5.3.1 Optimisation of Method for Protein Extraction

It was important to have one protein extraction method to provide protein homogenate from the skeletal muscle tissue of the SPIRIT study participant that could then be utilised for determining the activity of the three different mitochondrial enzymes of interest. On investigating several standard methods of protein extraction from skeletal muscle the following key things needed to be resolved for finding the best protocol for obtaining protein homogenate that provided optimal enzyme activity. Firstly the best technique for disrupting the muscle cell, tissue homogeniser or grounding under liquid nitrogen had to be determined. Secondly the amount of tissue required for detecting optimal enzyme activity had to be identified and thirdly, a detergent that would not interfere with the activity of the three mitochondrial enzymes and provided optimal activity had to be found. As shown in Figure 5.2, rat skeletal muscle tissue was used first for testing the different techniques above and the extraction was then trialled on the spare human tissue. The COX enzyme was assayed and used for helping with optimisation of the protein extraction protocol. Figure 5.2 outlines the overall process that was involved in optimising the method for extracting protein from skeletal muscle.

5.3.1.1 Determination of homogenisation technique

Muscle tissue (20 mg) was homogenised with 25 times volume of ice-cold extraction buffer (0.7M Sucrose, 0.01M Hepes, 1M EDTA, 2mM Mannitol, pH=7.4 appendix 1) containing protease inhibitor Tablet. It was homogenised for 20 second bursts three times with a 10 second interval in between each burst. Two separate 20 mg rat muscle samples were prepared in this manner. The same amount of muscle tissue was ground under liquid nitrogen then dissolved in the same cold extraction buffer used for tissue homogenisation. Again two separate 20 mg rat muscle samples were prepared in this manner. The four homogenates were placed on an orbital shaker for an hour at 4°C. Each sample was then further homogenised by passing it 20 times through a 1 ml syringe with 22 gauge needle and then 20 times through a 1 ml syringe with a 25 gauge needle. The four protein homogenates prepared by the two different methods were then centrifuged (Eppendorf centrifuge 5415D) at 600 rpm, 4°C, for 15 min and the supernatant was then measured for COX activity spectrophotometrically [14]. This experiment was further repeated two more times and Figure 5.3(a) represents the results from one experiment. It was observed that there was less variability in COX activity

measured in homogenate prepared using a homogeniser compared to the liquid nitrogen and greater activity of COX enzyme in the rat muscle tissue homogenised with tissue homogeniser (0.059 ± 0.001 , $n=3$) compared to the liquid nitrogen (0.04 ± 0.04 , $n=3$). On this basis tissue homogeniser was chosen for disrupting the muscle tissue.

How long the tissue homogeniser should be used to get optimum extraction of proteins was determined by trialling 10, 20, 30, 40 and 60 second bursts of homogenisation three times with 10 second intervals in between each burst as previously done. This experiment was repeated three times and Figure 5.3(b) represents the results of one experiment. Table 5.1 shows the protein concentration and COX activity determined for the 10 s, 20 s, 30 s, 40 s and 60 s homogenisation times.

Table 5.1: Protein Concentration and COX activity for skeletal muscle tissue homogenised for different periods of time.

Homogenisation times	Protein Concentration (mg/ml)	COX activity (units/ml)
10 s	2.17 ± 0.03	0.037 ± 0.002
20 s	2.30 ± 0.02	0.060 ± 0.001
30 s	2.50 ± 0.03	0.059 ± 0.002
40 s	2.23 ± 0.02	0.042 ± 0.002
60 s	1.96 ± 0.01	0.005 ± 0.001

Data expressed as mean \pm SD ($n = 3$). Protein concentration was determined using the standard BCA assay. COX activity at 25°C was determined spectrophotometrically [14], with absorbance measured every 30 seconds at 550 nm for 10 min. units/ml represents $\mu\text{mol}/\text{min}/\text{ml}/\text{mg}$ of protein.

Twenty second and 30 second homogenisation bursts exhibited greatest COX activity (Table 5.1). The protein concentration for the 30s burst was slightly higher than 20 s burst however they exhibited similar COX activity. Based on these results the 3 x 20 second homogeniser bursts with 10 second intervals in between the bursts was chosen as the best time period for disruption of the muscle.

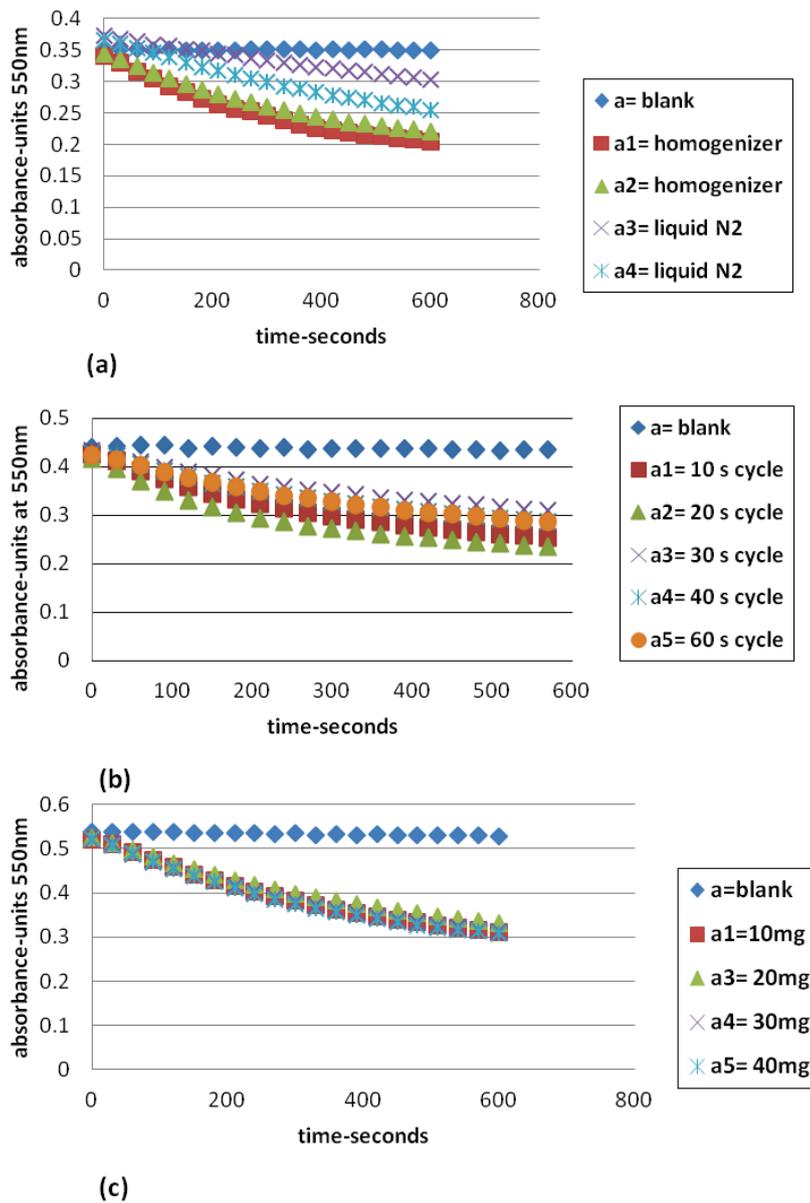


Figure 5.3: Optimisation of method for protein extraction. Detection of COX activity in muscle protein homogenate was used to examine best optimised procedure for extraction of protein. COX activity at 25 °C was determined spectrophotometrically [14], with absorbance measured every 30 seconds at 550 nm for 10 min. (a) Examination of best homogenisation technique with tissue homogeniser being compared to muscle crushed using liquid nitrogen. (b) Examination of best homogenisation time (10, 20, 30, 40 and 60 s burst cycles 3x with 10 s interval in between each burst) for disruption of muscle tissue. (c) Examination of amount of muscle tissue (10 – 40 mg) to be used for optimal COX activity.

5.3.1.2 Determination of amount of tissue homogenised

With the technique for disruption of the muscle established the next thing to determine was amount of muscle tissue to be homogenised. Different amounts of rat skeletal muscle, ranging from 10 mg - 40 mg were homogenised for 20 seconds three times with 10 second intervals between each burst. Protein concentration and COX activity was measured in each protein homogenate prepared. This experiment was repeated three times. Figure 5.3(c) represents results for one experiment. The mean COX activity and protein concentration for the three experiments are presented in Table 5.2. All four protein extracts provided protein concentration ranging from 2.10 mg/ml to 2.44 mg/ml (Table 5.2). COX activity in the protein homogenates ranged from 0.06 ± 0.03 to 0.09 ± 0.04 units/ml (Table 5.2). The 20 mg, 30 mg and 40 mg tissue showed almost similar protein concentration and COX activity while the concentration of protein and activity for 10 mg of tissue was very low. Therefore the median range of 20-25 mg of skeletal muscle was chosen for further experiments. This experiment was done on the rat tissue only and was not repeated with human tissue because of the limited availability of the tissue.

Table 5.2: Protein concentration and COX activity in rat skeletal muscle

Amount of tissue	Protein Concentration (mg/ml)	COX activity (units/ml)
10 mg	2.10 ± 0.17	0.06 ± 0.03
20 mg	2.33 ± 0.15	0.09 ± 0.02
30 mg	2.44 ± 0.13	0.09 ± 0.03
40 mg	2.26 ± 0.16	0.08 ± 0.04

Data expressed as mean \pm SD (n = 3). Protein concentration was determined using the standard BCA assay. COX activity at 25 °C was determined spectrophotometrically [14], with absorbance measured every 30 seconds at 550 nm for 10 min. COX activity is expressed as units/ml which represents $\mu\text{mol}/\text{min}/\text{ml}/\text{mg}$ of protein.

5.3.1.3 Selection of the right detergent

For the three enzymes to be measured in this PhD study, all three standard enzyme assays either used no detergent (for COX and BHAD) [11, 13] or Triton X100 detergent for CS assay. As mentioned previously, there was limited SPIRIT study muscle tissue for analysis of enzyme activity so the aim for this study was to have one protein homogenate that could be used to assay all three enzymes of interest. This meant that to achieve maximal activity, one detergent that did not interfere with enzyme activity had to be found. Six detergents were investigated (Figure 5.4). The results for CHAP and SDS are not shown as COX activity was not detectable, and Tween-80 was very low activity so these detergents were excluded from further experiments when these detergents were used. The other four detergents, Triton X100, Tween-20, and Brij-35 were examined. For this experiment, 5 µl of 1% detergent (Triton X100 or Tween-20 or Brij-35) was added to the extraction buffer (section 5.3.1.1), protein was extracted from 20 mg of rat muscle tissue using the optimised procedures discussed in sections 5.3.1.1 and 5.3.1.2, and COX enzyme activity was assayed. In this experiment the 1st pellet from the protein homogenate (1st supernatant) was resuspended in extraction buffer with appropriate detergent and centrifuged again (Figure 5.4). The 2nd supernatant and 2nd pellet along with the 1st supernatant were measured for protein concentration and COX activity (Table 5.3). This experiment was repeated three times. Figure 5.5(a) represents the results for one experiment and shows the activity for the 1st supernatant.

As seen in Table 5.3, Brij-35 and Tween-20 were the detergents that provided the best results having maximum COX activity (0.35 units/ml and 0.31 units/ml respectively for the first supernatant). Extraction buffer containing Brij-35 exhibited greatest concentration of protein. Comparing the results in Table 5.2, where no detergent has been used in the extraction buffer, to those in Table 5.3, where detergent has been used in extraction buffer, it can be seen that extraction of protein from rat skeletal muscle is enhanced in the presence of detergent. It was clear that for all detergents used, the first pellet (Figure 5.4) needs to be processed again for extraction of further protein (Table 5.3). It was extracted by adding 100µl of extraction buffer and after homogenisation and centrifugation the second extract was obtained. The activity assay was performed on the second supernatant. Activity of 0.21 units/ml was detected in the 2nd Brij-35 appeared to be the best detergent in obtaining maximal COX activity in rat skeletal muscle tissue

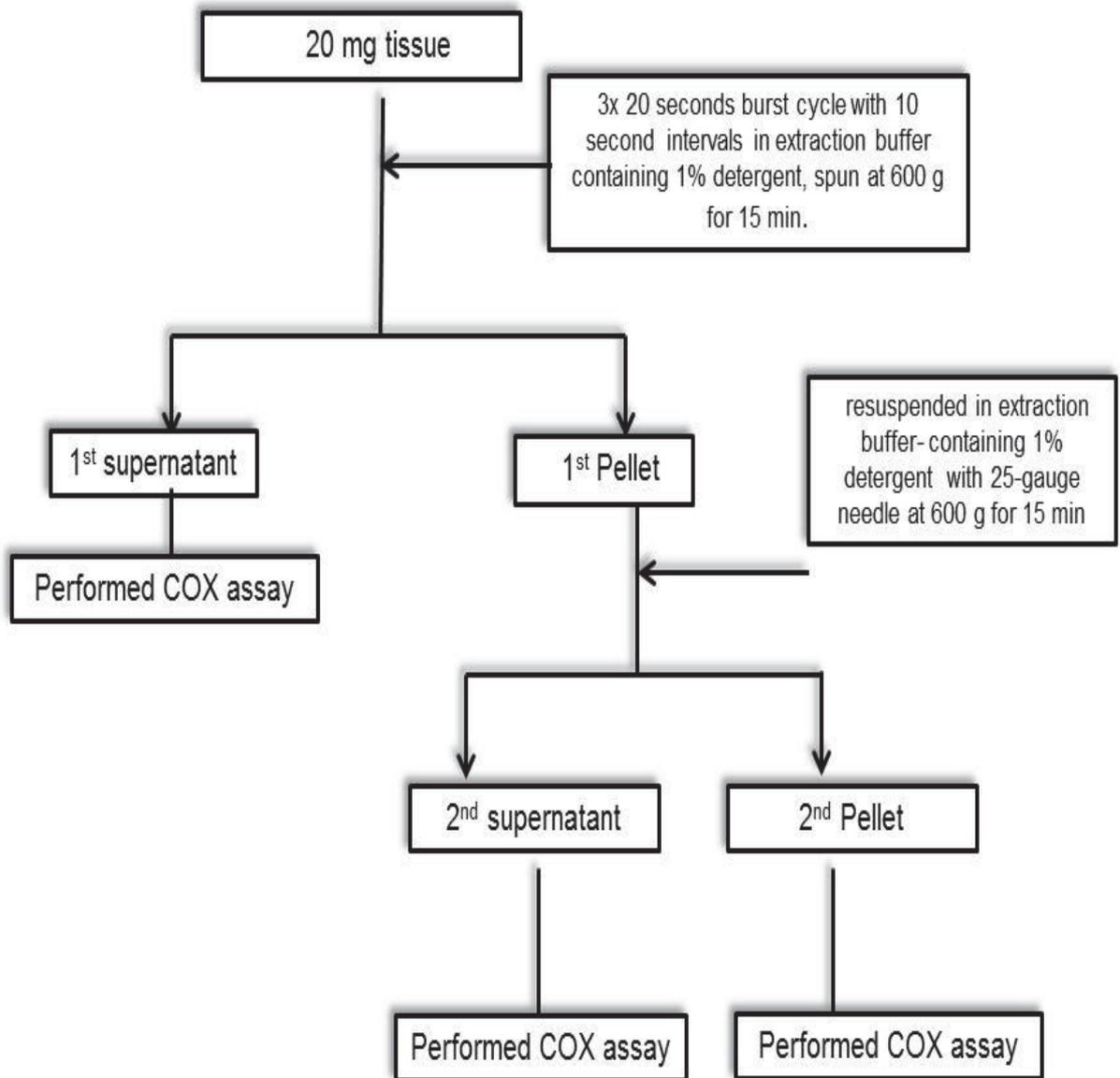


Figure 5.4: Flow diagram showing optimisation step by step process for the selection of most suitable detergent for all three assays. The second pellet was resuspended in extraction buffer containing 1% detergent and syringed 20 times through 25 gauge needle and then spun at 600 g for 15 minutes.

Table 5.3: Effect of different detergents on protein concentration and COX activity

Detergent Used	Muscle Protein Homogenate	Protein Concentration (mg/ml)	COX activity (units/ml)
Brij-35	<i>1st supernatant</i>	2.52 ± 0.31	0.35 ± 0.02
	<i>2nd supernatant</i>	0.81 ± 0.02	0.21 ± 0.02
	<i>2nd pellet</i>	0.32 ± 0.00	0.01 ± 0.01
Tween-20	<i>1st supernatant</i>	2.04 ± 0.34	0.31 ± 0.29
	<i>2nd supernatant</i>	0.88 ± 0.02	0.04 ± 0.02
	<i>2nd pellet</i>	0.044 ± 0.002	0.036 ± 0.002
Triton X100	<i>1st supernatant</i>	1.86 ± 0.29	0.16 ± 0.12
	<i>2nd supernatant</i>	0.25 ± 0.02	0.076 ± 0.02
	<i>2nd pellet</i>	0.21 ± 0.02	0.036 ± 0.002

Data expressed as mean ± SD (n = 3). Protein concentration was determined using the standard BCA assay. COX activity at 25 °C was determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. COX activity is expressed as units/ml which represents µmol/min/ml/mg protein.

Although rat muscle protein homogenate using Brij-35 was showing best results for COX activity, it was important to establish that Brij-35 did not interfere with the other mitochondrial enzyme assays of CS and BHAD. The rat muscle protein supernatants prepared for the previous experiment (results shown in Figure 5.5(a) and Table 5.3) were used for determining both CS activity (Figure 5.5(b)) and BHAD activity (Figure 5.8(c)). For each enzyme, the experiment was repeated three times and the assay results for one experiment are shown in Figure 5.5. The mean activities for the three enzymes using the different detergents for extraction of the protein from rat muscle are shown in Table 5.4.

Table 5.4: The effect of different detergents on the activities of three mitochondrial enzymes in rat skeletal muscle

Detergent	Total COX activity (units/ml)	Total BHAD activity (units/ml)	Total CS activity (units/ml)
Brij-35	0.52 ± 0.02	0.015 ± 0.010	0.14 ± 0.01
Tween-20	0.35 ± 0.16	0.009 ± 0.020	0.18 ± 0.01
Triton X100	0.24 ± 0.07	no activity	0.17 ± 0.08

Data expressed as mean ± SD (n = 3); COX represents cytochrome c oxidase; CS represents citrate synthase and BHAD represents beta-hydroxyacyl-CoA dehydrogenase. Enzyme activity has been determined from combined supernatant (The combined supernatant was formed by combining the 1st and 2nd supernatant as shown in Figure 5.4 and is expressed as units/ml which represents $\mu\text{mole}/\text{min}/\text{ml}/\text{mg}$ of protein).

As can be seen in Figure 5.5 and Table 5.4 both COX activity and CS activity was detected using all three detergents in the extraction buffer. BHAD activity was only detected in the presence of Brij-35 and Tween-20 indicating that Triton X100 interfered with the BHAD assay. Muscle homogenate with Brij-35 detergent showed greatest BHAD activity results (0.015 ± 0.010) as compared to Tween-20.

For CS activity, Triton X100 appeared to show greater variation between the three experiments. Tween-20 seemed to lead to greater CS activity however the activity when using Brij-35 was still in the same magnitude of activity observed for Tween-20. For BHAD activity Tween-20 in the extraction buffer seemed to lead to a 60% drop in activity compared Brij-35 in the extraction buffer. Due to this, Brij-35 was selected as the best detergent to use in the extraction buffer as all three enzyme activities were able to be measured with good enzyme activity being exhibited.

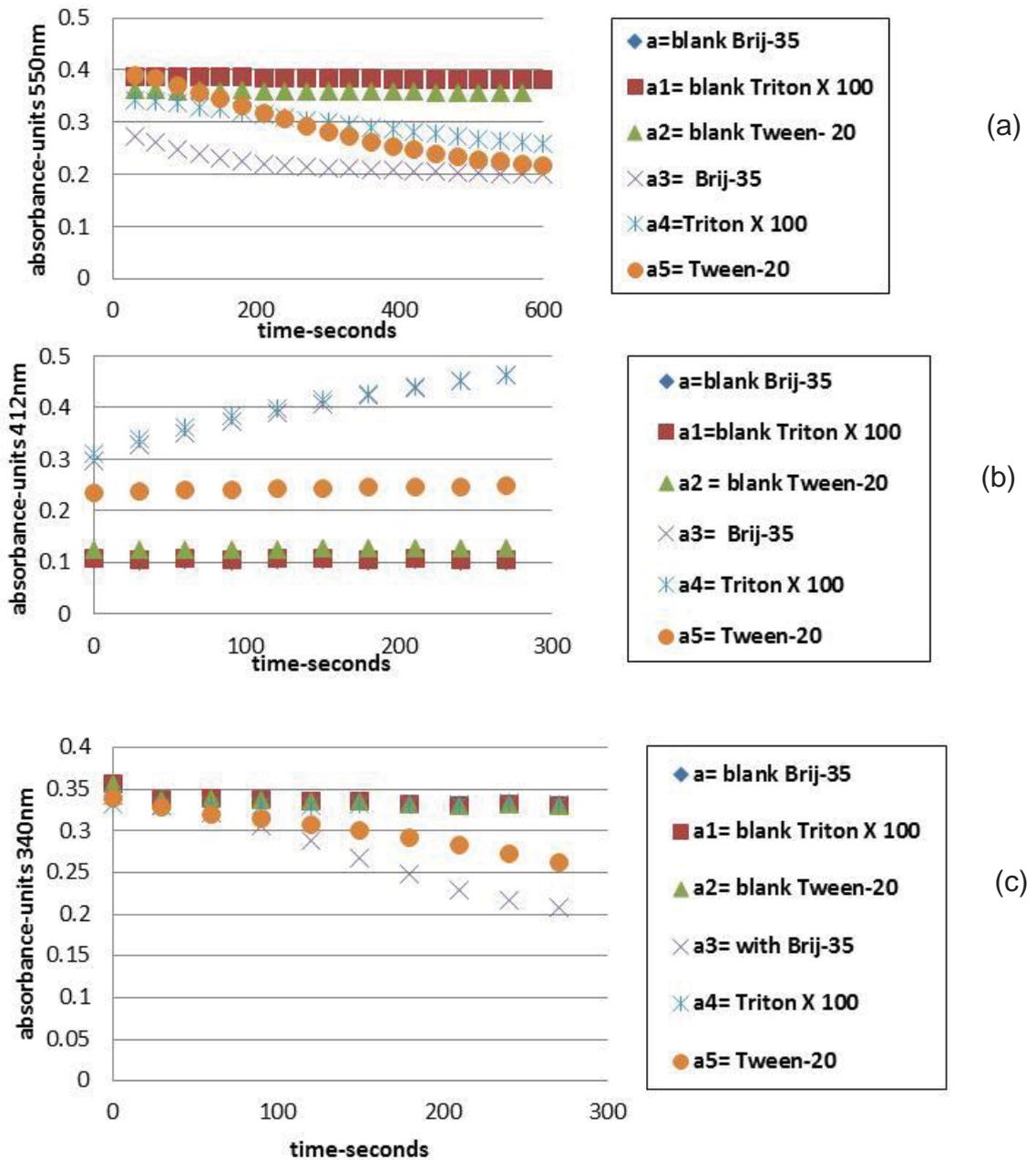


Figure 5.5: Determination of most suitable detergent for all three enzyme assays. Since three different detergents were used, three blanks were prepared for each detergent i.e. the blank was extraction buffer containing 1% of detergent. (a) COX activity [11] was measured at 25 °C every 30 seconds for 10 min with absorbance measured at 550 nm (b) CS activity [12] was measured at 25 °C every 30 seconds for 5 min with absorbance measured at 412 nm. (c) BHAD activity [13] was measured at 25 °C every 30 seconds for 5 min with absorbance measured at 340 nm.

The last experiment in optimisation of the protein extraction method was to examine how much Brij-35 should be used in the extraction buffer. The different concentrations of Brij-35 used ranged from 1% - 2.5%. The experiment was repeated three times and Figure 5.6 represents the results for one experiment. Table 5.5 presents the protein concentration and COX activity results for this experiment.

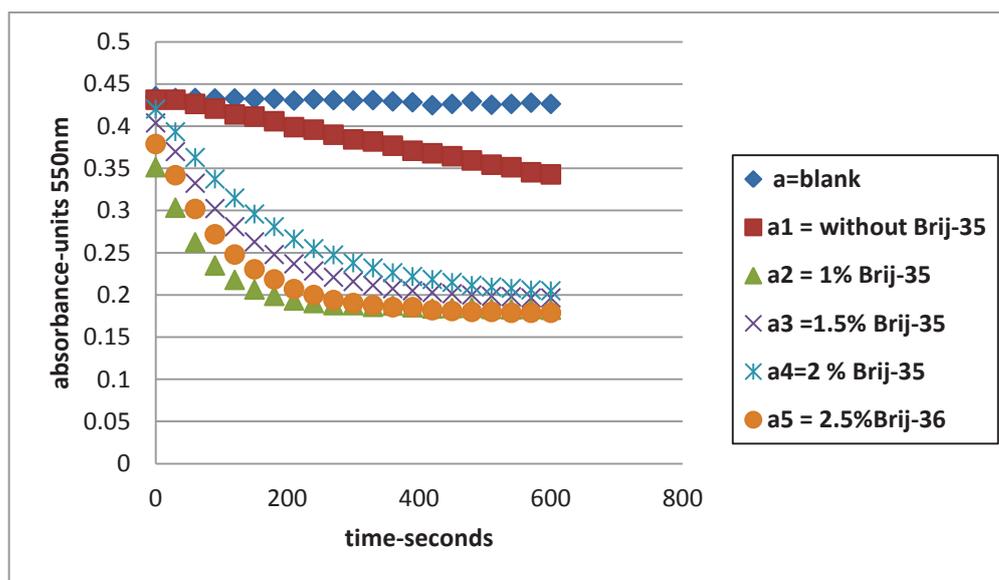


Figure 5.6: Determination of suitable concentration of Brij-35 in extraction buffer. COX activity at 25 °C was determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. Blank was extraction buffer containing 1% of Brij-35.

Table 5.5: Protein concentration and COX activity using different concentrations of Brij-35 in the extraction buffer

Percentage Brij-35	Protein Concentration (mg/ml)	COX activity (units/ml)
0%	2.17 ±0.001	0.037±0.001
1%	2.84 ±0.001	0.31±0.001
1.5%	2.82 ±0.002	0.19±0.002
2 %	2.80 ±0.001	0.17±0.001
2.5%	2.81 ±0.001	0.019±0.001

Data expressed as mean ± SD (n = 3). Protein concentration was determined using the standard BCA assay. COX activity at 25 °C was determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. COX activity is expressed as units/ml which represents $\mu\text{mole}/\text{min}/\text{ml}/\text{mg}$ of protein.

The results in Table 5.5 show that 1% Brij-35 is a suitable concentration of the detergent to use for extraction of protein from the muscle and measurement of optimal COX activity.

5.3.2 Optimisation of cytochrome oxidase activity

The optimisation of COX assay was simultaneously done with the optimisation of protein extraction technique (section 5.3.1). The final optimised COX enzyme assay is described in Chapter 3 section 3.3 and was adopted from [11]. COX activity is determined by a colorimetric assay. The change in colour of ferrocytochrome c (pale purple red) caused by its oxidation to ferricytochrome c (pale orange red) as catalysed by cytochrome c oxidase. The assay was performed at 25 °C and absorbance was measured at 550 nm wavelength every 30 seconds for 10 min. The important things for the optimisation of this assay were a) preparation of reduced cytochrome c b) to determine the volume of reduced cytochrome c to be used in the assay c) the amount of muscle sample to be used d) the linearity of the assay e) reproducibility of the assay and f) determination of intra and inter-assay CV (Chapter 3 section 3.4.3) for the COX assay to ensure reliability in repeatability of the assay. These assays were optimised on rat tissue, and tested on spare human tissue before being performed on the SPIRIT study participant muscle tissue.

5.3.2.1 Cytochrome c

The method used for reducing cytochrome c was the Sigma-Aldrich protocol [17] of adding 5µl of 0.1M DTT in 2.7 mg/ml of cytochrome c in phosphate buffer (0.05M potassium phosphate, pH 7.4) and leaving the solution mixing at room temperature. The ratio of absorbance at 550 and 565 nm needs to be between 10 and 20 as this represents cytochrome c in its reduced form. An experiment comparing COX activity determined from four freshly prepared reduced cytochrome c solutions (solution left mixing at room temp for 1 hour) to four overnight prepared reduced cytochrome c solutions was performed (Figure 5.7). Figure 5.7 is a representative example of the result of one experiment and this experiment was repeated three times. The 1 hour freshly prepared cytochrome c provided less COX activity (0.002 ± 0.0002 units/ml) compared to using the overnight prepared cytochrome c (0.019 ± 0.001 units/ml) indicating that the overnight prepared cytochrome c provided a higher percentage of reduced cytochrome c (the substrate needed for COX assay – see Chapter 3 section 3.4.2.2).

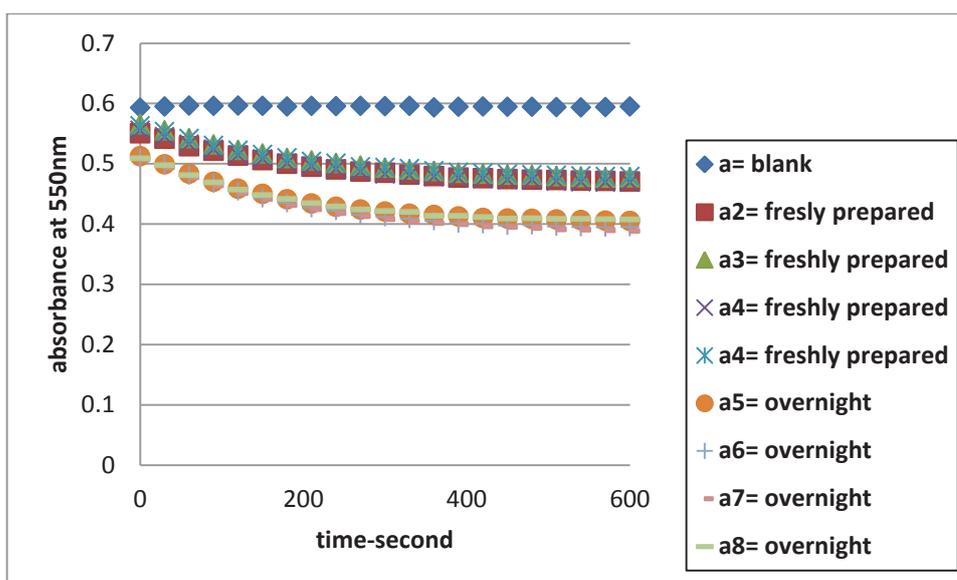


Figure 5.7: Comparison of COX activity using freshly prepared cytochrome c or overnight prepared cytochrome c. COX activity at 25 °C was determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. The same protein homogenate was used for the 1 hour freshly prepared cytochrome c and overnight prepared cytochrome c solutions. Blank was extraction buffer containing 1% Brij-35.

On comparing the A550/A565 ratio the 1 hour freshly prepared cytochrome c samples were 6.0 ± 1.24 (n=4), and the overnight prepared samples were 13.6 ± 0.46 (n=4). The overnight prepared cytochrome c solution had an A550/A565 ratio between 10 and 20 and hence indicative of fully reduced cytochrome c.

An experiment was performed to investigate how long to leave the cytochrome c and DTT solution mixing to obtain an A550/A565 ratio greater than 10. The A550/A565 ratio was compared for cytochrome c and DTT solutions kept for 1, 2, 3, 4, 5 and 6 hours and overnight (Table 5.6).

It was obvious that to obtain reduced cytochrome c suitable for measuring optimal COX activity, the cytochrome c had to be left overnight to obtain greatest reduction (Table 5.6). Freezing of the overnight prepared cytochrome c sample led to a decrease in A550/A565 ratio. This meant that the overnight prepared reduced cytochrome c sample had to be used immediately for determining COX activity

Table 5.6: Different periods of time for preparing reduced cytochrome c

Time	550/565 ratio
1 hours	6.0 ± 1.24
2 hours	6.3 ± 0.40
3 hours	6.7 ± 0.81
4 hours	7.0 ± 0.81
5 hours	7.0 ± 0.91
6 hours	8.0 ± 0.47
overnight	13.6 ± 0.46

For each time point a solution of cytochrome c and DTT was left mixing for the time indicated in the Table. The absorbance at 550 nm and 565 nm was measured at the time point conclusion. Three different solutions were prepared for each time point and data was expressed as mean ± SD (n = 3).

The next step was to determine the amount of reduced cytochrome c needed for maximal COX activity. An experiment measuring COX activity, in 10 µl of rat muscle homogenate, by adding different amounts of cytochrome c (0 µl up to 60 µl) was performed. An example of the results for one experiment is shown in Figure 5.8. This experiment was repeated three times. As can be seen in Figure 5.8, 10 µl of reduced cytochrome c is not suitable for determining maximal COX activity, whereas 20 µl – 60 µl is, with 40 µl, being the best. On this basis 40 µl of overnight prepared cytochrome c was used in the final optimised COX assay (Chapter 3 section 3.4.2.2).

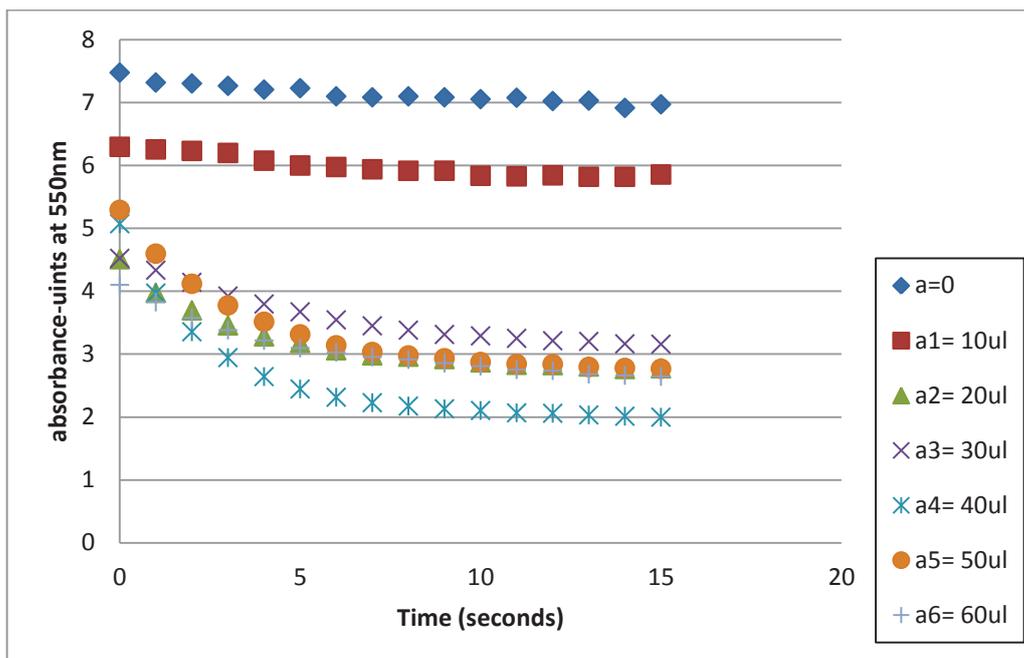


Figure 5.8: Effect of different volumes of reduced cytochrome c on COX activity in rat muscle homogenate. COX activity was measured in 10 μ l of rat muscle homogenate using 0 (blank), 10, 20, 30, 40, 50 and 60 μ l of overnight prepared cytochrome c as substrate. COX activity was measured at 25 $^{\circ}$ C and determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min.

5.3.2.2 Investigation of Linearity, Reproducibility and Precision of the COX Assay

An experiment to determine the amount of rat muscle homogenate to use for determining maximal COX activity was performed. Increasing volume of muscle protein homogenate (2.5 μ l to 15 μ l) with 40 μ l reduced cytochrome c was used for determining COX activity. This experiment was repeated three times and Figure 5.9(a) represents results from one experiment. Best activity was observed with 10 μ l (0.19 ± 0.001 , $n=3$) and 15 μ l (0.18 ± 0.002 , $n=3$) of muscle protein homogenate (a4 and a5 in Figure 5.9(a)) compared to 2.5 μ l (0.10 ± 0.02 , $n=3$) of muscle protein homogenate (a1 in Figure 5.9(a)). It was decided that 10 μ l muscle protein homogenate would be used in the final optimised assay (Chapter 3 section 3.4.2.2).

With the final COX assay now optimised using rat muscle homogenate, reproducibility and linearity of the assay needed to be investigated and examination of the assay in human tissue, before embarking on determining COX activity in the SPIRIT study participant muscle biopsy sample.

An experiment using the optimised conditions now established for COX activity was performed, where COX activity was measured in quadruplet using 10 μl of the same rat muscle protein homogenate (Figure 5.9(b)). As can be seen just by visualisation of the activity curves in Figure 5.9(b) the assay is highly reproducible. The intra-assay of coefficient variability (CV) (see Chapter 3 section 3.4.3) for the COX assay was 2.10%, The inter-assay coefficient of variability (CV) (see Chapter 3 section 3.4.3) for the optimised COX assay is shown in Table 5.7. The average coefficient of variation between microplate to microplate (n=5) was 1.38%.

Table 5.7 Inter-Assay Coefficient of Variability for the COX assay

Mean of means	0.38
Standard deviation	0.005
% CV of means	1.38

The inter-assay CV was measured between five microplates (used for the three experiments examining intra-assay CV)

Enzyme activity gets inhibited by enzyme saturation therefore it was important to make sure that the selected amount of muscle homogenate used in the enzyme assay allows enzyme activity to be in the linear portion of enzyme activity curve and to ensure that enzyme saturation has not been reached. To ensure COX activity being measured in the 10 μl of muscle protein homogenate was in the linear portion of the enzyme progress curve, COX activity was determined using increasing amounts of muscle protein homogenate (Figure 5.9(c)).

As can be seen in Fig 5.9(c) 5 μl muscle homogenate (COX activity= 0.031 ± 0.01 $\mu\text{mole}/\text{min}/\text{ml}/\text{mg}$; n=3) and 10 μl of (0.067 ± 0.002 $\mu\text{mole}/\text{min}/\text{ml}/\text{mg}$ (n=3)) are in the linear portion of the COX enzyme activity curve and saturation of enzyme activity is not occurring.

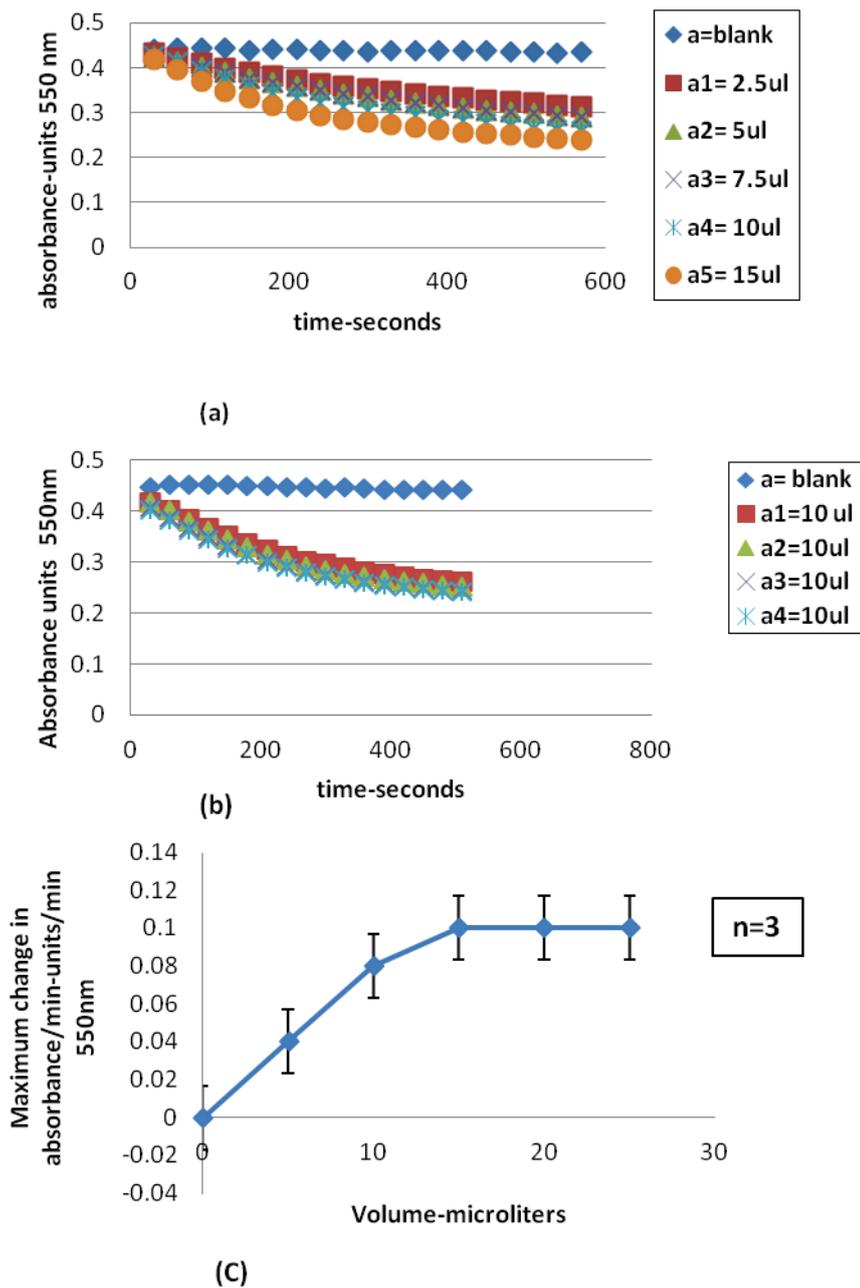


Figure 5.9: Three different experiments measuring COX activity to (a) determine suitable amount of muscle protein homogenate for the assay (b) examine reproducibility of the assay and (c) validate linearity of the assay. COX activity was measured in 10 μl of rat muscle homogenate for (a) and (b) and 5, 10, 15, 20 and 25 μl in (c), using 40 μl of overnight prepared cytochrome c as substrate. COX activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. The blank contained the extraction buffer in the same quantity of muscle homogenate plus all the reagents of the assay.

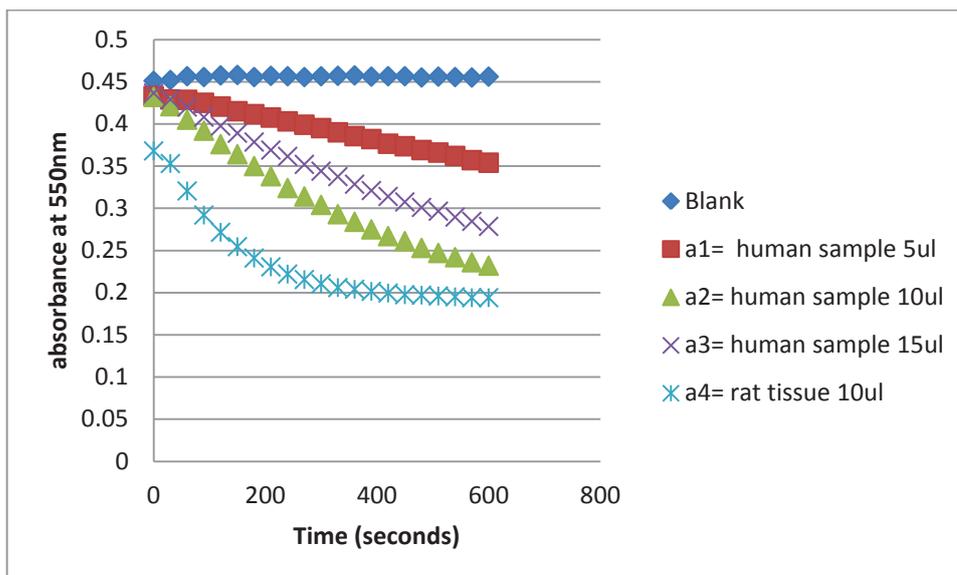


Figure 5.10: Comparison of activity in rat and human muscle protein homogenate. COX activity was measured in 10 μl of rat muscle homogenate and 5, 10 and 15 μl human muscle homogenate using 40 μl of overnight prepared cytochrome c as substrate. COX activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. The blank contained the extraction buffer in the same quantity of muscle homogenate plus all the reagents of the assay.

The final optimised COX assay needed to be tested in human tissue. The spare human tissue was used to test the assay. Protein homogenate was extracted from the spare human muscle tissue following the optimised protein extraction technique previously described in section 5.3.1. The COX assay was performed with increasing amounts of human muscle protein homogenate (5 to 25 μl) and 10 μl rat muscle protein homogenate was used as positive control for the comparison. Figure 5.10 represents results for one experiment and this experiment was done in triplicate. Table 5.8 shows the mean COX activity results for the three experiments.

Table 5.8: Cytochrome c oxidase (COX) activity in human and rat skeletal muscle

Sample	COX activity (units/ml)
Rat muscle homogenate (10 μ l)	0.06 \pm 0.012
Human muscle homogenate:	
5 μ l	0.03 \pm 0.001
10 μ l	0.079 \pm 0.012
15 μ l	0.076 \pm 0.012
20 μ l	0.073 \pm 0.012
25 μ l	0.075 \pm 0.012

Data expressed as mean \pm SD ($n = 3$). COX activity was measured in 10 μ l of rat muscle protein homogenate (positive control) and 5, 10, 15, 20, 25 μ l of human muscle protein homogenate. COX activity was measured at 25 $^{\circ}$ C and determined spectrophotometrically [11] with absorbance measured every 30 seconds at 550 nm for 10 min. COX activity is expressed as units/ml which represents μ mole/min/ml/mg of protein.

As shown in Table 5.8 the activity of 10 μ l human muscle protein homogenate was greatest and this was the amount used in the final optimised COX assay for human muscle tissue. The COX activity results for the SPIRT study cohort are presented in section 5.3.5.

5.3.3 Optimisation of Citrate Synthase Assay

The final optimised citrate synthase (CS) enzyme assay is described in Chapter 3 section 3.4.2.1 and was adapted from Huber *et al* [12]. CS activity is determined by a spectrophotometric assay where conversion of oxaloacetate to citrate is detected by measurement of absorbance at 412 nm wavelength every 30 seconds for 5 or 10 minutes. The important things for the optimisation of the CS assay were a) determination of the amount of muscle protein homogenate to use in the assay and b) the linearity, reproducibility and inter-assay and intra-assay coefficient of variability (CV) of the assay. In order to achieve these aims the standard assay for CS [12] was adapted using the rat muscle tissue first and then the spare human muscle tissue before measuring the CS activity in the SPIRIT study participants' muscle.

To determine the amount of rat muscle homogenate to use in the CS assay an experiment including increasing volume of muscle protein homogenate (5 μ l to 20 μ l) was performed.

This experiment was repeated three times and Figure 5.11(a) represents results from one experiment. CS activity observed with 10 μl (0.08 ± 0.02 units/ml, $n=3$), 15 μl (0.09 ± 0.02 units/ml, $n=3$) and 20 μl (0.10 ± 0.02 units/ml, $n=3$) of muscle protein homogenate provided comparable activity. It was decided that 10 μl muscle protein homogenate would be used in the final optimised assay (Chapter 3 section 3.3.2.1) because the multi-channel pipette allowed for quick and easy deliverance of 10 μl of sample to the microplate and it allowed for more assays to be performed (when compared to using 15 μl) which was very important with very little SPIRIT study human muscle tissue for measurement of enzyme activity.

With the final CS assay now optimised using rat homogenate, reproducibility and linearity of the assay (Figure 5.11(a) and (b)) and examination of the assay in human tissue (Figure 5.12 and Table 5.10) needed to be investigated before embarking on determining CS activity in the SPIRIT study participant muscle biopsy sample.

An experiment using the optimised conditions now established for CS activity was performed, where CS activity was measured in triplets shown in Figure 5.11(b) using 10 μl of the same rat tissue homogenate (Figure 5.11(b)). As can be seen just by visualisation of the activity curves in Figure 5.11(b) the assay is highly reproducible. The intra-assay CV (see Chapter 3 section 3.4.3) for the CS assay was 2%. The inter-assay CV (see Chapter 3 section 3.4.3) for the optimised CS assay is shown in Table 5.9. The average coefficient of variation between microplate to microplate ($n=5$) was 3.1%.

Table 5.9: Inter-Assay Coefficient of Variability for the CS assay

Mean of means	0.16
Standard deviation	0.005
% CV of means	3.116

The inter-assay CV was measured between five microplates (used for the three experiments examining intra-assay CV)

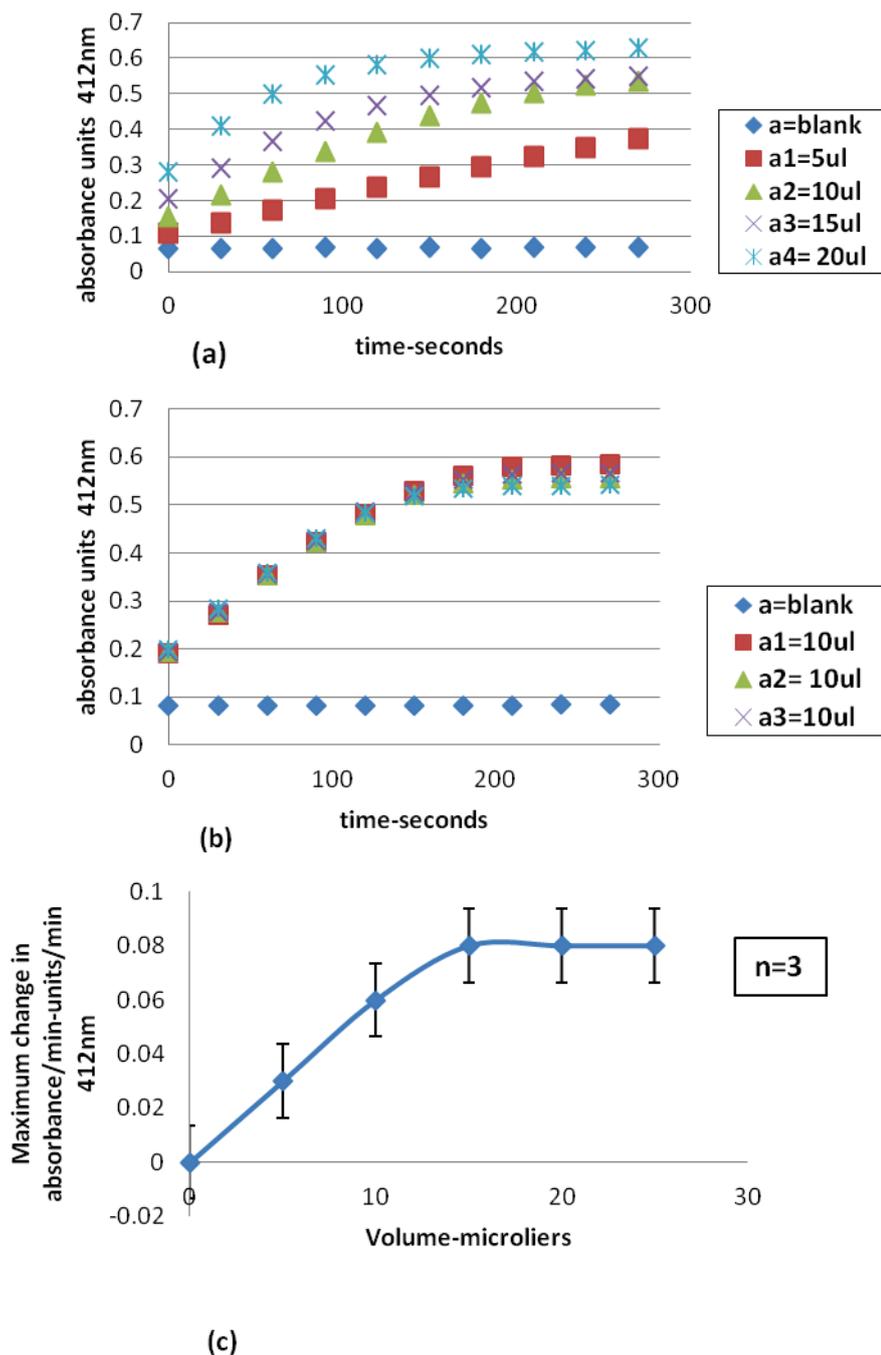


Figure 5.11: Three different experiments measuring CS activity to (a) determine suitable amount of muscle protein homogenate for the assay (b) examine reproducibility of the assay and (c) validate linearity of the assay. CS activity was measured in 5, 10, 15 and 20 μl of rat muscle homogenate for (a), 10 μl of rat muscle homogenate for (b) and 5, 10, 15, 20 and 25 μl rat muscle homogenate for (c). CS activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [12], with absorbance measured every 30 seconds at 412 nm for 5 minutes). 20 μl of 5mM oxaloacetic acid was used as substrate. The blank contained the extraction buffer in the same quantity of muscle homogenate used and all the reagents of the assay.

To ensure CS activity being measured in the 10 μ l of muscle protein homogenate is in the linear portion of the enzyme activity curve, increasing amounts of muscle protein homogenate as shown in Figure 5.11(c) were used to examine CS enzyme activity. The 5 μ l and 10 μ l muscle homogenate exhibited activity in the linear portion of CS activity curve showing 0.03 ± 0.01 units/ml (n=3) and 0.06 ± 0.01 units/ml (n=3) of CS activity respectively whereas at 15, 20 and 25 μ l CS activity was 0.08 ± 0.004 units/ml (n=3), 0.078 ± 0.002 units/ml (n=3), 0.076 ± 0.001 units/ml (n=3) respectively showing enzyme saturation has reached.

The final optimised CS assay needed to be tested in human tissue. The spare human tissue was used to test the assay. Protein homogenate was extracted from the spare human muscle tissue following the optimised protein extraction technique previously described in section 5.3.1. The CS assay was performed with increasing amounts of human muscle protein homogenate (5 to 15 μ l) and 10 μ l of rat muscle protein homogenate was used as a positive control for the comparison. Figure 5.12 represents results for one experiment and this experiment was done in triplicate. Table 5.10 shows the results for the mean CS activity for each homogenate for the three experiments.

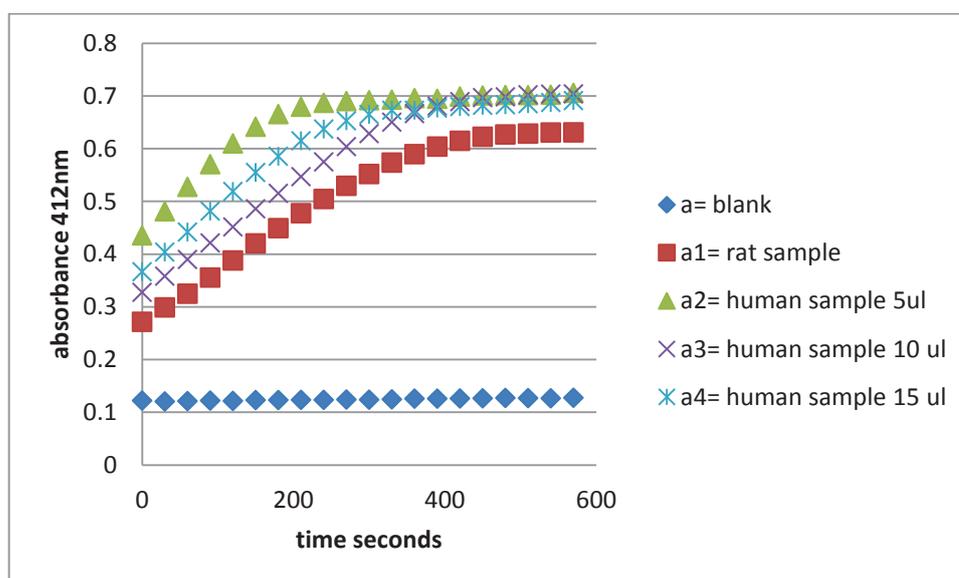


Figure 5.12: Comparison of CS activity in rat and human muscle protein homogenate. CS activity was measured in 10 μ l of rat muscle homogenate and 5, 10 and 15 μ l human muscle homogenate using 20 μ l of 5mM oxaloacetic acid as substrate. CS activity was measured at 25 $^{\circ}$ C and determined spectrophotometrically [11], with absorbance measured every 30 seconds at 412 nm for 10 min. The blank contained extraction buffer in the same quantity as muscle homogenate used and all the reagents of the assay.

Table 5.10: Citrate Synthase (CS) activity in human and rat skeletal muscle

Sample	CS activity (units/ml)
Rat muscle homogenate (10 μ l)	0.10 \pm 0.002
Human muscle homogenate	
5 μ l	0.03 \pm 0.002
10 μ l	0.06 \pm 0.001
15 μ l	0.08 \pm 0.004
20 μ l	0.078 \pm 0.002
25 μ l	0.076 \pm 0.001

Data expressed as mean \pm SD ($n = 3$). CS activity was measured in 10 μ l of rat muscle protein homogenate (positive control) and 5, 10, 15, 20, 25 μ l of human muscle protein homogenate. CS activity was measured at 25 $^{\circ}$ C and determined spectrophotometrically [13] with absorbance measured every 30 seconds at 412 nm for 10 min. 20 μ l of 5mM oxaloacetic acid was used as substrate. Units/ml represents μ mole/min/ml/mg of protein.

The 10 μ l human muscle protein homogenate was the amount used in the final optimised CS assay for human muscle tissue. The CS activity results for the SPIRT study cohort are presented in section 5.3.5.

5.3.4 Optimisation of BHAD

The final optimised BHAD enzyme assay is described in Chapter 3 section 3.4.2.3 and was adapted from [13]. BHAD activity is determined by a spectrophotometric assay where conversion of S-acetoacetyl-CoA to β -hydroxybutyryl-CoA is detected by measurement of absorbance at 340 nm wavelength every 30 seconds for 5 or 10 minutes. The important things for the optimisation of the BHAD assay were a) determination of the amount of muscle protein homogenate to use in the assay and b) the linearity, reproducibility and inter-assay and intra-assay coefficient of variability (CV) of the assay. In order to achieve these aims the standard assay for BHAD was adapted using the rat muscle tissue first and then the spare human muscle tissue before measuring the BHAD activity in the SPIRT study participants' muscle.

To determine the amount of rat muscle homogenate to use in the BHAD assay an experiment including increasing volume of muscle protein homogenate (7.0 μ l to 15 μ l) was performed. This experiment was repeated three times and Figure 5.13(a) represents results from one experiment. BHAD activity observed with 7 μ l (0.014 ± 0.002 units/ml, n=3), 10 μ l (0.015 ± 0.001 units ml, n=3) and 15 μ l (0.014 ± 0.001 units/ml, n=3) of muscle protein homogenate provided comparable activity. It was decided that 7 μ l muscle protein homogenate would be used in the final optimised assay (Chapter 3 section 3.4.2.3) because it allowed for more assays to be performed which was very important with very little SPIRIT study human muscle tissue for measurement of enzyme activity.

With the final BHAD assay now optimised using rat muscle homogenate, reproducibility and linearity of the assay (Figure 5.13) as well as examination of the assay in human tissue (Figure 5.14 and Table 5.12) needed to be investigated before embarking on determining BHAD activity in the SPIRIT study participant muscle biopsy sample.

An experiment using the optimised conditions now established for BHAD activity was performed, where BHAD activity was measured in quintuplet using 7 μ l of the same rat muscle homogenate (Figure 5.13(b)). As can be seen just by visualisation of the activity curves in Figure 5.13(b) the assay is highly reproducible. The intra-assay CV (see Chapter 3 section 3.4.3) for the BHAD assay was 2%. The inter-assay CV (see Chapter 3 section 3.3.3) for the optimised BHAD assay is shown in Table 5.11. The average coefficient of variation between microplate to microplate (n=5) was 3.65%.

Table 5.11: Inter-Assay Coefficient of Variability for the BHAD assay.

Mean of means	0.43
Standard deviation	0.015
% CV of means	3.65

The inter-assay CV was measured between five microplates (used for the three experiments examining intra-assay CV)

To ensure BHAD activity being measured in the 7 μ l of muscle protein homogenate is in the linear portion of the BHAD enzyme progress curve, increasing amounts of muscle protein homogenate as shown in Figure 5.13(c) were used to examine BHAD enzyme activity. The 7 μ l, 10 μ l and 12 μ l muscle homogenate exhibited BHAD activity in the linear portion of the BHAD activity curve showing 0.14 ± 0.01 units/ml (n=3) 0.027 ± 0.0001 units/ ml (n=3) and 0.04 ± 0.0001 units/ ml (n=3) BHAD activity respectively indicating saturation of BHAD enzyme had not been reached as compared to 15 and 17 μ l of muscle homogenate where the activities of enzyme are as follows) 0.06 ± 0.0001 units/ ml (n=3) and 0.056 ± 0.0001 units/ ml (n=3).

The final optimised BHAD assay needed to be tested in human tissue. The spare human tissue was used to test the assay. Protein homogenate was extracted from the spare human muscle tissue following the optimised protein extraction technique previously described in section 5.3.1. The BHAD assay was performed with increasing amounts of human muscle protein homogenate (5 to 15 μ l) and 10 μ l of rat muscle protein homogenate was used as a positive control for the comparison. Figure 5.14 represents results for one experiment and this experiment was done in triplicate. Table 5.12 shows the results for the mean BHAD activity for each homogenate for the three experiments.

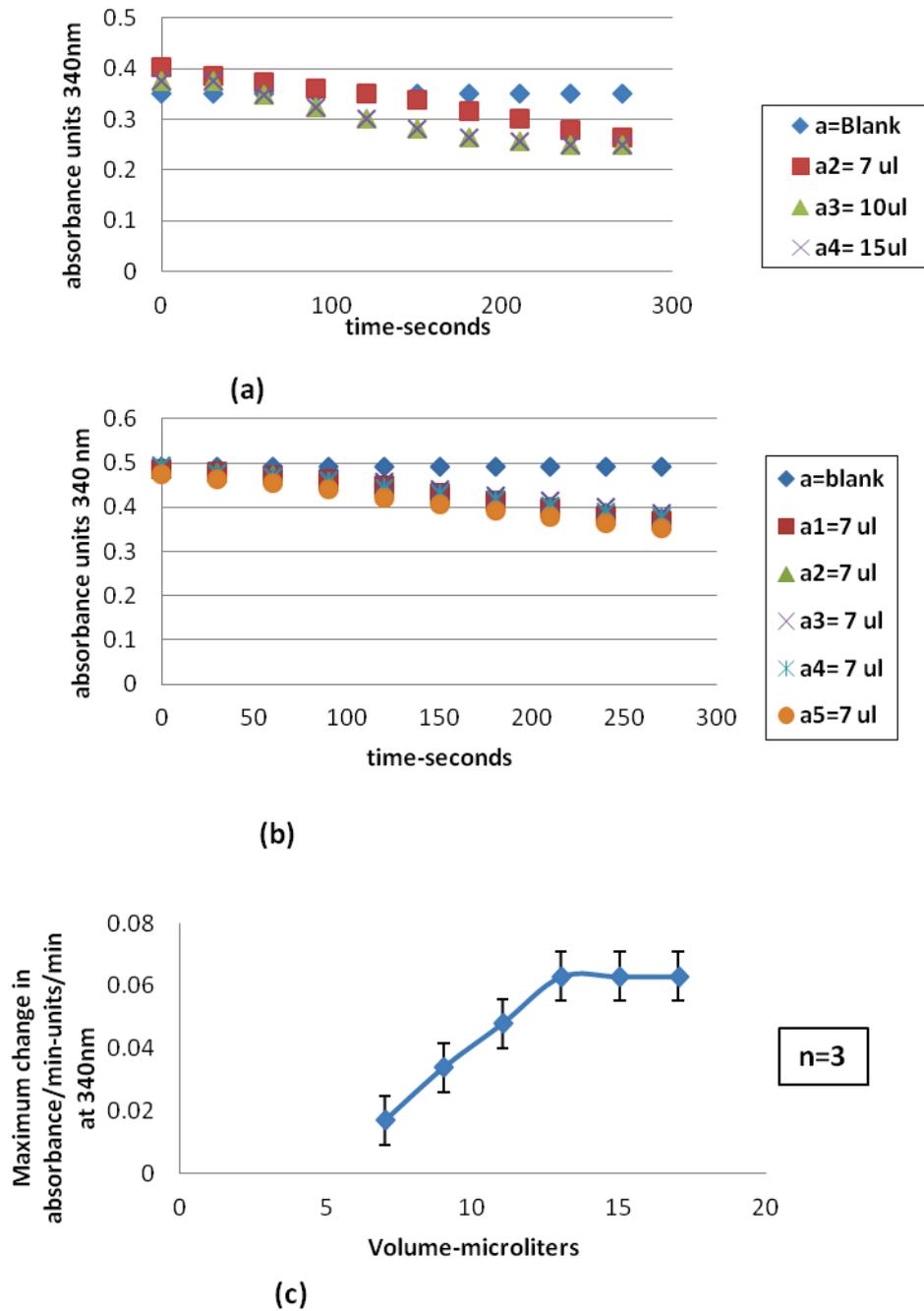


Figure 5.13: Three different experiments measuring BHAD activity to (a) determine suitable amount of muscle protein homogenate for the assay (b) examine reproducibility of the assay and (c) validate linearity of the assay. BHAD activity was measured in 7, 10 and 15 μl of rat muscle homogenate for (a), 7 μl of rat muscle homogenate for (b) and 7, 9, 12, 15 and 17 μl rat muscle homogenate for (c). BHAD activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [13], with absorbance measured every 30 seconds at 340 nm for 5 minutes. 5.4 mM S-acetoacetyl CoA was used as substrate. The blank contained the extraction buffer in the same quantity of muscle homogenate used and all the reagents of the assay.

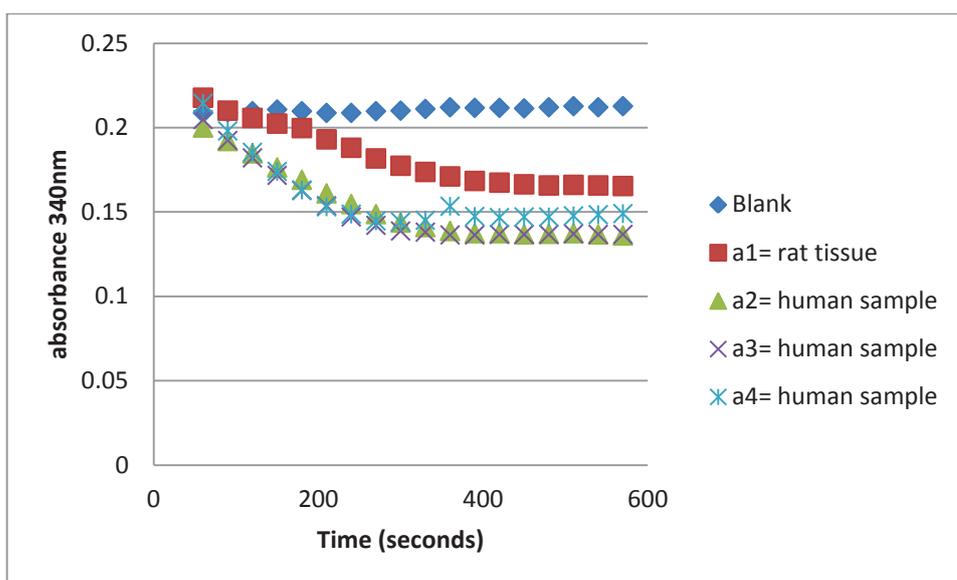


Figure 5.14: Comparison of BHAD activity in rat and human muscle protein homogenate. BHAD activity was measured in 10 μl of rat muscle homogenate and 7, 10 and 15 μl human muscle homogenate using 5.4 mM S-acetoacetyl CoA as substrate. BHAD activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [13], with absorbance measured every 30 seconds at 340 nm for 10 min. The blank contained the extraction buffer in the same quantity of muscle homogenate used and all the reagents of the assay.

Table 5.12: Beta-hydroxyacyl-CoA dehydrogenase (BHAD) activity in human and rat skeletal muscle

Sample	BHAD activity (units/ml)
Rat muscle homogenate (10 μl)	0.0140 \pm 0.0002
Human muscle homogenate	
7 μl	0.0140 \pm 0.0001
10 μl	0.0250 \pm 0.0001
12 μl	0.04 \pm 0.0001
15 μl	0.06 \pm 0.0001
17 μl	0.056 \pm 0.0001

Data expressed as mean \pm SD ($n = 3$). BHAD activity was measured in 10 μl of rat muscle protein homogenate (positive control) and 7,10, 12, 15, 17 μl of human muscle protein homogenate. BHAD activity was measured at 25 $^{\circ}\text{C}$ and determined spectrophotometrically [13], with absorbance measured every 30 seconds at 340 nm for 10 min. 5.4 mM S-acetoacetyl CoA was used as substrate. Units/ml represents $\mu\text{mole}/\text{min}/\text{ml}/\text{mg}$ of protein.

The 7 μ l human muscle protein homogenate was the amount used in the final optimised BHAD assay for human muscle tissue. The BHAD activity results for the SPIRT study cohort are presented in section 5.3.5.

5.3.5 Final enzyme results for SPIRT study cohort

Using the final optimised assay for COX (Chapter 3 section 3.4.2.2), CS (Chapter 3 section 3.4.2.1) and BHAD (Chapter 3 section 3.4.2.3), the respective enzyme activities were determined in the SPIRT study participant skeletal muscle at 0 and 16 weeks after exercise. An example of enzyme curves obtained for participants in the progressive training (PRT) group and aerobic (AER) group at 0 weeks and 16 weeks are shown in Figures 5.15 respectively. All experiments were repeated in triplicates. Figure 5.16 is a bar graph showing the average baseline (0 week) and 16 week mitochondrial enzyme activity results for the SPIRT study cohort. The effect of PRT and AER training on mitochondrial enzyme activity in the SPIRT study cohort is presented in Table 5.13.

Within the AER group there was a statistically significant increase in enzyme activity for all three mitochondrial enzymes examined (Table 5.13). For the PRT group there was a statistically significant increase in COX and CS activity within the group (Table 5.13). Between groups there were statistically significant differences for all three mitochondrial enzymes (Table 5.13). The effect size shows whether the increase in activity is a small or large effect. For the AER group, the increase in BHAD activity is a very large effect, whilst the increase in COX activity is a moderate effect and CS activity a small effect. For the PRT group the increase in activity for all three mitochondrial enzymes examined was large to very large effect.

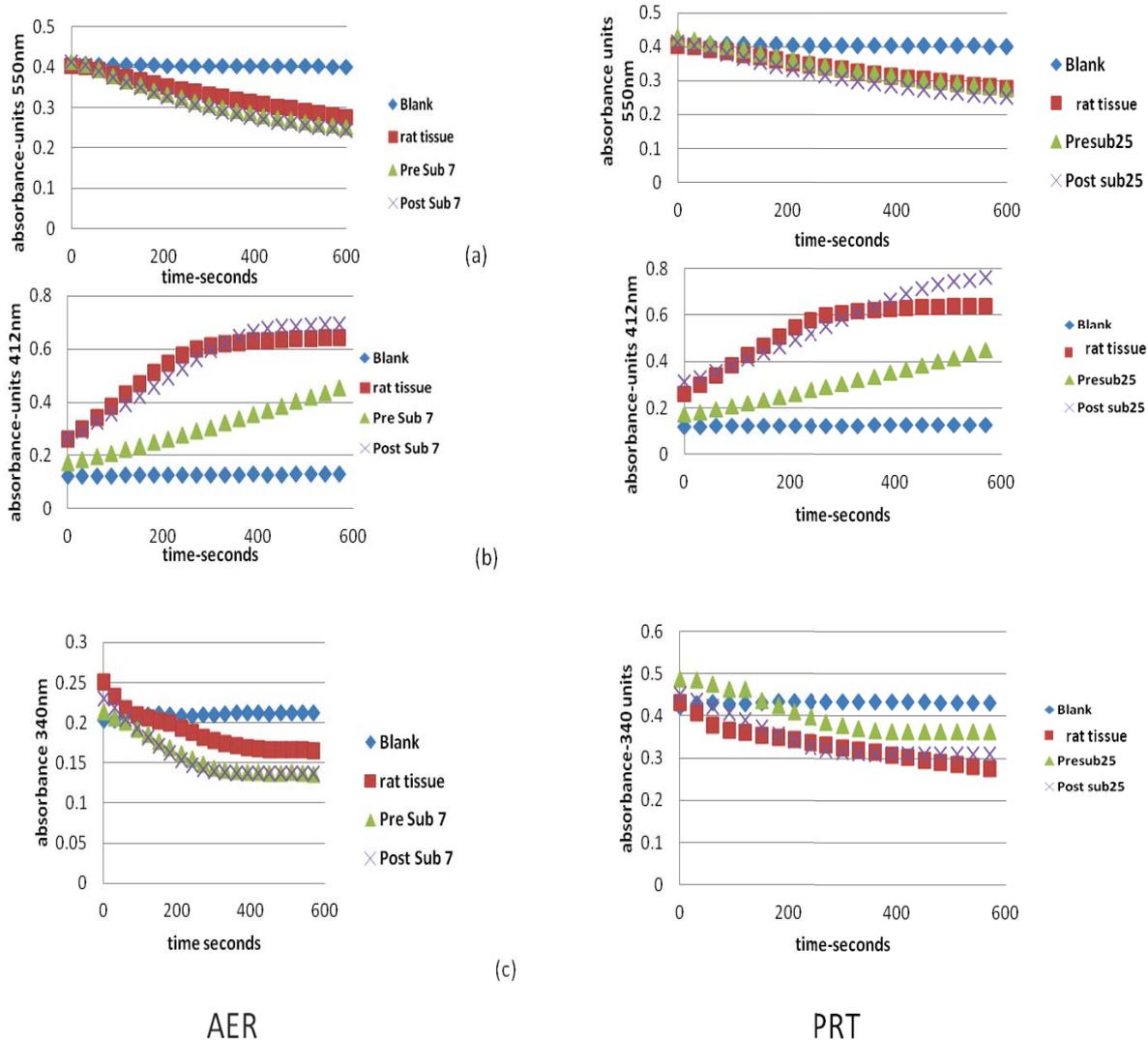


Figure 5.15: Example of enzyme activity curves for measurement of (a) COX activity (b) CS activity and (c) BHAD activity in skeletal muscle of SPIRIT study participant in aerobic (AER) or progressive resistance (PRT) intervention. COX activity at 25 °C was determined spectrophotometrically [11], with absorbance measured every 30 seconds at 550 nm for 10 min. CS activity was measured at 25 °C and determined spectrophotometrically [12], with absorbance measured every 30 seconds at 412 nm for 10 min. BHAD activity was measured at 25 °C and determined spectrophotometrically [13], with absorbance measured every 30 seconds at 340 nm for 10 min. The blank contained the extraction buffer in the same quantity of muscle homogenate used and all the reagents of the assay. Participant 25 was in the PRT group (right hand side of the Figure). Participant 7 in the AER group (left hand side of the Figure). Pre represents baseline (0 week) muscle enzyme activity and post represents 16 weeks muscle enzyme activity.

Table 5.13: Effect of Aerobic and Resistance Training on Mitochondrial Enzyme activity

Enzymes	AER		PRT		P value
Enzyme	Post-Pre difference	Effect size (ES)^a	Post-Pre difference	Effect size (ES)^a	Between group
	<i>P-value within group</i>		<i>P-value within group</i>		
COX	0.011	1.0 ± 5.2	0.005	2.3 ± 1.3	0.007
CS	0.03	0.4 ± 1.5	0.007	1.8 ± 1.3	0.004
BHAD	0.03	6.7 ± 1.2	0.078	2.7 ± 1.2	0.008

Data are the effect of exercise training expressed as standardised difference (effect size), where the within group baseline standard deviation was used as the denominator. ES Thresholds 0.2= small, 0.6= moderate 1.2= large, 2.0= V large. Threshold is for small standardised difference, according to Cohen, 1986. Data expressed as ES ± SD for n= 8 in AER group and n=8 in PRT group. COX represents cytochrome c oxidase; CS represents citrate synthase and BHAD represents beta-hydroxyacyl-CoA dehydrogenase. P< 0.05 is considered statistically significant.

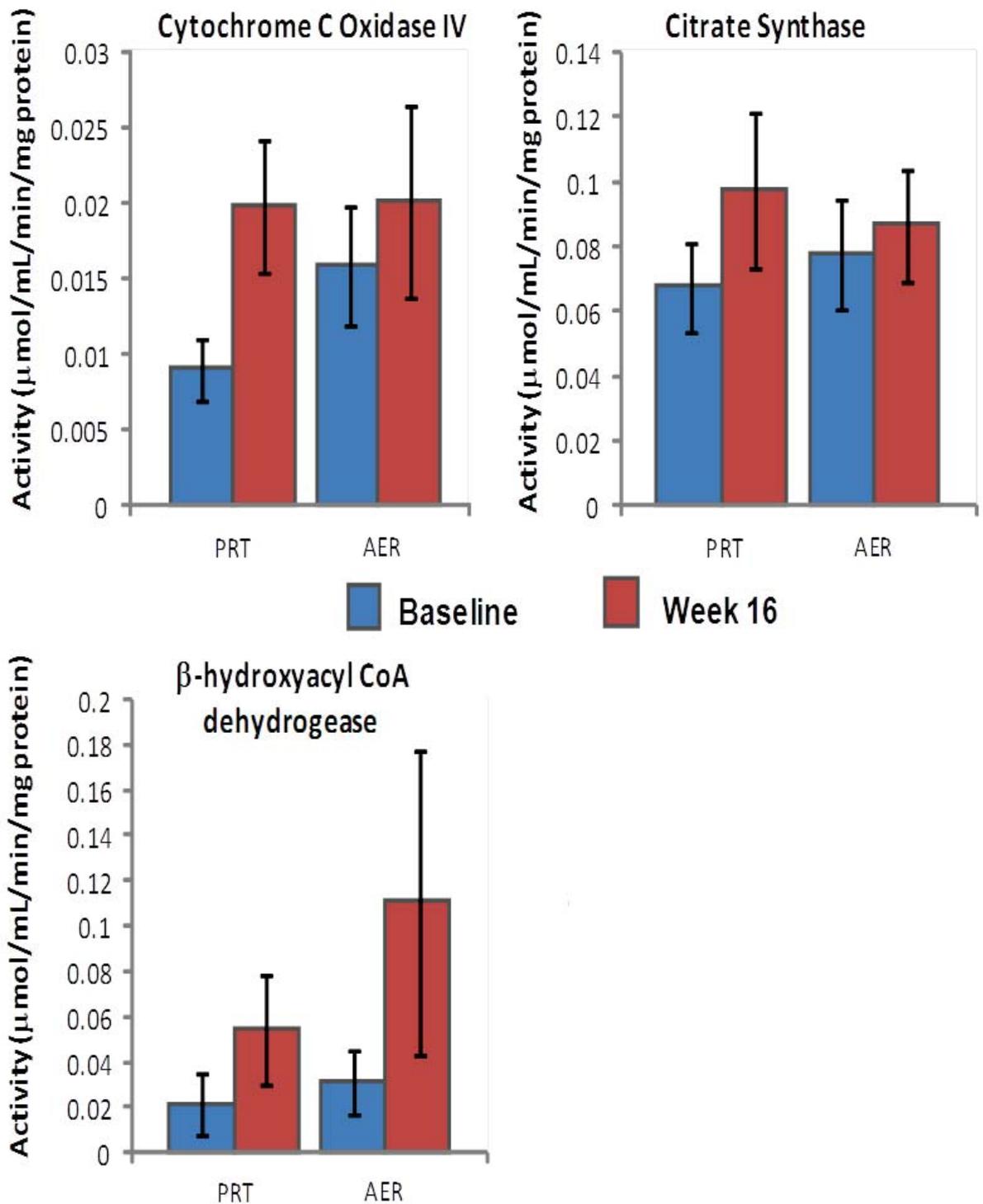


Figure 5.16: Activity of key mitochondrial enzymes in Aerobic (AER) and resistance (PRT) groups at baseline and at 16 weeks after intervention. The blue bars show the baseline (0 weeks) results and red bars show the results after 16 weeks. Data is expressed as mean \pm SD. $n=8$ for Aerobic group and $n=8$ for PRT group.

5.3.6 Intramyocellular Triglyceride (IMTG) content

An example of an electron microscopy (EM) image at 0 and 16 weeks of exercise for an AER SPIRIT study participant and PRT SPIRIT study participant are shown in Figure 5.17. As can be seen, examination of the EM image just by eye shows that the IMTG droplets were in greater number and larger in size at baseline (0 weeks) compared to 16 weeks for both the PRT and AER participants (Figure 5.17).

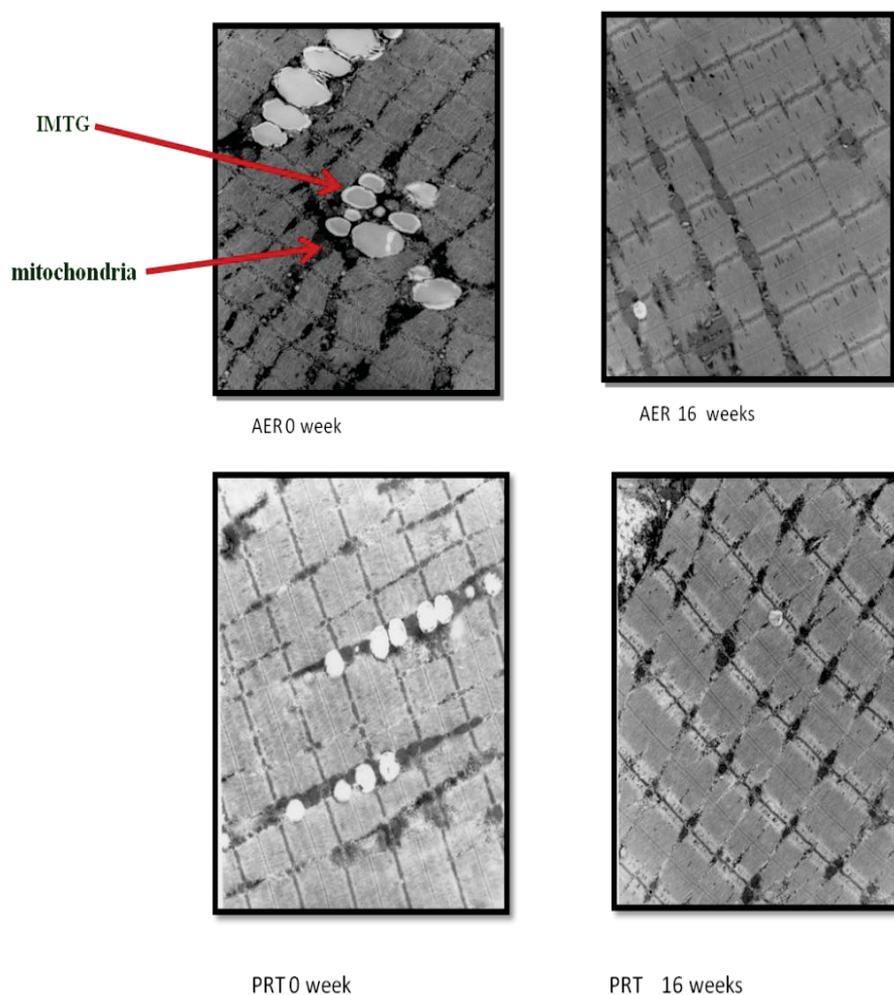


Figure 5.17 Changes in the muscle morphology after 16 weeks of exercise training. An example of a skeletal muscle electron microscopy image for SPIRIT study participant subject 23 who underwent progressive resistance training (PRT) exercise and subject 20 who underwent aerobic (AER) exercise and IMTG represents intramyocellular triglycerides.

As discussed in Chapter 3 section 3.5.2 each muscle biopsy sample had 5 to 8 images to process for determining total lipid content in the muscle sample. For the quantification of IMTG data, the total area for each sample was summed up and the area of the lipid was calculated from the total area of the image. As this data was very variable due to the variability within and between individuals the base line results were used as co-variate and the data was standardised by expressing as a fraction of the standard deviation for combined groups. The combined lipid measurement data for each group are presented in Table 5.14 showing the raw means, standard deviation and maximum and minimum size of the fat droplet. Table 5.15 presents the estimates about the qualitative and quantitative improvements of the exercise intervention on IMTG content.

Table 5.14: The percentage of muscle area from the EM image (μm^2) occupied by lipid droplets

No.	Group	Pre or Post 16 weeks	Mean% (μm^2)	Standard deviation	median	max	min	n
1	AER	Post	0.9	0.5	1.02	1.68	0.444	8
2	AER	Pre	1.7	1.05	1.6	4.07	0.663	8
3	PRT	Post	1.3	0.66	1.47	2.18	0.368	8
4	PRT	Pre	1.8	0.64	1.71	2.7	0.755	8

In the AER group after 16 weeks of intervention there was 1.89 fold decrease in the amount of IMTG present in the skeletal muscle (Table 5.14) and this decrease was statistically significant ($P = 0.007$) (Table 5.15) with the effect of change being moderate. In the PRT group there was a 1.38 fold decrease in the IMTG content which is statistically significant ($P=0.04$) with the change being of moderate effect (Table 5.15). In comparing the two exercise training regimes it can be seen that there was a very small difference between the changes noted for PRT and AER (16-0) weeks ($P= 0.87$) and showed small effect ($ES=0.23$). There was also very little difference in change at 16 weeks between PRT and AER, with small effect and no statistical significance ($P=0.9$) (Table 5.15).

Table 5.15: Effect of exercise training expressed as standardised difference and effect size

Effect	Fold change	Effect size	Confidence interval for effect size	P-value
AER change 16-0 weeks	1.89	0.9	0.69	0.007
PRT change 16-0 weeks	1.38	0.67	0.67	0.04
PRT-AER change 16-0 weeks	0.87	0.23	0.9	0.604
PRT-AER change at 16 weeks	1.02	0.02	0.64	0.939

Data are the effect of exercise training expressed as standardised difference (effect size), where the within group baseline standard deviation was used as the denominator. ES Thresholds 0.2= small, 0.6= moderate 1.2= large, 2.0= V large. Threshold is for small standardised difference, according to Cohen, 1986. n= 8 in AER group and n=8 in PRT group

5.3.7 Examination of Mitochondrial Morphology

A study by Kelley and colleagues [16] noted the presence of abnormal mitochondria in the muscle of people with T2DM. Kelley et al.[16] reported that the abnormal mitochondria exhibit less defined membrane structure, wider cristae and some vacuoles resembling lipid droplets. For this study the EM slices used for IMTG content were also used to examine mitochondrial morphology and presence of abnormally structured mitochondria. The normal structure of the mitochondria is characterised by double membrane with well-defined and dense network of cristae (See slide B-3 in Fig 5.18).

In studying the EM images, it was quite interesting to note that there were differences in the morphology and architecture of the muscle fibre before and after the 16 weeks exercise training. Figure 5.18 (A-2, A-3 highlighted by yellow and red arrows respectively) is an example of some “abnormal looking mitochondria” that look like what has been previously described in Kelley’s *et. al* [16] study. These abnormal mitochondria could be “degenerated or apoptotic mitochondria” in the muscle of the SPIRIT study participants. The mitochondrial structure was quite varied between individuals and at the same time within the same

individual as is shown in Figure 5.18 (A-1,A-2, B-1 and B-2 of subject 10 from PRT group and A-3,A-4, B-3 and B4 of subject 22 of AER group).

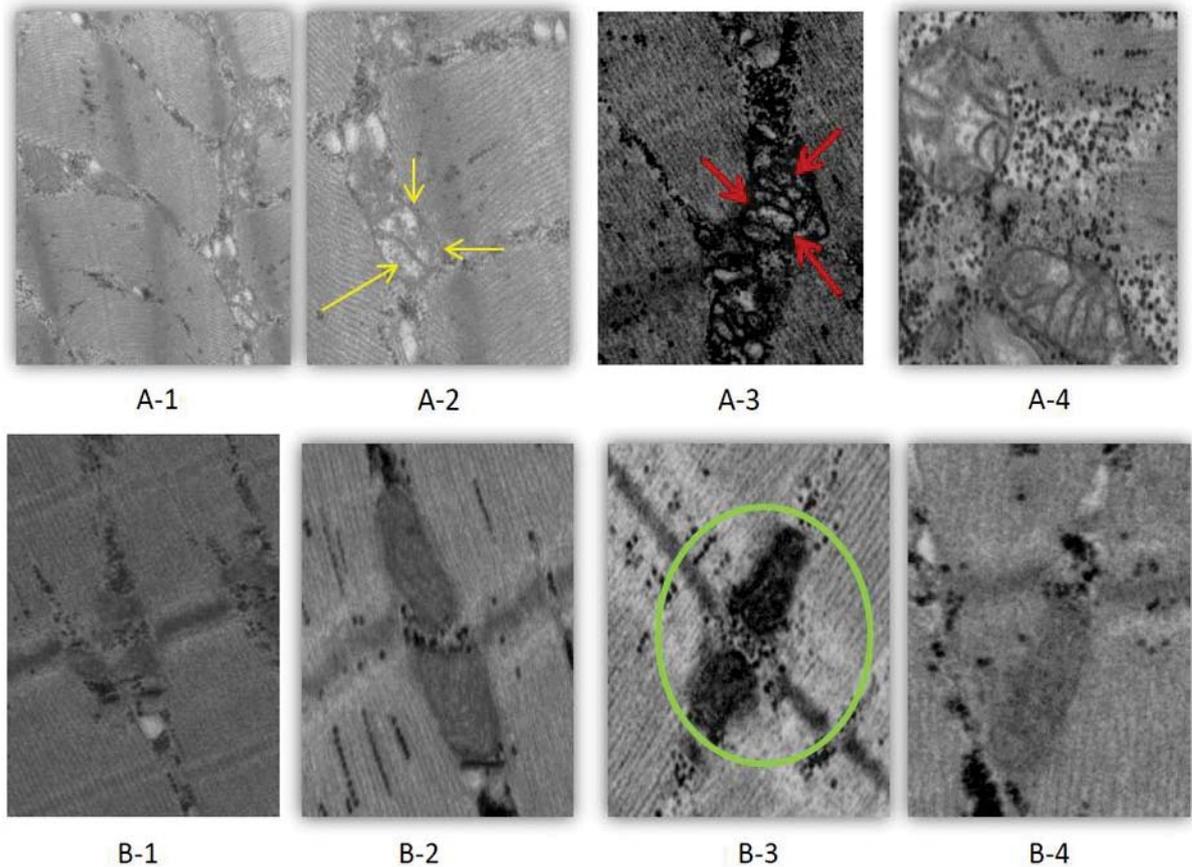


Figure 5.18: Example of electron microscopic images of skeletal muscle (SM) from AER and PRT participant at 0 weeks (A-1 to A-4 images) and 16 weeks (B-1 to B-4 images). A-1, A-2, B-1 and B-2 are images of SM from subject 10 (PRT) and A-3, A-4, B-3 and B-4 are images of SM from subject 22 (AER). Abnormal mitochondria (with wider cristae, less defined inner membrane structure (A-3) and having vacuoles resembling lipid droplets(A-2) are highlighted red arrows of A-3 image and yellow arrows in A-2. Normal mitochondria are highlighted via green circle of B-3 image.

5.4 Summary

With optimisation of any assay the aim is to obtain an intra-assay CV of less than 10% and inter-assay CV less than 15%. All three mitochondrial enzyme assays optimised in this study provided intra-assay CV and inter-assay CV of less than 5%, indicating excellent reproducibility. The enzyme results indicate increased mitochondrial function after 16 weeks of AER or PRT exercise as shown by the detection of 1.1- 3 fold increase in the enzyme activity of the three key mitochondrial enzymes in both exercise groups. There was a reduction in IMTG content or number of lipid droplets in the skeletal muscle of the SPIRIT study participants after 16 weeks of exercise, with the AER group showing a greater decrease in IMTG.

5.5 Discussion

The aim of this investigation was to examine skeletal muscle mitochondrial function and morphology and IMTG content in the SPIRIT study participants. In order to demonstrate the effect of 16 weeks of AER and PRT training on mitochondrial function the enzyme activity of three key mitochondrial enzymes, COX, CS and BHAD were examined. The results presented in Table 5.13 demonstrate that the activity of these key enzymes were significantly increased in the AER training group after 16 weeks of exercise intervention and in the PRT group only the CS and COX enzymes increased in activity significantly. The effect size was calculated to illustrate the magnitude of the change after AER and PRT training. There was a very large effect (6.7 ± 1.2) in the AER group for BHAD activity whereas in the PRT group a large effect (2.7 ± 1.2) for BHAD activity was observed. There was an increase in CS activity in both groups (PRT; $P = 0.007$, AER; $P=0.03$) however, the activity increase was more in the PRT group (effect size = 1.8 ± 1.3). COX activity was raised in both groups as well though the effect size in the PRT group was 2.3 ± 1.2 meaning a very large change with PRT exercise compared to a moderate effect (1.0 ± 1.2) with AER exercise.

The IMTG content was investigated by examining the skeletal muscle micrographs prepared by using electron microscope. Electron microscope images of the skeletal muscle were digitally analysed for determination of IMTG content. After 16 weeks of exercise there was a

statistically significant 28% decrease in IMTG content in the skeletal muscle of PRT participants and statistically significant 48% decrease in IMTG content in the skeletal muscle of AER participants (Table 5.15). The effect size (0.9) for the AER group suggests a moderate decrease in density of IMTG after 16 weeks AER exercise whereas the effect size of 0.6 for the PRT group, suggests a small to moderate change in IMTG density after 16 weeks resistance exercise. The novelty of this PhD study is that although there was no improvement in the glycaemic control and insulin resistance of the SPIRIT study participants after 16 weeks of aerobic or resistance training [17], the ability to investigate the muscle morphology and cellular functionality in this cohort could be performed. The results presented in this chapter support the notion that exercise is capable of inducing morphological and biochemical changes at cellular and molecular level. These cellular and molecular changes in the skeletal muscle, the main tissue for glucose disposal [10], are important as they are showing that previously metabolically inflexible skeletal muscle tissue has become metabolically flexible after 16 weeks of exercise training in this cohort. These cellular and metabolic changes could lead to macrophysiological changes in SPIRIT study cohort such as glycaemic control and may underscore the value of exercise in the insulin resistance related disorders such as obesity, T2DM and metabolic syndrome.

There is accumulative evidence that demonstrates there is lower activity of mitochondrial enzymes with insulin resistance in people with T2DM [1, 16] [18] [19]. It has been postulated that in insulin resistance states due to over-saturation of lipids in the muscle cells, mitochondria in the muscle cell gradually lose the capacity to oxidise fatty acids [20], decrease overall mitochondrial function and can lead to disturbed mitochondrial morphology. Exercise has been a very valuable tool to improve mitochondrial function and structure in skeletal muscle of individuals with T2DM [21-24]. The SPIRIT study is the first investigation that has demonstrated that 16 weeks of PRT and AER training in grade 3 obese Pacific Island people with T2DM leads to statistically significant increases in mitochondrial enzyme activities. This finding supports the hypothesis presented in (chapter 1 section 1.3) regarding the potential benefits of exercise at the cellular level and is also in agreement with previously reported studies demonstrating the effects of aerobic and resistance exercise on muscle tissue [21, 25-27]. This is an important skeletal muscle functional adaptation because insulin sensitivity is positively correlated with muscle oxidative capacity (as shown in Fig 5.1) and the derangements in the oxidative capacity has been linked with insulin resistance in

physically inactive tissue [1, 5, 28, 29]. Oxidative enzymes are abundant in type 1 muscle fibres exhibiting greater capillary density. Exercise has been shown to increase capillary density with an associated improvement in oxidative capacity of muscle. In the SPIRIT study cohort, an increase in capillary density was found after 16 weeks of aerobic exercise [30] which is also associated with the increase in oxidative enzyme function in this PhD study. These results suggest that both aerobic and resistance exercise can generate transformations at the cellular level in muscle overloaded with lipids in grade 3 obese type 2 diabetes participants.

IMTG have attained considerable attention in relation to insulin resistance [2, 31-34] Their role in the muscle cell along with mitochondria is considered to be distinctive in inducing metabolic derangements in the muscle cell [15, 20, 34]. In the last 10 years the pathological basis of T2DM has been viewed with a 'lipocentric' approach meaning that lipid stored inside the non-adipose tissue such as skeletal muscle can lead to insulin resistance leading to metabolic abnormalities [35]. Increased content of IMTG in the skeletal muscle of athletes was reported in 1979 by Lithel and colleagues [36] for the first time and was considered to be a valuable fuel for prolonged exercise in athletes. In 1999, Karssk and colleagues [37] associated the presence of increased amounts of IMTG with diminished insulin sensitivity leading to a paradigm shift from the positive role of IMTG to an uncertain disposition. After this the role of IMTG in the skeletal muscle has been a matter of debate. The subsequent studies after Karssk *et al.* [37], namely Schrawen *et al.* [2], Rattarsarn *et al.* [38], Schrawen [39], Amati *et al.* [32] and Bajpeyi [40] reported that IMTG levels were linked with insulin resistance. The meta-analysis by Rattarsam *et al.* [38] suggested that abnormal loci of fat in non-adipose tissue in T2DM individuals have an essential role in insulin resistance. In 2006, Schrawen and colleagues argued that "IMTG may be a valuable energy store during prolonged exercise, which however in the absence of regular physical activity and over consumption of fat can have detrimental effect on muscle insulin sensitivity"[2]. In the SPIRIT study participants the decrease in skeletal muscle IMTG after 16 weeks of exercise seems to be an early positive change at the cellular level. In this cohort there has been no change in insulin sensitivity [17, 30] as measured by HOMA-IR [41] however a decline in number of lipid droplets in the skeletal muscle could ultimately lead to improved insulin sensitivity.

There was a statistically significant increase in COX and CS activities (Table 5.13) in both AER and PRT groups and BHAD activity in AER group (Table 5.13), and a decrease in the IMTG content for both exercise groups (Table 5.15). These findings are in line with the findings of previous exercise studies of comparable duration (≥ 16 weeks) [29] but differs from some of the exercise studies (≥ 16 weeks) which clearly demonstrated improved insulin sensitivity along with improvement in the mitochondrial function and decrease in IMTG content [3, 27]. In SPIRIT study participants there was no significant change in the insulin sensitivity determined by HOMA-IR in either AER or PRT groups [17, 30]. In a clinical exercise trial with T2DM participants ($n=20$), Toledo *et al.* [29] reported that there was a $7.1 \pm 0.8\%$ weight loss observed with an associated $67 \pm 17\%$ decrease in the IMTG content and increased activity of mitochondrial enzymes. This study employed moderate to intensive daily exercise for 16-20 weeks however, the ethnicity of the participants was not identified. The difference of this study compared to the SPIRIT study was the BMI of the participants, which was 30 ± 1.5 (classified as grade 1 obesity), compared to BMI of 43.8 ± 9.5 (grade 3 obesity) in the SPIRIT participants (Table 1.2). Schrauwen and colleagues [39] compared the mitochondrial activity and IMTG in obese non-diabetic and BMI matched diabetic participants. They showed that there was similar IMTG content in the BMI matched T2DM relative to the obese individuals but the mitochondrial activity was markedly diminished in the T2DM individuals. These results may suggest that mitochondrial function as well as IMTG content may be important in people with T2DM.

Amati *et al* [32] reported that there was a twofold increase in IMTG content quantified by EM images in the sedentary obese subjects when compared to the lean counterparts and trained athletes. He found that the IMTG content was increased in both obese and trained athletes but the level of ceramide and DAG was higher in obese individuals. In this study the IMTG levels dropped in both AER and PRT groups after 16 weeks exercise however, the ceramide and DAG levels were not determined. Bajpeyi [40] compared effect of short term exercise for 10 consecutive days on lean, obese (BMI 38.8 ± 1.7) and T2DM subjects (BMI $=35.5 \pm 2.5$) and reported that the reduction of IMTG by 35% quantified by oil-o-red staining technique was only observed in T2DM individuals. No change in IMTG was observed in grade 3 obese individuals (BMI $= 38.8 \pm 1.7$) without diabetes [19]. These results are suggestive that the response to exercise is not universal; it seems quite variable and could be dependent on the metabolic status of the population under study. When these results are compared to the results

of the SPIRIT study participants who were grade 3 obese as well as having diabetes, 16 weeks of AER or PRT exercise has been able to produce morphological and functional changes in the skeletal muscle and reduction of IMTG (28% in PRT, 48% in AER) is equivalent to that observed in Bajpeyi *et. al* study [40]. These results may suggest that metabolic adaptation in the SPIRIT cohort is occurring although not immense enough to produce the macro physiological changes such as change in HbA1c, and it is possible that these could occur if exercise was continued for longer duration. Furthermore, the increase in mitochondrial enzyme activity after 16 weeks of exercise could indicate that skeletal muscle is regaining metabolic flexibility [28, 42].

The decreased density in lipid droplets (1.89 fold drop in AER group; 1.38fold drop in PRT group) in the muscle cell and increased activity of the BHAD enzyme (P=0.03 for AER group; P=0.078 for PRT group), a key mitochondrial enzyme involved in oxidation of fatty acids, could suggest that there was increased lipid handling and trafficking [1, 12, 43-45] in the skeletal muscle of the SPIRIT study participant. The microarray data does provide confirmation of changes in mRNA expression of genes coding for proteins involved in metabolism of lipid and this is discussed further in Chapter 6 section 6.3.

Exercise-induced changes in mitochondria and lipid metabolism can be beneficial for well-being of people with T2DM and obesity [20, 23, 28, 42]. The results from the SPIRIT study (Table 5.13) show that after 16 weeks of training the mitochondrial enzymes related with fatty acid oxidation (BHAD), electron transport chain (COX) and citric acid cycle (CS) are significantly increased in the PRT and AER exercise groups. These results are comparable with Baldi and Snowling [25] study where improvement in the mitochondrial enzymes activity was observed after resistance training of 10 weeks of moderate intensity in T2DM subjects.

Overall the mitochondrial enzyme and IMTG data suggests that exercise is inducing changes at the cellular level in spite of lack of improvement in glycaemic control and insulin sensitivity noted for the SPIRIT study cohort [17, 30]. Furthermore these results also support the notion that exercise can provoke some favourable changes in the metabolically unbalanced muscle fibre and is capable in altering the fatty acid oxidation in the muscle cell by improving the activity of the mitochondrial enzyme BHAD. However, to our knowledge there is no study found to date that has reported the change in morphology and function of

mitochondria after exercise in grade 3 obese individuals with T2DM. The morphological and functional transformation of mitochondria by exercise could open new horizons in investigation regarding the role of mitochondria in exercise rehabilitation. The change in mitochondrial function and fatty acid metabolism in the muscle cell after exercise supports that it is not only IMTG that are responsible for the metabolic disturbances in muscle tissue but the role of mitochondria is also critical as proposed by Martins and colleagues [15].

Lastly although the data regarding the role of mitochondria and intramyocellular lipids in Pacific peoples with T2DM and obesity is consistent with previous research in this area [2, 32, 40, 46] what was not investigated was the role of lipid products like ceramide and DAG in SPIRIT study participants. As is discussed in detail in Chapter 2, ceramide and DAG are metabolically active fat particles that interfere with insulin signalling [15, 47] and the study of these lipid products along with their transporter proteins such as Perlipin 2 and 5 would be important to further confirm the lipid trafficking within in the skeletal muscle building a relationship between mitochondria and lipid metabolism affecting insulin signalling pathway.

Overall these findings suggest that both PRT and AER exercise can be effective therapeutic modalities for the induction of changes at the cellular level in muscle of people with T2DM. These results supplement the current data available regarding the crucial role of mitochondria and IMTG in metabolic dysfunction observed in T2DM and obesity which are collectively named as “diabesity” in the relevant literature [12, 45, 48]. The key strength of this study was that this is the first study of its kind in which investigation of mitochondrial and muscle lipid biomarkers were performed on a T2DM cohort with grade 3 obesity and in a Polynesian population.

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CHAPTER 6

mRNA EXPRESSION CHANGES IN THE SKELETAL MUSCLE OF SPIRIT PARTICIPANTS AFTER 16 WEEKS OF AER AND PRT

6.1 Introduction

Type 2 diabetes mellitus (T2DM) has been viewed as an evolutionary disease of modern age linking its pathogenesis to the ‘thrifty genotype [1]’ and physical inactivity. The thrifty’ genotypes hypothesis states that when the individuals with these ‘thrifty’ genotypes are exposed to food excess and physical inactivity the genotype suffers from metabolically challenging alterations which promotes more energy being stored in the form of fat. The excessive fat cell deposition in the body leads to phenotypic changes such as obesity, thickening of blood vessel walls, muscle sarcopenia and atrophy of myocytes. These phenotypic changes can lead to chronic metabolic disorders such as T2DM which ultimately can make people with the “thrifty” genotype susceptible to morbidity and early mortality [2].

The thrifty genotype has been used to explain the increased incidence and prevalence of chronic non-communicable diseases such as T2DM in high risk ethnic populations such as Pima Indians, Latino and African Americans, Japanese and Pacific people [3-5]. The rate of incidence of T2DM and obesity are rapidly escalating in New Zealand [6] and its incidence and prevalence is more in Pacific Islanders [7]. Sukala *et al.* [8] reported in the SPIRIT study that 16 weeks of exercise training either in the form of aerobic (AER) or resistance (PRT) could serve as a promising intervention for addressing the symptoms related with obesity and T2DM in Pacific Islands people. The randomised clinical exercise trial that was performed (SPIRIT study) [9] showed improvement in quality of life in both AER and PRT groups [10] and improved GLUT4 translocation along with clinical significant improvement in systolic blood pressure in the AER group only [9].

The muscle in sedentary obese T2DM adults is characterised histologically by disorganised myofibrils, lipid accumulation [11], lower activities of insulin-stimulated enzymes [12,13], abnormal mitochondria [14,15], relative sarcopenia, thickened endomyosium [13] and low capillary density and insulin resistance [16]. Skeletal muscle is a major site in the body that responds to exercise training in people with T2DM, by increasing glucose disposal [17], increasing fatty acid oxidation [18] and enhancing mitochondrial function [19]. The improvement with exercise is related to the same concept of pathogenesis of diabetes as explained by Booth *et al.* [2] in terms of genetic interaction, where physical inactivity leads to a loss of function resulting from the silencing of genes. The improvement with exercise is

based on the concept that loss of function due to physical inactivity can be revived again with the introduction of exercise [17].

The improved muscle metabolism via non-invasive means such as exercise (AER/PRT) has also been associated with improved insulin sensitivity. Exercise affects the primary defects related with reduced metabolic capacity and fatty oxidation which are both factors related to the aetiology of obesity and insulin resistance (Chapter 2 section 2.3). An improved understanding of the molecular systems linked to changes after AER and PRT seems to be a significant step forward in understanding the impact of exercise on muscle plasticity which is reliant on the metabolic flexibility (Chapter 2 section 2.3) of the skeletal muscle and its structure. The weakened metabolic flexibility (see chapter 2 Figure 2.2) and increased lipid deposition and hence weakened muscle plasticity is associated with aetiology of T2DM (see chapter 2). Therefore the objectives of this chapter are to:

- a) Investigate the statistically significant changes in skeletal muscle mRNA expression in both exercise training groups after 16 weeks, using the microarray data.
- b) Identify the statistically significant genes related to energy metabolism and skeletal muscle remodelling in response to exercise in the SPIRIT study cohort.
- c) Utilise the network bioinformatics approach and examine significant connections between genes and their particular biological pathways and their associated impact in health and disease (T2DM).

6.2 Methods

The study design, participant characteristics, interventions and muscle sample collection are described in detail in Chapter 1 and Appendix A. The methods for RNA extraction, gene expression profiling and bioinformatics analysis and interpretation of results with statistical analysis have been described in Chapter 3 section 3.6. Statistically significant genes in the microarray data obtained are defined by $ROBP < 0.005$ (See Appendix E for complete microarray dataset).

Interrogation of the gene set, molecular and physiological function analysis and network construction was performed in Ingenuity Pathways Analysis (IPA) software (Winter Release 2011, Ingenuity® Systems, www.ingenuity.com). The IPA software identifies the cascade of

upstream transcriptional regulators in the gene database provided. These upstream transcriptional regulators are able to highlight the observed gene expression changes in the database of statistically significant genes (Appendix E) and can further illuminate the biological/physiological activities occurring in the tissue under investigation. IPA can help to examine these results further by interrogating the physiological processes, pathways and diseases that are controlled by these transcriptional regulators linked to the statistically significant genes (Appendix E). The IPA software provides a network or module that is based on the statistically significant gene set and associated upstream regulators such as cell surface receptors and kinase cascades.

The SPIRIT gene expression microarray data (ROBP<0.005) after unbiased examination was uploaded to IPA software for further analysis and biased selection occurred in relation to wanting identification of networks/modules associated with skeletal muscle, exercise, obesity and T2DM. The aim of this analysis was to interrogate the regulatory pathways affecting systemic biology/physiology of skeletal muscle in relation to energy metabolism (fatty acid and glucose metabolism) and skeletal muscle remodelling as stated above. IPA transcription factor regulator analysis utilises the prior knowledge of expected change between transcriptional regulators and their target genes stored in the IPA knowledge base. The analysis examines the known targets of each transcription regulator in the database and compares its direction (i-e upregulated or downregulated). When the direction of change of the genes in the dataset (Appendix E) is consistent with the literature across the majority of targets, then the transcriptional regulator is said to be active in the tissue. On the other hand if the direction of change cannot be correlated with the existing literature then the regulator is predicted to be inactive in the biological sample.

IPA system generates two types of statistical quantities; overlap P-value and regulation Z-score. These statistical quantities are designed to avoid the random changes leading to significant results. Overlap P-values are approximately uniformly distributed in the given pathway while regulation Z-score are approximately normally distributed with mean equal to 0 and variance equal to 1. The Z-score (one standard deviation) for a network shows magnitude of the expression of total genes involved and overall direction (up-regulation being a positive Z-score and down-regulation being a negative Z-score) of the system under investigation. The raw microarray mRNA data has been provided on a CD (Appendix E) with details regarding the ontological name, function, probe-ID, P-values and fold change.

6.3 Results

A total of 20,000 genes related with the human genome were investigated. An unbiased exploration for the statistically significant genes (ROBP < 0.005) was performed. In AER 786 (3.9 % of the human genome) and in PRT 653 genes (3.3 % of the human genome) were found to change in expression, with statistical significance, after 16 weeks of intervention. In the AER group a total of 130 genes showed fold change ≥ 1.2 . Out of these total 130 genes, 119 genes showed a fold change ≥ 1.3 and 11 genes had a fold change ≥ 1.4 . The top 11 genes with fold change ≥ 1.4 are presented in Table 6.1. All 130 genes are presented in Appendix E with yellow highlights. In the PRT group, of the 653 statistically significant genes identified, a total of 143 genes had a fold change ≥ 1.2 . Out of the 143 genes, 120 showed a fold change ≥ 1.3 and 20 genes had a fold change ≥ 1.4 . The 20 genes with ≥ 1.4 fold change are presented in Table 6.2. All 143 genes are presented in Appendix E with yellow highlights.

Tables 6.1 and 6.2 present the genes (ROBP < 0.005 and fold change ≥ 1.4) after unbiased exploration of the microarray dataset. Examining the ontological function of the top ranked genes presented in Table 6.1 it can be seen that in the AER group the genes related with lipid transport (FABP4), immune and inflammation (CD93, IF16), muscle remodelling associated with fibrosis and extracellular matrix (COL4A1, COL3A1, MYH11, BGN, ACTA2, CD300LG, A2M, GPR116) showed significant fold change after 16 weeks of exercise. By looking at the top ranked genes in the PRT group (Table 6.2), genes related with muscle remodelling associated with extracellular matrix and fibrosis (COL3A1, COL1A2, COL1A1, BGN, ACTC1, DDIT4L, COL6A3, COL4A1, MGP, LUM, ITGB1BP3, MXRA5, FNDC1, THBS4, EEF1A1, COL5A2), calcium action (S100A6, S100A4), cholinergic receptor (CHRNA1) and unknown function (LOC649150) were the genes exhibiting greatest fold change after 16 weeks of exercise.

The Ingenuity Software was used to interrogate the microarray gene selection to construct molecular system models from the functions related with skeletal muscle plasticity and glucose and lipid handling in the skeletal muscle tissue of SPIRIT participants. Networks/Modules identifying disease related functions were overlapped with biologically relevant functions to identify the key “gene hubs”. Hub genes are the genes related with more

than one function and represent the genes that are interconnecting different pathways/networks/modules.

Table 6.1: List of genes showing statistically significant change (ROBP < 0.005 and ≥ 1.4 fold change) after 16 weeks of AER exercise

SYMBOL	DEFINITION	ONTOLOGY FUNCTION*	Fold-Change(Po st vs. Pre)
COL4A1	H collagen, type IV, alpha 1 (COL4A1)	The action of a molecule that contributes to the structural integrity of the extracellular matrix (ECM)	1.82414
BGN	biglycan (BGN)	Interacting selectively with any protein or protein complex	1.66224
FABP4	fatty acid binding protein 4, adipocyte (FABP4)	Enables the directed movement of substances (such as macromolecules and fatty acids)	1.63762
ACTA2	actin, alpha 2, smooth muscle, aorta (ACTA2)	Interacting selectively with any protein or protein complex	1.48435
CD300LG	CD300 molecule-like family member g (CD300LG)	Combining with an extracellular or intracellular messenger to initiate a change	1.46471
COL3A1	type III, alpha 1 (COL3A1)	Interacting selectively with an integrin	1.45908
A2M	alpha-2-macroglobulin (A2M)	Stops, prevents or reduces the activity of serine-type endopeptidase (enzymes)	1.42063
IFI6	interferon, alpha-inducible protein 6 (IFI6)	Interacting selectively with any protein or protein complex	1.41344
MYH11	myosin, heavy chain 11, smooth muscle (MYH11)	Interacting selectively with a nucleotide	1.40903
CD93	CD93 molecule (CD93)	Interacting selectively with the C1q component of the classical complement cascade	1.40759
GPR116	G protein-coupled receptor 116 (GPR116), transcript variant 1	Combining with an extracellular or intracellular messenger to initiate a change	1.40205

*Ontological function has been obtained from IPA software and what was assigned to the identified genes (Appendix E)

In the AER group the top functions derived from IPA analysis, related with muscle remodelling and energy metabolism were angiogenesis (formation of new blood vessels), fibrosis, immune and inflammatory cell assembly, anti-fibrosis (new tissue development) and

lipid and glucose handling (Figures 6.1, 6.2 and Table 6.3). In the PRT group, fibrosis, angiogenesis and immune and inflammatory cell assembly were the top functions identified (Figures 6.3, 6.4 and Table 6.4).

Table 6.2: List of genes showing statistically significant change (ROBP < 0.005 and ≥ 1.4 fold change) after 16 weeks of PRT exercise

SYMBOL	DEFINITION	ONTOLOGY FUNCTION*	Fold Change (Post vs Pre)
COL3A1	collagen, type III, alpha 1 (COL3A1)	Interacting selectively with an integrin	2.32591
COL1A2	collagen, type I, alpha 2 (COL1A2)	The action of a molecule that contributes to the structural integrity of the extracellular matrix(ECM)	2.03223
COL1A1	collagen, type I, alpha 1 (COL1A1)	The action of a molecule that contributes to the structural integrity of the ECM	1.95253
BGN	biglycan (BGN), .	Interacting selectively with any protein or protein complex (a complex of two or more)	1.62605
ACTC1	actin, alpha, cardiac muscle 1 (ACTC1), mRNA.	Interacting selectively with a nucleotide, any compound consisting of a nucleoside	1.57351
DDIT4L	DNA-damage-inducible transcript 4-like (DDIT4L), mRNA.	Any process that stops, prevents or reduces the frequency, rate or extent of signalling	1.56332
COL6A3	collagen, type VI, alpha 3 (COL6A3), transcript variant 3	Stops, prevents or reduces the activity of serine-type endopeptidases, enzymes	1.54063
COL4A1	collagen, type IV, alpha 1 (COL4A1),	The action of a molecule that contributes to the structural integrity of the ECM	1.53885
MGP	matrix Gla protein (MGP),	The action of a molecule that contributes to the structural integrity of the ECM	1.48891
LUM	lumican (LUM)	The action of a molecule that contributes to the structural integrity of the ECM	1.48035
ITGB1BP3	integrin beta 1 binding protein 3 (ITGB1BP3)	Interacting selectively with a nucleotide, involved in myogenic differentiation	1.47676
MXRA5	matrix-remodelling associated 5 (MXRA5), mRNA.	Interacting selectively with any protein or protein complex (a complex of two or more)	1.47205
LOC649150	eukaryotic translation elongation factor 1	Exact function unknown	1.46642
FNDC1	fibronectin type III domain containing 1 (FNDC1)	integrity of ECM	1.46055
S100A6	S100 calcium binding protein A6 (S100A6)	Interacting selectively with calcium ions (Ca ²⁺)	1.44987
THBS4	thrombospondin 4 (THBS4)	The action of a molecule that contributes to the structural integrity of a compliment system	1.44421
EEF1A1	eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	Interacting selectively with a nucleotide, enzymatic delivery of t RNA to ribosome	1.42486
CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle) (CHRNA1), transcr	Catalyst	1.4206
COL5A2	collagen, type V, alpha 2 (COL5A2)	The action of a molecule that contributes to the structural integrity of the ECM	1.41414
S100A4	S100 calcium binding protein A4 (S100A4), transcript variant 2	Interacting selectively with calcium ions	1.40932

*Ontological function has been obtained from IPA software and what was assigned to the identified genes (Appendix E)

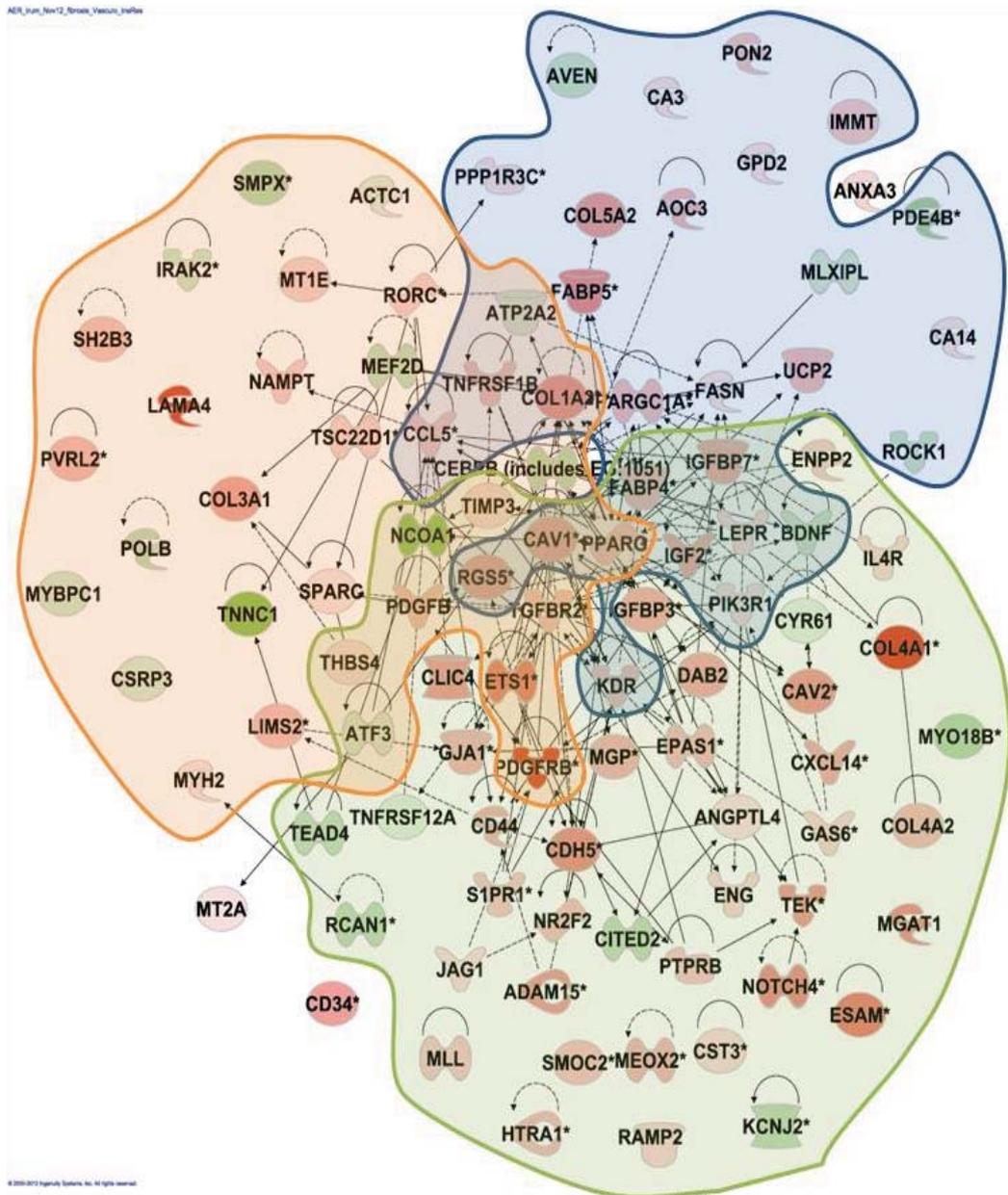
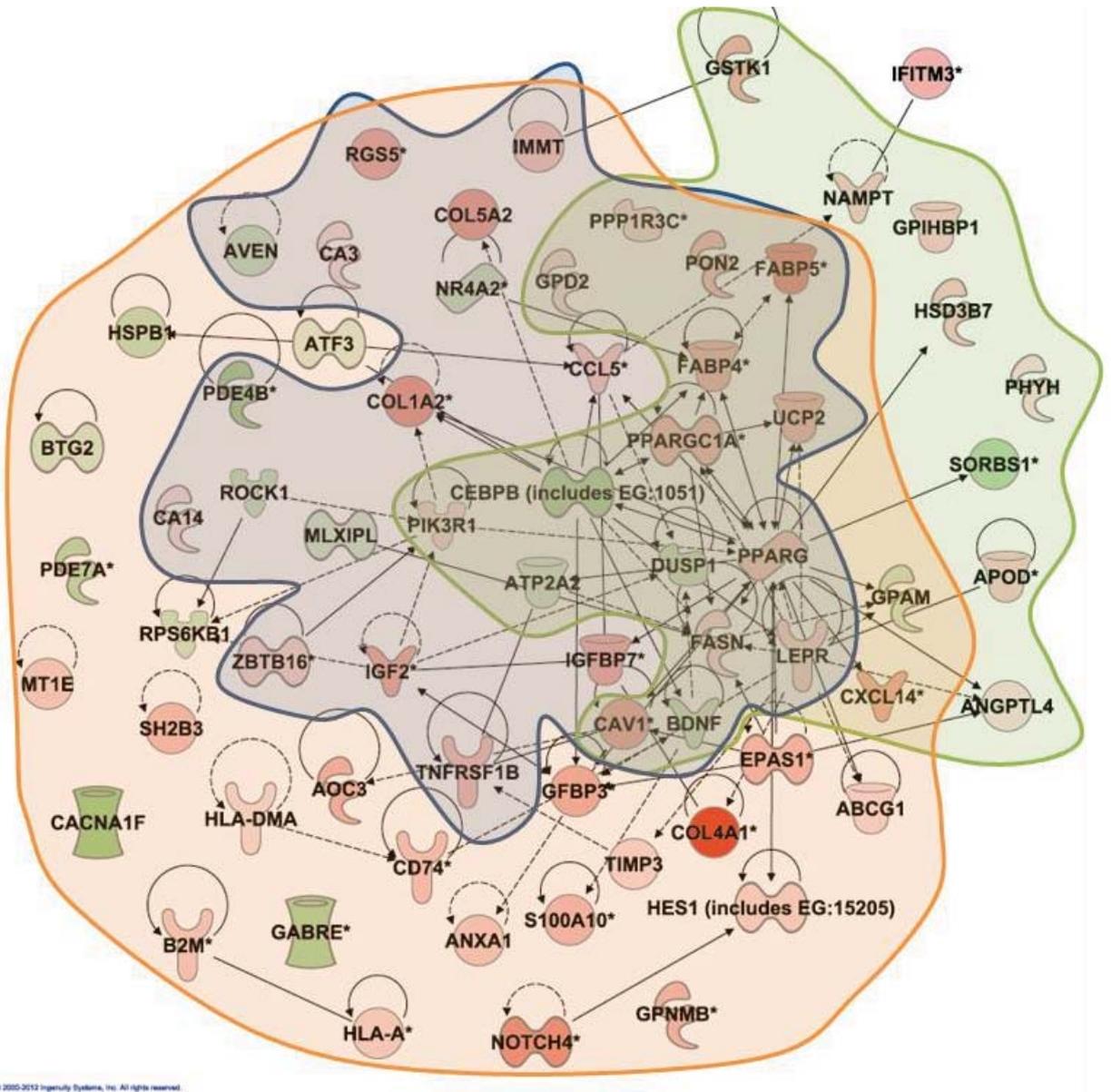


Figure 6.1: The disease module is fibrosis (regulation directional Z-Score -1.5, function p-value 4.57E-03, brown) and the functional remodelling module is vasculogenesis (Z-score 2.2, p= 1.23E-06, green). These modules are overlapped by the insulin resistance module (Z-Score, 0.2, p= 1.36E-02, blue), to show the functional connectivity between the disease and functional adaptation modules leading to molecular plasticity associated with improved tissue metabolic function. The pink colour of the symbol represents upregulation of the gene and green colour represents downregulation. A glossary of the symbols are shown in Appendix D.



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Figure 6.2: The disease module is glucose metabolism disorder (Z-Score 0.3, $p=1.10E-03$, brown) and triglyceride content (Z-score -0.5, $p=1.2E-03$, green) and the functional remodelling module is insulin resistance (Z-Score, 0.2, $p=1.36E-02$, blue). The pink colour of the symbol represents upregulation of the gene and green colour represents downregulation. A glossary of the symbols can be seen in Appendix D.

Table 6.3: Hub genes associated with the top-ranked functional networks determining molecular regulation of skeletal muscle plasticity to aerobic training in grade 3 obese T2DM adults

Network	Regulatory Hub Genes	fold change
Insulin resistance, Fibrosis and Vasculogenesis	PPARG	1.11257
	IGFBP7	1.35778
	CAV1	1.36114
	PIK3R1	1.07449
	COL1A2	1.25829
	BDNF	-1.06243
	IGF2	1.08238
Glucose Metabolism, Triglyceride Content and Insulin Resistance	PIK3R1	1.07449
	FABP4	1.63762
	BDNF	-1.06243
	ATP2A2	-1.19149
	TGFBR2	1.1683
	TNFRSF1B	1.07517
	UCP2	1.27635
	FABP5	1.33173
	PPARGC1A	1.20220
	CCL5	1.12566
	CAV1	1.36114
	MLXIPL	-1.13712
	COL1A2	1.25829
	LEPR	1.01001
	NR4A2	-1.08985

Abbreviations: ATP2A2 ATPase associated with Darier's disease and Acrokeratosis verruciformis ;BDNF Brain-derived neurotrophic factor;CAV1 Caveolin-1;CCL5 Chemokine (C-C motif) ligand 5;COL1A2 collagen, type I, alpha 2;FABP4 Fatty acid binding protein 4;IGF2 insulin-like growth factor 1 receptor;IGFBP7 Insulin-like growth factor-binding protein 7;PIK3R1 regulatory subunit of phosphoinositide-3-kinase.;PPARG peroxisome proliferator- activated receptor gamma. ;TGFBR2 transforming growth factor, beta receptor II;TNFRSF1B Tumor necrosis factor receptor superfamily member 1B;PPARG peroxisome proliferator- activated receptor gamma. ;PPARGC1A peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; CAV1 Caveolin-1; MLXIPL MLX-interacting protein ; COL1A2 Arginine for glycine substitution in the triple-helical domain of the products of one alpha 2(I) collagen allele; NR4A2 Nuclear receptor related 1 protein.

The two top networks representing the response to AER training comprised of decreased proliferation of fibroblasts and connective tissue (decreased fibrosis) and vasculogenesis and overlapped with genes involved in insulin resistance (Figure 6.1). The second top network implicated functional regulation of glucose metabolism and increased fatty-acid metabolism leading to decreased triglyceride content overlapped with the disease module insulin resistance (Figure 6).

The key hub genes that are involved in both the fibrosis and vasculogenesis modules and overlap with the disease module insulin resistance were PPARG, IGF1, IGF2, CAV1, PIK3R1, COL1A2, BDNF, IGF2 (Figure 6.1 and Table 6.3). The key hub genes that are involved in both glucose and lipid metabolism and overlap with the disease module insulin resistance were PIK3R1, FABP4, BDNF, ATP2A2, TGFBR2, TNFRSF1B, UCP2, FABP5, PPARGC1A, CCL5, CAV1, MLXIPL, COL1A2, LEPR, NR4A2 (Figure 6.2 and Table 6.3).

In response to 16 weeks of resistance training, cellular movement, haematological system development and function, immune cell trafficking, inflammatory response, tissue and cellular growth and development were the top ranked functional annotations resolved from interrogation of the transcriptome. The identified increased leukocyte migration, vasculogenesis, muscle development, and decreased fibrosis were highly indicative of connective tissue remodelling. The functional connectivity between diseased module fibrosis and vasculogenesis is shown in Figure 6.3 and diseased module fibrosis and leukocyte migration in Figure 6.4.

The hub genes that are involved in fibrosis and vasculogenesis are IGF1, SGK1, PDGFRB, CXCL12, CAV1, PDGFRB (Figure 6.3 and Table 6.4). The hub genes that are involved in both fibrosis and leukocyte migration are TIMP2, APOE, TGFBR2, CAV1, PDGFRB1, PSEN1, PRKCDBP, CXCL12, COL1A2, IL-4 (Figure 6.4 and Table 6.4).

The activated or inhibited upstream transcription regulators associated with the top networks in the skeletal muscle after 16 weeks of AER exercise (Figure 6.1 and 6.2) are shown in Table 6.5. The activated or inhibited upstream transcription regulators associated with the top networks in the skeletal muscle after 16 weeks of PRT exercise (Figure 6.3 and 6.4) are shown in Table 6.6.

Table: 6.4: Hub genes associated with the top-ranked functional networks determining molecular regulation of skeletal muscle plasticity to resistance training in grade 3 obese T2DM adults

Network	Regulatory Hub genes	fold change
Fibrosis and Vasculogenesis	PRKCDBP	1.14242
	IGF1	1.11163
	SGK1	1.19347
	PDGFRB	1.25442
	CXCL12	1.27264
	CAV1	1.30386
	PDGFRB	1.25442
Fibrosis and Leukocyte Migration	TIMP2	1.23804
	APOE	1.09792
	TGFBR2	1.19642
	CAV1	1.30386
	PDGFRB1	1.16
	PSEN1	1.04952
	PRKCDBP	1.14242
	CXCL12	1.27264
	COL1A2	2.03223
IL-4	1.04	

Abbreviations: PRKCDBP binding protein of the protein kinase C, delta; IGF1 insulin-like growth factor 1 receptor ; SGK1 serum/glucocorticoid regulated kinase 1 ; PDGFRB platelet-derived growth factor receptor, beta polypeptide; CXCL12 Stroma-derived factor (SDF/CXCL12) ; TIMP2 metalloproteinase inhibitor 2, a tissue inhibitor; APOE Apolipoprotein E ; TGFBR2 Transforming growth factor beta 2; CAV1 Caveolin-1 ; PSEN1 Presenilin-1 (development of the brain and spinal cord); FABP4 Fatty acid binding protein 4; COL1A2 collagen, type I, alpha 2 ; IL-5, interleukin 5.

The upstream regulators for AER training related to lipid metabolism and insulin resistance presented in Table 6.5 are ACOX1, IL-4, IL-6, MAPK1, PTEN, RELA and PPARA. The main upstream regulators related to glucose metabolism disorder and microvasculature are WISP2, CTGF and CEBPB (Table 6.5). The upstream regulators related to PRT training for antifibrotic development through immune and inflammatory mechanism are NFKB1A, HIF1A, ERBB2, SMAD3, FBN1, EDN1, HOXA9, IL1B and SMAD2 (Table 6.6).

Table 6.5: Predicted activation status of upstream regulatory factors associated with the top-ranked functional networks determining molecular regulation of skeletal muscle plasticity to aerobic training in grade 3 obese T2DM adults.

Network	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap
Lipid metabolism, insulin resistance	ACOX1	enzyme	Inhibited	-2	6.9E-03
	IL4	cytokine	Activated	2.1	5.20E-02
	IL6	cytokine	Activated	2.1	2.50E-05
	MAPK1	kinase	Inhibited	-2.2	2.20E-03
	PTEN	phosphatase	Inhibited	-2.2	1.70E-03
	RELA	transcription regulator	Inhibited	-2.2	1.40E-02
Glucose metabolism disorder, microvasculature	PPARA	ligand-dependent nuclear receptor	Activated	2.7	1.10E-05
	WISP2	growth factor	Inhibited	-2.2	5.10E-07
	CTGF	growth factor	Activated	2	4.30E-04
	CEBPB	transcription regulator	Activated	2.3	4.20E-06

Abbreviations: ACOX1, peroxisomal acyl-coenzyme A oxidase 1; IL4, interleukin 4; IL6, interleukin 6; MAPK1, mitogen-activated protein kinase 1; PTEN, phosphatase and tensin homolog; RELA, V-rel reticuloendotheliosis viral oncogene homolog A, PPARA, peroxisome proliferator-activated receptor alpha; WISP2, WNT 1-inducible-signalling pathway protein 2, CEBPB, CCAAT/enhancer binding protein beta; CTGF, connective tissue growth factor

Table 6.6: Predicted activation status of upstream regulatory factors associated with the top-ranked functional networks determining molecular regulation of skeletal muscle plasticity to resistance training in grade 3 obese T2DM adults.

Network	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap
Antifibrotic tissue development	NFKBIA	other transcription regulator	Activated	2.4	9.10E-06
	HIF1A	regulator	Activated	2	5.90E-05
	ERBB2	kinase transcription regulator	Activated	2.2	3.70E-10
	SMAD3	regulator	Activated	2.2	3.00E-06
	FBN1	other	Inhibited	-2.2	2.90E-07
	EDN1	cytokine transcription regulator	Activated	2.1	2.20E-06
	HOXA9	regulator	Activated	2	1.70E-04
	IL1B	cytokine transcription regulator	Activated	3.7	1.50E-13
	SMAD2	regulator	Activated	2.2	1.20E-04

Abbreviations : NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; HIF1A, hypoxia inducible factor 1, alpha subunit; alpha; ERBB2, epidermal growth factor receptor 2; SMAD3, mothers against decapentaplegic homolog 3; FBN1, fibrillin 1; EDN1, endothelin 1; HOXA9, homeobox protein Hox-A9; SMAD2, mothers against decapentaplegic homolog 2; IL1B, interleukin-1 beta (catabolin)

6.4 Discussion

Summary of the principal findings

An unbiased analysis of the mRNA expression microarray data results (Appendix E) was performed. The statistically significant genes ($ROBP < 0.005$) were ranked according to their fold change (Table 6.1, and 6.2, Appendix E for yellow highlights). The functional and disease module intra-tissue cellular network modelling approach was utilised using IPA software to produce modules/networks biased towards skeletal muscle structure and function (Figures 6.1 - 6.4). The hub genes (genes that are connected in both the disease and normal physiology networks) involved in skeletal muscle plasticity related to muscle structure and metabolic flexibility (Chapter 2 section 2.3) were identified in grade 3 obese T2DM Pacific Island adults in response to aerobic (Table 6.3) and resistance training (Table 6.4). The transcriptome

modules/networks were analysed to identify upstream regulators (Table 6.5 and Table 6.6), associated with the top network/modules identified for AER and PRT exercise, by using IPA statistics i-e Z-score and overlap P value for normal distribution of genes. The networks/modules presented in section 6.3 suggest antifibrotic vasculogenic plasticity in response to both forms of training. However it was observed that only in response to aerobic training there was the transcriptome changes (based on ROBP value <0.005) for glucose and lipid handling. The principal findings are summarised in Figure 6.5. Molecular functions and regulatory genes associated with the immune system and inflammatory response (Table 6.1 - 6.6), are potentially implicated in the early phase connective tissue remodelling response in both intervention groups.

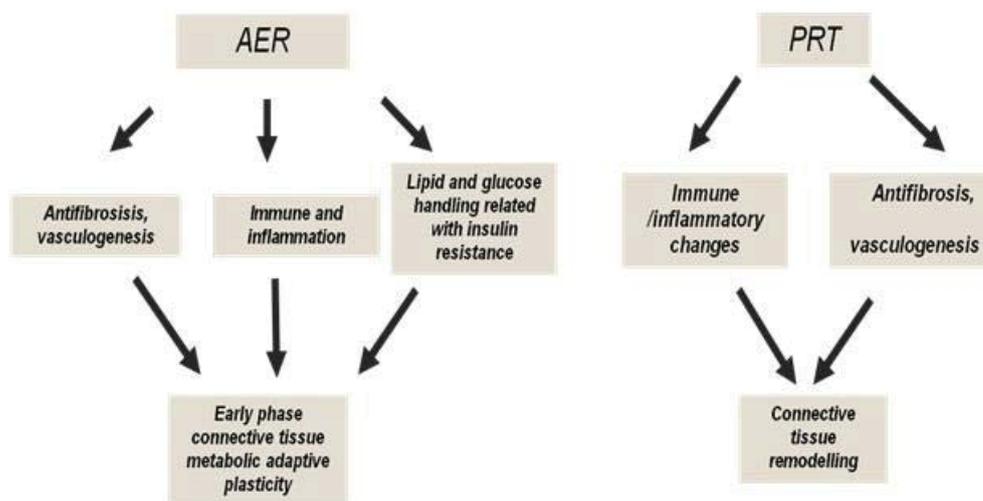


Figure 6.5: Aerobic and resistance training programmes regulate antifibrotic and proangiogenic plasticity in skeletal muscle.

Aerobic and resistance training programmes regulate skeletal muscle plasticity

The aim of the IPA software modelling approach was to identify the top ranked functional modules/networks. By overlapping the top modules/network for each exercise intervention, hub genes and upstream regulatory proteins were identified.

Peroxisome proliferator-activated receptor alpha coactivator (PGC-1 alpha) is a transcriptional coactivator and metabolic regulator which is the key regulator of mitochondrial biogenesis and lipid oxidation (chapter 2 section 2.3). This transcriptional coactivator PGC1-alpha (gene = PPARGC1A) was significantly up-regulated (1.2 fold

change) only in the AER group (Table 6.3). PGC1- α is the key regulator of energy metabolism [18] and upregulation of PGC1- α is linked with improvement in mitochondrial function, fatty acid oxidation, and metabolic capacity after exercise [18, 20] which was also observed in this PhD study (Chapter 5). The key transcriptional factors that are known to be upregulated by PGC1- α related with mitochondrial biogenesis are nuclear respiratory factors (NRF1, NRF2), which up-regulate mitochondrial transcriptional factors (mtTFA, mtTFB1 and mtTFB2) genes [20-25]. The nuclear receptor, protein peroxisome proliferator-activated receptor alpha (PPAR-alpha or PPAR- α) is also stimulated by PGC1- α and is linked to increased fatty acid oxidation [18, 25]. PPAR- α was identified as an activated upstream regulator with AER training in this PhD study (Table 6.5). Activation of PPAR- α promotes uptake, utilisation, and catabolism of fatty acids by upregulation of genes involved in fatty acid transport like fatty acid binding proteins (FABPs), peroxisomal and mitochondrial fatty acid β -oxidation [25]. For this PhD study, FABP4 and FABP5 were identified as key hub genes and FABP4 exhibited a 1.6 fold increase in expression and FABP5 a 1.3 fold increase after 16 weeks of AER training (Table 6.3). It seems very interesting that this upregulation can be correlated with the data about fatty acid oxidation presented in Chapter 5 i-e increased BHAD activity and reduced IMTG content in AER group. The increased fatty acid oxidation could explain the observed decreased lipid accumulation, which could in time lead to decreased tissue insulin resistance [13]. PGC1- α is also reported to activate the expression of GLUT4 in muscle cell [27, 28]. Sukala *et al.* [9] demonstrated increased expression of GLUT4 (by immunological analysis), in the AER group of the SPIRIT cohort. Data suggests that AER exercise was more effective for producing early plasticity changes consistent with better glucose and fatty acid handling as compared to resistance training, where the changes in glucose metabolic disorder related gene functions and phenotype (no change in GLUT4 [9]) were not evident.

Other interesting hub genes identified for the AER training networks were PIK3R1, IGFBP7, CAV1, COL1A2, BDNF, IGF2 ATP2A and MLXIPL (Table 6.3). Phosphatidylinositol 3-kinase regulatory subunit alpha an enzyme encoded by the *PIK3R1* gene, is associated with insulin resistance due to its important role in insulin action [32] (Chapter 2 section 2.3). So in this PhD study we showed an upregulation of PIK3R1, which could lead to improved insulin sensitivity. Insulin-like growth factor-binding protein 7 is a protein that in humans is encoded by the *IGFBP7* gene. The major function of this protein is the regulation of availability of

insulin-like growth factors in tissue as well as in modulating IGF binding to its receptors [33]. For this PhD study we had 1.35 fold increase in *IGFBP7*, which could lead to improved insulin sensitivity. The hub gene *MLXIPL* (MLX interacting protein like), a transcription repressor, was recently reported to have a role in regulation of glucose metabolism [30, 31]. This PhD study showed a 1.2 fold decrease in mRNA expression of *MLXIPL* after 16 weeks of AER exercise and needs to be investigated further. Another interesting gene in this network (Figure 6.2 and Table 6.3) is *ATP2A* which is ATPase associated with Darier's disease and Acrokeratosis verruciformis. *ATP2A2* is related with muscular excitation and contraction [34, 35] however its regulation and effects are not well-known in humans. For this PhD study we demonstrated a 1.2 fold decrease in mRNA expression of *ATP2A2* and this needs to be investigated further.

The other hub genes identified for the AER group *CAV1* (gene that encodes for a scaffolding protein), *BDNF* (involved in regeneration of neurons in CNS and PNS), and *COL1A2* (gene encoding collagen an essential extracellular matrix protein) (Table 6.3) are involved in different cell functions related to structural remodelling. Caveolin-1 (*CAV1*), was upregulated by 1.3 fold. This protein links or binds a number of other proteins in the plasma membrane. For instance *TGFBR2* (receptor II for TGF-beta) which was exhibited 1.16 fold change after 16 weeks AER exercise, is connected with *CAV1*. Both of these proteins have a very important role in cell migration, cell proliferation [36] and vasculogenesis i.e formation of new blood vessels. *TGFBR2* was also upregulated in the resistance training group and is discussed in further detail below.

With respect to the resistance training group, the main upregulated genes related to immune function and inflammation and insulin resistance, were insulin-like growth factor-1 (*IGF-1*), transforming growth factor beta receptor 2 (*TGFBR2*), platelet-derived growth factor receptor beta (*PDGFRB*), mothers against decapentaplegic homolog 3 (*SMAD3*) and interleukin-4 (*IL-4*) (Figure 6.3, Table 6.4 and Table 6.6). *IGF-1* plays an important role in growth and continues to have anabolic effects in adults [33]. Likewise, defects in *IGF-1* have well known associations with pathogenesis of T2DM [37]. Furthermore, *IGF-1* variations are associated with adiposity [38] and it is recently reported that it has receptors on endothelial cells [33] and thus can be involved in multiple processes associated with muscle.

Of the upstream regulatory factors identified that have a role in regulation of ECM remodelling, the SMAD genes, SMAD2 and SMAD 3 are of importance (Table 6.6). SMADs are a central regulator of fibroblast and myofibre formation which is implicated in the inhibition of fibrotic differentiation in myoblasts through down-regulating both extracellular matrix and cell adhesion genes [49]. SMAD3, is the primary mediator for Transforming Growth Factor- beta 1 (TGF- β 1)-induced transactivation of the SM22 (Smooth Muscle specific protein 22) promoter [40]. Receptor II of TGF-beta controls the sensitivity of myostatin/TGFB1 ligand binding and activity of SMADs. In this PhD study a 1.2 fold increase in TGFBR2 mRNA expression occurred after 16 weeks of resistance training. TGFBR2 was also identified as a hub gene for fibrosis and leukocyte migration (Table 6.4) TGF-beta is a known inhibitor of terminal differentiation of cultured myoblasts [41]; however, the functional contribution of TGF- β 1 signalling to disease pathogenesis is associated with various inherited myopathic states *in vivo* [39]. Systemic antagonism of TGF- β 1 has been observed through administration of TGF- β 1 neutralizing antibody or the angiotensin II type 1 receptor blocker, losartan, normalises muscle architecture, repair and function *in vivo* [39, 40, 41].

Skeletal muscle has the ability to achieve rapid repair in response to injury or disease. Myofibroblasts play an important role in a variety of developmental and pathological processes, such as vascular remodelling, atherosclerosis and wound healing [43]. This PhD study provides evidence for gene expression changes associated with increased extracellular matrix remodelling, common to wound-healing biology (Figure 6.4 and Table 6.2). Thirteen of the 20 genes showing greater than 1.4 fold change in mRNA expression are associated with ECM remodelling (Table 6.2). Immune and inflammatory associated remodelling was shown by the activation of some cytokines as upstream regulators in both AER and PRT training groups. The key upstream regulators related with immunity and inflammation were IL-4 and IL-6 which were activated in AER group (Table 6.5) while key activated cytokines in PRT were IL-1B and EDN1. All of the above cytokines have well known association with immune and inflammatory processes [42-44]. By looking at the list of genes with > 1.4 fold increase in both groups (Table 6.1 and Table 6.2), 2/10 were associated with immune inflammation in the AER group and 4/20 in the PRT group.

Both anti-fibrotic and pro-angiogenic modules were top ranked disease and functional processes evident in response to aerobic and resistance training. Increased tissue fibrosis is a well-defined component contributing to disease state in aging, obesity and T2DM [45]. In skeletal muscle, excessive connective tissue accumulation is thought to be triggered by disease associated mechanisms including stimuli like TGFB1. TGFB1 is a protein structurally related similar to a cytokine [43]. It performs multiple functions related with production, proliferation and differentiation of immune cells. It is associated with immunity and chronic low-grade inflammation [45]. The increased endomyosin thickening that increases the physical transit distance between the capillary lumen and the myocellular insulin receptor, hypovascularity and impaired insulin signalling in capillary endothelial cells appear to be key components of skeletal muscle tissue insulin resistance [13]. Meanwhile, ECM and cytoskeleton remodelling are central to aerobic training adaptation as it is discussed in previous paragraphs and consistent with the literature [46-48], changes that may lead to reduced insulin resistance through the connective tissue mechanism. In this PhD study, an important upstream regulator associated with AER training that was identified, was CEBPB (Table 6.5). CEBPB, a transcription regulator, was activated after 16 weeks of AER training, and is an important protein in the macrophage mediated muscle fibre regeneration [51]. This gene is also involved with cellular programme cell death [52]. The interesting aspect of CEBPB is that it has recently being reported [51] to be related with microvascular injury in a mouse model [50] and also reported to have association with the extracellular matrix [53]. an activation of CEBPB after 16 weeks of AER training could mean that the increased expression of CEBPB could lead to immune and inflammatory induced structural remodelling in the skeletal muscle after exercise. Therefore, understanding the complex molecular mechanism(s), driving reduced fibrosis and vasculogenic plasticity could be instrumental in discovery of the cellular processes regulating improved tissue functional capacity in skeletal muscle with metabolic dysfunction.

The involvement of leukocytes in skeletal muscle regeneration from injury, fibrosis, and myogenesis [54] is well established, but identification of an inflammatory-related immune-cell associated regulatory molecular programme directing extracellular matrix remodelling from a within-subject longitudinal exercise-training intervention in grade 3 obese adults with T2DM is a new finding. Sedentary obese and diabetic adults have been reported to exhibit increased collagen deposition and profound thickening of the endomysium (the extracellular

matrix layer surrounding individual muscle fibres) [55]. Increased collagen and other extracellular matrix gene expression were seen in skeletal muscle from healthy adults [47] but has not been demonstrated in T2DM subjects until this PhD study.

The SPIRIT cohort had elevated blood lipid content shown by elevated levels of low density lipoproteins and triglycerides [56]. Improved insulin sensitivity results have been shown by lowering of blood lipids [58]. The microarray data for the AER group of the SPIRIT cohort support changes in lipid handling in the skeletal muscle and support increased fat oxidation and decreased lipid deposition. Therefore, it could be proposed that aerobic exercise may be altering metabolic flexibility in the skeletal muscle by improving lipid handling.

Significance of network exploration

Strengths of the current network approach via the IPA software include construction of reliable disease and functional modules/networks based on the comprehensive human tissue specific transcriptome. A consequent weakness is the dataset complexity. The modular disease network bioinformatics approach utilises the existing knowledge of interaction and imitates the bioinformatics tools to explore the roles of those involved networks in diseases. It further explores the related pathways particularly with respect to the effective molecular and phenotypically functional magnitude (Z-score and P-value) altered by intervention. This approach was used for the first time in T2DM participants with grade 3 obesity (SPIRIT study) to explore the mRNA changes in relation to exercise intervention (AER and PRT) in South Pacific Islanders and has provided new information regarding the cellular and molecular pathways underpinning the skeletal muscle plasticity in relation to exercise.

Proposed Hypothesis related to the gene expression changes observed in the SPIRIT cohort

The involvement of leukocytes in skeletal muscle regeneration from injury, fibrosis, and myogenesis is well established, but identification of an inflammatory-related immune-cell associated regulatory molecular programme directing extracellular matrix remodelling from a within-subject exercise-training intervention in grade 3 obese adults with type-2 diabetes is new. The discovery of this new pathway can lead to the hypothesis that exercise can induce antifibrotic provasculogenic changes in the skeletal muscle leading to increased structural

plasticity and metabolic flexibility. Furthermore, exercise may be recommended as an intervention for the pathogenic model of fibrosis induced skeletal muscle metabolic inflexibility proposed by Coletta and Mandarino [13].

Implications

The key hypothesis underlying the network modular approach was that the disease modules/networks may represent dysfunctional-tissue phenotype associated with disease. Moreover, these phenotypic changes/dysfunctions associated with disease could be a consequence of changes in the key genes intersecting or overlapping upon several cellular and molecular processes of physiological significance. As a result of this interdependency, key functional and disease regulators, and associated gene regulatory connectivity was readily identified from centrally connected hubs. Indeed, the repeated occurrence of known important regulators of skeletal muscle adaptation to exercise within the networks (e.g. *PPARGCIA*, *PDGF*, *PPARA*) presented support to the concept that exercise either AER or PRT in the SPIRIT cohort was able to induce mRNA changes [55]. Additionally, the functions utility in IPA analysis provided further valuable insight into the control networks matching with muscle plasticity. It also informs on the complexity of the multiple molecular systems governing disease-attenuating plasticity to chronic exercise training in type-2 diabetic skeletal muscle. However these results should be considered with caution due to small number of participants (17 in total) and low attendance with the prescribed exercise sessions (5/9 (56%) completed 75% of sessions in AER group and only 3/8 (37%) for the PRT group). Furthermore the significant mRNA changes observed in this study from the microarray data were not further validated by real-time PCR, due to unavailability of the muscle tissue. Some protein expression work has been done and validates some of the mRNA expression data (see Chapter 5).

To summarise, these results suggest that exercise could be an important rehabilitation tool to stimulate skeletal muscle plasticity in subjects with T2DM and grade 3 obesity. Both resistance and aerobic forms of exercise can induce plasticity in terms of their role in antifibrosis and vasculogenesis gene stimulation. However, aerobic exercise seems to be the superior exercise intervention in terms of its role in lipid and glucose handling in this SPIRIT cohort. These results should be dealt with caution due to the limited number of participants, less compliance to the exercise regime and future studies should examine both a longer

exercise duration and make direct measures of tissue and whole-body insulin sensitivity or glucose uptake to validate the present molecular inferences. Future work is also needed to validate the microarray data.

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CHAPTER 7

CONCLUSION

7.1 Introduction

Obesity is a major risk factor in the pathogenesis of T2DM [1]. When the amount of circulating lipids exceed the white adipose tissues' ability for uptake and storage of lipids, fatty acids will find other tissues that have a limited capacity for lipid storage, such as liver and skeletal muscle, to accumulate in. Such abnormal ectopic lipid accumulation (lipotoxicity) is strongly associated with insulin resistance and International Diabetes Federation reported [2] that approximately 80% of patients with T2DM have associated obesity.

In light of this evidence, development of strategies to promote better health such as purposeful and targeted exercise programmes could be advantageous in the battle against obesity and T2DM. In fact in the past 15 years, exercise has become an integral tool in the prevention and management of T2DM [3,4]. However there is very limited evidence available to look at the impact of exercise for all populations and most RCT exercise studies have been concentrating on Caucasians with T2DM as participants in the study. For instance Sukala and colleagues [5, 6] have reported that colonised, indigenous populations such as Pacific Islanders that emigrate to live in New Zealand are the most severely affected by the diabetes-obesity epidemic, yet the evidence base for exercise efficacy in these cohorts remains virtually non-existent.

This PhD project has focused on the role of exercise either aerobic (AER) or progressive resistance training (PRT) in Pacific Islanders and Maori people and is part of the South Pacific Islands Resist Diabetes with Intense Training (SPIRIT) study. The SPIRIT study was initiated by Bill Sukala for his PhD [7] and its purpose was to evaluate and compare the effectiveness of two conventional exercise modalities for improving glycosylated haemoglobin (HbA1c) and related physiological and psychological outcomes in Polynesian adults with T2DM and grade 3 obesity [5-10]. Dr Sukala found that 16 weeks of either AER or PRT had no effect on improving glycaemic control as noted by no change in HbA1c [6, 7]. This was an unexpected outcome due to the fact that the inclusion of exercise as a tool in management of T2DM has been based on evidence from exercise studies showing a drop in HbA1c in T2DM participants undergoing AER and PRT exercise [11 - 13]. This led to discussions as to why the SPIRIT study participants demonstrated no change in HbA1c levels. The proposed hypothesis as outlined in Chapter 1, Section 1.3 was that the metabolic

adaptations such as a drop in HbA1c levels in the SPIRIT study cohort are delayed due to metabolic inflexibility in this cohort. This led to the question “If the metabolic adaptations are delayed are there other changes in the SPIRIT participant that could be identified to demonstrate that exercise for the grade 3 obese T2DM participants is having a beneficial effect?” Functional, genetic and structural changes in the skeletal muscle, the major tissue for disposal of glucose [14] and where development of insulin resistance plays an important role along with looking at changes in known obesity markers in the SPIRIT cohort were potential targets for proving or disproving the proposed hypothesis and five specific objectives outlined for this study (Chapter 1 Section 1.3). The results of this investigation are summarised and discussed below.

7.2 Summary of Principal Findings

For the purpose of this study, the impact of 16 weeks exercise on the obesity biomarkers leptin, adiponectin, cortisol, CBG and SHBG were investigated (Chapter 4 section 4.3). These obesity markers were chosen because they cover a wide range of physiological functions connected with obesity and are associated with insulin resistance either directly or indirectly by virtue of their role in glucose and fatty acid metabolism. There was significant increase in the SHBG levels in the PRT group which can be a marker of better insulin sensitivity. The results of rest of the obesity markers in this study show that there was no improvement in the obesity markers i-e leptin, CBG, Cortisol and their related mRNA levels (Chapter 4 section 4.3).

Some statistically significant and interesting correlations were observed in both training groups in relation to obesity markers (Chapter 4, Tables 4.4- Table 4.7). In the AER group there were a number of positive statistically significant correlations associated with the obesity markers investigated (Chapter 4: Table 4.5). An increase in SHBG levels was associated with increased number of exercise sessions attended which suggests that attendance at the available sessions for the exercise was a key factor in increasing SHBG levels(a surrogate marker of insulin resistance) and hence possibly improving insulin sensitivity. Increased leptin levels were associated with increased BMI and fat mass, and increased cortisol levels were associated with increased waist-to-hip ratio (Chapter 4 Table 4.4). Cortisol levels were statistically negatively correlated with the number of exercise sessions attended meaning that if the participant attends more exercise sessions this should

lead to decreased cortisol levels and possibly a decrease in gluconeogenesis and lipid deposition, both factors important in T2DM. In the PRT group there were a number of positive statistically significant correlations associated with the obesity markers investigated as well. Increase in SHBG levels was associated with increased number of exercise sessions attended, an increase in leptin levels was associated with increased fat mass, and decreased cortisol levels were associated with increased SHBG (Chapter 4: Table 4.4 and Table 4.5). These results may illustrate that the number of exercise sessions could be important to induce any change in the SHBG levels and cortisol levels in both training group. Moreover leptin levels may be affected by change in fat-mass and waist-to-hip ratio.

Functional changes in the SPIRIT study skeletal muscle tissue after 16 weeks of exercise, was examined by investigating mitochondrial activity. Three key mitochondrial enzymes cytochrome c oxidase (COX- involved in electron transport chain), citrate synthase (CS- involved in Krebs cycle) and beta-hydroxyacyl-CoA dehydrogenase (BHAD- involved in beta oxidation of fatty acids) were examined (Chapter 5, section 5.3.1). The activities of the three key enzymes were significantly increased in both groups. The effect size was calculated to illustrate the magnitude of the change after AER and PRT training. There was a very large effect ($r = 6.7 \pm 1.2$) in the AER group for BHAD activity whereas in the PRT group a large effect (2.7 ± 1.2 and 2.3 ± 1.3) for BHAD activity and COX activity respectively was observed. There was a statistically significant increase in CS activity in both groups (PRT; $p = 0.007$, AER; $p=0.03$) however the activity increase was more in the PRT group (effect size = 1.8 ± 1.3). COX activity was raised in both groups as well though the effect size in the PRT group was 2.3 ± 1.2 meaning a very large change with PRT exercise compared to a moderate effect (1.0 ± 1.2) with AER exercise. The significant increase in the mitochondrial activity reflects that exercise is causing a change in the metabolic capacity of the skeletal muscle, especially metabolic pathways associated with energy metabolism (Krebs cycle and electron transport chain) and fatty acid oxidation. This would mean that exercise is causing muscle adaptation in the SPIRIT cohort but yet to provide observable changes at the macrophysiological level. It is believed that a longer duration of exercise and intensity are needed before the physiological changes such as reduced HbA1c may occur.

To look for the morphological changes in the SPIRIT participants' skeletal muscle, the IMTG content and mitochondrial structure was analysed (Chapter 5 section 5.3.2). Significant reduction in IMTG content in both AER (48% decrease in IMTG) and PRT (28% decrease in

IMTG) exercise group was observed. This indicated that both exercise groups were able to induce changes in lipid metabolism in the muscle. The changes in IMTG content has been previously reported with exercise trainings [15, 16] but the cohorts were not grade 3 obese. The observed decrease in IMTG content for this study could indicate a change in tissue structure lending towards healthy tissue formation. It would have been helpful to determine mitochondrial density (as IMTG density was done) but unfortunately it was due to time restraints. For future work the EM images need to be investigated further and changes in abnormal mitochondria after 16 weeks of exercise as well as alterations in number of mitochondria pre and post 16 weeks exercise needs to be determined.

To look for the genetic modifications after 16 weeks of exercise in the SPIRIT participant skeletal muscle, microarray data was interrogated and changes in gene expression related to fatty acid metabolism, insulin resistance, fibrosis and angiogenesis were investigated (Chapter 6 section 6.3). For the PRT training group the following upstream regulators (Table 6.6) and hub genes (Table 6.4) were identified: TGFBR2, SMAD2, SMAD3 and IL1-B and for the AER training group the following upstream regulators (Table 6.5) and hub genes (Table 6.3) were identified: PIK3R1, BDNF, COL1A2, MLXIPL, CAV1, IL4, IL-6 and TGFBR2. The aforementioned genes in both exercise groups suggest antifibrotic and vasculogenic plasticity has occurred after 16 weeks of exercise. Furthermore in response to aerobic training there was further evidence for induced glucose and lipid handling. The hub genes and key upstream regulators related to glucose and lipid handling were, PIK3R1, FABP4, PPARG, and PGC1-alpha (Table 6.3 and 6.5).

The key findings for this PhD study are summarised in Figure 7.1. These findings may support the hypothesis stated in Chapter 1, that cellular changes in the skeletal muscle of the SPIRIT cohort are occurring. The increased activity of mitochondrial enzymes, decrease in IMTG content, and up-regulation of pathways related to anti-fibrosis in both training modalities indicate improvement in the plasticity of skeletal muscle after AER and PRT exercise. AER seems to be a better intervention than PRT in terms to the cellular and gene changes occurring in the muscle that are related to lipid and glucose metabolism and insulin signalling.

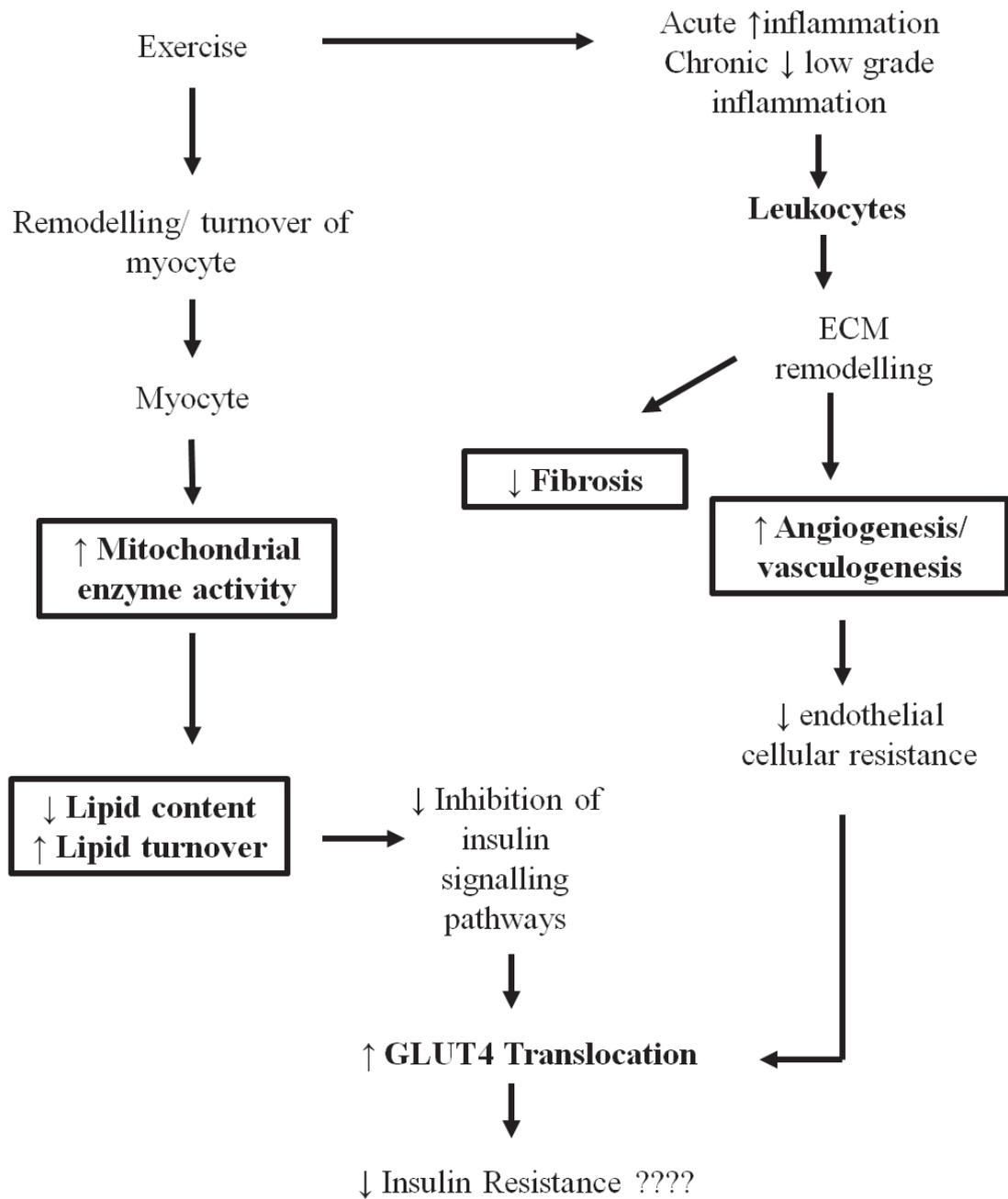


Figure 7.1 Principal findings of SPIRIT cohort in relation to morphology, function and genetic changes of skeletal muscle after 16 weeks of AER or PRT exercise. The boxes in bold are high-lighting principal findings of this study.

The SPIRIT cohort undergoing AER exercise had moderate increase in capillary density and a drop in the resting blood pressure [6, 7] however molecular systems indicate increased vasculogenesis and tissue remodelling in both training groups. After examining the morphological/phenotypic data to genetic data obtained, it can be suggested that vasculogenic

and connective tissue remodelling in the SPIRIT cohort is responding to AER exercise more quickly than PRT training. These findings are summarised in Figure 7.1.

For this study the functional data and the genetic data support skeletal muscle plasticity after 16 weeks of both AER and PRT trainings however the lipid tissue handling in the PRT was not evident from the genetic data.

7.3 Limitations and Difficulties

There are a few limitations of this study. Firstly the cohort size was very small and these results should be considered with caution. Secondly obesity and T2DM have strong pathogenesis related with inflammation and pro-inflammatory cytokines. However detection of the pro-inflammatory cytokines e.g. TNF- α and interleukins was not done. A further study with more focus on other associated parameters of inflammation and immunity is therefore suggested. Further work is required to establish the intervention strategies for T2DM.

Thirdly the lack of diet and other physical activity data is another limitation of this investigation. Existing data suggests that the diet with carbohydrate or lipid will affect formation, storage and breakdown of intramyocellular lipids [17-19]. It has been proposed that lipid and carbohydrate infusions can hamper glucose metabolism in the state of hyperglycaemia [20] and high fat levels in the blood. The studies with the effect of exercise also suggest that diet plays a paramount role in the selection of what fuel to use during the recovery period in the muscle cell [21]. Thus the diet could be a confounding factor in this study.

Thirdly the participants in our study were mostly females (AER=6 females, PRT= 6 females). As the evidence suggests that the lipid metabolism in the female body is under the control of hormones such as oestrogen and can protect females in the reproductive age group against heart disease [22]. The lack of menstrual data and female hormones present pre and post 16 weeks intervention has not allowed exploration into impact of exercise on lipid metabolism in women with diabetes and obesity.

There were several difficulties with the studies. Firstly optimisation of mitochondria and lipid data the human muscle sample was in small quantity so the assays had to be optimised on the rat tissue first and after that trialled on the human spare tissue. The critical issues regarding the optimisation were the amount of tissue to be utilised as there was less tissue available so it

was necessary to find one method for extraction of muscle protein which then allowed the muscle protein homogenate to be used for all three enzyme assays. It was time consuming to optimise the protein extraction method and three enzyme methods before being able to determine the mitochondrial enzyme activity in the 18 participants' pre and post exercise. Secondly the data regarding the electron microscope was enormous and it was very difficult to quantify all the images. Thirdly the genetic data was very difficult to analyse statistically due to the huge amount of information available. It was important to scrutinise and make inferences from that data set. Regarding the genetic changes, several key gene hubs have been identified however the microarray data should be validated by real time PCR work.

Further research is required to investigate the factors that hinder the body's adaptation to exercise, including obesity which promotes leptin resistance and a state of chronic inflammation. This relationship of cortisol, leptin and adiponectin in relation to exercise has not been addressed before and it adds new insights to the complexity of the intricate mechanisms linking obesity, cortisol and exercise. The challenge is to understand why exercise was unable to affect adipocytokines and what pathways should be targeted in future to see the improvements. So the research questions that could be asked include a) whether SHBG is the early indicator of improvement in the insulin resistance b) that a decrease in CBG levels after exercise without change in the cortisol, is a positive adaptative phenomenon with exercise of 16 weeks in morbidly obese individuals c) what is the type and length of exercise that improves leptin sensitivity in obese individuals with T2DM d) can the changes in the leptin resistance be correlated to insulin sensitivity and e) can cortisol be a contributor of leptin and insulin resistance in morbidly obese individuals. Further studies on current topic of obesity as the first hurdle in improving insulin and leptin resistance in people with type 2 diabetes are needed.

Figure 7.2 illustrates the mechanism which is related to the disturbed metabolism associated with obesity. This figure shows that the obesity, insulin resistance and stress can be related to each other. Increased lipid content or obesity leads to increased release of leptin and it is releases proinflammatory cytokines if these signals exceed the physiological limit, they can result into leptin resistance. The inflammatory markers cause systemic low grade inflammation that produces insulin resistant states ultimately leading to T2DM. The neuro-

endocrine pathway related with HPA axis releases cortisol that leads to increased glucose and fatty acids release and can be lead to obesity.

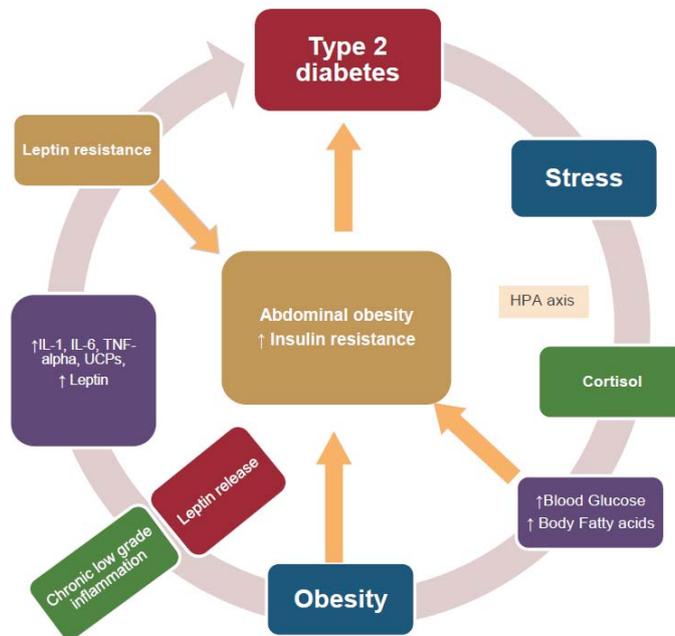


Figure 7.2 Mechanisms at the cross roads of obesity, stress and T2DM working in vicious cycle

7.4 Consideration for Future Research

This PhD study proposes/highlights several pathways and factors that may be targeted in order to prescribe the exercise trial to contribute to better improvement and may positively influence obesity markers.

It prepared the ground for the novelties of skeletal muscle research that has already produced a huge amount of data and is clarifying a number of physiological and pathophysiological realities. Rising prevalence of T2DM and obesity along with the burden of its associated cardiometabolic complications makes exercise intervention a most timely one.

A recent review by S.R.Bird & Hawley [23] recommends a new strategy of high-intensity interval training (HIT) and argues that such type of training could be time-efficient and well-tolerated in pre clinical and clinical diabetes population. So such types of exercise training

programme could produce better results in a prompt manner. Lastly as outlined by Sukala *et al.* [10] investigations to more fully understand the socio-economic, cultural and psychological barriers to exercise adoption in indigenous populations are warranted. Furthermore it can also be suggested that exercise of longer duration would be more effective.

7.5 Conclusion

Although 16 weeks of AER and PRT trainings were unable to produce change in HbA1c and related parameters the cellular and metabolic findings from this PhD project indicate that the changes occurring in the SPIRIT study cohort are subtle. This study has shown that AER or PRT exercise is causing changes in skeletal muscle morphology and function. 16 weeks of either AER and PRT can increase mitochondrial activity (increased CS, COX and BHAD enzyme activity), increase activity of metabolic pathways involved in energy metabolism (increase CS and COX activity) and increase fatty acid β oxidation (increased BHAD activity), reduce IMTG content and thus can improve functional plasticity of the skeletal muscle. Secondly interrogation of the genetic pathways in the SPIRIT cohort skeletal muscle reveal that AER and PRT exercise are stimulating changes in gene expression and induce plasticity in the skeletal muscle in terms of their anti-fibrotic and angiogenic roles. AER intervention also appears to stimulate changes in lipid and glucose handling in the muscle of grade 3 obese people with T2DM. The take home message from this study is that exercise does work and is effective in causing cellular and morphological changes in skeletal muscle tissue that is previously metabolically inactive.

7.6 References

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APPENDIX A:

SPIRIT STUDY MATERIALS AND METHODS

A- Participants

A1. Sample size

The SPIRIT study was initially intended to be a randomised controlled trial comparing a resistance training group to a non-exercise control group. A sample size of 12 per group was determined *a priori* based on a previous study of similar design by Castaneda *et al.*[1] and is illustrated below.

T-tests: Means: Difference between two independent means (two groups)
Analysis: A priori: Compute required sample size

Input	Tail(s)	=	Two
	Effect size d	=	1.2162423
	α err prob	=	0.05
	Power (1- β err prob)	=	0.80
	Allocation ratio N2/N1	=	1
Output	Noncentrality parameter δ	=	2.979173
	Critical t	=	2.073873
	Df	=	22
	Sample size group 1	=	12
	Sample size group 2	=	12
	Total sample size = 24		

However, upon initiation of subject recruitment, many subjects stated they would drop out if randomised to a non-exercise control group because they knew exercise could potentially improve their diabetes and facilitate weight loss. After further consultation with cultural liaisons, the substitution of an aerobic exercise group in place of the control group was deemed a feasible and acceptable option to participants.

A2 Subject screening

Individuals were selected for participation in the trial if they met the following inclusion criteria: 1) self-identified Maori or Pacific Islands descent; 2) diagnosis of type 2 diabetes by physician [54]; 3) waist circumference of 88 cm for women and 102 cm for men; 4) physically inactive for six months; 5) no change in diabetes medications for previous two months; and 6) no acute or chronic medical conditions for

which exercise would be contraindicated as outlined by the American College of Sports Medicine (ACSM) [2]. All protocols and safety procedures were developed in partnership with consulting diabetes specialists and were in accordance with established international safety guidelines for exercise set forth by the American Diabetes Association [3] and ACSM [2].

After initial telephone pre-screening, potential participants were invited along with family and friends for a private consultation to learn more about the SPIRIT study and ask questions. Home and office visits were made by the principal investigator (WRS) to accommodate busy work and family schedules. Written informed consent was obtained from all subjects and their respective general practitioners, with the latter responsible for conducting a thorough medical history review of each participant. All procedures and protocols were approved by the Central Regional Ethics Committee (CEN/07/08/054), and the trial was registered with the Australian New Zealand Clinical Trials Registry (number: ANZCTR12609001085268).

A3 Recruitment

The SPIRIT recruitment process differed markedly from the usual procedure of placing a newspaper advert or strict reliance upon referrals from medical management teams. Health care professionals affiliated with clinics in the Porirua region initially provided the SPIRIT study contact details to potentially eligible diabetic patients but this resulted in few referrals. This was addressed by instead having general practitioners and practice nurses forward the names and phone numbers of interested and eligible candidates (with permission) to WRS, at which time first contact was initiated. The first wave of enrolled subjects referred friends, family, co-workers, and fellow church congregants to the study similar to the “snowballing” effect previously described by Murphy and colleagues [4]. Some subjects contacted WRS after viewing several media stories on the study.

A4 Randomisation

Following baseline testing, participants were randomly assigned *via* computer generated randomisation list [5], stratified by gender in blocks of four to receive 16 weeks of either PRT or AER. Randomisation assignments were generated by an investigator who was not involved

in testing or training and delivered to patients in opaque sealed envelopes on the completion of all baseline testing.

B- Exercise venue

Exercise sessions were initially planned to take place at the fitness centre on Massey University's Wellington campus, but several issues including traffic, distance, lack of parking, and scheduling arrangements in relation to existing programs would have adversely impacted upon recruitment and implementation. Instead, the trial was conducted at Porirua City Fitness gym. The club is located in the largely Polynesian suburb of Porirua (21 km north of Wellington) and has a high Maori and Pacific membership base. Participants were welcomed by members and employees, many of whom were already close personal contacts from the community. Porirua City Fitness management allocated staff time and resources (offices, equipment) at no cost to the SPIRIT study to help ensure the smooth execution of the study protocols. Participants received free memberships for the duration of the study intervention period (16 weeks) and were encouraged by investigators to bring family and friends free of charge to support their efforts. During initial consultation with cultural leaders and allied health professionals, there was concern that a commercial fitness centre would be a potential barrier to M_ori and Pacific Islands people. However, participants anecdotally stated their satisfaction with Porirua City Fitness, and that the large Polynesian membership and cultural influence within the venue created a comfortable environment which enhanced the experience and facilitated the study's execution. For example, some participants spoke their native language and engaged in prayer with fellow study participants and club members before or after training sessions.

C. Intervention

Group exercise training sessions were held three times per week (Monday, Wednesday, and Friday) in the morning and afternoon and lasted no longer than 50-66 minutes, including warm-up and cool-down procedures. Pre- and post-exercise pulse rate, blood pressure, and capillary blood glucose (via Accu-Chek Performa glucometer, Roche Diagnostics, Auckland, New Zealand) were monitored and recorded at each session. William Sukala maintained ongoing communication with each participant's diabetes management team (e.g., endocrinologist, general practitioner,

diabetes nurse specialist) and reported any untoward signs or symptoms. Subjects continued to receive their usual medical care and were instructed to maintain their current dietary and physical activity habits. Weekly status checks were conducted and any adverse signs or symptoms were reported to participants' general practitioner.

C1-Exercise leaders

All exercise sessions were personally supervised by William Sukala, a qualified clinical exercise physiologist with extensive experience working with obese, diabetic, and cardiac patients. Four advanced (year 3) exercise science students from Massey University and three New Zealand Registry of Exercise Professionals-certified personal trainers from Porirua City Fitness were familiarised with the intervention protocols and assisted in providing one-on-one attention to participants before, during, and after exercise in order to ensure both safety and compliance to study protocols. Four of the seven exercise leaders were of Maori and Pacific Islands heritage and regularly reaffirmed cultural and ethnic identities. Furthermore, a number of Maori and Pacific religious and community leaders involved in establishing the study were also Porirua City Fitness members and periodically provided on-site encouragement to participants.

C2 Progressive resistance exercise

After a five minute warm-up, subjects performed eight machine-based resistance exercises in a circuit format targeting all major muscle groups: seated leg press, knee extension, knee flexion, chest press, lat pull downs, overhead press, biceps curls, triceps extension (Cybex International, Medway, MA). Due to the level of physical deconditioning and class III morbid obesity (Body Mass Index [BMI] = 43.8 ± 9.5 kg/m²; n = 18) observed in this cohort, a one-repetition maximum (1RM) lift at baseline was deemed inappropriate for safety reasons. Initial weights were therefore determined as a percentage of the extrapolated 1RM as previously described by Brzycki [6]. A graduated periodised regimen was employed as illustrated in Figure 3.3 and 3.4. Subjects progressed from 65% to 85% of their extrapolated 1RM over the course of phase 1, two sets at 85% during phase 2, three sets at 85% during phase 3, and a continuation of three sets at 85% until conclusion of the intervention. This regimen was chosen because it is known to promote muscular hypertrophy and therefore enhance insulin sensitivity and glucose uptake. Subjects performed six to eight repetitions

with a one minute rest between sets. Workloads were increased by five percent when subjects could perform 10 repetitions. Exercise leaders encouraged subjects to exercise at a perceived exertion of “hard,” or 15, on the Borg scale (6 – 20 scale) [8]. In order to minimise the risk of overtraining, weeks four, eight, and 12 were designated active recovery weeks in which subjects performed one set on each exercise at a weight 10% less than the previous week’s peak workload.

C3 Aerobic exercise

Subjects randomised to the AER group performed a graduated cycle ergometry (Life Fitness, Schiller Park, IL, USA) protocol (Figure 3.5 and 3.6) in parallel with the PRT group. An equivalent training frequency, duration, and intensity were prescribed to evaluate the training response between modalities. AER not only improves cardiovascular fitness, but is also known to enhance insulin sensitivity and consequently glycaemic control [7]. After familiarisation with the equipment at week 0, subjects gradually progressed from 65 to 85% of their heart rate reserve during subsequent sessions. They were encouraged to sustain a rating of perceived exertion of “hard,” or ‘15’ on the Borg scale [8]. Heart rate and blood pressure were monitored and recorded at peak steady state workloads. Watts and duration at peak intensity were increased to accommodate improved fitness levels over time. Similar to the resistance training group, weeks four, eight, and 12 were designated active recovery weeks in which subjects exercised at an intensity 10% less than the previous week’s peak workload achieved during the previous week.

D Outcome Measurements

Outcome measure assessments were performed at baseline and after 16 weeks of exercise training. Health history assessment forms and the Medical Outcomes Study Short-Form General Health Survey version 1 (SF36) quality of life (QOL) questionnaire were completed in a quiet room at Porirua CityFitness gym. Blood draws, anthropometric, haemodynamic, and muscle biopsy procedures were performed at Kenepuru Hospital in Porirua after an overnight fast. Many subjects were familiar with the hospital because they (or family) had previously been admitted as a patient or attended health education classes. Moreover, it is also a trusted local healthcare institution with multilingual signage and

culturally-diverse staff. SPIRIT study investigators or hospital staff greeted participants upon arrival and guided them to the exam rooms.

D1 Biochemical measurements

Fasting blood draws were performed by hospital phlebotomists and sent to the medical laboratories at the Capital and Coast District Health Board and Canterbury District Health Board for analysis. Glycosylated haemoglobin was the primary outcome measure and was determined by ion exchange high pressure liquid chromatography using the Bio-Rad D-10 analyser (Bio-Rad Laboratories, Hercules, CA, USA) with a coefficient of variation (CV) of 3%. Plasma glucose was determined by the hexokinase enzymatic method on the Roche Modular Analyser (Roche Diagnostics, Indianapolis, IN, USA) with a CV of 3%. Serum free insulin concentration was determined by electrochemiluminescence immunoassay using an Elecsys 2010 immunoanalyser (Roche Diagnostics, Indianapolis, IN, USA) with a CV of 3.8%. Insulin resistance was estimated by two methods: 1) from fasting glucose and insulin *via* homeostasis model assessment (HOMA2-IR software, version 2.2.2, Oxford University), a method previously validated against the euglycaemic clamp ; and 2) the McAuley Index (MI) (calculated as $MI = EXP [2.63 - 0.28LN(\text{fasting insulin}) - 0.31LN(\text{fasting triglycerides})]$), which has also been validated against the euglycaemic insulin clamp . Serum free fatty acids were determined enzymatically by the ACS-ACOD method (Wako Chemicals, Neuss, Germany). Serum C-peptide concentrations were determined by electrochemiluminescence immunoassay using an Elecsys 2010 immunoanalyser (Roche Diagnostics, Indianapolis, IN, USA) with a CV of 4.5%. Serum total cholesterol, high density lipoprotein (HDL) cholesterol, and triglyceride concentrations were measured using standard enzymatic methods (Roche/Hitachi lipid assay kits) with a Roche Modular Analyser with a CV of 3% on each assay. Low density lipoprotein cholesterol was mathematically determined as $\text{total cholesterol} - \text{HDL} - (0.45 \times \text{TG})$. C-reactive protein was determined by latex agglutination method on the Roche Modular 72. Analyser with a CV of 4%. Adiponectin was measured by radioimmunoassay (Linco)

D2 Anthropometric and haemodynamic measurements

Height and weight were measured to the nearest 0.1 cm and 0.1 kg on a calibrated hospital stadiometer and scale, respectively. BMI (in kg/m²) was calculated

from these measures. Lean body mass, fat mass, and percent body fat were estimated *via* bioelectrical impedance analysis (Tanita TBF-310 analyser, Tanita Corporation, Arlington Heights, Illinois, USA). Waist circumference was measured to the nearest 0.1cm at the end of normal expiration and the midpoint between the lower costal margin and the iliac crest using a retractable steel tape measure (model F10-02. KDS Corporation, Japan). Resting blood pressure was measured in duplicate from the left arm after 5 minutes of seated rest on a standard hospital sphygmomanometer, with the lowest blood pressure being recorded. Large adult cuffs were used if the subject's arm was too large for the standard cuff size.

D3 Muscle biopsy procedure

Muscle biopsy samples (~200 mg) were harvested from the right vastus lateralis under local anaesthesia (1% Xylocaine, Astra Zeneca Ltd, Auckland, New Zealand) using a 5 mm Bergstrom needle with applied suction at baseline and 16 weeks. Consistent with previous research follow-up testing was standardised to 72 hours after completion of the final exercise session to minimise the potential confounding effect of the acute exercise response from the last training session. Participants were given the opportunity to speak with the physician performing the biopsy, ask questions, and reserved the right to refuse the procedure. All subjects gave separate written informed consent for the biopsy procedure. Muscle samples were oriented longitudinally in Tissue-Tek optimal cutting temperature (OCT) embedding medium (Sakura Finetek Ltd, Tokyo, Japan) and snap frozen in liquid nitrogen-cooled isopentane and stored in Eppendorf cryotubes at -80°C until analysed.

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APPENDIX B:

SPIRIT PARTICIPANT DATA SHEET

Report

Name of participant and number have been hidden for confidentiality.

GLUCOSE FASTING

Glucose	18.6	mmol/L
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Authorised by : Filippo Faiga at 6:06 PM on 07-11-2008

FASTING LIPIDS

Cholesterol	5.6	mmol/L
Triglycerides	2.06	mmol/L
LDL	3.7	mmol/L
HDL Cholesterol	0.96	mmol/L
Chol:HDL ratio	5.8	

Authorised by : Filippo Faiga at 6:06 PM on 07-11-2008

%HbA1c	12.2 H	%	(<6.0)
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HbA1c REFERENCE RANGE AND INTERPRETATION :

<6.0 Normoglycaemia
 6.0 - 7.0 Near normoglycaemia
 7.0 - 8.0 Good glycaemic control
 8.0 - 9.0 Fair to inadequate glycaemic control
 9.0 - 10.0 Poor glycaemic control
 >10.0 Very poor glycaemic control

Authorised by : Evelyn Tompson at 4:59 PM on 12-11-2008

Total Testosterone	Not required.		
SHBG	36.2	nmol/L	(26.1 - 110.0)
%Free Testosterone (calc)	Not required.		
Free Testosterone (calc)	Not required.		

Authorised by : Elizabeth Keightley at 4:12 PM on 11-11-2008

Insulin (free)	512 H	pmol/L	(10 - 80)
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This INSULIN assay has been performed by Canterbury Health Labs.

The DEFINITIVE report is the ORIGINAL issued by Canterbury Health Labs.

Ref. Range is for adult, fasted overnight, BMI<25 . Non fasting ref range: up to 400 pmol/L

This report printed: 20-Jul-2009 at 09:51

Page: 1 of 3

Report

Name of participant and number have been hidden for confidentiality

If patient hypoglycaemic, an elevated fasting insulin is suggestive of inappropriate insulin excess secondary to endogenous hypersecretion, sulphonurea use or exogenous insulin administration.

The measurement of insulin, either fasting or random, is not recommended in the investigation of PCOS or the insulin resistance syndrome.

Authorised by : Vivienne Hughes at 10:36 AM on 12-11-2008

Sent away **ADIPONECTIN**
This assay not performed at Wellington Hospital Laboratory.
Adiponectin: 5.5 ug/ml (Interim range >4)
Test performed by CHL.

Authorised by : Vivienne Hughes at 09:20 AM on 03-12-2008

Sent away **LEPTIN**
This assay not performed at Wellington Hospital Laboratory.
Plasma Leptin: 34.0 ug/L

Ref Range:
Female: 3-15 ug/L (BMI= 20)
 7-35 ug/L (BMI= 25)
Male: 1-3 ug/L (BMI= 20)
 2-10 ug/L (BMI=25)
Reference interval related to BMI and gender. Graph available on request.
Test performed by CHL.

Authorised by : Vivienne Hughes at 1:08 PM on 13-02-2009

Sent away **CBG**
This assay not performed at Wellington Hospital Laboratory.
Cortisol binding globulin: 768 nmol/L
Ref Range: 300-1800
Test performed by CHL.

Authorised by : Vivienne Hughes at 12:55 PM on 19-11-2008

Report

Name of participant and number has been hidden due to confidentiality

Serum held for this Episode is due for discard in : **SEE NOTE** weeks.
 FOR PICK UP

Authorised by : Vivienne Hughes at 1:28 PM on 10-11-2008

FULL BLOOD COUNT

HAEMOGLOBIN	152	g/L	(120 - 155)
PACKED CELL VOLUME	0.455 H		(.340 - .450)
RED CELL COUNT	4.87	10 ¹² /L	(3.90 - 5.35)
MEAN CELL VOLUME	93.4	fL	(78 - 100)
MEAN CELL HAEMOGLOBIN	31.2	pg	(27.0 - 32.0)
PLATELET COUNT	186	10 ⁹ /L	(150 - 500)
MEAN PLATELET VOLUME	12.8 H	fL	(7.4 - 10.4)
WHITE CELL COUNT	6.11	10 ⁹ /L	(4.0 - 11.0)
NEUTROPHIL ABS AUTO	2.80	10 ⁹ /L	(2.0 - 7.5)
LYMPHOCYTE ABS AUTO	2.52	10 ⁹ /L	(0.9 - 3.6)
MONOCYTES ABS AUTO	0.43	10 ⁹ /L	(0.1 - 0.8)
EOSINOPHIL ABS AUTO	0.33	10 ⁹ /L	(0.1 - 0.5)
BASOPHIL ABS AUTO	0.03	10 ⁹ /L	(0.0 - 0.1)

Authorised by : Emilie Payne at 11:56 AM on 07-11-2008

C-Reactive Protein (High Sensitivity) 7.38 H mg/L (0 - 3)

Performed by CHL

In the absence of overt inflammation there is a gradient of cardiovascular risk across the normal range and at higher levels.

Test performed by CHL.

Authorised by : Vivienne Hughes at 09:37 AM on 11-11-2008

APPENDIX C:
BUFFERS AND SOLUTIONS

Buffers / Solutions

Name	Preparation
0.05M Phosphate Buffer, pH 7.4	<ul style="list-style-type: none"> • 0.011M Acid (KH₂PO₄)(MW=136.09) • 0.0389M Base (K₂HPO₄) (MW=174.18) • 2 Liter distilled water <p>Adjust pH with H₂PO₄</p>
Muscle Extraction Buffer1, pH 7.4	<ul style="list-style-type: none"> • 70mM Sucrose • 10mM Hepes • 1mM EDTA • 220mM Mannitol <p>Adjust pH with NaOH or HCl</p>
Muscle Extraction Buffer2, pH 7.4	<ul style="list-style-type: none"> • 50mM HEPES • 4mM EGTA • 10mM EDTA • 100mM b-glycerophosphate • 5mM Sodium orthovandate • 15mM Tetrasodium pyrophosphate • 25mM Sodium Fluoride • 1 Complete Mini-EDTA free protease Inhibitor tab(Roche)
Tris-HCl Buffer, pH 8.0	<ul style="list-style-type: none"> • 50mM/L Tris • Dissolve in 1Liter of distilled water <p>Adjust pH with 0.1M HCl</p>
100mM Phosphate Buffer, pH 7.3	<ul style="list-style-type: none"> • 100mM (KH₂PO₄)(MW=136.09) <p>Adjust pH with 1M KOH</p>

Cytochrome C solution	<ul style="list-style-type: none"> • Weigh 2.7mg of cytochrome C • Dissolve in 1ml of 0.05M phosphate buffer • Add 5ul of 0.1M DTT • Place overnight on bench to reduce
0.1mM Acetoacetyl-CoA solution	<ul style="list-style-type: none"> • Weigh 0.0439g of acetoacetyl CoA • Dissolve in 1ml of Tris-HCl buffer
0.2mM DTNB	<ul style="list-style-type: none"> • Weight 0.39535g of DTNB • Dissolve in 1ml of Tris-HCl buffer
5mM Oxalo-acetic Acid	<ul style="list-style-type: none"> • Weight 0.00330175g of oxaloacetic acid • Dissolve in 1ml of Tris-HCl buffer
6.4mM b-NADH solution	<ul style="list-style-type: none"> • Weight 0.004245952g of b-NADH • Dissolve in 1ml of 100mM phosphate buffer
5.4mM S-acetoacetyl coenzyme A solution	<ul style="list-style-type: none"> • Weight 0.000952299 of s-acetoacetyl coenzyme A • Add 1ml of molecular water

APPENDIX D:

LIST OF SYMBOLS USED IN IPA PATHWAY ANALYSIS

Symbol

Name



Cytokine/ Growth Factor



Enzyme



G-protein Coupled Receptor



Ion channel



Kinase



Ligand-dependent Nuclear Receptor



Peptidase



Phosphatase



Transcription regulator



Translation regulator



Transmembrane receptor



Transporter

APPENDIX E:

MICROARRAY RAW DATA
(Attached as CD)